Sukhes Mukherjee ·
Jagat Rakesh Kanwar *Editors*

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Preface

Oncology is in a thrilling period right now! Because we now know a lot more about the genetic and epigenetic changes that drive the neoplastic process, we have many more ideas on how to make more effective and less harmful cancer treatments than current cancer treatments. Research in the last several decades has dramatically increased cancer's reactions to hypoxia. Most malignant tumors experience hypoxia, a level of oxygen tension that is not physiological. The epithelial-to-mesenchymal transition phenotype and progressive but defective vascularization, both caused by tumor hypoxia, result in cell motility and metastasis. Hypoxia changes the way cancer cells work by making them stop dividing. This makes them resistant to treatment. However, the range of knowledge is expanding due to the expanding field of study. Information on cancer cells' reactions to and adaptations to hypoxia has increased. It goes beyond what can be covered in one article. The focus on which researchers have new research on the interaction between cancer cells and the host is beginning to pay off with methods of transformative therapy that strengthen the patient's immune system and cause diseases that are challenging to cure with current medicines.

The contact between immune cells and cancer cells occurs with the involvement of numerous elements that have a significant impact on the tumor microenvironment. This highlights the importance of an all-encompassing adaptive mechanism or adjustment to changes. Oxygen levels are considered a critical biological factor in the tumor microenvironment. The remarkable development in understanding the tumor's molecular mechanisms of hypoxia has sparked interest and increased efforts to comprehend the biological implications and use any therapeutic possibilities. This book aims to compile this wealth of knowledge into a clear and thorough collection. Eminent researchers and scientists working on fundamental, clinical, and translational research have contributed. Despite the undeniable advancement in growth, finding cures for metastatic cancer remains an elusive and desired objective despite the development of innovative cancer therapies. It has been an honor for me to have the chance to work with such a skilled team of researchers and experts, and I want to thank them all for their contributions.

This book has received a lot of help. Without their help, this book would not have been able to be put together. We sincerely appreciate everything our research team has done for their considerate, enlightened, and passionate contributions to this

volume. Additionally, we appreciate all of the reviewers' helpful comments. We are grateful to Dr. Suman Kumar Ray for giving us many helpful background pieces that helped us change the script in many chapters and make it easier to print. His generosity especially moves us to take the time to read the entire script and edit it before providing the foreword. Lastly, we want to thank the Springer Nature publication team for all of their help.

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Acknowledgment

Writing a book is harder than we thought and more rewarding than ever imagined. Having an idea and turning it into a book is as hard as it sounds. The experience is both internally challenging and rewarding. I especially want to thank the individuals that helped make this happen. None of this would have been possible without Dr. Suman Kumar Ray. He conceptualized, formatted, and designed all the book chapters. The world is better, thanks to people like Dr. Suman, who want to develop and lead others. We also thank him for his exercise in making creative juices needed to bring new ideas to the book on Hypoxia and its biological implications for Cancer Therapy. What makes it even better are people like him who share the gift of their time to design a Book. Thank you to everyone who strives to grow and help others. We also thank our esteemed reviewers for this book, who in spite of their busy schedule performed their work meticulously.

We are indebted to our talented and conscientious contributors, for this book would not exist without them. We are grateful for the many people at Springer Nature who supported us through their encouraging words. Many of those who shepherded this book through the complex publication process worked behind the scenes. Finally, we want to acknowledge our family members who supported this time-consuming labor of love. Untold hours were spent away from the family, sitting in front of our computers to bring this project to fruition. We would also like to thank laboratory staff members, scholars, and researchers who patiently listened to my endless rambling about the project.

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Sukhes Mukherjee is working as an Additional Professor in the Biochemistry Department of AIIMS Bhopal, India. Dr. Mukherjee has published more than 60 papers in peer-reviewed journals and presented his work at several national and international conferences. He has also authored or co-authored numerous books and book chapters. Dr. Sukhes got the best paper award from the "Association of Clinical Biochemists of India" in 2008. He got the international travel fellowship to attend the Neurochemistry conference at Busan, South Korea, in August 2009 and another AACC fellowship in 2011. Dr. Mukherjee has been awarded by the International Society for Neurochemistry in 2008 and is a member of the Who's Who as International Executives. He is elected as a member of the National Academy of Medical Sciences (MNAMS). He has served as executive committee member of various national and international scientific organizations and is also in editorial board and reviewer of several journals**.**

Jagat Rakesh Kanwar is Professor and Head Department of Biochemistry in All India Institute of Medical Sciences (AIIMS), Bhopal, MP, India. He is the highly cited (Globally top 2% highly cited ranked) researcher in three disciplines of science (field on oncology, medical chemistry, and nanotechnology). Dr. Kanwar earned his PhD from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India in 1993. In 2002, he joined as Senior Scientist/Senior Research Fellow in The University of Auckland. Pro-

fessor Kanwar is the group leader and laboratory head of Nanomedicine-Laboratory of Immunology and Molecular Biomedical Research in School of Medicine, Faculty of Health at Deakin University. Prof. Kanwar is currently working on nanotechnology/nanomedicine-based protein/peptide, aptamers, and his research approach employs monotherapy (gene therapy, immunotherapy) and combinational therapy with commercially available chemotherapeutic agents including LNA-aptamers (RNA/DNA), peptides, and other biomolecules such as siRNA, miRNA, aptamers, proteins, siRNA, miRNA, and their chimera in cancer and chronic inflammation.

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Hypoxia and Its Biological Implications for Cancer Therapy

1

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Abstract

Cancer is one of the leading public health issues with poor prognosis, high mortality rate, and limited effective treatment strategies. Hypoxia, a common characteristic feature of solid tumors, is caused by structural and functional modifications in microvasculature. Hypoxia-inducible factor-1 (HIF-1) is the principal regulator of physiological adaptations to hypoxia that activates the expression of battery of target genes, leading to the development of cancer, stromal angiogenesis, metastasis, and drug resistance by targeting MMPs, VEGF, LOX, and STAT3. It also stimulates complex cancer signaling networks, including PI3K and MAPK pathways. We have discussed how hypoxia regulates progression of various cancers, including breast, ovarian, cervical, and prostate, and their metastasis, angiogenesis, and drug resistance for better understanding of the implications of hypoxia in cancer therapy.

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Keywords

Hypoxia · Cancer · Tumor progression · Angiogenesis · Drug resistant · Cancer therapy

1.1 Introduction

Cancer causes about ten million deaths annually worldwide and is among the leading causes of deaths in humans. According to the GLOBOCAN report 2020, about 19.3 million cases of cancer were reported in 2020. In 2040, worldwide, an estimated 28.4 million cancer cases are expected to occur, an increase of 47% (Sung et al. 2021). For several cancers, etiology has been strongly linked to specific environmental factors, including cervical cancer and human papilloma virus (HPV), gastric cancer and *Helicobacter pylori,* lung cancer and smoking, skin cancer and exposure to the sun, hepatocellular cancer and viral hepatitis (Wu et al. 2018). Several factors, including food habits, alcohol consumption, genetic factors, obesity, late pregnancy, and miRNA dysregulation, also play a significant role in the development of cancer. Hypoxia is a common physical factor promoting cancer progression. In normal cells, oxygen is required to maintain the metabolic processes. In tumor cells, oxygen supply is also needed to fulfill metabolic requirements. Due to inadequate oxygen, tumors make their own vascular systems through angiogenesis. In hypoxic condition, activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1), is the principal regulator and helps in physiological adaptation to hypoxia (de Heer et al. 2020). HIF is a heterodimer consisting of HIF-1β subunit, which is inactive as a monomer and O_2-dependent HIF-α subunit (HIF-1α or HIF-2α). It mediates its function by binding to the hypoxia-responsive elements (HREs) in promoters of its downstream target genes, which further contributes to adaptation to hypoxic conditions. The activity of HIF is stringently regulated by two O_2-sensors—asparaginyl hydroxylase factor-inhibiting-HIF-1 (FIH-1) and prolyl hydroxylase domain proteins (PHD1–3)—with PHDs being characterized to have a low K_M value for O_2 than FIH (Samanta and Semenza 2018). PHDs hydroxylate proline residues on HIF subunits in normoxia (Eales et al. 2016). This post-translational modification of HIF-α subunits flags them for recognition by the von Hippel–Lindau (VHL), which further recruits ubiquitin ligases and mediates its proteasomal degradation (Eales et al. 2016). Hydroxylation of HIF-α by FIH-1 impairs the interaction between HIF-α subunits (HIF-1α or HIF-2α) and its co-activator (p300 or CBP), which in turn results in the inhibition of HIF-α at the mRNA level (Sebestyén et al. 2021). In hypoxia, due to low oxygen concentration, the activities of FIH-1 and PHDs are reduced because of which HIF-α subunits become stabilized. The stabilized HIF-α protein then translocates into the nucleus and induces the expression of target genes by binding to the promoter region of HREs. HIF-α regulates the expression of multiple genes that are involved in metabolic adaptation, survival, angiogenesis, and migration of cancer cells through this (Fig. 1.1; Table 1.1). We have discussed here hypoxia and its roles in several cancers, including breast,

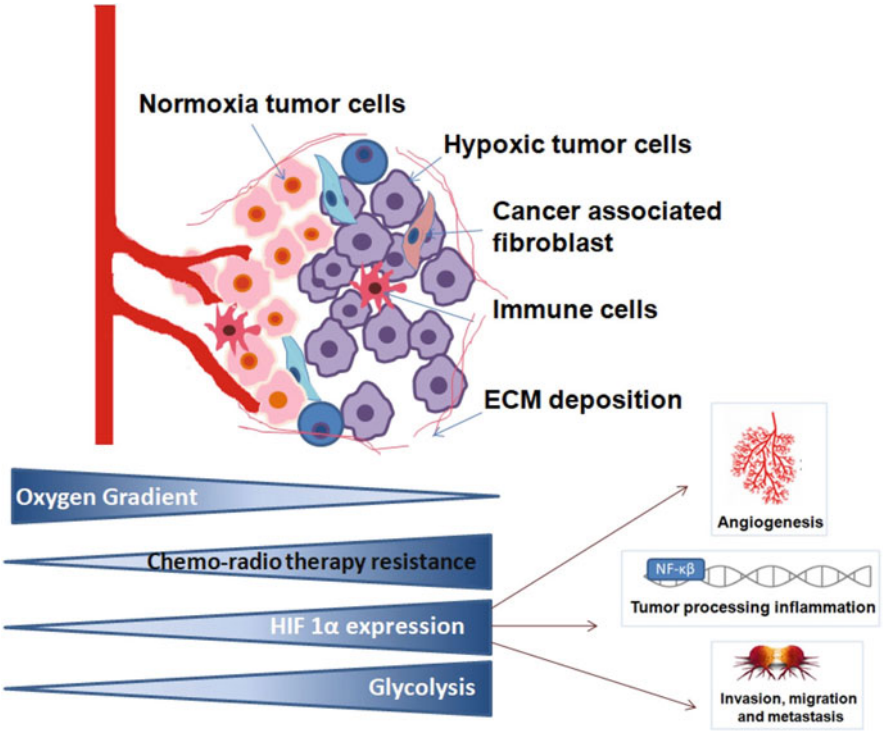

Fig. 1.1 The elements of the tumor microenvironment are affected by hypoxia in numerous ways in cancer

ovarian, cervical, and prostate, along with their metastasis, angiogenesis, and drug resistance for an in-depth understanding of the implications of hypoxia in cancer therapy.

1.2 Hypoxia in Breast and Other Cancers

1.2.1 Breast Cancer

Breast cancer ranks fifth among all forms of cancer-associated mortality worldwide and was estimated to be the leading cause of cancer-related deaths in 2020. Besides other factors, hypoxia is a common factor that helps in the development of breast cancer (Brown and Bicknell 2001). The hypoxic niche triggers tumor growth by inducing a variety of tumor-promoting oncogenes and signaling pathways through HIF-1. In breast tumors, HIF-1α is predominantly overexpressed in ductal carcinoma in situ (DCIS) and early stages of breast cancer, and its expression levels are highly correlated with tumor grade and invasiveness (Mehraj et al. 2021). HIF-1α's

Table 1.1 Role of HIF and many other genes in progression and metastasis of various cancers in response to hypoxia

Factors	Role in cancer	Role in hypoxia
HIFs	• HIF-1 promotes tumor growth and malignant progression, as well as gene regulation and pathological genomic alterations. • HIFs trigger the expression of a battery of genes involved in initial tumor development and vascularization, stromal cell recruitment, ECM, cell motility, invasion, migration, metastasis, and cancer stem cell maintenance. HIFs regulate the activity of P4HA1, P4HA2 and PLOD2.	Activity of HIFs induced by the hypoxic environment that elevate the other transcription factors
MMPs	• MMPs help in the remodeling of extracellular matrix that promotes invasion, migration, and ultimately metastasis.	In hypoxic microenvironment, HIF activates the expression of MMP
VEGF	• VEGF is the mediator of the angiogenesis in cancer.	Hypoxia induces the expression of HIF-1 that upregulates the expression of VEGF
LOX	• Components of extracellular matrix remodeling. • Contributes to the metastasis niche formation.	Produced from hypoxic breast cancer cells
TGF-β	• Prognostic markers.	Upregulation of TGF-β-associated proteins in response to hypoxia

stability, transcription, or translation is also regulated via genetic alterations commonly found in breast cancer cells, inhibition of the tumor suppressors' activity (PTEN, p53, or BRCA1), and activation of the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt)/mTOR pathway (Sharma et al. 2019; de Heer et al. 2020). Activation of HIF-2α decreased the mitochondrial reactive oxygen species (ROS) production and upregulated the expression of SOD2, which subsequently inhibited the expression of PDI. Suppressed PDI dissociated the aggregation between GRP78 and UPR, which leads to the activation of UPR. Recent studies have demonstrated that UPR was activated by the hypoxic condition and induces stemness in breast cancer cells through the HIF-2α/mitochondrial ROS-dependent signaling network (Katseff et al. 2021). Prolyl residues of HIF-1 are hydroxylated by PHDs in the presence of oxygen, and the hydroxylated HIF-1 binds to pVHLs, which recruit E3 ubiquitin ligase, causing proteasomal destruction of HIF-1. In anoxic environment, unhydroxylated HIF-1 translocates to the nucleus, dimerizes with HIF-1, and binds to the HRE promoter, consequently activating multiple genes associated with tumor formation, cell proliferation, and metastasis. Moreover, PHDs are inactivated by HIF-1 (Zagórska and Dulak 2004). Hypoxia and HIF-1 upregulated the matrix metalloproteinase (MMP)-2 and MMP-9 expression (Muñoz-Nájar et al. 2006), which is associated with metastasis and poor prognosis (Krishnamachary et al. 2006). HIF-1 is also important for collagen oogenesis by elevating the expression of lysyl hydroxylases (PLOD2) and procollagen prolyl

(P4HA1 and P4HA2), which are reported as breast cancer metastasis markers (Gilkes et al. 2013). Several oxidases of the lysyl oxidase (LOX) family proteins (LOX, LOXL2, LOXL4) were produced from hypoxic breast cancer cells (Wong et al. 2011). Activities of LOX proteins catalyze collagen crosslinking at hydroxylated lysine residues and help in remodeling extracellular matrix (ECM) at both primary and distant sites, which ultimately lead to the formation of metastatic niche (Kai et al. 2019).

Numerous studies have reported that in hypoxic environments cancer cells show Warburg effect, which is considered the hallmark of cancers. Because cancer cells proliferate at a rapid rate, which require more ATP, this increased energy generation leads to an increase in ROS enrichment. As a result, the levels of ROS in tumor cells are shown to be higher than those in normal cells. Furthermore, endogenous and exogenous ROS activates pathways such as PTEN, PI3K/Akt/mTOR, and MAPK, which in turn activate HIF-1, thereby promoting angiogenesis by elevating the expression of vascular endothelial growth factor (VEGF), MMPs, and other cytokines (Courtnay et al. 2015). By stimulating the EGFR/ERK/c-Fos signaling pathway, ROS has been demonstrated to enhance the production of VEGF, HIF-1, and G-protein-coupled estrogen receptor (GPER) in breast cancer cells (Rigiracciolo et al. 2015). In a hypoxia-independent mechanism, ROS may potentially stimulate angiogenesis by activating NF-κB via TLRs (Aggarwal et al. 2019). In addition, hypoxia impairs the activity of cytochrome b oxidoreductase, a component of the mitochondrial respiratory chain, as well as the activity of macrophage NADPH oxidase, which contributes to the production of ROS and the aggravation of oxidative stress in breast cancer (Nourazarian et al. 2014). In hypoxic environment, tumor-associated macrophages (TAMs) are accumulated and develop a tumorigenic phenotype. TAMs produce angiogenic growth factors that were involved in the formation of angiogenesis and associated with poor prognosis in invasive breast cancer (Leek et al. 2002). Susceptibility to hypoxia-induced apoptosis is maintained by the integrated regulation of a number of pro- and anti-apoptotic pathways in both HIF-independent and -dependent manner in specific cell types (Mylonis et al. 2017). Hypoxia lowered cytochrome c release, caspase 3 activity, and Bax/Bcl-2 ratio by inducing VEGF (Baek et al. 2000).

1.2.2 Ovarian Cancer

Hypoxia in ovarian cancer is well reported for ruling the tumor microenvironment, resulting in tumor progression. Tumor cells in ovarian cancer are highly hypoxic dependent. Hypoxia modulates oncogene HIF-1α, which plays a significant role in the development of ovarian cancer, invasive progression, and metastasis. Hypoxia induces HIF-1α, which upregulates COX2 expression, further promoting ovarian cancer proliferation and metastasis (Ding et al. 2021). Overexpression of HIF-2α is positively correlated with enhanced stemness of ovarian cancer stem cells (OCSCs) and drug resistance (He et al. 2019), whereas knockdown of HIF-1α induces autophagy and inhibits the PI3K/AKT/mTOR signaling pathway in ovarian cancer

cells. The study suggests the role of HIF-1α in therapeutic resistance by inducing NADPH oxidase 4 (NOX4), which results in an increase in reactive oxygen species (Liu et al. 2021). HIF-1 and HDAC4 could mediate the interplay between p53 and RAS signaling to regulate the cisplatin (CDDP) resistance through dysregulation of autophagy and apoptosis (Zhang et al. 2019). Hypoxia declines the intensity of immune response and enhances immunosuppressive and immune checkpoints genes in ovarian cancer. Under the hypoxic environment, HIF-1 promotes the transcription and expression of neuronal pentraxin II (NPTX2), resulting in malignant phenotype in epithelial ovarian cancer (EOC) (Behboudi-Gandevani et al. 2021). Hypoxic environment upregulates the TLR4/NF-κB signaling via the HIF-1α in human EOC cell lines (Zhao et al. 2022).

1.2.3 Cervical Cancer

The dynamic nature of hypoxic environment within the tumor microenvironment results in the combination of adaptive responses. Tumor shows specific metabolic, cellular, and molecular changes in response to hypoxic environment. In cervical cancer, metastasis is a multistage process by interaction with various cells within the tumor microenvironment. Epithelial-to-mesenchymal transition (EMT) induced by hypoxia increases the potential to invade neighboring tissues. Zhang et al. reported that knockdown of hCINAP decreased the EMT and migratory capacity of cervical cancer cells, which suggests that hCINAP drives apoptosis and hypoxia-induced EMT (Zhang et al. 2020). Furthermore, studies show that Plantamajoside decreases hypoxia-induced invasion and migration with increased expression of E-cadherin and decreased N-cadherin expression through PI3K/Akt and NF-κB pathways in cervical cancer (Zuo et al. 2021). Metabolic reprogramming is another important process and is controlled by tumor hypoxic environment. In hypoxic environment, CNPY2 is upregulated, which further supports glycolysis in cervical cancer through activation of Akt pathway (Tian et al. 2021) while cancer cells adapt partly through HIF-1α and HIF-2α promotes glycolysis during slowdown of mitochondrial activity (Denko 2008). Moreover, hypoxia enhances cervical cancer cell migration and invasion by the co-stimulation of Rab11 and Rac1 (Xu et al. 2017). Furthermore, hypoxia-induced ZEB1 enhances tumor-associated macrophage (TAM) recruitment via CCL8 (Chen et al. 2019).

1.2.4 Prostate Cancer

Prostate cancer (PCa) is one of the most frequently detected cancer types in men, and HIFs are responsible for the progression and aggressiveness of tumors in prostate cancer. Iwasaki et al. suggested that hypoxia-induced Slug promoted the invasive behavior of prostate cancer by activating ephrin-B1 (Iwasaki et al. 2018). It has also been reported that the hypoxic microenvironment induces the expression of EZH2 and miR-93, which ultimately initiate H3K27me3 in TGFBR2 that promotes tumor

development (Zhou et al. 2018). Chronic hypoxia also has a critical role in the augmentation of migration potential in androgen-independent prostate cancer progression (Yamasaki et al. 2013). The interaction between miR-182 and HIF-1 results in the activation of the HIF pathway, which helps the tumor cells to bear the hypoxic stress during the progression of prostate cancer (Li et al. 2015a, b). Another study revealed that autophagy is also induced by hypoxia and the hypoxia-induced autophagy is somewhat regulated by miR-96 (Ma et al. 2014). Furthermore, hypoxic environment influences miRNA activity for migration and EMT in PC3 and DU145 prostate cancer cells (Zhang et al. 2020). A similar study reported that hypoxia induces microRNA-301b-3p overexpression, which promotes migration, proliferation, and invasion in PCa (Munteanu et al. 2020). Hypoxia-associated genes such as LOX, glucose transporter-1 (GLUT-1), and carbonic anhydrase (CA) IX positively correlate with Gleason scores in prostate cancer (PCa) (Ambrosio et al. 2016). Moreover, cell surface receptors, including fibroblast growth factor receptor 2 (FGFR2), control migration and invasion of PCa cells under hypoxic environment by suppressing the HIF-driven gene expression and hypoxia-triggered metastasis is negatively regulated by FGFR2 (Lee et al. 2019). In addition, HIF-1α independently promotes PCa progression.

1.3 Hypoxia in the Regulation of Tumor Microenvironment

Hypoxia is a hallmark feature of the tumor microenvironment (TME). In total, 50–60% of solid tumors consist of hypoxic and/or anoxic regions that develop due to disrupted balance between oxygen supply and oxygen intake (Vaupel and Mayer 2007). In TME, oxygen supply is compromised because of aberrant tumor vasculature, including inflated capillaries characterized by leaky and slow blood flow (Brown and Giaccia 1998). At the same time, oxygen demand is high due to tumor proliferation and infiltration of immune cells (Radharani et al. 2021). To deal with their energy need, instead of metabolizing glucose through oxidative phosphorylation, hypoxic tumor cells start metabolizing glucose through glycolysis (Warburg effect) (Koppenol and Bounds 2009; Qiu et al. 2017). However, in addition to ATP, glycolysis also produces lactic acid and carbonic acid that reduces intracellular pH and thus increases the possibility of acidosis (Chiche et al. 2009). To surpass these effects, the HIF family of transcription factors plays a vital role in tumor cell transcriptional reprogramming. HIFs control the expression of more than 150 genes and contribute to hypoxia-inducing adaptive responses in tumor microenvironment (Emami Nejad 2021).

VEGF, transferrin, transferrin receptors, anti-apoptotic factors, glycolysis enzymes, multiple growth factors like transforming growth factor beta (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor-B (PDGF-B), insulin-like growth factor-2 (IGF-2), and other proteins involved in normal homeostasis are all activated by HIF-mediated transcription. As a part of adaptive response, these factors compensate for the nutrients and reduced oxygen tension; therefore, they overexpress these to extreme cell growth, aberrant angiogenesis, increase survival,

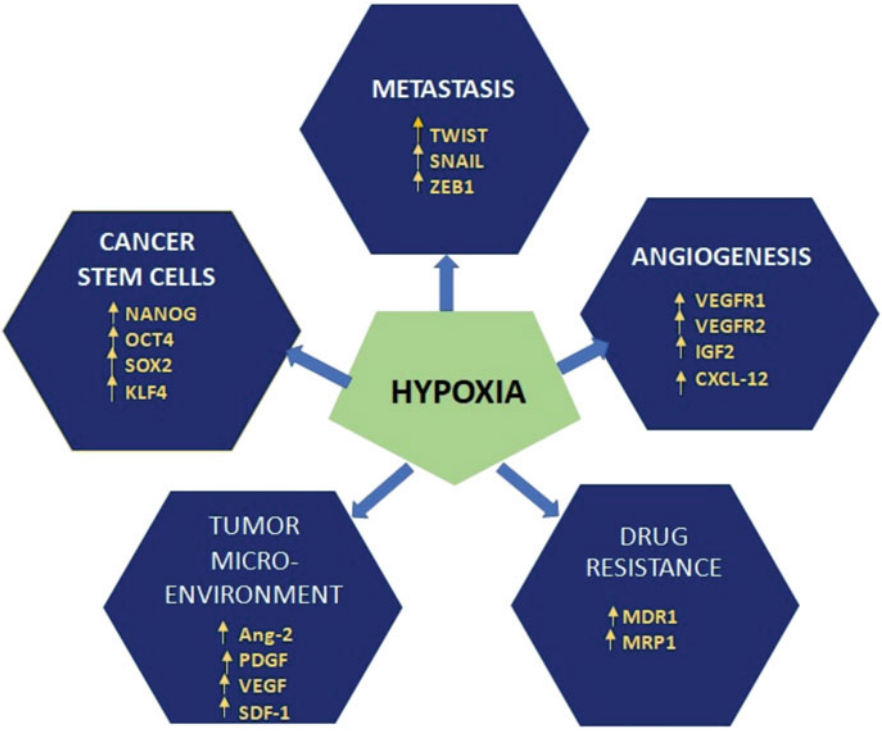

Fig. 1.2 Impact of hypoxia on cancer progression, angiogenesis, metastasis, and drug resistance

and metastasis. Thus, the hypoxic tumor microenvironment with upregulated HIF-1α plays a critical role in proliferation, apoptosis, cellular metabolism, metastasis, and angiogenesis (Matuszewska et al. 2021) (Fig. 1.2).

1.4 Hypoxia in Cancer Metastasis

Metastasis is a dynamic progression where aggressive tumor cells traverse from their original tissues, survive in the foreign tissue microenvironment, and proliferate at distant sites. Clinically, upregulated expression of HIF-1 and HIF-2 is correlated with distant metastasis and poor survival of cancer patients. Likewise, hypoxia and HIF signaling promote early as well as late stages of metastasis in different steps of the process.

Hypoxia regulates immune evasion to promote metastasis: One of the important parts in metastatic progression is the efficacy of cancer cells to evade immune attacks. Hypoxia facilitates immunosuppression in tumor cells as well as penetrating immune cells (Neophytou et al. 2021).

HIF signaling regulates invasion, migration, and angiogenesis to support the early stages of metastasis: tumor cells have invasive and migratory features that allow them to migrate from their initial site into nearby tissues. This process is associated with the epithelial–mesenchymal transition (EMT). HIF regulates the expression of EMT-associated transcription factors, including Twist, Snail, and ZEB1 (Marconi et al. 2021). Besides, HIF pathway also promotes EMT through many signaling cascades like TGF-β, Notch, Hedgehog, integrin-linked kinase (ILK), certain tyrosine kinase receptors and Wnt (Zada et al. 2021), and osteopontin (OPN), playing a prime role in hypoxic environment adaptation that controls breast cancer progression and angiogenesis (Raja et al. 2014). For example, AXL, a receptor tyrosine kinase, is a crucial player of HIF-dependent invasion and metastasis process.

HIF signaling promotes the late stages of metastasis by supporting premetastatic niche formation: in the late stage, metastatic tumor cells are required to colonize, survive, and proliferate in the foreign microenvironment of distal tissues. HIF signaling elevates secreted factors like LOX and exosomes to establish a premetastatic setting (Deasy and Erez 2021).

HIF signaling facilitates adaptation of tumor cells at the distant site: tumor cells are required to adapt to the foreign tissue microenvironment that could be very different from the original microenvironment of the primary tumor. Hypoxia at the distant tissue selects metastatic cells that can survive in the hypoxia-related metabolic stress.

1.5 Hypoxia in Tumor Angiogenesis

To overcome the oxygen deficit in hypoxia, tumor cells form new blood vessels through angiogenesis (Emami Nejad 2021; Seo et al. 2014). HIFs are master regulators of angiogenesis. HIF signaling promotes angiogenesis through HIF transcription factors that activate pro-angiogenic factors and their receptors like VEGF and VEGF receptor1 and 2, IGF2, angiopoietin-2 (ANGPT-2), chemokine C-X-C motif ligand12 (CXCL-12), basic fibroblast growth factor (bFGF), placenta growth factor (PGF), EGF, platelet-derived growth factor B (PDGF), and adrenomedullin (Carmeliet 2005). Besides, adrenomedullin, iNOS, endothelin, and heme oxygenase 1 are also involved in modulating local blood flow by controlling the vascular tone (Wenger 2002).

VEGF-A is the most important HIF-regulated pro-angiogenic factor (Lugano et al. 2020; Ferrara 2003). VEGF-A, as well as its subtypes VEGF-B, C, D, PGF, is released and acts on endothelial cells by binding to VEGF receptor-1 and -2 (Schito 2019). This activates pathways involved in endothelial cell survival and proliferation, such as the extracellular regulated kinase (ERK) and PI3K/Akt pathways (Vaish and Sanyal 2012); endothelial cell migration, such as Rho GTPases (Lamalice et al. 2007); and extracellular matrix (ECM) degradation for sprouting and invasion (Vaish and Sanyal 2012; Weis and Cheresh 2005). VEGF-A is also important for maintaining tissue homeostasis by increasing vascular permeability (Weis and Cheresh 2005). Because of the increased interstitial pressure in the tumor,

vascular hyperpermeability promotes tumor cell extravasation and metastasis into the bloodstream (Azzi et al. 2013). While physiological angiogenesis results in functioning vessels, they are aberrant in form and function in malignancies, resulting in inadequate tumor perfusion (Schito 2019; Schito and Semenza 2016; Carmeliet and Jain 2011). The sprouting of capillaries from preexisting blood vessels is termed angiogenesis, which is vital for tumor growth, invasion, and metastasis (Emami Nejad 2021). The anomalies in tumor vascularization lead to hypoxia, which mediates many of the cancer hallmarks. These hypoxia-derived cancer hallmarks arise when the tumor size prevents diffusion of the oxygen and induces angiogenesis to recompense the oxygen level. The secretions of the cancer stem cells present in the tumor microenvironment help endothelial cells recruit through angiogenic factors, ultimately creating tumor vascularization (Seo et al. 2014). The majority of HIF transcriptional responses are attributed to HIF-1 and HIF-2. Co-expression of both HIF-1 and HIF-2 has been shown in many cell types. But there is evidence claiming the divergent nature of HIF-1α and HIF-2α in the same cell type (Carmeliet 2005). For example, deletion of endothelial HIF-1α weakens tumor vascularization and tumor progression (Wenger 2002), while deletion of endothelial HIF-2α results in unsystematic vasculation, which makes the tumor more hypoxic and develop angiogenesis (Lugano et al. 2020, Ferrara 2003). Hahne et al. suggested that the macrophage migration inhibitory factor (MIF) is driven by HIF-1 and HIF-2, which is also crucial in hypoxia-induced angiogenesis (Hahne et al. 2018). They studied the crosstalk between HIF-1α and VEGF. They showed that HIF-1α and VEGF-A were upregulated in hypoxic condition. Further upregulation of HIF-1α leads to increased expression of VEGF-A and activation of HIF-1α/VEGF pathway results in hypoxia-induced angiogenesis. Several other pathways are also involved in tumor vascularization in hypoxic condition such as IGF1R and STAT3 that get activated and upregulation of HIF-1α due to exogenous IGF1, further lead to release of VEGF and ultimately result in vascularization.

1.6 Mechanism of Drug Resistance in Cancer in Response to Hypoxia

Hypoxia employs drug resistance to tumor cells. Cellular sensitivity to radiation depends on free radical formation in the presence of oxygen. This causes hypoxic tumor cells to be relatively radioresistant (Tan et al. 2015). Hypoxia also induces chemoresistance to many drugs like doxorubicin, cisplatin, melphalan, etoposide, gemcitabine, 5-flouoruracil, and docetaxel in multiple malignancies. HIF-1 contributes to drug resistance in various cancers (Doktorova et al. 2015). HIF-1 inhibition is reported to reverse the multidrug resistance in colon cancer (Chen et al. 2014). Furthermore, HIF-1α knockdown cells show more sensitivity to cytostatics and irradiation compared to wild type (Rohwer and Cramer 2011). However, hypoxia-induced drug resistance in cancers may occur in an HIF-1-dependent as well as HIF-1-independent manner.

Many HIF-1-responsive genes like MDR, VEGF, Bcl-2, and Glut-1 are directly or indirectly related to drug resistance (Liu et al. 2008). HIF-1 target genes may promote chemoresistance by increasing expression of drug export pump P-glycoprotein (MDR1, multidrug resistance 1) (Comerford et al. 2002; Wartenberg et al. 2003). Hypoxia can downregulate DNA topoisomerase II so that chemotherapeutic drugs like etoposide and doxorubicin that function as DNA topoisomerase II-inhibitor will no longer be effective (Ogiso et al. 2000). Hypoxia is correlated to the loss of tumor suppressor p53 protein that may further reduce apoptosis and promotes angiogenesis and invasiveness (Graeber et al. 1996; Haensgen et al. 2001). Hypoxia promotes autophagy, which is a cellular survival strategy under stress (Azad et al. 2008; Rouschop et al. 2010). Moreover, hypoxic tumor cells become chemoresistance due to acidosis and nutrient starvation that inhibits proliferation of cancer cells. Slow proliferation of tumor cells also causes resistance to cell-cycle inhibitors (Trédan et al. 2007). Furthermore, enzyme carbonic anhydrase is overexpressed in hypoxia, causing elevated extracellular acidification (Svastová et al. 2004). Some basic anticancer drugs (e.g., doxorubicin) diffuse across the cell membrane in uncharged form. However, acidic extracellular environment charges them, resulting in inferior uptake into cells. Hypoxic tumors with aberrant vascularization also cause bioavailability of the drugs (Rohwer and Cramer 2011).

1.7 Hypoxia and Cancer Therapy

HIF-1α is upregulated in multiple solid tumors like colon, breast, lung, skin, gastric, pancreatic, prostate, ovarian, and renal carcinomas compared to the corresponding normal tissues (Jing et al. 2019; Mayer et al. 2008; Simiantonaki 2008; Sun 2007). Therefore, it has been recognized as a potential biomarker for specific cancers. For example, many reports indicate that HIF-1α can be considered a biomarker for evaluating prognosis and survival of pancreatic cancer patients (Zhang et al. 2010; Hoffmann et al. 2008; Zhitomirsky and Assaraf 2016).

As discussed earlier, pH in the TME is decreased due to hypoxia. However, some cancer drugs are pH dependent. For example, melphalan is a mild acidic drug for ovarian cancer and myeloma. It shows enhanced efficacy in preclinical as well as clinical trials due to local hypoxic acidic TME (Li et al. 2015a, b).

The available cellular enzyme converts a prodrug into an active molecule in the target cell. In anoxic tissues, an anoxic prodrug is activated, killing anoxic tumor cells selectively. Meanwhile, hypoxic prodrugs are activated by enzyme reductases, reoxidized into original drug progenitors in anoxic tumor cells, and transformed to cytotoxic active drug. For example, the results of a phase II clinical trial combining the hypoxic progenitor TH-302 with gemcitabine for the treatment of pancreatic cancer are promising (Phillips et al. 2013)

Furthermore, targeting HIF and downstream of HIF signaling are also important in hypoxic tumors. VEGF is targeted by monoclonal antibody (bevacizumab) or small molecule inhibitors, and clinical outcome is promising for advanced cancers. Furthermore, HIF-1α upregulates transketolase (TKT) and cytidine triphosphate

synthase (CTPS1) and thus promotes gemcitabine resistance in pancreatic cancer. However, if digoxigenin is applied to inhibit HIF-1α, pancreatic cancer cells become gemcitabine sensitive (Xiong et al. 2018). Likewise, multiple strategies are undertaken by various research laboratories to target hypoxia, HIF-1, and its downstream targets to mitigate cancer progression and chemoresistance.

1.8 Conclusion

Hypoxia is a characteristic feature of all solid tumors that promotes cell progression, angiogenesis, metastasis, and resistance in cancer therapy. It was observed that hypoxic microenvironment increased the expression of HIF that helps in the impairment of oxidant/antioxidant homeostasis and influences tumor initiation by activating a group of signaling networks, including PI3K, VEGF, and JAK-STAT3. Hypoxia promotes tumor vasculogenesis through mobilization of endothelial progenitor cells by VEGF, VEGF-R2, FGF, PDGF, and SDF-1 and angiogenesis via sprouting of preexisting vessels along with increased secretion of VEGF, VEGF-R1, as well as MMPs. Hypoxic cancer cells are also associated with EMT by upregulating transcription factors like Slug, Snail, and Twist while downregulating adhesion molecules like β-catenin and E-cad. Hypoxia is also correlated with the loss of p53 activity that promotes angiogenesis and invasiveness by reducing apoptosis. Hypoxia also induces chemoresistance to many drugs like doxorubicin, cisplatin, melphalan, etoposide, gemcitabine, 5-flouoruracil, and docetaxel in multiple malignancies by interacting with p-glycoprotein, DNA-topoisomerase II, MDR1, and MRP1. Based on the molecular functions, HIF-1α can be considered as early diagnostic and prognostic biomarker in cancer. Hence, the role of hypoxia in various processes must be extensively studied, which may lead to developing hypoxia and hypoxia-regulating genes as a potential target(s) in cancer therapy.

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Hypoxia's Function in Cancer

2

Avani Kulkarni, Neha Masarkar, Suman Kumar Ray, and Sukhes Mukherjee

Abstract

Hypoxia is defined as the inadequate supply of oxygen to the tissue that can occur due to a multitude of causes and is called by various names such as hypoxemic, anemic, ischemic, diffusional, and cytotoxic hypoxia. Cancer-induced hypoxia is an interplay of ischemic, diffusional, and anemic hypoxia, and plays an important role as a prognosticator of the disease and also as a target for treatment modalities. The major mediator of hypoxia in tumor cells is hypoxia-inducible factor (HIF), which is a heterodimeric protein that is upregulated in hypoxic conditions. The consequences of HIF action are the activation and upregulation of several enzymes, transporters, and factors that modulate the neoplastic cell's metabolic functions that result in functional responses to the hypoxic stressor, which resists apoptosis/necrosis, in addition to modifying and refashioning the local microenvironment to suit the neoplastic cell's survival. Metastasis—one of the most feared outcomes of neoplasm—has almost all of its steps upregulated or controlled by hypoxia and HIF. Hypoxia is also responsible for drug resistance to various chemotherapeutic agents by different mechanisms. This makes it harder to treat neoplasms that are susceptible to these drugs. Therefore, treatment modalities acting by blocking HIF, in addition to the standard chemotherapeutics, target the neoplasm from all aspects, making it more comprehensive and more effective.

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Keywords

Hypoxia · HIF · Cancer · Tumor · Chemotherapy · Tumor metastasis · Chemoresistance

2.1 Introduction

Hypoxia refers to the inadequate oxygenation of tissues. Put simply, the amount of oxygen reaching the tissues is less than what the ideal range of oxygen supply should be. This leads to derangement in the tissue's local microenvironment, leading to adaptive functional alterations and, if persistent, apoptosis (McKeown 2014). At a biochemical level, hypoxia is evident in the form of oxygen-deficient electron transport. There are 4 major mechanisms for the development of hypoxia:

1. Hypoxemic hypoxia: decrease in arterial oxygen partial pressure secondary to causes like poor oxygenation because of pulmonary diseases, poor oxygen tension in the environment (high altitudes), etc.
2. Anemic hypoxia: decrease in the ability of blood to carry oxygen due to decreased oxygen-carrying capacity.
3. Ischemic hypoxia: decrease in the perfusion of the tissues.
4. Diffusional hypoxia: decrease in the diffusion of oxygen to the cells in the tissue due to alterations in the diffusion membrane, turbulence of flow within the microvasculature, etc.
5. Cytotoxic hypoxia: alteration in the cellular metabolism that leads to difficulty in the utilization of oxygen within the cell, and causes include cellular intoxication due to cyanide poisoning.

Cancer-induced hypoxia is a combination of acute (ischemic), chronic (diffusional), and systemic forms (anemic) of hypoxia (Sebestyén et al. 2021) (Fig. 2.1).

The "gold standard" for the measurement of O_2 partial pressures in the tumor microenvironment is by using an intratumor polarographic microsensor technique. The critical O_2 partial pressure in tumors, below which the detrimental changes

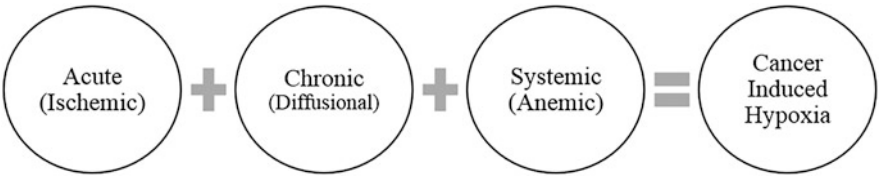

Fig. 2.1 The determinants of cancer-induced hypoxia: cancer-induced hypoxia is not a single entity; rather, it arises from a complex interaction of different types of hypoxias. Acute/ischemic hypoxia is due to inadequate vascular perfusion to the tumor. Chronic hypoxia occurs due to limitations in diffusion and changes in the diffusion permeability. Systemic hypoxia occurs as a response to the anemic state the patients with a tumor present with

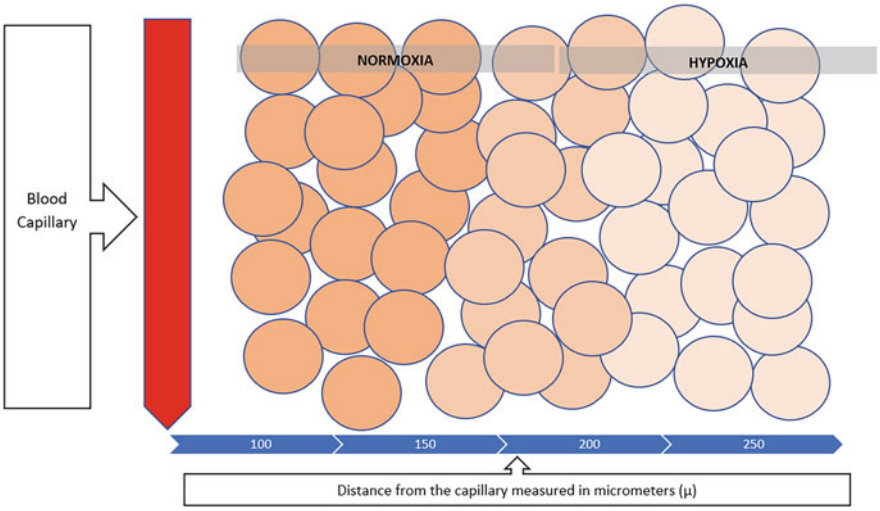

Fig. 2.2 Depiction of Folkman's theory. For a 250 μ radius of capillaries, 1 mm^3 of tissue can survive without the growth of new vessels. In a neoplastic proliferation of cells, the growth exceeds well beyond 3 mm^3 and can thus result in a state of *acute hypoxia,* which, due to the failure of resolution, can lead to necrosis, but is prevented in a tumor by mechanisms like faulty angiogenesis triggered by hypoxia

associated with hypoxic changes and derangement have been observed, was found to be 8–10 mmHg.

The cells in the tissue meet their oxygen requirements by diffusion. In neoplastic tumor, there is uncontrolled, unregulated cell growth and proliferation. This results in the development of a situation wherein lots of neoplastic cells move from the source of oxygen. According to Folkman's theory at a distance of more than 200–250 μ radius of the capillaries (Sebestyén et al. 2021), the cells are affected by hypoxia (Höckel and Vaupel 2001) (Fig. 2.2).

Exposure to hypoxic environments results in functional abnormalities, which leads to the activation of the adaptive functions of the cell, and if the stressor of hypoxia is chronic, may lead to apoptosis/necrosis. At the same time, this stressor leads to the selection of more resistant and tolerant tumor cells and their preferential growth (Höckel and Vaupel 2001; Jing et al. 2019; Emami Nejad et al. 2021). Hypoxia increases glycolysis within the neoplastic cell and angiogenesis by promoting the activation and release of growth factors (like VEGF) and other survival responses. It also promotes invasion of the neoplastic cells into the surrounding tissue, promoting metastasis by the activation of applicable gene expressions through hypoxia-inducible factors (HIFs) (Lu and Kang 2010).

One of the causes of cancer hypoxia is inadequate and incongruous intratumoral vascularization along with the compression of the existing ones because of unregulated proliferation. This refers to the haphazard angiogenesis in response to the growth factors released by the growing neoplastic cells that fail to adequately perfuse

the tissue. This, along with systemic hypoxia of the patient (often because of anemia), leads to a unique form of genetic reprogramming by hypoxia-induced transcription factors (HIFs). Another cause is the constitutive activation of oncogene-driven signaling pathways that activate hypoxia signaling independent of oxygen supply (pseudohypoxia). Thus, cancer-induced hypoxia and the adaptation mechanisms are two of the major causes of therapy resistance (Sebestyén et al. 2021).

Cancer hypoxia is considered one of the important hallmarks of any neoplastic process. The process of a rapidly proliferating tumor requires an equally rapidly developing vasculature to support the growth and metabolic processes mentioned above. This means that the tumor cells far (around 200–250 μ) from the vasculature will develop features of hypoxia and thus activate the heterodimeric protein HIF.

Hypoxia in the local tumor environment is associated with poorer outcomes in established cancers and is also associated with higher rates of recurrences. Hence, several targets have also been developed to combat the hypoxic response and its implications as discussed later.

Hypoxia can thus be considered an independent prognostic marker for cancer mortality. Hypoxia and hypoxia-inducible factors (HIFs) regulate various characteristic features of neoplasia:

1. Genetic instability.
2. Dedifferentiation.
3. Metabolic alterations.
4. Neovascularization.
5. Invasion–metastasis cascade.
6. Drug resistance.

2.2 Importance of HIF in Cancer Therapy

Hypoxic cells are considered resistant to many routinely used chemotherapeutics, and this can be partially associated with the selection of cells that have lost sensitivity to p53-mediated apoptosis (which is one of the ways by which alkylating agents act) in addition to many other mechanisms of resistance.

Hypoxia is, thus, an important entity (Fig. 2.3) that is being targeted to develop newer modalities as adjuvants to chemotherapeutics and also as the mainstay treatment. Combining drugs that act under hypoxic conditions [hypoxia-activated prodrugs (HAPs)] or act by inhibiting the working of HIF directly or indirectly by acting on its downstream pathways with standard radiotherapeutic modalities and chemotherapeutics has been shown to eliminate the most malignant cells, with maximum mutations, only causing a limited increase in systemic toxicity.

Therefore, targeting hypoxia and its associated features either directly or indirectly by inhibiting the downstream signaling pathways can be an efficient way to overcome chemotolerance/chemoresistance and supplement the activity of the

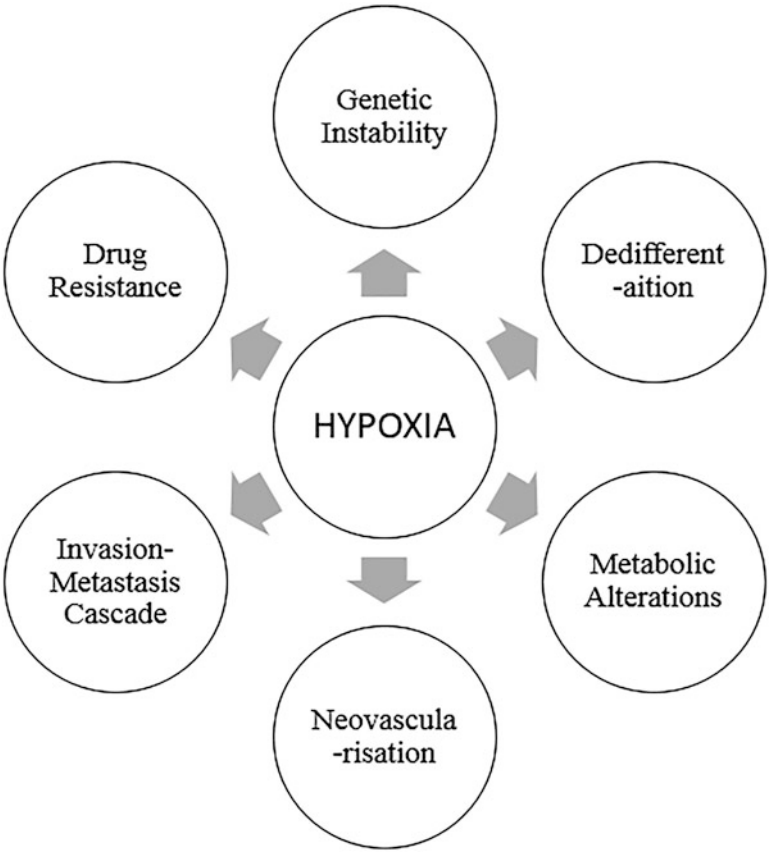

Fig. 2.3 Hypoxia and its consequences. Hypoxia, acting by the induction of hypoxia-inducible factors, regulates and affects the tumor microenvironment and has the abovementioned effects. It promotes the selection of more resistant cells with more neoplastic potential and its survival and metastasis. Hypoxia promotes the sustenance of the tumor microenvironment by promoting vascular angiogenesis and metabolic alterations. The neoplastic cell is more susceptible to the invasion–metastasis cascade because of the HIF-induced metabolic alterations and activation of factors (TWIST) that promote metastasis. Additionally, the tumor may become resistant to the drugs being used for the treatment, a phenomenon called chemoresistance

chemotherapeutics. Researchers are trying to obtain the ideal HIF1 inhibitor that can be successfully used for clinical application.

2.3 Hypoxia-Inducible Factor (HIF)

Hypoxia-inducible factor (HIF) is responsible for the development of all changes that help the neoplastic cell survive in the hypoxic microenvironment of a solid tumor. However, it also plays a key role in the maintenance of oxygen homeostasis

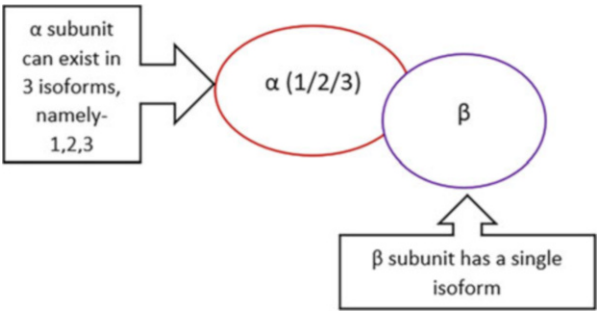

Fig. 2.4 Hypoxia-inducible factor-a heterodimeric protein composed of an alpha (α) and a beta (β) subunit. The alpha subunit can exist in three different isoforms giving specific actions to the protein. The alpha subunit is used for the nomenclature of HIF into HIF-1, HIF-2, and HIF-3. The clinically relevant isoforms are HIF-1 and HIF-2. HIF-3 has a more regulatory effect and promotes gene expression in addition to inhibiting HIF-1 and HIF-2

under physiological conditions. This protein is found in almost all human tissues. It is imperative to understand that it is a physiological response that is exploited and misused by the neoplastic cells to promote their metabolic activities and survival that ultimately makes it responsible for malignancies.

As a response to the decrease in O_2 tension due to the aforementioned mechanisms, the hypoxia-inducible factor (HIF) is activated to mediate the primary adaption at a transcriptional level in neoplastic cells (Huang et al. 2014). HIF is a heterodimeric protein with an alpha (α) subunit and a beta (β) subunit. The alpha subunits have three isoforms: HIF-1α, HIF-2α, and HIF-3α (Fig. 2.4).

HIF-1α and HIF-2α, and their overexpression, are linked with metastasis and unfavorable clinical outcomes (Lu and Kang 2010). The increase in the expression of HIF can be seen in various solid tumors (Simiantonaki et al. 2008) such as carcinoma of the

1. Oral cavity.
2. Breast.
3. Stomach.
4. Prostate.
5. Endometrium.
6. Cervix.

The α subunit is oxygen-labile, that is, hypoxia stabilizes and promotes the activity of the alpha subunit and normoxic states destabilize the subunit's activity, whereas the β subunit has a constitutive action (Fig. 2.5).

HIF-1 (HIF-1α/β) and HIF-2 (HIF-2α/β) have some common targets and some specific targets of action. The common factors include the activation of the genes coding for vascular endothelium-derived growth factor (*VEGF)* and glucose transporter- 1 (*GLUT1*), which promote angiogenesis and glucose uptake, respectively (Figs. 2.6 and 2.7). HIF-1 has a specific action for the activation and upregulation of

Fig. 2.5 The depiction of the oxygen lability of the alpha subunit of the HIF heterodimeric protein. The α subunit is stabilized by inhibition of post-translational hydroxylation of the α subunit and is promoted by the absence of oxygen, whereas the β subunit has a more constitutive action, independent of the oxygen states in the microenvironment

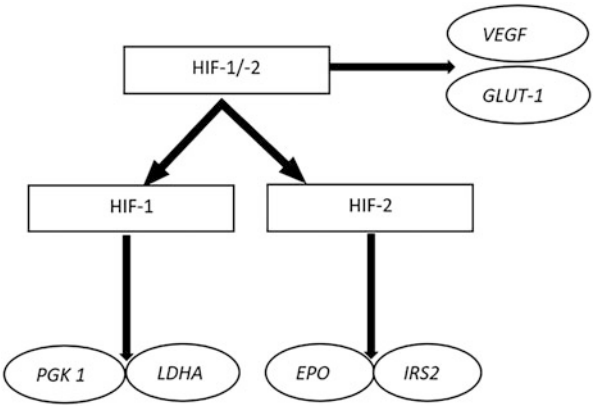

Fig. 2.6 Flowchart describing the varied actions of the HIF heterodimeric protein. HIF-1 and HIF-2 differ in their α subunit isoform, which imparts specific properties and results in differential activation of genes. Both HIF-1 and HIF-2 activate the genes encoding vascular endothelium-derived growth factor (*VEGF*) and glucose transporter-1 (*GLUT1*). HIF-1 selectively activates the genes encoding the glycolytic pathway enzymes: phosphoglycerate kinase-1 (*PGK1*) and lactate dehydrogenase A (*LDHA*), whereas HIF-2 selectively activates the genes encoding erythropoietin (*EPO*) and insulin receptor substrate-2 (*IRS2*)

glycolytic pathway enzymes (*PGK1, LDHA*), whereas HIF-2 acts on the genes coding for *EPO* and *IRS2*. HIF-1 is also implicated in the upregulation of enzymes like carbonic anhydrase-9 and -12, hexose kinase 2 (HK2), and transport proteins like monocarboxylate transporter 1 (MCT1).

2.4 Implications of HIF Activation in Tumors

It is established that solid tumor progression and recurrence are closely related to HIF, which is activated under hypoxic conditions. As discussed above, hypoxia stabilizes the alpha subunit by inhibiting the post-translational hydroxylation of the alpha subunit, which leads to heterodimerization and binding to hypoxia response

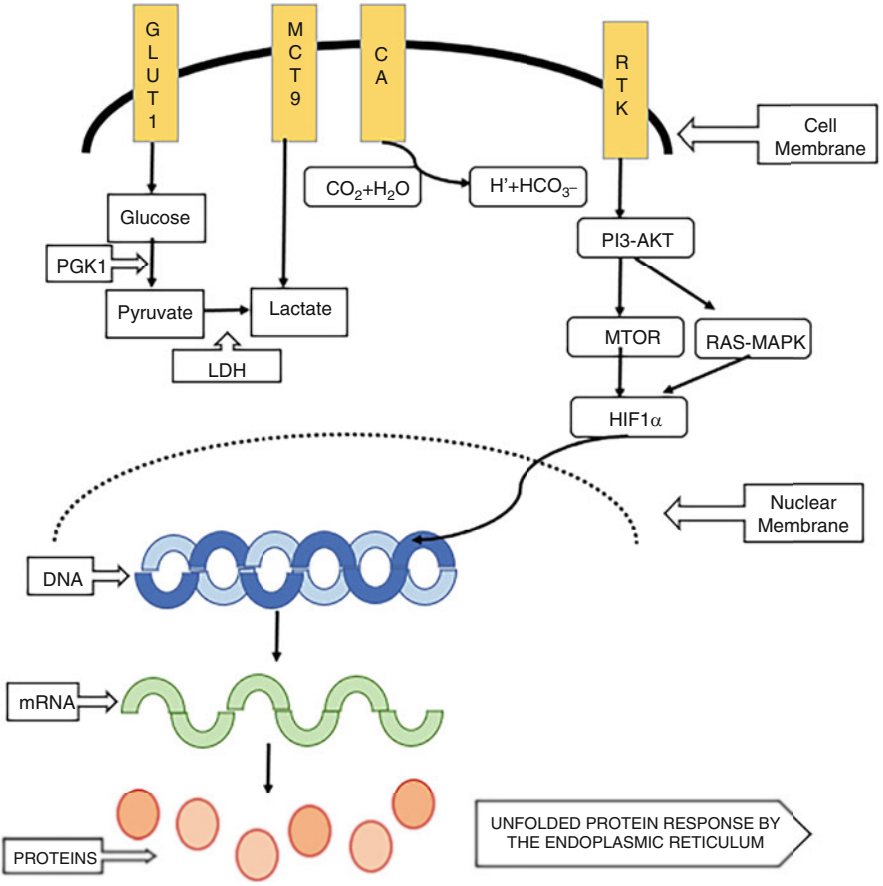

Fig. 2.7 A schematic representation of the potential targets of chemotherapeutics. GLUT1 is upregulated by HIF-1, which causes the entry of lots of glucose into the cells, further promoting the phenomenon of "aerobic glycolysis" or the Warburg phenomenon. This results in rapid conversion to pyruvate by PGK1, which produces ATP production that combats hypoxic free radical and ROS insults. MCT also facilitates the transport of lactate into the cells. Carbonic anhydrase also favors the promotion of a local acidic environment. RTK via the PI3-AKT pathway activates and promotes further HIF1 upregulation by the RAS-MAPK pathway and mTOR pathway. The other level at which it can be regularized is at the genetic level by preventing the unwinding of A, the transcription of the DNA segments to mRNA, or the translation of mRNA into proteins. GLUT1, glucose transporter; MCT, monocarboxylate transporter; PGK1, phosphoglycerate kinase-1; CA, carbonic anhydrase; RTK, receptor tyrosine kinase

elements (HRE) in target genes. This results in the activation of genes that oppose the hypoxic stress and counter the hypoxic insults with metabolic and functional changes at a genetic and biochemical level that is evident in the tumor microenvironment as the effects of HIF.

The implications of HIF activation in tumors can thus be classified as (Sebestyén et al. 2021)

1. Tumor angiogenesis.
2. Metabolic derangement.
3. Altered tumor immune response.

2.4.1 Tumor Angiogenesis

As discussed above, Folkman's theory states that cells beyond the radius of 200–250 μ develop hypoxia, and any tissue that grows beyond 2–3 mm^3 requires new blood vessels. It has been shown that HIF-1 is a major regulatory factor of angiogenesis (Kelly et al. 2003). The upregulation of angiogenesis is one of the characteristic features of hypoxia. This is due to the transcriptional activation of genes encoding platelet-derived growth factor-β (PDGF-β), vascular endothelial-derived growth factor (VEGF), and angiopoietin-2 (ANG-2), and others like fibroblast growth factor (FGF), tumor necrosis factor-α (TNFα), and transforming growth factor-β (TGFβ). They can be activated by both HIF-1 and -2. This leads to endothelial proliferation and, thus, angiogenesis.

This is different from *vasculogenesis*, a process that occurs in the embryonic stage of life, wherein angiogenic precursors from the bone marrow are organized into new, developing tissues. In contrast, angiogenesis refers to the newly formed capillaries that develop by sprouting from preexisting peritumoral capillary networks.

This concept becomes clinically relevant because these angiogenic factors and their receptors are potential targets for chemotherapeutic agents. One instance wherein it has been put into action includes bevacizumab, a monoclonal antibody targeted against VEGF, one of the products of activation of the HIF downstream signaling pathway, and other small-molecular compounds against VEGF-receptors have shown clinical benefits in cases of advanced care.

2.4.2 Metabolic Derangement

One of the hallmarks of neoplasia is the Warburg effect. The Warburg effect, or aerobic glycolysis, is the preferential utilization of the glycolytic pathway rather than oxidative phosphorylation of the intermediate produced even under normoxic conditions. This results in the production of two molecules of ATP for every molecule of glucose utilized in comparison to the 32 molecules of ATP produced for every molecule of glucose utilized. This can be attributed to the overexpression of HIF-1, which is seen in almost 50% of the tumor cells under normoxia. HIF-1 activation leads to the transcriptional activation of genes upregulating GLUT-1-3, pyruvate-dehydrogenase-kinase 1 (PDK1), and pyruvate kinase isoform 2 (PKM2). In addition, this further suppresses the TCA cycle. Activation of the gene encoding PDK1 inactivates the TCA cycle enzyme, pyruvate dehydrogenase (PDH), which catalyzes the conversion of pyruvate to acetyl-CoA.

Rapid production of ATP by the Warburg effect decreases hypoxic reactive oxygen species (ROS) generation and thus protects the neoplastic cells from hypoxia-induced apoptosis. The hypoxia-induced metabolic change redirects glucose metabolites from the mitochondrial oxidative phosphorylation to the cytoplasmic glycolytic pathway to maintain steady ATP production and prevent toxic ROS production (Kim et al. 2006).

The clinical implications are as follows.

Rapid ATP production by "aerobic glycolysis" can increase the resistance to chemotherapeutic drugs like doxorubicin and ara-c (Kim et al. 2006). New drugs like mTOR inhibitors can be developed to downregulate HIF-1-induced metabolic changes. mTOR kinase comes from the family of regulatory proteins that control translational and post-transcriptional modifications of proteins. mTOR hyperactivity is associated with HIF-1 stabilization and, thus, disruption of this pathway can promote the prevention of metabolic derangements and, thus, hypoxia-induced apoptosis (Sebestyén et al. 2021).

2.4.3 Tumor Immune Response

Hypoxia is implicated in tumor resistance. Evasion of the innate immune response elicited by the body against the developing tumors is one of the hallmarks of neoplasia. This evasion is assisted by the hypoxic conditions generated in the tumor's microenvironment. The innate immune response is primarily mediated by two cell types:

1. Natural killer cells (NK- cells).
2. Macrophages.

Macrophage activity is hypoxia-sensitive. There is a shift in the usual antitumoral polarization (M1) under normoxic conditions into the immunosuppressive phenotype (M2) under hypoxic conditions (Díaz-Bulnes et al. 2020). NK cells, on the other hand, retain their tumoricidal activity in hypoxic conditions too (Taylor and Colgan 2017). Moreover, HIF-1-induced hypoxia promotes the immunosuppression activity of suppressor cells derived from the myeloid cell lineage (Vetsika et al. 2019).

The release of VEGF because of HIF-1 further promotes CD4 + T cell differentiation into T-regulatory cells that are immunosuppressive. Cytotoxic CD8 + T cells also undergo immunosuppressive modulation mediated by HIF-1.

2.5 Tumor Metastasis and Hypoxia

Each step of the metastasis process, from the initial epithelial–mesenchymal transition to the final organ-specific colonization, can be regulated by hypoxia, indicating a master regulator role of hypoxia and HIFs in metastasis. Hypoxic conditions promote the invasive potential of tumor cells. HIF-1/-2 activation is associated

with loss of E-cadherin, a component of cellular junctions that protects the cells from undergoing invasion and metastasis. It was observed that antiangiogenic therapy, which was thought to reduce metastasis and invasiveness by blocking neovascularization, rather promoted metastasis and invasiveness in preclinical trials because lack of neovascularization created and further exacerbated hypoxic conditions in the tumor microenvironment.

TWIST1, a regulator of epithelial–mesenchymal transition, is induced in hypoxia, which leads to abnormal EMT. Promotion of the invasion–metastasis cascade occurs partly by the activation of HIF-upregulated proteins responsible for matrix remodeling, like lysyl oxidase (LOX, an extracellular enzyme that covalently modifies collagens to increase focal adhesion kinase activity, cell migration, and metastasis), and metalloproteases; they disrupt cell–cell and cell–matrix (ECM) interactions. Other proteins implicated are cathepsin D, the urokinase-type plasminogen activator receptor.

HIF also activates other genes known to be involved in metastasis and invasion (Table 2.1) such as the c-met proto-oncogene, the chemokine receptor CXCR4, and the autocrine motility factor (AMF).

As discussed previously, cells that survive acidosis and a hypoxic environment not only develop a growth advantage but also become more resistant and invasive by activation of adaptive mechanisms and collection of more genetic mutations with every passing abnormal cell division (Brahimi-Horn et al. 2007; Chan and Giaccia 2007). Multiple approaches to target hypoxia and HIFs may become effective treatment to prevent or reduce metastasis.

2.6 Tumor Hypoxia and Chemoresistance

Chemotherapy is one of the mainstays for the treatment of cancers. In a significant proportion of cases, tumors are either intrinsically resistant or develop resistance during chemotherapy. Despite hypoxic conditions, the apoptotic processes are inhibited by metabolic compensatory mechanisms, resulting in a reduction in the sensitivity to chemotherapeutic drugs that block cell division. Additionally, in neoplastic cells, hypoxic conditions induce the upregulation of drug transporter proteins, further enhancing the cell's chemoresistance. Furthermore, perfusion hypoxia develops due to dysregulated angiogenesis that impairs the distribution and delivery of chemotherapeutics. Some chemotherapeutics require optimal O_2 concentrations for its cytotoxic effects (Sebestyén et al. 2021). Resistance to chemotherapy can thus be predicted by estimating the expression of HIF1α and its upregulated genes in some types of squamous cancers (Muz et al. 2015).

2.6.1 Hypoxia and Drug Resistance

Initially, in hypoxia, HIF1α induces the upregulation of the MDR1/ABCB1 efflux transporter, which leads to the development of resistance to chemotherapeutic drugs

Table 2.1 Details of the steps of the invasion–metastasis cascade and hypoxia at different levels

Metastasis–invasion cascade		
Stage	Mediators	Action of hypoxia
Epithelial–mesenchymal transition (EMT)	E-cadherin is a functional requirement for cell connection and loss of E cadherin is a hallmark of EMT	Transcription of repressors like SNAIL, TWIST1, TCF3, ZEB1, and ZEB2.
Invasion, extracellular matrix modulation, and cell motility	Basement membrane dysregulation	HIF-1α-dependent upregulation of CTSD[1], uPAR[2], and MMP2[3] via a proteolytic cascade. Hypoxia also upregulates the expression of LOX[4]. Activation of MET by HIF-1- increases cell motility. Induction of AMF[5], a tumor-secreted cytokine by HIF-1 and VEGF under hypoxia to enhance proliferation, migration, and angiogenesis through autocrine or paracrine mechanisms.
Intravasation, circulation, and extravasation	Aggressive, neoplastic cells separate from the tumor and enter the circulation, and travel to the target metastatic site	In addition to promoting angiogenesis and lymphangiogenesis, VEGF induced by hypoxia is also associated with increased microvascular permeability and interstitial fluid pressure (IFP), both of which contribute to an increased chance of intravasation.
Homing	Chemokine receptor CXCR4 plays a key role in metastatic homing of tumor cells to organs expressing a high level of its ligand SDF1	Hypoxia may increase metastatic homing by inducing CXCR4 expression
Proliferation at the metastatic site	Sustenance and growth of secondaries	The secondary sites may develop hypoxic states due to sudden compromise in the vasculature due to metastatic proliferation, but neoplastic cells with already upregulated hypoxia genes due to hypoxic conditions in the primary site have a better chance of survival and establishment of a secondary metastatic site.

like doxorubicin that are its substrates (Sebestyén et al. 2021). Activation of nuclear factor, erythroid 2 like (NrF2) by hypoxia, further activates HIF1α, which upregulates multidrug resistance genes such as *MDR1/ABCB1, MRP1/ABCC1*, and *BCRP/ABCG2,* resulting in resistance to a variety of other chemotherapeutics (Belisario et al. 2020). Mechanisms of resistance and sensitivity to chemotherapeutic agents under hypoxic conditions are shown in Tables 2.2 and 2.3.

Table 2.2 The mechanism of resistance to various treatment modalities

Mode of therapy		Hypoxia	
Mechanism of action/property of drug	Examples	Effect of hypoxia	Mode of resistance to agent
Ionizing radiation		No free radical generation due to lack of O_2 – No DNA oxidation	Failure to induce breaks in the DNA
Antibiotics that induce DNA breaks	Bleomycin		
Cycle selective chemotherapeutic drugs	5-Fluorouracil	Cell cycle arrest in G1/G2 phase	Repair of the cell before progression to the S or M phase
Drugs extensively bound to tumor cells	Taxanes	Distance from the vasculature increases (indirect effect)	Compromised drug exposure
Basic drugs	Doxorubicin	Acidosis in the extracellular environment (indirect effect)	Decreased uptake
Multiple		Resistance to apoptosis	Genetic selection of TP53 mutants
Multiple	Etoposide		Downregulation of BID, BAX (pro-apoptotic proteins)
Multiple	DHFR[1] amplification, methotrexate	Genomic instability	Mutagenesis
DNA methylating agents		Suppression of DNA repair	Downregulation of MMR
ABC transporter substrates	MDR-1, doxorubicin	HIF-1 stabilization	Expression of ABC[2] transporters
Agents that induce DSBs[3]	Etoposide		Downregulation of NHEJ[4]

Table 2.3 Sensitivity to chemotherapeutic agents under hypoxic conditions

Mode of therapy		Hypoxia	
Mechanism of action/ property of drug	Examples	Effect of hypoxia	Mode of sensitivity to agent
PARP inhibitors	Veliparib	Cell cycle arrest in the S phase	The collapse of stalled replication forks
Acidic drugs	Chlorambucil	Acidosis in the extracellular environment (indirect effect)	Increased uptake
Bulky DNA monoalkylating and crosslinking agents	Cisplatin	Suppression of DNA repair	Downregulation of NER[2], HR[3]

2.7 Hypoxia and New Treatment Modalities

From the discussion so far, it is established that hypoxia and the resultant local acidosis are very characteristic of solid neoplastic tumor pathology. In summary, hypoxia plays an important role in the promotion of the invasion–metastasis cascade, the development of chemoresistance, and alteration in the innate immune response the body lodges against the tumor. Treatment modalities that work by using the hypoxic condition for the destruction of the tumor can prove to be a useful paradigm. Using the following strategies with a combination of immunotherapy can be a better therapeutic approach.

The most important strategies/targets to use the hypoxic conditions and HIF signaling for the treatment of the tumor include

1. Hypoxia-activated prodrugs (HAPs).
2. HIF-1α expression.
3. HIF-1 transcription.
4. HIF-1 target gene products.
5. Tyrosine kinase receptors.
6. RAS-MAPK signaling.
7. mTOR,
8. Unfolded protein response (UPR).

2.8 Hypoxia-Activated Prodrugs

Five different chemical groups (nitro groups, quinones, aromatic N-oxides, aliphatic N-oxides, and transition metals) have the potential to be metabolized by enzymatic reduction under hypoxic conditions, and thus provide the basis for the development of bioreductive prodrugs or hypoxia-activated prodrugs for exploiting tumor hypoxia. These are drugs that gain activation under hypoxic conditions, thereby limiting the action of the drugs beyond the site at which the action is required, ensuring high selectivity.

Tirapazamine is 50–200 times more toxic to hypoxic than normoxic cells, and is selectively activated by reductases in hypoxic cells inducing DNA damage due to breaks in base pairs, leading to cell death (PubChem [Internet] 2004); however, clinical trials are not very promising (DiSilvestro et al. 2014). The most recent use has been in the form of a phase 1 trial as an intra-arterial embolization with hypoxia-activated tirapazamine for unresectable hepatocellular cancer (b).

The prodrug apaziquone (E09), a mitomycin C derivative, showed positive preclinical trials but did not prove effective in clinical trials. Its use in vesicular cancer as a phase 2 clinical trial showed promising effects. (c) Phase II clinical trials for another prodrug TH302 in combination with sunitin have shown favorable results against metastatic neuroendocrine pancreatic cancer. (d) Other prodrugs have been included in Table 2.4 discussing the target, clinical trial, and the type of cancer it was studied for.

Table 2.4 A brief description of the various drugs acting at different steps, their mechanism of action, and their current clinical status

Drug details			Clinical trial (latest)		
Strategy/target	Class	Name	Phase	Status	Type of carcinoma under study
HAP	Aromatic N-oxide	Tirapazamine	2	Active, not recruiting	Hepatocellular carcinoma
	Quinone	Apaziquone	3	Terminated	Bladder cancer
	Nitro	TH302	2	Terminated	Metastatic neuroendocrine pancreatic cancer
	Nitro	PR104	2	Completed	Advanced hepatocellular cancer
	Aliphatic N-oxide	AQ4N	2	Unknown	Glioblastoma multiforme
	Nitro	Caricotamide and tretazicar	2	Terminated	Advanced hepatocellular cancer
HIF-1α expression	Synthetic RNA oligonucleotide	EZN-2968	1	Completes	Liver metastases
	Topoisomerase inhibitors	Topotecan (along with melphalan)	3	Ongoing	Retinoblastoma
	HSP90 inhibitor- benzoquinone ansamycin antibiotics	Geldanamycin, 17-AAG (tanespimycin)	1	Completed	Unspecified adult solid tumor, protocol specific
HIF-1 transcription	Dithiodiketopiperazines	Chetomin	–	–	–
	DNA intercalators	Echinomycin	–	–	–
HIF-1 target gene products	GLUT1	Phloretin	–	–	–
		Fasentin	–	–	–
	MCT1	α-Cyano-4-hydroxycinnamate	–	–	–
	CA-9, CA-12	Aryl sulfonamides	–	–	–
Tyrosine kinase receptor	Monoclonal antibody- anti-VEGF	Bevacizumab	1/2/3	Ongoing	Several different malignancies
RAS-MAPK	BRAF- ATP kinase competitive inhibitor	Sorafenib	2	Ongoing	Pancreatic cancer
mTOR		Rapamycin, everolimus	1/2/3	Ongoing	Several different malignancies

(continued)

Table 2.4 (continued)

Drug details			Clinical trial (latest)		
Strategy/target	Class	Name	Phase	Status	Type of carcinoma under study
	mTORC1 allosteric binders of rapamycin-binding domain				
UPR	Proteasome inhibitor	Bortezomib	1/2/3	Ongoing	Several different malignancies
	HSP90 inhibitor- benzoquinone ansamycin antibiotics	Geldanamycin, 17-AAG (tanespimycin)	1	Completed	Unspecified adult solid tumor, protocol specific
	IRE1	Salicylaledehydes	–	–	–
	SERCA	Celecoxib	–	–	–

2.9 HIF-1α Expression

HIF-1α expression can be inhibited by using the anti-sense mRNA oligonucleotide developed against the mRNA coding sequence for HIF-1α (drug EZN-2968) that prevents its translation into proteins in a dose-dependent fashion (Jing et al. 2019). Drugs like topotecan and irinotecan, which are topoisomerase inhibitors, inhibit HIF1α translation and reduce the expression of the same (Bertozzi et al. 2014).

Heat shock proteins (HSPs) act as chaperones in the cell, for instance, by guarding against the transport of proteins to proteasomes. Inhibitors to these proteins like geldanamycin promote proteasomal degradation of HIF1α under hypoxic conditions. GA analogs like 17-AAG (tanespimycin) and 17-DMAG (alvespimycin) and EC154 are under evaluation in clinical trials. Ubiquitin acts as an important tagging protein that in turn acts as a marker for protein degradation. Alteration in this function is implicated in several cancers. Deubiquitinase (DUBs) can be used to combat ubiquitylation. DUBs undergo reciprocal regulation by hypoxia, and, thus, this can act as a good target for treatment modalities (Mennerich et al. 2019).

2.10 HIF-1 Transcription

The HIF transcription is dependent on several co-activators like p300/CBP. Using these as targets for inhibiting transcription of HIF can thus aid in enhancing the efficacy of other chemotherapeutics. Dithiodiketopiperazines like chetomin, a metabolite derived from fungi, inhibit the binding of HIF with its co-activator (p300) and show antitumor effects. Other drugs like DNA intercalators also inhibit the unwinding of DNA strands and thus prevent transcription of HIF-1.

2.11 HIF-1 Target Gene Products

HIF1-regulated facultative glucose transporter is a potential target for chemotherapeutic drugs. Elevated GLUT1 levels have been outlined in a variety of tumor types and have been demonstrated to be a negative prognostic indicator. Many experimental GLUT1 inhibitors, such as phloretin, act indirectly, but fasentin targets GLUT1 directly.

The lactate transporter monocarboxylate transporter 1 (MCT1) has been viewed as a potential target for eliminating hypoxic cells by glucose starvation. The metabolic derangement observed is the Warburg effect – the shift to glycolysis – and it is facilitated by an increase in the generation of pyruvate (PGK2- upregulated by HIF-1) and its conversion to lactate by lactate dehydrogenase A (LDHA-upregulated by HIF-1).

Aerobic tumor cells expressing MCT1transporter can use lactate as a preferred substrate for respiration, and inhibition of MCT1 by α-cyano-4-hydroxycinnamate increases glucose consumption in vitro. The proposed model is that the stimulation

of glucose consumption in aerobic tumor cells compromises glucose penetration into hypoxic regions, leading to the selective death of hypoxic cells in tumors.

MCT4 is upregulated in an HIF1α-dependent manner, and there is an increase in the expression of MCT4 in tumor cells. MCT4 export of lactate and H^+ prevents intracellular acidification and assists in the remodeling of the extracellular environment, but specific inhibitors of MCT4 are yet to be reported.

Carbonic anhydrases are metalloenzymes that catalyze the reversible reaction of carbon dioxide to carbonic acid. The expression of CA9 and CA12 is controlled by HIF1, and CA9 is also regulated through the UPR. Silencing both CA9 and CA12 results in marked inhibition of the growth of LS174 human colon carcinoma cell xenograft tumors. Extensive drug development efforts have identified a range of compounds with varying selectivity for CA9 and CA12; several compounds inhibited tumor growth and metastasis selectively in CA9-positive tumor models.

2.12 Drugs Targeting Hypoxic Signaling Tyrosine Kinase Receptors, RAS-MAPK Pathway, and mTOR Pathway

Targeting the actions of HIF by either direct inhibition or by indirectly targeting the downstream pathways activated/upregulated by HIF can act as suitable therapeutic modalities. VEGF, as discussed previously, has significant effects on the neoplastic cell's physiological functions. Monoclonal antibodies developed against VEGF (bevacizumab) and VEGF-receptors have been proven to be clinically beneficial, especially in advanced cases. mTOR inhibition can decrease the levels of HIF1α and HIF2α by modulating the translation of IF mRNA, which is under the control of the PI3k/AKT/mTOR pathway. Similarly, the RAS-MAPK pathway can also be inhibited and can help downregulate the HIF-1 expression.

2.13 UPR Targets

The role of unfolded protein response (UPR) in oxygen sensing and hypoxic cell survival has provided many potential molecular targets for combating hypoxic cells. Severe hypoxia leads to an increase in the levels of unfolded proteins in the endoplasmic reticulum (ER), leading to the induction of the unfolded protein response (UPR). The UPR is mediated by three signaling pathways:

1. PERK–eukaryotic translation initiation factor-2A (eIF2A)-activating transcription factor 4 (ATF4) pathway.
2. Inositol-requiring enzyme-1 (IRE1)–X-box binding protein-1 (XBP1) pathway.
3. ATF6 pathway.

These pathways activate responses to suppress protein synthesis, stimulate protein degradation in the ER, and activate apoptosis and autophagy to resolve ER stress. Two drug strategies are being pursued to kill hypoxic cells selectively through

UPR targets. One approach seeks to inhibit the UPR by targeting PERK, ATF4, and IRE1. A second approach seeks to heighten the ER stress to overload the UPR on the assumption that the UPR has reached its maximum capacity in hypoxic cells. Evidence that the ER stressors thapsigargin and bortezomib elicit hypoxia-selective cytotoxicity in vitro supports this approach.

2.14 Conclusion and Future Aspects

Hypoxia is one of the key players in neoplasm and metastatic development. Hypoxic conditions in the tumor trigger a response from the neoplastic cells, which make it more resistant. It allows the selection of those neoplastic cells with upregulated genetic expressions that in turn allow the neoplastic cell's proliferation and sustenance. These include modified metabolic pathways, release of growth factors promoting angiogenesis, and modifying the local cellular interaction to promote metastasis. The major pathway by which hypoxia acts is by hypoxia-inducible factor (HIF), which acts at a genetic level by selectively upregulating enzymes and factors. This has major implications such as major metabolic derangements, altered tumor immune response, and neovascularization in a disorderly fashion. Metabolic derangements allow the neoplastic cells to generate energy with low oxygen availability and create metabolites that allow the hypoxic cells counter the reactive oxygen species and other harmful metabolites. Alterations in the tumor immune response result in the evasion of these proliferating cells from under the scanner of the immune cells in circulation and tissue either by deactivating/blunting their responses or by preventing their activation. The invasion–metastasis cascade is also positively upregulated under hypoxic conditions by various mechanisms, which includes activation of transcription factor repressors like TWIST and upregulation of proteins implicated in metastasis. Hypoxia has a negative impact on drugs routinely used for chemotherapy by various mechanisms, and to overcome this, development of drugs that inhibit the hypoxic response is essential. Several drugs have been used to target the pathways at different levels, and their results are promising. While some drugs are already being used, the potential use of other drugs warrants our attention as a mainstay as well as an adjuvant chemotherapeutic agent.

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Consent for Publication No.

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Role of Hypoxia and Reactive Oxygen Species in Cancer Biology

3

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Abstract

Cancer is a critical community health problem. In the future, it is foreseen that there will be more aged people with more diseases, especially diseases like cancer. The critical roles of hypoxia and reactive oxygen species (ROS) on human health have been investigated comprehensively over the years. Numerous scientific evidence from various laboratory and clinical investigations points toward the role of hypoxia in cancer. Besides, ROS also plays a significant role in cancer initiation and progression itself. In living organisms, ROS production is unavoidable because they consume oxygen for their metabolic activity, which generates ROS. Many research outcomes clearly showed that hypoxia and ROS play an essential role in cancer disease, which also is associated with oxygen. This chapter will discuss the crucial role of hypoxia and ROS in cancer initiation and progression. Besides, this chapter also will provide a comprehensive overview, focusing on the contribution of hypoxia and ROS in cancer biology. This chapter also summarizes the updated information on hypoxia and ROS.

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Keywords

Hypoxia · Reactive oxygen species · Free radical · Cancer

3.1 Introduction

Cancer is a leading community health problem worldwide, with few efficient treatment options, poor prognosis, and high death rates (Chen et al. 2016). It is foreseen that in the year 2050 there will be more than 20% of aged people with age over 60 years worldwide (Noordin et al. 2020). Meanwhile, the World Health Organization (WHO) estimated in 2019 that cancer will be the first or second important reason of mortality of people with age below 70 years in 112 of 183 countries and ranks third or fourth in the remaining 23 countries (WHO 2020). This crucial statistical data panicked us on the requirement of prompt consideration to increase people's health internationally. Hypoxia and reactive oxygen species (ROS) play an essential role in cancer biology (Tafani et al. 2016). Hypoxemia is defined as reduced oxygenation of the blood. Numerous scientific evidence from various laboratory and clinical investigations points toward the role of hypoxia in cancer. Furthermore, ROS are incredibly reactive chemicals derived from oxygen, namely superoxide, peroxides, singlet oxygen, alpha-oxygen, and hydroxyl radical. ROS is an unavoidable happening in living organisms that use oxygen for their metabolic activity, followed by ROS production. In addition, an excessive amount of ROS is an introductory course of cancer initiation. Besides, the ROS also plays numerous roles through the normal development of malignant tumors, such as the initial growth of transformed cells, which subsequently help develop the initial tumor mass with or without angiogenesis. When the tumor tissue grows bigger, it might lead to limited diffusion until the tumor tissue becomes hypoxic. These scientific pieces of literature reported the interaction of ROS and hypoxia in cancer origination and development. Although there are many reports on the crucial role of ROS and hypoxia in cancer, there is a lack of literature that combines the subjects on cancer initiation and progression itself. In this chapter, we focus on the relationship between reactive oxygen species (ROS) and hypoxia in cancer in particular on their vital role in cancer initiation and progression. Moreover, this chapter will also summarize the updated information on ROS and hypoxia.

3.2 Cancer

Cancer is an illness in which some of the normal cells grow uncontrollably and spread to other parts of the body. Cancer can be initiated in any part of the human body, which consists of trillions of cells. Usually, human cells grow and multiply in an orderly process of the progression named cell division to create new cells in the

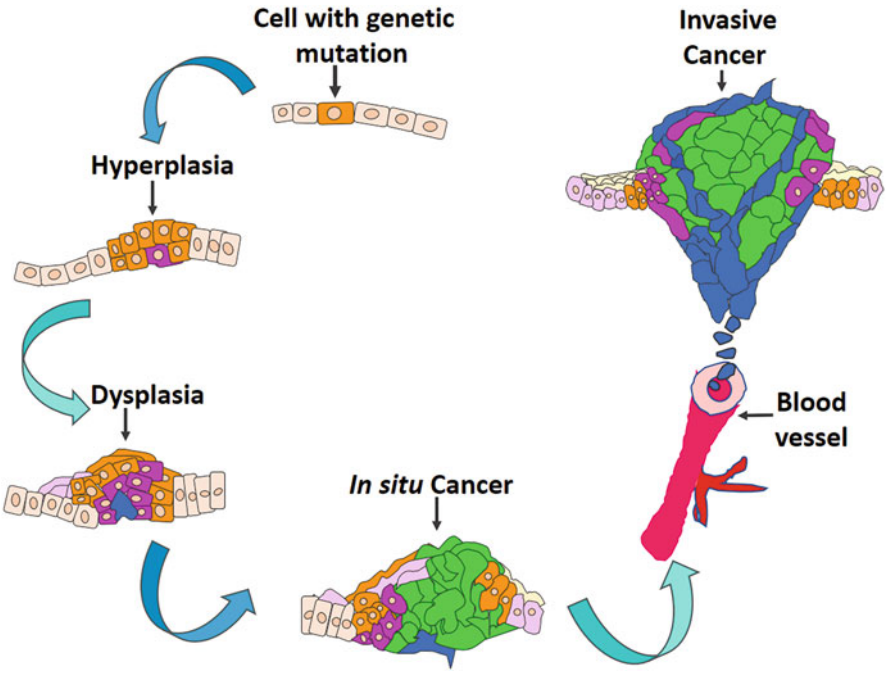

Fig. 3.1 Stages of tumor development

body. Sometimes, this orderly process will be disturbed and lead to uncontrollable cell growth and starts to proliferate to form cancerous or noncancerous (benign) tumors, which are lumps of tissue. The mass of cells or a tumor formed of these abnormal cells may stay within the tissue of their origin (in situ cancer) or it may start to invade adjacent tissues (invasive cancer). The invasive tumor is called malignant, and the cells that flow into the bloodstream or lymph from a malignant tumor have the potential to spread to other parts of the body and form new cancers (metastases) (Fig. 3.1). Mortality occurred when the growth of the tumors destroys the vital tissues and organs that are responsible for the individual's survival (Jena et al. 2012).

3.3 Hypoxia

Hypoxia is a condition where insufficient amounts of oxygen are available in the tissue to preserve satisfactory homeostasis. Hypoxia could result from inadequate oxygen distribution to the tissues either due to low blood supply or low oxygen content in the blood (hypoxemia). Many cellular responses are triggered in the chronic moderate hypoxia stage, such as ion homeostasis, hypoxia-inducible factors (HIFs), erythropoiesis, angiogenesis, cell proliferation, cell differentiation, energy metabolism, ROS generation, and cell death. Hypoxia affects significantly ionic homeostasis, which use much ATP to uphold the ionic gradient (Erecinska and

Silver 2001) During hypoxia, a reduction in the intracellular ATP/ADP ratios leads to a series of biological disorders and ROS generation (Bickler and Donohoe 2002; Corbucci et al. 2005; Seta et al. 2004). While activating ion channels at the acute response, at the chronic level, hypoxia implicates differences in the gene expression (Semenza 1999) by activating and stabilizing the hypoxia-inducible factors (HIFs) (Bracken et al. 2003). Chronic hypoxia also upregulates the genes involved in erythropoiesis, which is required for the formation of red blood cells and leads to the increment in the ability of red blood cells to transport oxygen (Farrell and Lee 2004). Furthermore, hypoxia also regulates the iron-metabolizing gene, namely transferrin, transferrin receptor, and ferroxidase, by increasing the expression of these genes to increase the iron resource to the erythroid tissues (Ke and Costa 2006).

Besides, chronic hypoxia also persuades angiogenesis, a multistep process by which new blood vessels grow from present vasculature, responsible for preserving a satisfactory blood flow to areas of inadequate oxygen supply (Liao and Johnson 2007). Chronic hypoxia also encourages the expressions of the numerous growth factors involved in cell proliferation. The promotion of cell proliferation leads typically to cell migration and regeneration after acute or chronic hypoxia injury (Harris 2002). Various scientific evidence also proposes that hypoxia encourages numerous types of cell differentiation, and a strong connection has been established between hypoxia, HIFs, and molecules that are important in cell differentiation, such as Notch, Oct-4, and MYC (Simon and Keith 2008). In normoxic situations, the energy in ATP for cell metabolism is mainly produced via the oxidative metabolism of carbohydrates, fats, and amino acids. However, under the chronic hypoxia stage, the energy production will switch from oxidative phosphorylation to anaerobic glycolysis, which will lead to a noteworthy decrease in the ATP/ADP ratios (Kristian 2004; Hochachka et al. 1996). In normoxic situations, the ROS is produced at numerous cell sites, and the electron transfer chain at the mitochondria produces a significant amount of cellular ROS (Fruehauf and Meyskens 2007). However, although it is uncertain how ROS is formed under the hypoxia stage, researchers have reported both reductions and upsurges of ROS levels (Michiels et al. 2002; Chandel and Budinger 2007; Fandrey and Genius 2000). Nevertheless, it is generally accepted that chronic moderate hypoxia cells induce a relative addition in ROS productions (Chandel et al. 1998), which is toxic to the cells. Unexpectedly, the cells' reaction to the hypoxia not only leads to the cell proliferation/survival but also the apoptotic cells' death by regulating the antiapoptotic protein such as BCL-2 proteins to promote apoptosis (Youle and Strasser 2008).

3.4 Role of Hypoxia in Cancer

Hypoxia in cancer can be mainly categorized into two stages: perfusion-restricted (acute) hypoxia and diffusion-restricted (chronic) hypoxia. Acute hypoxia refers to short-term hypoxia (between a few minutes and up to 72 h), with inadequate transport of oxygen as a result of abnormal blood vessels that close and reopen repeatedly to sluggish blood flow and lead to a fluctuation of oxygen supply

(Challapalli et al. 2017; Emami Nejad et al. 2021). It usually occurs during the vacillation in tumor perfusion accompanying defective structural and functional vascular network in tumor and is often associated with high-interstitial pressure of extracellular matrix (Challapalli et al. 2017). Chronic hypoxia arises when the diffusion of oxygen is restricted by the aberrant vascular network (Emami Nejad et al. 2021). It usually occurs when the tumor expands and exceeds a diameter of 70 μm, which result in inadequate oxygen consumption for the cells further away from the preexisting nutritive blood vessels (Emami Nejad et al. 2021). Sometimes, anemic hypoxia will occur associated with a reduction in the oxygen transport capacity by the blood. Anemic hypoxia is the consequence of the presence of the tumor or therapy-induced (Emami Nejad et al. 2021).

Folkman was the first to provide seminal evidence regarding the importance of the availability of oxygen and nutrient that can affect the growth of tumors leading to the prediction of the existence of a molecular mechanism allowing tumors to co-opt vessels (Folkman 1995, 1971). This hypothesis was subsequently proved as angiogenesis (a process of development of new blood vessels from preexisting vasculature) is an adaptive pathobiological response that is commonly found in hypoxic tumor cells to promote oxygen delivery (Schito 2019). This is because angiogenesis is required to sustain tumor cells that proliferate rapidly with an adequate supply of oxygen and metabolism. Hence, hypoxia is the key regulator of angiogenesis in cancer. Hypoxia-inducible factor (HIF), a family of transcriptional regulators, had been identified as the primary mediator in response to cellular adaptation to hypoxia (Muz et al. 2015). Intratumoral hypoxia stimulates the expression of several pro-angiogenic and angiogenic factors and their receptors such as vascular endothelial growth factor (VEGF), VEGF receptor-1,-2 (VEGFR-1,-2), platelet-derived growth factor B (PDGF), epidermal growth factors (EFG), stromal-derived growth factor-1 (SDF-1), basic fibroblast growth factor (bFGF), and insulin-like growth factor II (IGF2) in cancer cells via HIFα-dependent transcriptional activity to facilitate tumor angiogenesis (Carmeliet 2005; Emami Nejad et al. 2021). In particular, gene products, including VEGF-A and angiopoïetun-2 (ANG-2), which are mediated by HIF in the hypoxic microenvironment are able to induce the growth of the vascular network and develop new blood supplies (Brahimi-Horn et al. 2007; Seo et al. 2014). Hypoxia also activates the HIFα-independent pro-angiogenic pathways including the unfolded protein response (UPR) and mechanistic target of rapamycin (mTOR) (Schito 2019). Additionally, angiogenesis-related gene products such as endothelin, heme oxygenase 1, inducible nitric oxide synthase (iNOS), and adrenomedullin are also important in modulating local blood flow via regulation of the vascular tone (Emami Nejad et al. 2021).

In tumors, new vessels that develop under hypoxia conditions are often abnormal, immature, and leaky. They are either inadequate or excessive depending on the type of tumor (Muz et al. 2015). These newly developed vessels often displayed substantial abnormalities such as contractile wall components deficiency and physiological or pharmacological receptor deficiency (Emami Nejad et al. 2021). Besides, reported literature showed that these newly developed vessels with insufficient smooth muscle layer, lack of endothelial cell lining and basement membrane, or broken

endothelium may increase their dilation and permeability, whereas the newly developed vessels with abnormal elongated, tortuous, or disorganized shape and often containing blind ends may lead to the disruption in blood flow and intermittent stasis due to the formation of geometric resistance (Emami Nejad et al. 2021). Hence, the tumor tissue response to alleviate defective oxygen supply still fails. Consequently, hypoxia-induced tumor angiogenesis will associate with a more invasive tumor phenotype due to more access of metastatic cells to blood vessels. This further allows the cancer cells to escape from the adverse microenvironment and disseminate into secondary sites (Schito 2019). Therefore, hypoxia-induced tumor angiogenesis can also be recognized as one of the important steps in carcinogenesis as it increased tumor progression and impeded the survival rate of cancer patients.

It was demonstrated previously that tumor oxygenation is the primary factor of cancer progression. Cancer stem cells (CSCs) that possess the self-renew ability are considered one of the important agendas for tumor growth and metastasis as they can produce all the heterogeneous cells in tumors (Sun et al. 2020). In a hypoxic environment, the expression of HIF-1α and HIF-α transcriptional regulators can maintain the stemness of cancer stem cells (CSCs) by activating the Notch and Wnt signaling pathway so that the CSCs can continue to proliferate and self-renew (Sun et al. 2020). Besides, hypoxic tumor cells with overexpression of HIF-α transcriptional regulators and enhanced angiogenesis are often associated with metastasis as the newly developed heterogeneous and permeable vessels are able to facilitate the circulation, extravasation, and relocation of tumor cells (Muz et al. 2015). This may cause the tumor cells to metastasize in order to escape from the adverse hypoxic environment and move to new and unaffected tissues (Muz et al. 2015). Furthermore, hypoxic tumor cells also possess a more aggressive and invasive phenotype and have a better ability to metastasis. For instance, the expression of HIFs in hypoxic cancer cells will generate numerous signaling pathways, further resulting in the disruption of the basement membrane and reconstruction of the extracellular matrix (EMC) that causes the tumor tissue to invade and passage the lumen of lymphatic vessels or blood vessels (Schito and Semenza 2016). The expression of HIFs will also inhibit anoikis, which is a programmed cell death occurring upon the detachment of tumor cells from the ECM when transiting in blood or lymph. In addition, the HIF transition factors also induce epithelial-mesenchymal transition (EMT) whereby the tumor epithelial cells lose their polarity and convert into mesenchymal cells, which are more intrusive and invasive and regarded as a critical event in morphogenic changes during cancer metastasis (Jiang et al. 2011; Schito and Semenza 2016). This EMT process can be done by decreasing the expression of epithelial-associated genes including the β-catenin, E-cad, and increasing the expression of mesenchymal-like genes such as SMA, N-cad, CXCR4, and vimentin (Muz et al. 2015).

Moreover, hypoxia is also responsible for modulating the immunosuppressive tumor microenvironment (TME). TME, which consists of immune effector cells and stromal such as $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells, $FoxP3/CD25^+$ regulatory T cells, myeloid-derived suppressor cells (MDSC), natural killer (NK) cells, NK-like T (NKT) cells, and M1 and M2 macrophages, are sensitive to hypoxia

(Multhoff and Vaupel 2020). Several shreds of evidence suggest that a hypoxic microenvironment may inhibit antitumor immune effector cells to protect tumors from the antitumor response and facilitate immune escape (Lee et al. 2010). For example, hypoxia (1) induces cell shedding of immune recognition molecules, (2) inhibits the release of immunostimulatory cytokines, (3) induces the production of immunosuppressive cytokines, (4) induces the expression of the immune checkpoint, and (5) supports the immunosuppressive activity of M2 macrophages (Lee et al. 2010; Multhoff and Vaupel 2020). Besides, hypoxia also increases the production of immunosuppressive Treg cells flowed by decreasing the monocytes and dendritic cells (DC) motility, which further impairs the stimulation of T cells (Ohta 2016).

Otto Warburg was the first to describe the increased conversion of glucose to lactate in cancer cells, leading to the exploration of the enhanced anaerobic glycolysis effect in cancer cells (Warburg 1956). A number of studies have proven that enhanced glucose uptake is one of the hallmarks of cancer. It is also agreed that the "Warburg effect" is part of the essential metabolic changes in cancer cells in order to proliferate uncontrollably and inhibit apoptosis (Matsuura et al. 2016). The alteration of cancer metabolism closely relates to the cancer microenvironment. Under hypoxic conditions, tumor cells will switch their energy-producing metabolism from mitochondria oxidative phosphorylation to glycolysis with the help of HIF-1 transcription factors (Matsuura et al. 2016). This is because the glycolysis pathway provides the precursors for the nucleotides and phospholipids synthesis, which are critical for cells to grow rapidly (Li and Rich 2010). In hypoxic conditions, the von Hippel–Lindau (VHL) protein that is responsible for the degradation of HIF-1α is inhibited. This further results in the accumulation of HIF-1α and lead to the upregulation of HIF-1-related targets such as glucose transporter GLUT1 and GLUT3, hexokinases HK1 and HK2, to promote glycolysis (Matsuura et al. 2016). Besides, HIF-1 transcription regulators also induce the target genes, i.e., (1) lactate dehydrogenase A (LDHA) that is responsible for the conversion of pyruvate to lactate; (2) pyruvate dehydrogenase kinase 1 that is responsible for the inhibition of acetyl-CoA conversion from pyruvate as well as (3) BNIP3 and BNIP3L that is responsible for the mediation of damaged mitochondria clearance in tumor cells (Matsuura et al. 2016).

Additionally, hypoxia also plays an important role in therapy resistance and had been recognized as one of the biggest obstacles in cancer therapy. For example, oxygen deprivation may cause the cancer cells' resistance to ionizing radiation, multiple forms of chemotherapy, and photodynamic therapy (Emami Nejad et al. 2021). In this case, hypoxia confers treatment resistance to cancer cells by inhibiting apoptosis and senescence of cells, promoting cell cycle arrest, controlling autophagy, and affecting drug delivery and cellular uptake (Muz et al. 2015). Accumulating evidence shows that HIF-1 transcriptional factors play a crucial role in the apoptosis resistance of tumor cells. HIF-1, which is expressed in the hypoxic environment, is able to upregulate the antiapoptotic genes, including MCL-1, BCL-xL, BCL-2, surviving and NF-κB, to protect the cancer cells from hypoxia-induced cell death and confers tumor cells the ability to resist various cancer

treatments (Lin et al. 2014). Besides, hypoxia promotes cell cycle arrest and quiescence cellular state that make tumor cells less susceptible to external stress during chemotherapy or radiotherapy, which is commonly targeted at the bulk of rapidly proliferating tumor cells (Emami Nejad et al. 2021; Muz et al. 2015). Under a hypoxic environment, the expression of HIF-2 in tumor cells may result in the promotion of HIF-induced autophagy and inhibition of p53-mediated apoptosis, which increases the ability of cancer cells to resist chemotherapy (Emami Nejad et al. 2021). Hypoxia also leads to the formation of low pH (acidic) tumor microenvironment (TME). The changes in TME will affect the accumulation of chemotherapeutic drugs that are highly pH-dependent in terms of their cellular targets and further reduce the efficacy of drugs, thereby eventually leading to drug resistance (Jing et al. 2019).

The components in the tumor hypoxic microenvironment are associated with the poor prognosis in cancer patients as hypoxia allows the tumor to survive, disseminate, and be invasive. The HIF transcription factors play an important role in the regulation of the hypoxic tumor microenvironment. In hypoxic conditions, the activation of HIF transcription factors will result in the expression of specific target genes encoding proteins to promote angiogenesis, maintain the stemness of cancer cells, switch energy-producing metabolism from mitochondria oxidative phosphorylation to glycolysis, induce metastasis, and resist cancer therapy. Besides, tumor hypoxia may result in an immunosuppression effect in cancer cells as hypoxia promotes the production of cytokines and chemokines that are capable of recruiting pro-tumor immune cells and block tumor immune response. Furthermore, the hypoxia-induced acidic tumor microenvironment is also important for chemoresistance. Since hypoxia significantly increases tumor survival and reduces the survival rate of cancer patients, a better understanding of the hypoxic phenomenon and hypoxia signaling cascade is crucial to develop new strategies to improve treatment outcomes.

3.5 Free Radicals

Oxygen is a vital element of life that cells utilize in the process of generating energy. It is during the production of adenosine triphosphate (ATP) by mitochondria that results in the formation of free radicals. Free radicals can be defined as reactive molecules or molecular fragments containing one or more unpaired electron(s) in their external shell (Valko et al. 2007). Free radicals are said to be unstable and highly reactive because of the odd number of electron(s) in their atomic or molecular shell. The presence of the unpaired electron tends to donate or attract electrons from other compounds to attain its stability. This process results in a chain reaction cascade as the attacked molecule loses its electron and becomes a free radical itself that brings about damage in living cells (Phaniendra et al. 2015). Since these free radicals are produced by losing or accepting a single electron, therefore, they are also known as reductants and oxidants, respectively. The existence of free radicals in biology was discovered less than 50 years ago (Commoner et al. 1954; Droge 2002).

Free radicals were described as Pandora's Box of evil due to their possibility to account for cellular damage, cancer, as well as the degenerative process of biological aging. In the later years, the discovery of enzyme superoxide dismutase (SOD) brought about the evolution of free radicals in living organisms that convinced most researchers regarding its importance in biology (McCord and Fridovich 1969). Ever since, the mention of free radicals has gained an ever-increasing curiosity due to their pivotal role in various physiological conditions and a diverse range of diseases (Phaniendra et al. 2015). Free radicals typically include both reactive oxygen and nitrogen species referred to as reactive oxygen-nitrogen species (RONS). Both reactive oxygen and nitrogen species are categorized into two compound groups: radicals and nonradicals. Radicals primarily consist of those species that contain at least one unpaired electron in the external shells and are capable of independent existence (Phaniendra et al. 2015). One excellent example of a radical is the oxygen molecule. However, because of the presence of two unpaired electrons in the shell surrounding its atomic nucleus, it is referred to as biradical. Besides oxygen, superoxide, hydroxyl, alkoxyl, peroxyl radical, nitric oxide, and nitrogen dioxide are some other examples of radicals. Nonradical species, on the contrary, are not free radicals but are capable of bringing about their reactions in living organisms. Some examples of nonradical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen (O_2), nitrous acid (HNO_2), nitrosyl cation (NO+), nitroxyl anion (NO−), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), and peroxynitrite (ONOOH) (Phaniendra et al. 2015).

ROS and RONS in the human body are produced primarily by employing essential cellular metabolism and also external sources. In other words, these sources of free radicals are conveniently known as endogenous and exogenous sources. Endogenous sources simply refer to internally generated sources of free radicals like mental stress, excessive exercise, infection, inflammation, phagocytosis, reperfusion injury, mitochondria, and peroxisomes (Lobo et al. 2010). Continuous formation of free radicals takes place in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions contributing to this process include those involved in the respiratory chain, prostaglandin synthesis, phagocytosis, and cytochrome P-450 system. Nonenzymatic reactions include the reaction of oxygen with organic compounds and those initiated by ionization (Lobo et al. 2010). Exogenous free radicals, on the other hand, result from cigarette smoke, air pollutants, industrial solvents, radiation, ozone, heavy and/or transition metals such as mercury, lead, and iron, as well as some drugs like cyclosporine and gentamycin. Upon the absorption/penetration of these external compounds into the body, they undergo decomposition or metabolization, which brings about the formation of free radicals. There are various physiological and pathological conditions in which free radicals play their fundamental part. By targeting and attacking essential macromolecules, reactive oxygen-nitrogen species (RONS) leads to cell damage and disruption of homeostasis (Lobo et al. 2010). Major essential molecules targeted in the body by RONS include nucleic acids (RNA and DNA), lipids, and proteins. Severe damage to these macromolecules occurs abundantly with the

accumulation of free radicals as a consequence of antioxidants and oxidants imbalance. This leads to tissue damage in various disease conditions such as diabetes mellitus, neurodegenerative diseases, cancer, cardiovascular diseases, rheumatoid, arthritis, and asthma, therefore speeding the progression and growth of the disease (Phaniendra et al. 2015). Although both reactive oxygen and nitrogen species are frequently studied for their ability to cause extensive damage to some major molecules in the body, these free radicals also are undoubtedly recognized for their dual role as both deleterious and beneficial species (Valko et al. 2007). The harmonious balance between both antagonistic effects plays a significant role in the body. Reactive species, at low or moderate levels, portray beneficial effects in cellular signaling systems and immune function (Pham-Huy et al. 2008). On the contrary, its overproduction leads to the exertion of undesirable effects by inducing oxidative stress that damages the cell.

3.6 Oxidative Stress

The term "stress," initially used in the literature, was described as the hyperactivity of the hormone system particularly concerning corticosteroids (Breitenbach and Eckl 2015). After about two decades, the understanding of "stress" was brought to considerable attention once again regarding its importance in the study of diseases as well as general physiology. In other words, "stress" was primarily seen as a disease-causing factor. With the evolution of theories over the years, the term "oxidative stress" was proposed based on the profound knowledge of its mechanism and involvement in disease studies. Oxidative stress is the detrimental effect of free radicals that brings about possible biological damage. Oxidative stress occurs because of an imbalance between reactive oxygen species (ROS) formation and cell capacity to eliminate them (Liguori et al. 2018). This arises when the production of ROS overpowers the content level of intrinsic antioxidants, rendering the antioxidant defense mechanism unfavorable. The generation and elimination of ROS are usually very well balanced by extensive regulatory systems to maintain an equilibrium state of ROS level (Lushchak 2014). However, this critical balance can be interrupted by several sources/factors. The factors include depletion of low molecular mass antioxidant reserves, inactivation, and/or decreased production of antioxidant enzymes, as well as the combinations of the stated factors (Lushchak 2014). The degree of outcomes due to the imbalance depends on the location of ROS production, the efficiency of antioxidant defense systems, and the cellular targets with which the free radicals interact (Lushchak 2014).

As mentioned, oxidative stress is a destructive process that negatively affects cellular components like membranes, lipids, proteins, and nucleic acids. This process leads to the oxidation of the cellular components, resulting in their structural and functional changes (Pizzino et al. 2017). Thus, the critical balance between beneficial and deleterious effects of free radicals is vital and is achieved by a mechanism known as redox regulation (Droge 2002). Maintenance and regulation of ROS homeostasis play a crucial role in cellular growth, survival, and metabolism. The

presence of ROS at a low level is important in maintaining cellular functions such as viability and apoptosis while the extreme level, on the other hand, causes biological systems to incompletely detoxify the reactive intermediates and prevent the normal functions of biomolecules. Both reactive oxygen species and oxidative stress have been proposed to play significant roles in various illnesses and health conditions. They contribute mainly to the process of aging and diseases such as cancer, inflammatory disorders (arthritis, vasculitis, and systemic lupus erythematosus), ischemic disorders (heart diseases, stroke), acquired immunodeficiency syndrome, hypertension, neurological conditions (Alzheimer's, Parkinson's disease, muscular dystrophy), and many more (Bajpai et al. 2014). The metabolism of molecular oxygen by cells brings about the formation of reactive oxygen species that interact with vital macromolecules. This interaction becomes the basis of most diseases and conditions stated. Currently, it is a challenge to mention any illness for which the roles of oxidative stress and ROS have not been postulated (Ghezzi et al. 2017). Many researchers, with the help of strong evidence, suggest that oxidative stress can be associated with numerous diseases.

With virtually three decades passed since the primary definition of oxidative stress was introduced, there still has been no accepted categorization to date. Consequently, in an effort to understand the possible degrees of stress and effects, a basic classification based on intensity was presented. This intensity-based categorization includes basal oxidative stress (BOS), low-intensity oxidative stress (LOS), intermediate intensity oxidative stress (IOS), as well as high-intensity oxidative stress (HOS) (Lushchak 2014). In addition, another classification of potential stress degree too was proposed consisting of three simple terms, namely mild oxidative stress (MOS), temperate oxidative stress (TOS), and severe (strong) oxidative stress (SOS) (Lushchak 2014). Normally, the level of ROS fluctuates to an extent outlined by their generation and elimination. However, because of various endogenous and exogenous factors, its usual level increases beyond the normal range. In circumstances in which the antioxidant defense systems are capable dealing with increased free radical amounts, they are known as "acute oxidative stress" (Lushchak 2012). On the contrary, "chronic oxidative stress" takes place when cells are unable to counteract the enhanced ROS content. At such a state, even an improved expression of antioxidant and related enzymes would be unable to return ROS to its initial range. Stabilization at the new increased ROS level (quasi-stationary level) occurs and modification of cellular components is enhanced, significantly interrupting the redox homeostasis (Lushchak 2012).

3.7 Role of Reactive Oxygen Species in Cancer

Reactive oxygen species (ROS) are highly reactive oxygen species containing a single unpaired electron in their outermost shell of electrons. Among the various types of endogenous and exogenously generated ROS, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) are the most well-studied ROS in cancer (Liou and Storz 2010). Today, it is well-known that increased amounts of

ROS culminate in oxidative stress, a state of a cell defined by the oxidation of critical biomolecules, that alters cell function, homeostasis, and cell structure. This condition potentially leads to the development of numerous pathologies, including inflammatory, cardiovascular and neurodegenerative disorders, as well as age-related diseases and deadly cancer disease (Snezhkina et al. 2019; Zhang et al. 2016). Though the present antioxidant system, along with the enzymatic and nonenzymatic systems, protects the cells from the deleterious effects of ROS when they are in a state of the homeostatic microenvironment, during an overproduction of ROS, an imbalance between the ROS and antioxidant factors occurs (De Sá Junior et al. 2017). This interruption to the redox balance imbalance is related to the progression of many diseases, especially cancers.

In particular, excessive generation of ROS in cancer cells, both in the various cellular compartments and in the cancer cell microenvironment, are able to disrupt the genetic stability of cells and a variety of other cellular processes. ROS and oxidative stress are important factors in the development of carcinogenesis and have an impact on all the cancer hallmarks (Aggarwal et al. 2019). Indeed, when compared to nearby normal tissues, tumor tissues generation of H_2O_2 is elevated. Furthermore, in the blood and urine of cancer patients, it is common to detect higher levels of 4-hydroxynonenal (4-HNE) (lipid peroxidation) and 8-oxoguanine (8-oxoG) (oxidative DNA damage) and are linked to a poor prognosis (Assi 2017). Cancer cells have high steady-state ROS levels as a result of both external and internal processes. High metabolic activity, cellular signaling, peroxisomal activity, mitochondrial dysfunction, oncogene activation, and increased enzymatic activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylases have all been linked to elevated ROS levels in cancer cells (Kumari et al. 2018). The final impact on cancer biology is dependent on the overall balance of ROS and the combined positive and detrimental effects of ROS. Thus, a better understanding of the intricate roles of ROS in cancer biology could provide insights into the underlying molecular mechanisms and assist in the development of more effective anticancer strategies.

In the course of cancer, the role of ROS is considered a double-edged sword due to their characteristic features in which many studies have defined that the intensity of ROS levels could either play a role of tumor-promoting (carcinogenic role) or tumor-suppressing (antitumorigenic role) depending on several factors (Fig. 3.1). In fact, the intricate roles of ROS in cancer growth are complicated. For normal cell survival, a physiological concentration of ROS must be maintained in balance. Generally, in almost all malignancies, the reprogramming of redox metabolism causes an ectopic ROS accumulation level, therefore functioning as signaling molecules favoring various aspects of tumor growth and progression. Nevertheless, the role of ROS in cancer is not one-sided. ROS functions as anti-tumorigenic at excessive ROS levels triggering oxidative stress-induced cancer cell death due to their detrimental, genotoxic, or even proapoptotic effect on cancer cells (De Sá Junior et al. 2017). The latter is attributable to an increase in antioxidant protein levels in tumor cells in response to the overproduction of ROS, implying that cancer cell function depends on a precise balance of intracellular ROS levels. Taken

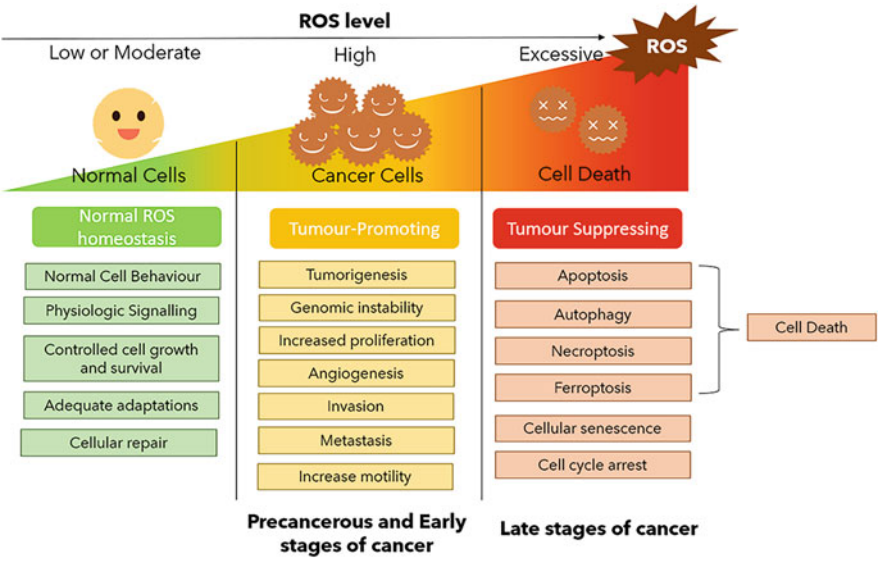

Fig. 3.2 The paradoxical role of reactive oxygen species (ROS) in cancer

together, the multifaceted roles of ROS in tumor growth, metastasis, and death are according to the ROS types, various distributions, concentrations, and lifetime in specific subcellular structures (Wang et al. 2021). Besides, recent literature has proposed categorizing the paradoxical roles of ROS in cancer cells depending on two groups: early and late stages of cancer progression. That is to say that intracellular ROS plays a variable role in cancer cell survival depending on the stage of cancer growth (Liao et al. 2019). Moderate ROS levels stimulate carcinogenesis, tumor propagation, angiogenesis, metastasis, and survival in precancerous/early stages of tumor growth. Whereas increased ROS levels beyond the hazardous threshold prompt cell death, apoptosis, and senescence with tumor progressions (Fig. 3.2) (Galadari et al. 2017).

3.7.1 ROS as Tumor-Promoting Agent (Carcinogenic Role)

Substantial research over the last two decades has clearly suggested that cancer cells have increased aerobic glycolysis (Warburg effect), which is correlated with augmenting ROS (Hart et al. 2015), and that these elevated ROS levels are thought to play oncogenic roles and act in multiple signaling cascades related to various behaviors in the cancer cell. Increased ROS is usually accompanied by proto-oncogenes activation, which is the initial step of malignant transformation and inactivation of tumor suppressor genes. ROS are thus essential and responsible for the tumorigenesis initiation, development, progression, invasion, as well as

metastasis of cancer in several ways (Wang and Yi 2008; Kirtonia et al. 2020). The increase in ROS will boost the mutation rate and promote the transformation of normal cells into tumor cells. ROS can also promote the stability of important signal molecules that drive tumorigenesis and progression (Xie et al. 2021). Overall, these tumor-promoting ROS levels can lead to cell cycle progression, increased proliferation and survival signaling, epithelial-to-mesenchymal transition (EMT), increased motility, genomic instability, and increased angiogenesis and may be negatively regulated by therapeutic antioxidants. ROS plays a vital role at every stage of cancer development in which the prooncogenic role for ROS is usually associated at precancerous and early stages of cancer. ROS has been demonstrated to activate a number of canonical pathways implicated in tumor-promoting inflammation and cell proliferation. The following sections go over some of ROS's most essential tumor-promoting actions.

3.7.2 ROS Role in Tumorigenesis

ROS can affect cellular proteins, lipids, and DNA, leading to genomic instability and activation of various tumor-related signaling pathways, depending on the concentration and duration of exposure (Galadari et al. 2017). Increased intracellular ROS is widely thought to enhance cancer initiation by triggering oxidative and base-pair substitution mutations in pro-oncogenes that target GC bases or inactivation of tumor suppressor genes, all of which ROS can promote carcinogenesis. One of the crucial phases in carcinogenic mutagenesis and tumor transformation is the accumulation of mutations, which impact genome stability and dynamics of gene expression, resulting in irreversible changes in genetic material. Examples of these mutations can be seen in active mutant Ras, Bcr-Abl, and c-Myc, which are involved in cell proliferation and tumor suppressor genes such as p53 that provokes abnormal mitosis, and promote cancer development (Assi 2017). 8-Hydroxy-2 deoxyguanosine (8-oxo-dG) is a major oxidative DNA damage product that is commonly used to test intracellular oxidative stress levels. It is highly expressed in a variety of malignant tumor tissues compared to matched normal tissues and is well known for inducing adjacent DNA base mutations (Tudek et al. 2010). ROS also functions as signaling molecules that are controlled by oncogene activation or anti-oncogene inactivation apart from the direct carcinogenic consequences of DNA damage and chromosomal instability. For instance, highly expressed proto-oncogene $p21^{RAS}$ in NIH3T3 fibroblast cells produces superoxide through RAC1 activation in NOX complexes in huge amounts, which proceeds to increase the mitogenic activity (Wang et al. 2021). Hence, ROS may contribute to cancer growth and progression by inducing signaling pathways that enhance the rate of mutations (Ramalingam and Rajaram 2021). Apart from triggering mutations, ROS can also affect the side chains of certain amino acids, altering the structure and function of proteins (Kumari et al. 2018), which is usually through oxidizing the disulfide bonds of cysteine residues. Due to the sheer presence of a thiol group, cysteine (Cys) is more susceptible to oxidation by ROS than the other amino acids.

Cys appears to be the most important player in redox signaling, working as a reversible regulatory molecular switch.

Furthermore, ROS has the ability to influence the activities of a number of proteins and signaling pathways involved in tumor cell proliferation of several malignancies, including lung, liver, and breast cancers. ROS drives proliferation by activating protein kinase D (PKD), mitogen activated-protein kinase/extracellular-regulated kinase 1/2 (MAPK/ERK 1/2), and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signaling pathways (Moloney and Cotter 2018). Increased ROS levels, for example, impede MAPK by oxidizing cysteine residues in the active site, while degradation of MAPK phosphatase 3 (MPK3) significantly lowers ERK 1/2 activity (Chan et al. 2008). Besides, the constitutive activation of cell proliferation-related transcription factors such as nuclear factor-B (NF-B) and activator protein-1, which promote cancer cell proliferation during cancer initiation and development by activating numerous genes, are also activated by the elevated ROS levels (Raza et al. 2017). These findings back up the theory that ROS-induced mutagenesis is a key driver of tumor initiation and progression.

3.7.3 ROS Role in Invasion and Metastasis

Some of the critical requirement events in the metastasis and prognosis of cancer are cell migration and invasion. The crucial characteristics of tumor metastasis processes that are linked to ROS include the loss of cell-to-cell adhesion, survival after matrix dissociation, mitigating capability, and breakthrough into the cell basement membrane (Chiang and Massagué 2008). Metastasis, the primary cause of morbidity and mortality, is a complicated process in which cancer cells migrate from the primary tumor to the surrounding tissues and distant organs. This happens as a result of cancerous cells' intrinsic mutational burden and bidirectional interaction between nonmalignant and malignant cells (Brooks et al. 2010). The activation of various transcriptional factors, including NF-kB, ETS-1 (ETS proto-oncogene 1, transcription factor), Twist, Snail, AP-1, and Zeb (zinc finger E-box binding homeobox); metalloproteases, viz., MMP-9 and, MMP-2; and chemokines or cytokines like transforming growth factor-beta (TGF-β) results in metastasis (Aggarwal et al. 2019). The epithelial to mesenchymal transition (EMT), in which epithelial cells lose their polarity, cell–cell adhesion, and motility, is the most common cause and beginning of tumor metastasis. Many studies have found that ROS, which is mostly formed as by-products of mitochondrial electron transport in aerobic respiration, plays a crucial role in the migration and invasion of malignant cells, particularly as the primary cause of EMT. ROS activate the Distal-less homeobox-2 (Dlx-2)/Snail axis, triggering the EMT, the glycolytic switch, and mitochondrial suppression, all of which are important in metastasis (Lee et al. 2019). Besides, ROS enhances tumor migration via activating hypoxia-mediated MMPs and cathepsin production, according to another study (Kamiya et al. 2016; Shin et al. 2015). One of the most important players in the EMT induction is TGF-β1, which employs the ROS-dependent pathways to regulate uPA (urokinase type plasminogen activator)

and MMP-9 to enhance cell migration and invasion (Tobar et al. 2010). As more information became available, it became clear that ROS plays a diverse role in EMT. The fact that ROS is involved in multiple pathways that are directly linked to many important EMT-inducing pathways emphasizes its significance and key function in EMT.

3.7.4 ROS Role in Angiogenesis

There will be more developments of fledgling blood vessels as highly proliferative tumors outgrow, assisting oxygen and nutrition delivery to the center of the tumor. Angiogenesis is the development of new blood vessels from preexisting capillaries during the early stages of carcinogenesis, which helps tumor proliferation, survival, and metastasis (Liou and Storz 2010). ROS appears to have a role in enhancing angiogenesis for tumor survival and maintenance by modulating important events in tumor angiogenesis such as endothelial cell (EC) proliferation, migration, and tube formation, according to numerous lines of evidence (Potente et al. 2011). Datla et al. (2007) observed that NOX4 mediates EC proliferation by generating H_2O_2, whereas NOX2 inhibits apoptosis and encourages EC survival. The angiogenesis process is mediated by an angiogenic switch that may be opened and closed by adjusting the balance between angiogenic [vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGFB)] and anti-angiogenic [angiopoietin-1, leptin, endoglin, prominin-1, transforming growth factor beta (TGF-beta), integrins, and matrix metalloproteinase (MMP) enzymes factors] (Varol 2020). The direction of the angiogenic switch is predicted to be regulated in conjunction with the activation of angiogenic or anti-angiogenic factors within cells and microenvironments where ROS and oxidative stress are present through the regulation of transcriptional factors, the release of some growth factors, and the alteration of cellular signaling cascades. ROS, for example, has been linked to numerous pathways that stimulate VEGF-mediated phosphorylation of the cadherin/catenin cell–cell adhesion complex. These ROS-mediated phosphorylation of cadherin/catenin causes EC disassembly, which promotes tumor vascularization and rapid expansion of the tumors (Monaghan-Benson and Burridge 2009). In addition, Redox factor-1 (Ref-1), NF-B, p54, matrix metalloproteinases (MMPs), and cyclooxygenase-2 (COX-2) are all transcription factors and genes involved in angiogenesis that are regulated by ROS (Galadari et al. 2017). The tumor-induced angiogenesis and the re-oxygenation of tumor cells through the development of new capillaries, however, is contradictory to the assumptions as this only exacerbates the problem of cancer cells rather than resolving it (Varol 2017).

3.7.5 ROS as Tumor-Suppressing Agent (Cytotoxic Role)

With the growing plethora of evidence pointing to ROS as a tumor-promoting agent, there is also mounting research demonstrating that in conjunction with the enhanced tumorigenesis, ROS is responsible for tumor degradation by inducing cell death and reversing chemoresistance in tumors (Yang et al. 2018). This apparent contradiction between the positive and negative roles of ROS in tumors stems from the fact that antioxidant therapy, which supposedly eliminates cancer-boosting ROS, bizarrely corresponds with lower survival in clinical trials (Goodman et al. 2011). Although it is rational to treat ROS-triggering tumors with antioxidants, the mechanism underlying the effects of many chemotherapeutic agents and ionizing radiation on tumor cell death is not linked to an upsurge in antioxidants, instead of with irreversible oxidative stress due to an increase in ROS (Wang and Yi 2008). Indeed, numerous therapeutic techniques have been shown to not only rely on ROS but also boost cellular ROS levels could in fact efficiently kill more cancer cells (Ozben 2007). Another explanation for the antioxidant therapy results could be that antioxidants reduce ROS to a level that promotes tumor propagation and migration while diminishing some of ROS's negative effects in cancer cells (Wang et al. 2016). These events of tumor degeneration occur when ROS levels rise above the tipping threshold, increasing cellular oxidative stress. The increased levels of ROS come as a result of chemotherapy, which affects ROS formation in cells while also inhibiting cellular antioxidant detoxification (Yang et al. 2018). This results in a shift in the role of ROS in cancer from carcinogenic to antitumorigenic by increasing regulated cell death (RCD) programs such as apoptosis, necroptosis, and ferroptosis, as well as triggering cell cycle arrest and senescence, all of which can impede tumor growth (Liao et al. 2019). The development of ROS' antitumorigenic role is attributed to the phases of cancer, in which ROS suppresses tumor during the late stages of cancer or as cancer develops. This is because the accumulation of excess intracellular ROS might trigger apoptosis as cancer advances (Assi 2017). Apoptosis, however, can be avoided by preserving the delicate balance in ROS level in tumor cells by creating high concentrations of intracellular antioxidants that allows them to proliferate and survive. Meanwhile, metastatic tumors acquire mechanisms that regulate ROS as a stimulant for cancer cell dispersion by lowering the cell's antioxidant capacity in the late stages of tumor progression. This general impact suggests that therapeutic techniques that either boost ROS production or weaken antioxidant defense may drive cancer cells past their snapping point, activating various cell death pathways and therefore slowing cancer growth. Nonetheless, due to the cell death-inducing action, disproportionately enhanced ROS emerges as a significant strategy for cancer therapeutic techniques.

3.7.6 ROS Role in Cellular Apoptosis

Increases in intracellular ROS that are disproportionally high can cause cancer cell cycle arrest, senescence, and apoptosis. The most prevalent form of cell death is

apoptosis, commonly known as type I programmed cell death, and is regulated by extrinsic (death receptor-dependent) and intrinsic (mitochondrial) pathways, which are performed by caspases, specialized cysteine proteases (Hengartner 2000). The ligand–receptor interaction between death-inducing ligands like Fas ligand (FasL) and tumor necrosis factor (TNF) and their respective receptors, Fas receptor (FasR) and TNF receptor, drives the extrinsic apoptotic pathway (TNFR) (Meynier and Rieux-Laucat 2019; Minchenko et al. 2016). Following the ligand–receptor contact, the death-inducing signaling complex (DISC) is formed, which includes an adaptor protein (FADD for FasR and TRADD for TNFR), receptor-interacting protein kinase 1 (RIP1), and procaspase-8 (Wang et al. 2021). On the other hand, the intrinsic apoptotic pathway is activated in a mitochondria-dependent manner by the release of the proapoptotic factors such as cytochrome-c (Cyt-c) and apoptosis-inducing factor (AIF) from mitochondria via the mitochondrial permeability transition pore (MPTP), which then increases the mitochondrial membrane permeability (Burke 2017). Overproduction of ROS by endogenous as well as exogenous sources stimulates both extrinsic and intrinsic apoptosis pathways. ROS activates the extrinsic apoptosis route by speeding up the ubiquitination of the cellular FLICE-inhibitory protein c-FLIP, which then increases the binding between the adaptor protein and pro-caspase-8, facilitating the extrinsic apoptosis process (Wang et al. 2008). ROS, on the other hand, promotes intrinsic apoptosis by accelerating the production of the proapoptotic factor cytochrome-c (Cyt-c). Cyt-c moves from the mitochondria to the cytoplasm, where it forms an apoptosome with caspase-9 and APAF-1, activating the caspase-9 signaling cascade and triggering apoptosis (Dorstyn et al. 2018).

Since the discovery of ROS's double-edged sword nature, there has been a shift in the understanding of its involvement in cancer. This multifaceted role of ROS in cellular homeostasis and carcinogenesis has been investigated for possible therapeutic effects is critically significant. The perplexing duality of ROS that may either impair antioxidant function or upregulate the apoptosis signaling pathway could be a promising approach in the active search for cancer therapies. The fine-tuning of intracellular ROS signaling to successfully deprive cells of ROS-induced tumor-promoting events in the direction of overturning the balance to ROS-induced apoptotic signaling will be a challenge for emerging therapeutic techniques.

3.8 Conclusions

This chapter provides detailed information on the role of hypoxia and reactive oxygen species in cancer biology. By providing detailed information on the role of hypoxia and reactive oxygen species in cancer biology, we aim to support scientists and young researchers conducting cancer-related studies and serve as resources for researchers working on the development of new strategies to treat cancer by targeting hypoxia and reactive oxygen species.

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Hypoxic Tumor Microenvironment: Driver for Cancer Progression

4

Sneha Dutta and Sanjeeb Kumar Sahoo

Abstract

Hypoxia or low oxygen concentration is one of the major physiological parameters within tumor microenvironment (TME). It has a profound effect in critical steps of tumor progression ranging from angiogenesis, lymphangiogenesis to metastasis and resistance to cancer therapies. It is regulated by a family of transcription factor HIF-1α, which is a master regulator controlling a wide variety of genes involved in cancer progression. In recent years, it has proved to be a major targeted therapy owing to its poor prognosis and failure of cancer therapies such as chemotherapy, radiotherapy, and photodynamic therapy, which are mostly oxygen dependent. But several factors have proved to be obstacles in targeting hypoxic cells such as delivery of drugs due to aberrant blood vessels, poor selectivity, and severely hypoxic cells that have stopped dividing. Therefore, high selectivity, efficient delivery, and selective cytotoxicity are necessary while targeting hypoxic cells. Hence, understanding the biology of tumor hypoxia is quintessential in suppressing its effect, increasing the efficiency toward current cancer treatment. This chapter aims to describe several biological and molecular aspects of hypoxia, its clinical aspect and diagnosis, along with the recent cancer therapies specifically targeting hypoxic tumor microenvironment.

Keywords

Hypoxia · Tumor microenvironment · Metastasis · HIF-1α

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4.1 Introduction

Cancer is the second leading cause of death globally, accounting for nearly ten million deaths worldwide, i.e., nearly one in six deaths in 2020. 1 in 5 people by the age of 75 will develop cancer as reported by WHO. While lungs, prostate, colorectal, stomach, and liver cancer are the most common types found in men, breast, colorectal, lungs, cervical, and thyroid cancer are commonly found in women (WHO 2020). The term *cancer* is derived from the Greek word having two meanings: one is crab and the other is tumor, which comprises a large group of diseases involved in uncontrolled cell growth with the potential to invade or metastasize in other parts of the body. ("Cancer | Origin and meaning of cancer by Online Etymology Dictionary". www.etymonline.com). Cancer develops from the initial lesions of the neoplastic or tumor cell that when fails to differentiate and disappear, becomes intermediate lesions, and enters into clonal transformation stage usually termed in situ malignancy having temporally unrestricted growth. This intermediate lesion with the tendency to persist and progress loosens its contact from the basement membrane zone and begins to interact with nonbasement membrane extracellular matrixes. This continuous interaction between extracellular matrix then selects only a subset of cells having heritable genetic and epigenetic characteristics that ultimately acquires metastatic competence to dominate the primary cancer cells (Clark 1991). As the tumor progresses, several factors contribute toward its heterogeneity, such as genetic and epigenetic changes, cancer stem cells, and non-neoplastic cells within tumor microenvironment all of which forms intra- and intertumoral heterogeneity having a profound impact cancer therapies (De Sousa et al. 2013). Because of such vast heterogeneity, there are almost more than 100 different type of cancers and within each organ subtypes of tumor can also be found. These cancer cells have deregulated circuits different from the ones found in normal cells involved in the proliferation and homeostasis. Together all such alterations within cancer cells collectively dictate the cancer growth in six major ways: self-sufficiency in growth signals, evading apoptosis, insensitivity to anti-growth signals, uncontrolled replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These six adaptations acquired by cancer cells are shared and common in all types of human tumors (Hanahan and Weinberg 2000). The physiological state regulated by various components of TME has a profound effect in cancer development. Despite the vast heterogeneity found in tumors from same or different organ locations, there are some common features found in TME regulated by genetic and epigenetic factors, leading to metabolic shift toward anaerobic glycolysis, uncontrolled growth, resistance to apoptosis, etc. This results in remodeling of TME creating events such as hypoxia, oxidative stress, acidosis, tumor fibrosis, etc., which in turn induced tumor migration, invasion, and angiogenesis, ultimately leading to metastasis. Recent studies have shifted their focus from targeting cancer cells to specialized niche of TME such as metabolism microenvironment, acidic niche, and innervated and mechanical niche. Hypoxia is one of the best characterized and most persuasive specialized microenvironments within TME that results due to rapid and uncontrolled proliferation of tumors limiting the oxygen

availability and blood supply. It gives plasticity and heterogeneity to tumors owing to its intratumoral oxygen gradients, thereby promoting more aggressive and metastatic phenotype. It is regulated by hypoxic-inducible factors (HIFs) such as HIF-1α, HIF-1β, HIF-2α, etc., which gets stabilized under low oxygen tension. It is an independent prognostic factor and found to be overexpressed in most solid tumors, especially head and neck cancer patients. Hence it can be used as a potent targeted therapy owing to its contribution in creating drug resistance, chemo- and radioresistance, etc. Considerable preclinical and clinical efforts have been focused on alleviating hypoxia by assessing hypoxia levels in tumors through various direct and indirect methods as explained later in the chapter.

4.2 Tumor Microenvironment

In 1889, Stephen Paget first proposed the "seed and soil" theory to explain the concept of tumor microenvironment, stating that cancer cells disseminate from their origin site (the seed) and will metastasize to the distant organs where the environment will be favorable (soil) (Ribatti et al. 2006). Tumor microenvironment is a heterogeneous and dynamic entity having complex interaction of tumor cells with their adjacent microenvironment that comprises various acellular components of extracellular matrix (ECM) altering their physical and chemical properties as well as communication with nearby cellular components such as endothelial cells, cancer-associated fibroblasts (CAFs), mesenchymal stem cell, and also a variety of different immune cells such as lymphocytes, tumor-associated macrophages (TAMs), etc. (Brábek et al. 2010). These contribute to tumor cell detachment, migration, invasion, adaptation and reattachment leading to cell adhesion, change of cell fate, cell movements, and mobility (Calorini and Bianchini 2010). A brief overview of the various cellular and acellular components has been described later.

4.2.1 Cellular Components

Tumor cells not only contain cancer cells but also a host of noncancer cells that interact with cancer cells acting as an active promoter during cancer progression. These majorly included immune cells and stromal cells. Tumors get infiltrated with various innate and adaptive immune cells mostly during chronic inflammation, leading to either pro- or antitumorigenic responses such as B cells, T cells, macrophages, dendritic cell, natural killer cells, neutrophils, etc. Stromal cells such as endothelial cells, cancer-associated fibroblasts (CAFs), adipocytes, and stellate cells are recruited by cancer cells from nearby endogenous tissue stroma that secretes many factors such as growth factors, cytokines, chemokines, etc., promoting critical steps in cancer progression such as angiogenesis, proliferation, invasion, and metastasis.

4.2.2 Acellular Components

Extracellular matrix (ECM) and exosomes are major acellular components of tumor microenvironment. ECM is a three-dimensional network composed of collagen, fibronectin, elastin, and laminin, which provides mechanical support as well as promotes tumor cell dissemination. It stiffens during the cancer progression due to large deposits of CAFs, growth factors, cytokines released by proteases like matrix metalloproteases (MMPs) (Anderson and Simon 2020) altering several characteristics of cancer cells such as uncontrolled proliferation, angiogenesis, hypoxia, aberrant signaling pathways, immunosuppressive TME, resistance against various cancer treatments, etc. (Huang et al. 2021).

4.2.3 Physical and Chemical Properties

Cancer development leads to profound changes physiologically at cellular and functional levels in tumor microenvironment. The functional parameters include hypoxia, extracellular pH, interstitial fluid pressure (IFP), tumor fibrosis, etc. Such dynamic changes provide sufficient growth factors and material conditions for tumor progression, treatment resistance, and immune suppression.

4.2.3.1 Acidosis or Extracellular pH

It occurs when there is lactic acid production due to increased glycolytic metabolism (known as Warburg effect) creating a pH as low as 6.5 in many solid tumors (Rohani et al. 2019). This lactic acid production promotes the synthesis of hypoxanthine and CD44, its transmembrane receptor, whose binding reduces the adhesion between tumor cells. Further, it also enhances the production of MCT-4 (monocarboxylated transporter 4) and sodium potassium transporters producing CO_2, which further adds to the acidosis process (Corbet and Feron 2017). It is reported that apart from hypoxic regions acidosis also overlaps with the tumor stromal surface (Rohani et al. 2019). Normal stromal cells absorb large amounts of lactic acid to generate pyruvate and restrict extracellular over acidification (Riemann et al. 2016). Poor blood and lymphatic vessels are mainly responsible for limiting acid metabolism contributing to tumor invasion, immune escape, metastasis formation, treatment resistance, anoikis resistance through mTor/NF-κb signaling, etc. (Ibrahim-Hashim and Estrella 2019). These signaling pathways transduce extracellular acidosis to gene reprogramming, opening the channels of calcium and H+ influx (Corbet and Feron 2017; Koukourakis et al. 2006).

4.2.3.2 Hypoxia

Hypoxia is known to be the driving force in cancer progression regulated by a family of transcription factor called hypoxia-inducible factor (HIF-1), which upon activation under low oxygen tension translocates inside nucleus and regulates a wide variety of genes and other signaling pathways involved in all steps of cancer progression. It is found in almost 60% of human tumors and sometimes even anoxia

(complete absence of oxygen) is found in tumor tissues. Hypoxia leads to imbalance in the pro- and antiangiogenic factors that disrupts the blood vessel formation due to which they are unable to restore and transport oxygen in hypoxic area, further perpetuating the hypoxia (Chan et al. 2009). Due to lack of oxygen, it leads to poor prognosis and failure of oxygen-dependent cancer therapies such as chemotherapy, radiotherapy, photothermal therapy, etc. (Muz et al. 2015).

4.2.3.3 Interstitial Fluid Pressure (IFP)

In normal tissues, the transcapillary flow of water and molecules is determined by hydrostatic and colloidal osmotic pressures together with hydraulic conductivity and plasma protein reflection coefficient that creates a slightly negative transcapillary pressure gradient in normal interstitium, but in solid tumors, both osmotic and hydrostatic interstitial fluid pressures are found to be increased (Dvorak et al. 1995). This increased or positive interstitial fluid pressure is one of the major barriers for drug delivery. Although the mechanism of this increased interstitial fluid pressure is not completely understood, but most probably it involves leaky and abnormal blood and lymphatic vessels, interstitial fibrosis, and increased contractility of fibroblast like cells in stroma (Heldin et al. 2004). The activated stroma around tumor cells that is characterized by modulated extracellular matrix, increased microvessel density, and activated fibroblasts results in the infiltration of macrophages and other immune cells secreting a wide variety of cytokines such as PDGF, TGF-β, VEGF, TNF-α, etc., which acts on several cells within tumor, thereby affecting IFP (Baluk et al. 2003).

4.2.3.4 Tumor Fibrosis

It is the excess deposition of ECM components leading to thickening and scarring of connective tissue that normally occurs in response to injury and tissue damage (Wei et al. 2020). In TME, this fibrosis leads to compression of blood vessels, diminished perfusion, and tumor oxygenation (Heldin et al. 2004). Such compressions not only hinder the transport of drugs and others but also cause mechanical activation of signaling pathways promoting survival and metastatic cascade of cancer cells (Nia et al. 2016)., (Jain 1990).

4.3 Hypoxia

Hypoxia is one of the key parameters in TME. It is a nonphysiological condition that happens due to lower level of oxygen tension compared to normal cells. In rapidly proliferating and expanding cancer cells, the decrease in oxygen and nutrient supply and increase in distance from blood vessels create hypoxic condition due to irregular blood vessel formation (Muz et al. 2015). This leads to a gradient of oxygen concentration within tumor cells. Although experimentally there is no clear distinction between acute vs. chronic hypoxia, in general, it is generally accepted that chronic hypoxia occurs when prolong hypoxic conditions create distorted blood vessels, which leads to an increase in intercapillary distance and decrease in oxygen

diffusion capacity (Kato et al. 2011). Such tumor cells are usually within 180 μm from the periphery of blood vessels eventually undergoing necrosis (Bayer and Vaupel 2012; Hsieh et al. 2010). Such long exposures lead to high frequency of DNA breaks, disruption in DNA repair system such as homologous recombination, and mismatch repair, which leads to genetic instability and mutagenesis (Luoto et al. 2013; Chan et al. 2008). On the other hand, acute hypoxia, also known as perfusion-limited or diffusion-limited hypoxia, occurs when there is temporary blockade or variable blood flow within tumor blood vessels. This causes reoxygenation injury, increase in free radicals, activation of several stress response genes, and tissue damage, making the diffusion of drugs to tumor sites even more difficult (Patel and Sant 2016). It is regulated by a family of transcription factors called hypoxic-inducible factors (HIFs), mainly HIF-1α, HIF-2α, HIF-1β, and HIF-2β. Whereas HIF-1β is constitutively expressed, HIF-1α gets stabilized under low oxygen concentration and translocates a broad array of genes involved in multiple steps of tumorigenesis such as epithelial to mesenchymal transition (EMT), invasion, migration, angiogenesis, metabolic reprogramming, metastasis, etc.

4.3.1 HIF Pathway

Cellular adaptation to hypoxia is mediated by hypoxia-inducible factor HIF-1α. The two isoforms of hypoxia-inducible factors, HIF-1α and HIF-1β, often known to be the master regulators of hypoxia, upon activation under low oxygen concentration, translocate in the nucleus. HIF-1α upon activation regulates the global network of signaling pathways regulating a wide variety of genes involved in angiogenesis, invasion, metastasis, apoptosis, and metabolism. Under normoxia, HIF-1α is short-lived, with half-life of less than 5 min, due to ubiquitin-dependent proteasomal degradation. This is mediated by three isoforms of PHDs, especially PHD2 and FIH, which are oxygen sensor enzymes. PHDs catalyze the post-translational hydroxylation of proline residues Pr-402 and Pro-564 located within the oxygen-dependent degradation domain (ODDD) of HIF-1α. Upon hydroxylation, ODDD shows a strong affinity toward von Hippel–Lindau protein (pVHL), a component of E3 ubiquitin ligase complex leading to polyubiquitination and degradation of HIF-1α by 26S proteasome. Another oxygen sensor FIH catalyzes the asparaginyl hydroxylation in the C-terminal TAD of HIF-1α, resulting in change in the local hydrophilic/hydrophobic balance of the protein and impairing the interaction between the hydroxylated C-TAD and one of the essential coactivators – p300/CREB binding protein (CBP), thereby abolishing the C-TAD activity (Fig. 4.1). Under hypoxic condition, the PHD enzymes lose their enzymatic activity and are targeted by E3 ubiquitin ligases Siah1a and Siah2, which prevents the association of HIFs and p VHL. PHDs, mainly PHD1 and PHD3, are phosphorylated by Siah2, which are downstream of p38 and Akt signaling. As a result, HIF-1α translocates in the nucleus and gets transcriptionally active. Inside the nucleus, it forms heterodimer complex with HIF-1β by binding to their promoter element HRE, which is located around the promoter region of HIF-1α-regulated gene. Although both PHD and FIH

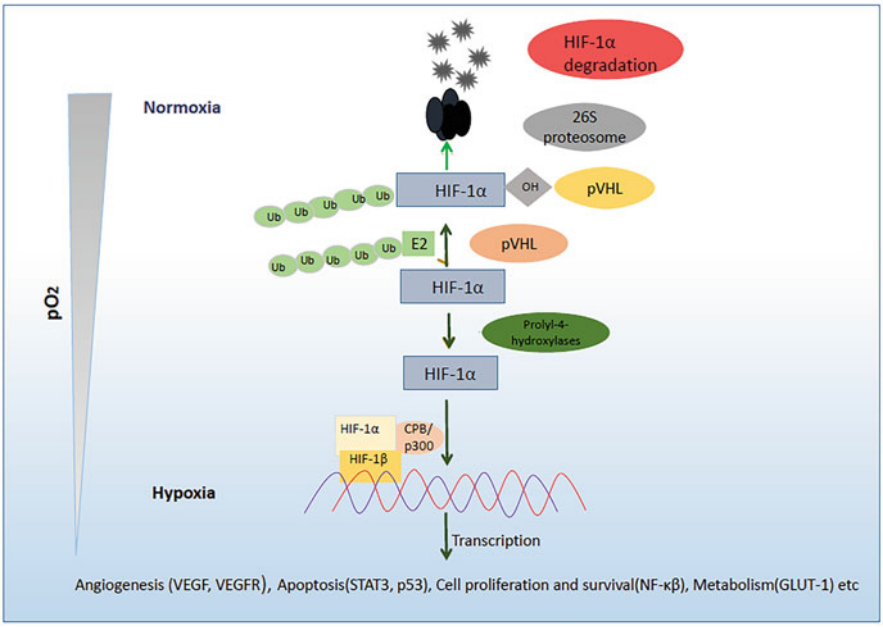

Fig. 4.1 Regulation of HIF-1 a during normoxia and hypoxia. Under normoxic conditions, HIF-1 a is recognized by the von Hippel–Lindau protein (pVHL) through binding to hydroxylated proline residues on HIF-1 a, executed by proline hydroxylases (PHDs), thereby ensuring ubiquitination and degradation of HIF-1 a by the proteasome. Another hydroxylation of HIF-1 a is performed by factor inhibiting HIF-1 (FIH-1), which abrogates the interaction between HIF-1 a and the coactivator CBP/p300. Thus, during normoxia HIF-1 a levels and transactivation activity are low, resulting in no transcription of target genes. On the other hand, during hypoxia, inactivation of oxygen-dependent PHDs and FIH-1 stabilizes HIF-1 a, which translocates to the nucleus and interacts with its partner HIF-1 b and the CBP/p300 protein athypoxia-response elements (HREs), leading to transcription of hypoxia-driven target genes. HIF-1 α, hypoxia-inducible factor-1; HIF-1b, hypoxia-inducible factor1b; p VHL, von Hippel–Lindau; Ub, ubiquitylation; PHD, prolyl hydroxylase domain; CBP/p300, CREB-binding protein/E1A binding protein p300; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; NF-Kb, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3; GLUT-1, glucose transporter 1

acts as oxygen sensors, their Km values differ markedly (Tian et al. 2011). Initially, HIF-1a accumulates under mild hypoxic conditions because of decrease in enzymatic activities of PHD, which has markedly high Km values than FIH. Under severely hypoxic conditions, it then shows its maximum transcriptional activity by interacting with p300/CBP as a result of inactivation of HIF-1α (Koivunen et al. 2004). Apart from PHDs and FIH, HIF1-α is also regulated by wide variety of other factors such as alpha-ketoglutarate, ferrous ions Fe2+ and also by genetic, mechanistic, or functional alterations in many genes, which either influence intracellular oxygen levels, alpha ketoglutarate, ferrous ions, or de novo synthesis and degradation of HIF-1α (Koyasu et al. 2018)(Table 4.1). Overexpression of HIF-1α and

Table 4.1 Positive and negative regulators of HIF-1α (partly adapted from Koyasu et al. 2017)

Environment conditions		Reference
Hypoxia	Increases stability and transcriptional activity	Lee et al. (2004)
Normoxia	Decreases stability	Metzen and Ratcliffe (2004)
High temperature	Increases stability and expression	Elming et al. (2019)
Co-factors		
2-OG, ascorbate, Fe2+	Decreases stability	Koyasu et al. (2018)
NO	Modulates expression	Malyshev et al. (1999)
NAD+	Decreases transcriptional activity	Luczak et al. 2021)
Copper	Increases expression	Feng et al. (2009)
Transcriptional initiation		
ISGF3 (STAT1/STAT2/IRF9), STAT3, NF-Ķβ	Increases stability by binding to promoter region	Gerber and Pober (2008), Dang et al. (2011), and Rius et al. (2008)
LY6E, HIF-1, PI3K/Akt/PKC/HDAC pathway, NRF-1	Decreases stability	Koslowski et al. (2011), Yeom et al. (2016), and Wang et al. (2016)
Enzymes		
VHL, PHD, ARD-1	Decreases stability	Ohh et al. (2000 and Epstein et al. (2001)
FIH-1, Sirtuin-1	Decreases transcriptional activity	Lando et al. (2002) and Lim et al. (2010)
PARP-1	Increases transactivation	Gonzalez-Flores et al. (2014)
NOS	Increases stability	Ho et al. (2012)
PHD and FIH-1 inhibition	Increases stability and enhances transcription of target genes	Hirota and Semenza (2005)
Transcription stability		
P-bodies (USP52/PAN2)	Positively regulates by interacting with the 3-UTR	Bett et al. (2013)
Translational initiation		
PI3K/Akt pathway, YB-1, ATR	Increases stability	Harada et al. (2009), Fallone et al. (2013), and El-Naggar et al. (2015)

ISGF3 interferon stimulated gene factor 3, *STAT* signal transducer and activator of transcription, *IRF9* interferon regulatory factor 9, *NF-κB* nuclear factor kappa B, *LY6E* lymphocyte antigen 6 complex locus E, *PI3K* phosphoinositide 3-kinase, *PKC* protein kinase C, *HDAC* histone deacetylase, *NRF-1* NF-E2-related factor 1, *VHL* von Hippel–Lindau, *2OG* 2-oxoglutarate, *FIH-1* factor-inhibiting HIF-1, *PHD* prolyl-4-hydroxylase, *YB-1* Y-box-binding protein 1, *ATR* ataxia telangiectasia and Rad3-related protein, *USP52* ubiquitin-specific protease 52, *HDAC* histone deacetylase, *PAN2* poly(A) nuclease 2

HIF-1β induces hypoxia, causing key modulations in the vasculature, immune system, and cell metabolism of tumor microenvironment helping cancer cells to adapt themselves by various complex mechanisms.

HIF-1α is regulated on multiple levels such as at transcriptional, translational, posttranslational modifications, protein–protein degradation, or interaction. Negative regulators of HIF-1α such as PHDs, VHL, p53, GSK3β, etc., are present both in normoxic and hypoxic conditions. However under normoxia, it is important to ensure that there is no aberrant HIF-1α angiogenesis. Similarly, positive regulators like PI3-K/AKT, mTor, p300, etc., make sure that once hypoxic stress signals have been communicated, it will turn off HIF-1α to ensure any proliferative or angiogenic signaling induced by HIF-1α under hypoxia are tightly controlled (Bárdos and Ashcroft 2005). The multifaceted aspect of HIF-1α involving signaling cascades merging growth factor and oncogenic signaling, tumor suppressor loss, post-translational modifications, subcellular localization, cell metabolism, apoptosis, and inflammation, has made it a potential therapeutic target.

4.3.2 Hypoxia and Metabolism

"Reprogramming of cancer cell metabolism," as stated by Otto Warburg in 1920s, implies that cancer cells uptake glucose even under aerobic glycolysis to produce lactate, thereby suppressing oxygen-dependent oxidative phosphorylation (OXPHOS) (Hanahan and Weinberg 2011). Although in glycolysis there is production of only 2 ATPs per glucose molecule compared to 36 ATPs in OXPHOS, but to meet the energy requirement, macromolecule biosynthesis, and redox needs, high level of glucose is required, which leads to overexpression of glucose transporters (GLUT-1) and downstream glycolysis transporters in most solid cancers (Barthel et al. 1999). It is mainly regulated by HIF-1α and c-Myc or in combination. Apart from glycolysis, there is also an alternative pathway such as pentose phosphate pathway (PPP) whose products help cells to counteract oxidative stress, facilitate DNA damage repair, and confer resistance to chemotherapy and radiation (Boros et al. 2000; Tian et al. 1999). Studies have shown that lactate is known to produce VEGF by its receptor VEGF2 by tumor and endothelial cells mostly through the activation of HIF-1a due to production of pyruvate, an indirect inhibitor of HIF-prolylhydroxylases (PHDs) (Sonveaux et al. 2012). Lactate is also involved in cancer cell motility by increasing the production of certain factors such as transforming growth factor-β2 (TGF-β2), hyaluronan, and CD44, which are involved in integrin activation, angiogenesis, stemness, ECM remodeling, etc. (Goetze et al. 2011; Baumann et al. 2009). In terms of immune modulation, lactic acid is known to strongly inhibit anticancer immune response by decreasing cytotoxic activity of human T lymphocytes and natural killer cells (Feder-Mengus et al. 2007; Husain et al. 2013). Hence, hypoxia broadly influences many metabolic pathways, especially glucose metabolism, where it inhibits glucose from entering tricarboxylic acid (TCA) cycle, a less efficient but more rapid and safer way.

4.3.3 Hypoxia and Its Role in EMT and Metastasis

Epithelial to mesenchymal transition (EMT) is proposed to be the initial step in metastatic pathway. It is a multistep process mediated by activation of a wide variety of signaling pathways like Notch, PDGF, VEGF, Snail, TGF-β, Wnt/catenin, etc., and transcription factors like TWIST, Snail, Slug, SIP1, and zeb1 (Kim et al. 2014). HIF-1a interacts with signaling pathways and transcription factors, which are known to regulate EMT (Yang et al. 2008). For example, TGF-β is known to regulate transcription of EMT-associated genes. TGF-β can also suppress both miRNA and protein expressions of PHD2 and consequently increase HIF-1a activity (Ruan et al. 2009). Under hypoxic conditions, major cell adhesion molecules like E-cadherin, tight junction proteins gets downregulated, whereas mesenchymal markers such as vimentin, fibronectin, and N-cadherin are upregulated (Lu and Kang 2010). Once the cells acquire mesenchymal characteristics, they get detached from the primary tumor, undergo local migration and invasion of stromal tissue, intravasate and transit through blood vessels, arrest capillary bed and extravasate, undergo local crawling and invasion, attachment, formation of micro metastases, survival, and eventually proliferation reaching to the secondary organs (Ruan et al. 2009).The downregulation of epithelial markers increases the cell motility by degrading the extracellular matrix components and eventually penetrate into the blood vessels (intravasation) and becomes circulating tumor cells (CTCs). The CTCs then undergo extravasation at the secondary organs like lungs, bones, kidneys, etc., where they get adapted in the premetastatic niche (Lu and Kang 2010). Hypoxia in primary tumors regulates the premetastatic niche formation in secondary organs by hematopoietic bone marrow-derived cells under the influence of soluble factors released from primary tumors. One of such critical mobilizing factors is LOX (Fig. 4.2) (Kaplan et al. 2005; Erler et al. 2009). Angiogenesis also plays an important role as increased expression of VEGF, which increases the heterogeneous blood vessel formation and permeability that enhances the extravasation, circulation, and dissemination of tumor cells to new and unaffected tissues by escaping the aggressive hypoxic environment (Carmeliet and Jain 2011).

4.3.4 Hypoxia and Angiogenesis

Angiogenesis is the process of formation of new blood vessels from preexisting blood vessels. Rapid proliferation of tumor cells rapidly consumes large amount of nutrients, leading to rapid oxygen consumption, lack of nutrients, and accumulation of metabolic wastes (Zhao et al. 2015). This leads to the formation of hypoxic condition, which induces an imbalance between pro- and antiangiogenic factors production, leading to abnormal and disordered formation of blood vessels.

VEGF is one of the most important proangiogenic factor, which is transcriptionally activated by HIFs. Some other HIF-regulated angiogenic targets include angiopoetin-2, adrenomedullin, cyclin G-2, endothelin-1, platelet-derived growth factor-β, etc. (Hickey and Simon 2006). HIF plays a significant role in all steps of

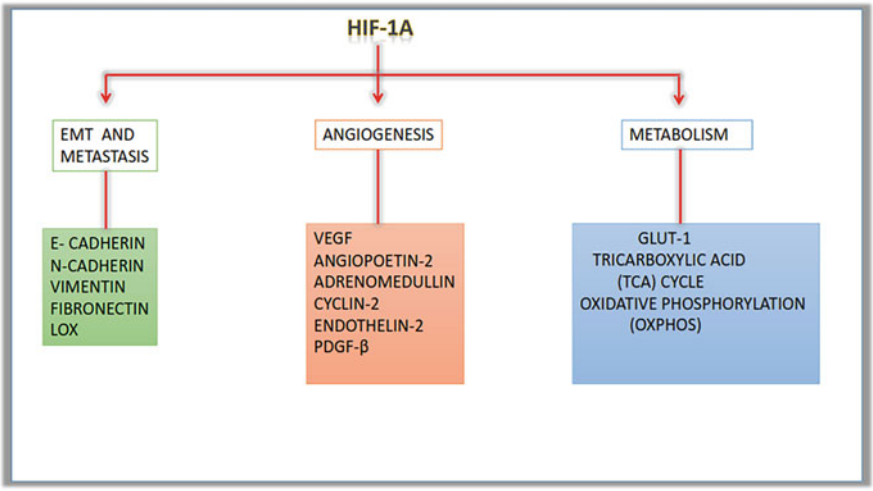

Fig. 4.2 HIF-1a is the master regulator in driving cancer progression. Once it gets stabilized and translocates inside nucleus, it regulates a wide array of genes that are involved in the critical steps of tumorigenesis such as epithelial to mesenchymal transition (EMT) and metastasis, angiogenesis, metabolism, etc. LOX, lysyl oxidase; VEGF, vascular endothelial growth factor; PDGF-β, platelet-derived growth factor β

blood vessel formation: (i) hypoxia and HIF-1α mediate the stimulation of pro-angiogenic molecule production such as VEGF-R2 (Flk-1), members of the FGF family and PDGF during the formation of primitive vascular network and in the recruitment of endothelial progenitor cells (EPC) from bone marrow and further inducing its differentiation to endothelial cells (EC) that is regulated by VEGF. (ii) It also induces the enzymatic expression of metalloproteases (MMPs) involved in sprouting and splitting of preexisting vessel. The neovessels form as a result, helps in the migration of ECs through chemoattractants across ECM (Conway et al. 2001). Hypoxia also induces the proliferation of ECs by regulating VEGF-R1 (Flt-1), Ang-1, and Ang-2 expression. (iii) Finally, hypoxia induces the expression of Ang-1, PDGF, and TGF-β to support blood vessel formation for recruiting of supporting cells such as smooth muscle cells and pericytes for creating mature and stable blood vessels (Carmeliet 2005). As more tumor cells mean more demand, hence hypoxia further stimulates angiogenesis to enhance the hypoxic conditions and the vicious circle continues.

4.4 Clinical Impact of Hypoxia in Cancer Progression

Hypoxia is one of the major pathophysiological factors in most locally advanced solid tumors. It results in radio−/chemoresistance and metastasis, which eventually leads to tumorigenesis, contributing to poor prognosis in cancer patients. The heterogeneity in tumor oxygenation status is mainly due to severe structural and

functional abnormalities of tumor microvessels, diffusion-limited oxygen delivery, and tumor-associated or therapy-induced anemia, which leads to reduced oxygen transport capacity of the blood (Vaupel and Mayer 2007). Tumor hypoxia represents two faces of the same coin. On the one hand, in its initial stages, it reduces its overall protein synthesis, which inhibits cell proliferation and eventually cell death by inducing apoptosis or necrosis. But as the tumor aggravates and reaches the advanced stages, it is known to promote aggressiveness, invasiveness, and metastasis by enabling cell to overcome nutrient and oxygen deprivation, to escape from the hostile microenvironment and favoring unrestricted growth. So, depending on the level and duration of oxygen level in cells, under acute hypoxia, cells get adapted to the environmental stress and survive, leading to cancer progression; on the other hand, under chronic or severe hypoxia, cells undergo apoptosis or immediate arrest in cell cycle. Cancer metastasis is induced by epithelial to mesenchymal transition (EMT) process where cells lose their epithelial properties by the downregulation of epithelial marker such as E- cadherin and upregulation of mesenchymal markers such as fibronectin and vimentin. Hypoxia-induced EMT has been reported to be an important factor in many such cases. Hypoxia also leads to therapeutic resistance through increased ROS and DNA damage, making many drugs and radiation ineffective in giving maximum cytotoxicity due to lack of oxygen, altered cellular metabolism, and enhanced genetic instability.

Hypoxia is also known to activate specific transcription factor such as Oct-4, Nanog, etc., and signaling pathways such as Notch that control stem cell renewal and pluripotency by blocking spontaneous cell differentiation and regulating the proliferation and survival of cancer stem cells. Hence, a wide range of etiological, genomic, and other host tumors factors interact with hypoxia to influence its clinical outcome.

4.5 Diagnosis of Tumor Hypoxia

Hypoxia is the driving force in regulating the various mechanisms and pathways involved in modulating the TME. The heterogeneity in oxygen tension within tumors makes the cancer treatments complicated by creating resistance and multiple side effects. Researchers have explored various ways to assess and diagnose hypoxia within tumors. Since the condition differs from person to person, it is necessary to classify patients based on different stages of hypoxia. Various assays have been developed, both invasive and noninvasive, that will enable both proper patient selection and therapy management.

4.5.1 Invasive Direct Methods

Invasive direct method involves insertion of an electrode or needle directly into tumor or metastatic lymph node to measure oxygen from several points using a selective oxygen sensor that will explicitly interact with oxygen within the vicinity

of the probe (Ljungkvist et al. 2007). *Oxygen electrodes* are considered to be the gold standard for measuring hypoxia in tumors, Eppendorf polarographic needle electrode is one of the first of its kind to be used at clinical level that allows the direct real-time measurements of oxygen tension in tissues (Ljungkvist et al. 2007). It consumes a small amount of oxygen and is moved throughout the tissue by giving multiple samplings. Oxylite probe, on the other hand, does not consume oxygen during measurement and can be kept at one place for a longer time. It can detect temporal fluctuations of oxygen tension within tumors (Hompland et al. 2021) (Milosevic et al. 2004). Although they give direct and rapid real-time measurements of absolute pO2 levels, but due to their invasive nature along with low spatial resolution they are only limited to easily accessible solid tumors. Hence, these are not feasible for routine analysis and are not commonly applied today (Seddon et al. 2001).

4.5.2 Noninvasive Direct Measurements

Noninvasive methods do not involve disruption of tumor lesions or metastatic lymph nodes and can detect oxygen directly within tissue or indirectly by administrating tracers that can activate or accumulate within hypoxic cells or by using endogenous or exogenous oxygen markers.

4.5.2.1 Phosphorescence Quenching

It involves the interaction between oxygen molecules with phosphorescent dyes. Upon illumination, the dyes emit their own light and the intensity of its emission depends upon the local oxygen concentration. The oxygen feedback here is independent of tracer concentration and its temporal resolution is also high, providing real-time tissue oxygenation profile. Phosphorescence quenching are molecular reporters with various kinds of palladium-containing porphyrins or physical needle probes.

4.5.2.2 Electron Paramagnetic Resonance (EPR)

In EPR, an exogenous probe with an unpaired electron like India ink or charcoal is injected, which is selective in its interaction with oxygen. It can be used for 3D imaging of pO_2 in tissue depths of up to 5–10 mm. Only one injection is sufficient for repeated measurements over a long period of time. Some recent EPR probes used to measure tumor hypoxia are implantable and are metabolically inert like paramagnetic lithium phthalocyanine crystals. These are used clinically, but their use is limited.

4.5.2.3 Overhauser-Enhanced MRI (OMRI)

It is an imaging technique that measures both tumor oxygenation and microvascular permeability at the same time using a paramagnetic oxygen-sensitive contrast agent that works by polarizing the water protons. It is a highly sensitive technique but has low spatial resolution (Daimiel 2019; Walsh et al. 2014).

4.5.2.4 Magnetic Resonance Imaging (MRI)

It uses a large external magnetic field and tuned coils for detecting the relaxation of atoms with magnetic moments after delivery of radiofrequency pulses. For detecting changes in blood oxygen saturation level, the blood oxygen level-dependent (BOLD) technique is used. It exploits the difference in the paramagnetic properties of deoxygenated (deoxy Hb) and oxygenated hemoglobin to generate a signal. The presence of deoxy Hb in blood vessels causes darkening in tissues due to decrease in T2 (changes in longitudinal relaxation time T1 and transverse relaxation T2). It has the advantage of having both spatial and temporal resolution and can detect spontaneous oxygen fluctuations (Daimiel 2019; Bonnitcha et al. 2018).

4.5.2.5 Endogenous and Exogenous Markers

Exogenous markers are basically bioreductive drugs that get bioactivated under reduced oxygen tension. These are administered systemically and are deducted in tumors using appropriate antibodies. Two major exogenous markers used extensively are pimonidazole and EF-5, which are usually combined with immunohistochemistry for clinical purposes. Endogenous markers, on the other hand, are normal cellular proteins whose expression is associated with hypoxic stress. For example, HIF-1α, carbonic anhydrase IX, etc., get activated under hypoxic stress. These can also be evaluated using immunohistochemical techniques (Williams et al. 2005).

4.5.2.5.1 Pimonidazole and Pentafluoropropyl (EF5)

These are exogenous 2-nitroimidazole-based markers that are used to assess hypoxia in tumors by undergoing chemical reduction in hypoxic cells) (Gross et al. 1995). It then irreversibly binds to the macromolecules that can be further detected by immunofluorescence or immunohistochemistry. This binding then increases exponentially with decreasing oxygen concentration about 1.3% O_2 (10 mmHg) (Hompland et al. 2021).

4.5.2.5.2 Hypoxic-Inducible Factor (HIF-1α)

It is an oxygen-dependent transcription factor that is constantly expressed under low oxygen pressure and is an important molecule that regulates a wide variety of hypoxia-targeted genes.

4.5.2.5.3 Glucose Transporter 1 (GLUT-1)

It is a membrane protein that facilitates the translocation of glucose across cell membrane. Under hypoxic condition when glycolysis increases, its expression level increases to fulfill the demand of glucose in cancer cells (Walsh et al. 2014).

4.5.2.5.4 Carbonic Anhydrase IX

It is an enzyme that regulates the cellular pH by acting as a catalyst in reversible transformation between bicarbonate anion and carbon dioxide. Under hypoxic conditions, its expression is significantly upregulated under hypoxic conditions due to tumor acidity (Betof et al. 2012; Loncaster et al. 2001).

4.5.2.5.5 Osteopontin

It is a secreted phosphorylated acidic protein that belongs to a small integrin binding ligand N-linked glycoprotein family (Chen et al. 2009). It is normally expressed in various cells such as macrophages, endothelial cells, smooth muscle cells in modulating cell adhesion, etc. Under hypoxic conditions, it gets overexpressed through activated AKT and Ras activated enhancer (RAE) in OPN promoter (Ziemer et al. 2003).

4.6 Current Cancer Therapies Targeting Hypoxic Tumor Microenvironment

Hypoxic cells are intrinsically more resistant to cancer treatments owing to lower intratumoral oxygen level, its distance from blood vessels, slow proliferation rate, and low blood flow. It is either intrinsic or acquires resistance. Both give rise to a population of tumor cells that do not respond to the radio- or chemotherapeutic treatment and continue to proliferate.

4.6.1 Enhancing Radiotherapy

Ionizing radiation requires the oxygen-derived free radicals to target cells. The oxygen responds to ionizing radiation by accepting free radicals generated due to radiotherapy. These radicals further induce DNA damage by strand breaks and thereby kill cancer cells. In hypoxic cells, due to the lower oxygen concentration, it contributes nearly three times more radio resistance compared with other factors. This mechanism of oxygen-driven increase of cellular radiosensitivity is called "oxygen fixation hypothesis," which states that the probability of permanent IR-induced DNA damage is higher in the presence than in the absence of oxygen. At present, a number of strategies have been developed that resensitizes the radioresistive hypoxic cells to radiotherapy using drug therapy such as NO donors, enzymatic inhibitions, metabolic inhibitors, HIF-1a inhibition, etc.

4.6.2 Enhancing Chemotherapy

In hypoxic cells, regions with lowest oxygen availability are found to be distant from the blood vessels; as a result, there is less diffusion of oxygen that restricts the movement of drug in the bloodstream (Roy et al. 2020). The acidic environment produced as a result of increased extracellular pH due to increase in lactate accumulation makes the drugs electrostatically charged and limits their ability to cross the hydrophobic plasma membrane. A number of other factors such as low drug bioavailability in hypoxic tumors, HIF-1α-mediated MDR-1 overexpression and decreased expression of drug targets such as topoisomerase II contributes to the hypoxic-mediated chemotherapeutic resistance in cancers. Chemotherapeutic drugs

such as doxorubicin, cisplatin, etoposide, etc. (Yeldag et al. 2018), have shown hypoxic-mediated chemoresistance in neuroblastoma. Chemoresistance is known to be induced by various mechanisms such as upregulation of P-glycoprotein expression, downregulation of topoisomerase, blockade of apoptosis, induction of autophagy, and upregulation of telomerase (Daijo et al. 2011). Along with the conventional cancer therapies, with the dawn of nanotechnology, numerous strategies have been developed and designed to combine it with various other therapies and drugs to increase their efficacy, mainly by (i) modifying tumor microenvironment by increasing oxygen concentration in tissues, (ii) nanoparticle acting as oxygen carriers, (iii) decomposition of substances to generate O2, and (iv) using hypoxia prodrugs to assist treatment.

4.6.3 Hypoxia-Targeted Therapy

4.6.3.1 Modifying Tumor Microenvironment by Increasing Oxygen Concentration in Tissues

The rapidly proliferating and expanding tumor cells surpass their oxygen demands as a result of which it creates hypoxic niche, leading to the formation of abnormal blood and lymphatic vasculature called angiogenesis. But due to their leaky and chaotic organization, they are still unable to transport sufficient blood and oxygen supply, resulting in circulating hypoxia (Jain 2013). Also, the stiffness of extracellular matrix makes it difficult to diffuse deep inside the tumor and hence increasing their distance from the blood vessels. Therefore, modifying the tumor microenvironment can be a promising strategy to improve oxygen concentration. Several factors such as temperature, ECM components, cancer-associated fibroblasts (CAFs), tumor vasculatures, TAMs, and HIF-1α can be of potential targets to modulate the tumor microenvironment to improve the tissue oxygenation (Table 4.2) (Dang et al. 2017).

4.6.3.2 Nanoparticles Acting as Oxygen Carriers

Nanoparticles acting as oxygen carriers to provide oxygen supply directly to the hypoxic site to enhance sensitization toward therapies such as photodynamic therapy (PDT) and radiation therapy (RT). Various carriers such as perfluorocarbon, RBC, Hb, etc., have proven to be a promising method to deliver oxygen directly to the hypoxic sites for PDT, RT, or chemotherapy, etc.

4.6.3.3 Decomposition of Substances to Generate Oxygen

The in situ decomposition of various endogenous substances like H_2O_2, H_2O, CuO, Au_2O_3, etc., can be carried out by many nanozymes acing as catalysts such as MnO_2, catalase, nano ceria, etc., to generate oxygen inside the tumor microenvironment upon getting the proper stimulation to prevent the premature release of their carried substances.

Table 4.2 Strategies for modifying tumor microenvironment (partly adapted from Xu et al. 2020)

Conditions	Targets	Nanoparticles	Reference
By improving tissue oxygenation	Temperature e.g., PTT for mild hyperthermia	Core–shell MnSe@Bi2Se3	Song et al. (2015)
	Hyaluronan –e.g., hyaluronidase to break down hyaluronan in tumor ECM	NM-Ce6	Gong et al. (2016)
	CAFs e.g., cyclopamine (CYC) to disrupt the tumor ECM barrier	ABN@HA-SeSe-Ce6/CYC	Feng et al. (2019)
	TAMs Bisphosphonates (BP) to deplete TAMs to enhance intratumoral perfusion	CaBP(^{32}P)-PEG	Tian et al. (2018)
	HIF-1α HIF-1α siRNA to inhibit HIF-1α	GdW10@CS nanosphere	Yong et al. (2017)
By utilizing nano carriers to transport O2	PFC (perfluorocarbon)	PEG-Bi2Se3@PFC@O2	Xu et al. (2020)
	PFP (perfluoropentane)	O2–PFP@HMCP	Lu et al. (2018)
	Red blood cell	P-FRT-RBCs	Tang et al. (2016)
	Hb (hemoglobin)	ICG-loaded artificial red cells (I-ARCs) 91 BP@RB-Hb	Luo et al. (2016a)
	Cutz-1	CuTz-1-O2@F127	Cai et al. (2019)
By decomposing substances to generate O_2	Decomposing H_2O_2, H_2O, C_uO, Au_2O_3 Using catalase, MnO2,C3N4, etc., to generate O2	TaOx@Cat-PEG HSA-MnO2 -Ce6&Pt (HMCP)	Song et al. (2016 and Chen et al. (2016)
Targeting hypoxia-assisting treatment	(i) Releasing in response to tumor hypoxia (ii) inducing hypoxia cytotoxicity for synergistic therapy	Bacteria-UCNRs TPZ-UC/PS	Luo et al. (2016b) and Liu et al. (2015)

4.6.3.4 Using Hypoxia Prodrugs to Assist Treatment

Hypoxia within tumor microenvironment can itself facilitate treatment through various drugs that are specifically sensitive to either to low oxygen or to certain compounds that are highly expressed in the hypoxic conditions, enabling the nanosystems to target hypoxic tissue or by inducing cell injury through synergistic therapy. For example, anaerobic and facultative bacteria favoring hypoxic tumor microenvironment can be decorated or encapsulated within the nanoparticles to directly target hypoxic niches, helping to guide accumulation of hypoxic prodrugs to their specific sites.

4.6.3.5 Bioreductive Drugs

These are deactivated cytotoxins that are reduced to toxic, active metabolites by endogenous human cellular oxidoreductases. This process takes place in the absence of oxygen, thereby imparting specificity for the hypoxic tumor microenvironment. Tirapazamine (TPZ) and AQ4N are such leading compounds in this class of agents (Guise et al. 2014).

4.6.3.6 Gene Therapy

Several hypoxia-responsive genes such as phosphoglycerate kinase 1 (PGK-1), enolase (erythropoietin), vascular endothelial growth factor (VEGF), etc., have been used to construct hypoxic response promoters using their hypoxia-responsive elements (HRE) to achieve high specificity to hypoxic tumor cells. Other methods of targeted gene therapy are gene-directed enzyme prodrug therapy (GDEPT) where they deliver foreign genes using adenovirus as vector encoding a nontoxic enzyme that gets activated only at the site of conversion. Others such as combination bacteriolytic therapy (COBALT) use anaerobic bacteria as vectors such as *Escherichia coli, Bifidobacterium longum,* etc., and macrophages as hypoxic prodrug -containing vector that gets infiltrated in many solid tumor malignancies (Kizaka-Kondoh et al. 2003).

4.7 Conclusion and Future Perspectives

Hypoxia and its master regulator HIF-1α are the major driving force in the cancer progression, leading to invasion, migration, metastasis, drug- and chemoresistance, etc. Various signaling mechanisms interact as well as oppose each other, creating a complex network, leading to antiapoptotic, chemo- and radio-resistance, as well as uncontrolled growth of tumor cells. Together, these mechanisms modulate the overall cellular and biological response of hypoxia. HIF-1α, upon getting stabilized under anaerobic conditions, translocates within the nucleus and regulates a wide array of genes responsible for maintaining the various functions within tumor microenvironment such as angiogenesis, apoptosis, p53, DNA repair pathways, EMT, etc. All these factors significantly lead to poor prognosis in cancer patients due to dependency of conventional cancer therapies on oxygen concentrations within tumor tissues such as chemotherapy, radiotherapy, etc. Various theranostics approaches have been put forward to evaluate the condition and severity of the hypoxic conditions within tissues, enabling clinicians to diagnose as well as treat patients. Since surgical resection and chemotherapies have proved to be creating more side effects eventually facilitating the tumor metastasis, different approaches have been designed to directly target the hypoxic regions within tumor microenvironment to increase the efficacy and decrease systemic toxicity such as by modulating tumor microenvironment, increasing the oxygen concentration, hypoxic prodrugs, anticancer drugs loaded with nanoparticles, etc. Hence, understanding the biology of tumor hypoxia can be of great importance as it will open new avenues that will help in targeting hypoxia and controlling hypoxic TME, thereby (i) reducing the

tumor growth and proliferation, (ii) overcoming drug and chemoresistance, and (iii) preventing metastasis and relapse.

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Hypoxia and Senescence: Role of Oxygen in Modulation of Tumor Suppression

5

Mehtap Kilic Eren

Abstract

Cellular senescence is a state of growth arrest implicated in both physiological and pathophysiological conditions. In aging cells, while senescence is induced via replicative exhaustion due to telomere shortening, in preneoplastic cells it emerges as a cellular failsafe program provoked by oncogenic activation and serves as an initial barrier constraining the malignant progression. Regulation of senescence is influenced by various intrinsic and extrinsic factors including tissue hypoxia, which apparently helps premalignant cells to evade instigation of "oncogene-induced senescence" (OIS). For a better understanding of the pathological consequences of senescence bypass, it is crucial and of great interest to explicit the hypoxia-related mechanisms and factors contributing to the modulation of oncogene-induced senescence. This chapter reviews the previous and recent data that contribute to the understanding of the fundamentals of cellular senescence as well as the mechanisms of hypoxia-induced modulation of OIS.

Keywords

Hypoxia · Senescence · OIS · Tumor suppression · HIF-1 · p53 · p21 · p16

5.1 Introduction

Cellular senescence emerges as one of the major and indispensable cell behavior that has critical importance in the regulation of the life span of the living organisms.

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More than a half-century ago, cellular senescence was first described as an in vitro phenomenon by Hayflick and Moorehead, who demonstrated that primary fibroblasts grown in culture have a finite proliferative capacity. Hayflick's observation was intriguing as the primary fibroblasts in culture were initially able to divide rapidly, but progressively cell division slowed down and eventually stopped (Hayflick and Moorhead 1961; Hayflick 1965). Currently, Hayflick's "in vitro phenomenon" is acknowledged as "replicative senescence" defining a permanent cell growth arrest state and an ultimate cellular stress response to telomere shortening reflecting the cell aging (Hernandez-Segura et al. 2018).

In the last two decades, cellular senescence has gained its reputation by its key roles in various physiological processes such as embryogenesis, tissue renewal and homeostasis, aging, and tumor suppression (van Deursen 2019; Rhinn et al. 2019; Nehme et al. 2020). Although much progress has been made regarding the understanding of the physiological and pathological significance of senescence, still much remains to be done to clarify the cellular dynamics and processes contributing to the regulation of senescence. For example, hypoxia is a typical feature of almost all tissues and organisms that display varying levels of oxygen in different tissues (Pouyssegur and Lopez-Barneo 2016), though the influence of hypoxia on senescence is not completely understood (Otero-Albiol and Carnero 2021; Welford and Giaccia 2011). Considering the substantial role of hypoxia in promoting tumor progression, angiogenesis, and metastases, it is of great interest to understand whether hypoxia acts as a stress factor triggering senescence or whether it leads to bypass of senescence, and thus promotes malignant transformation.

In this chapter, we first describe the fundamentals of cellular senescence as well as the mechanisms involved in the activation and regulation of senescence, then we associate senescence and hypoxia together and examine their relationship in terms of tumor suppression or acquisition of malignant properties.

5.2 Fundamentals of Cellular Senescence

After Hayflick's observation in diploid fibroblasts, replicative senescence was shown in various types of cells, including vascular endothelial cells, epidermal keratinocytes, lymphocytes, adrenocortical cells, chondrocytes, and smooth muscle vascular cells (Blasco 2005). Replicative senescence occurs as a result of telomere shortening that resembles the structure of repetitive nucleotide sequence of "TTAGGG" in DNA including the accompanying proteins residing at the end of the chromosomes. Telomeres are responsible for protecting chromosomes from degradation and/or fusion with nearby chromosomes (Martinez and Blasco 2011; Masutomi et al. 2003; Greider and Blackburn 1989). As cells proliferate, telomeres shorten by each cell division, during the DNA replication process due to the incomplete replication of the end of DNA strands (Martinez and Blasco 2011; Martinez et al. 2009). Accumulations of short telomeres produce genomic instability, leading to a premature senescence phenotype, and (Greider 1993) thus, shorten life span (Blasco 2005; Olovnikov 1973). This phenomenon and its contribution to

cellular senescence were established in the 1990s in diploid fibroblasts and afterward were also proved in vivo in different tissues, including lymphocytes, liver, skin, blood, and colon (Greider 1993; Greider 1990; Harley et al. 1994).

Later on, evidence from in vitro studies suggests eukaryotic cells also possess an acute "stress-induced premature senescence phenotype" (SIPS) similar to the replicative senescence but acting as a cellular failsafe program that is induced by a variety of cellular stress factors, including DNA damaging chemotherapy agents, telomere dysfunction, mitochondrial dysfunctionality, oxidative stress or reactive, oxygen species, and cytokines (de Magalhaes et al. 2002; de Magalhaes and Passos 2018; Dierick et al. 2002). Induction of senescence in cancer cells by cytotoxic chemotherapy or radiotherapy is recognized as "therapy-induced senescence" (TIS), as well (Zhang et al. 2019; Gorgoulis et al. 2019).

Overexpression of oncogenes such as *RAS* or *BRAF* is also known as a potent inducer of cellular senescence. Initially, the constitutively activated oncogenes provoke a hyper-proliferative phase intrinsically, resulting in a DNA hyper-replication stress, employing DDR signaling and induction of senescence (Barradas et al. 2002; Collado and Serrano 2006; Pantoja and Serrano 1999; Dhomen et al. 2009). This type of oncogene-provoked senescence is currently well-known as "oncogene-induced senescence" (OIS) acting as an early tumor suppressor barrier against malign transformation (Collado and Serrano 2006; Collado et al. 2005). Initiation of senescence programs either by various stress factors or telomere shortening is triggered by the cell's intrinsic DNA damage-sensing ability eventually manifesting the activation of the DNA damage response (DDR) signaling (Hernandez-Segura et al. 2018; Di Micco et al. 2008; von Zglinicki et al. 2005).

5.2.1 Morphological Alterations of Cellular Senescence

Cells undergoing senescence are defined as viable and metabolically active but stably arrested in the cell cycle and unable to respond to the mitogenic signals stimulating cell proliferation (Hernandez-Segura et al. 2018). Senescent cells undergo intense changes in gene expression displaying typically morphological and biochemical changes associated with induction of a secretory phenotype (SASP) with the involvement of extensive epigenetic regulations (Hernandez-Segura et al. 2018; Alster and Korwek 2014; Kosar et al. 2011). Under stress conditions, senescent cells acquire a unique morphology by exerting an abnormally enlarged, flattened cell structure as a result of the increased expression of Caveolin1, which is a part of the cholesterol-rich microdomains of the cell membrane (Dasari et al. 2006). Further, senescent cells display a disproportional increment in the cytoplasm-to-nucleus ratio concomitant with vacuole-rich cytoplasm and more granular appearing. This bulky state of the cytoplasm was initially defined as emerging with the manifestation of cell senescence, but recently has been proposed as it may exert a fundamental action in triggering the growth arrest in the course of senescence (Neurohr et al. 2019). Likewise, the generation of cytoplasmic chromatin fragments (CCFs) caused by the loss of nuclear filament protein lamin B1 (LMNB1)

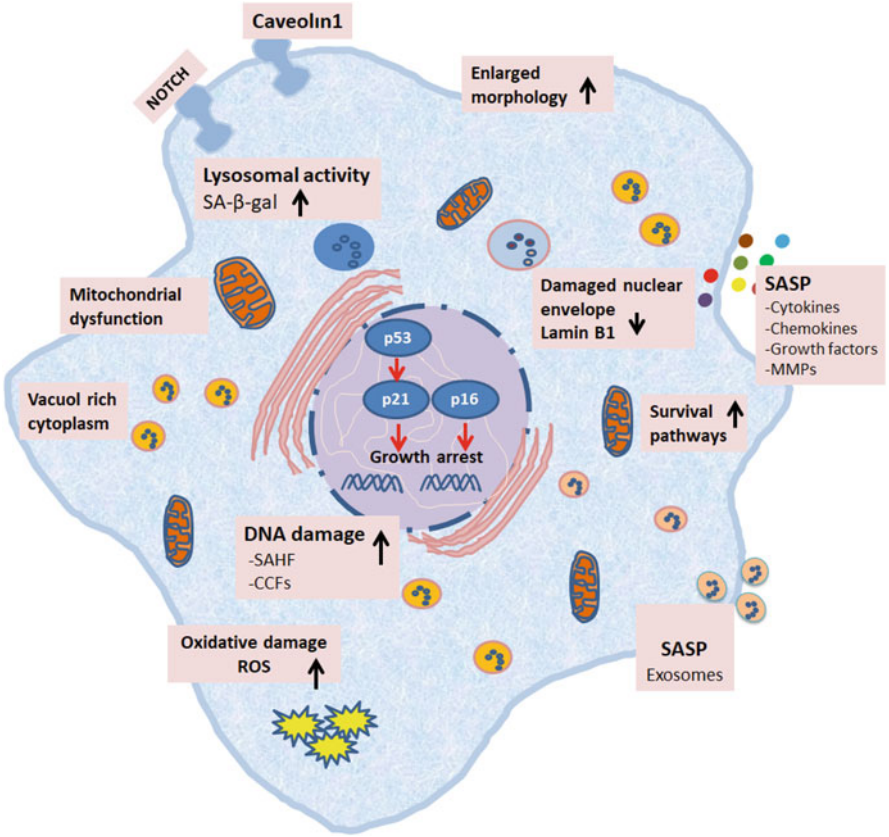

Fig. 5.1 Hallmarks of senescent cells. Senescent cells are viable and metabolically active but stably arrested in the cell cycle displaying morphological and biochemical and molecular changes including abnormally enlarged, flattened cell structure, a vacual-rich cytoplasm, dysfunctional mitochondria, and damaged nuclear envelope. Senescence is also associated with increased lysosomal β-galactosidase activity, SASP factors, epigenetic changes, including SAHFs, CCFs, as well as increased survival pathways

has been remarked as a characteristic of senescence and associated with the governing of the secretory phenotype of cells undergoing senescence (Fig. 5.1) (Freund et al. 2012; Wang et al. 2017).

Recent studies have provided substantial data that the increased activity of the lysosome-specific enzyme β-galactosidase (SA-ß-gal, senescence-associated ß-galactosidase) at pH 4.0–4.5 is the most distinguishing feature of senescent cells that also allows them to turn out to be blue when tested with a colorimetric assay using the substrate X-gal (Dimri et al. 1995; Dimri 2004; Debacq-Chainiaux et al. 2009). SA-β-gal activity test is the first accepted and currently valid gold standard test for detection of any type of senescent cells at suboptimal pH of 6.0, which generally is not present in pre-senescent, quiescent, immortal, or transformed cells

(Dimri 2004). Within the last decade, various methods have been developed for measuring SA-β-gal activity applying cytochemical or histochemical X-gal staining protocols (Debacq-Chainiaux et al. 2009; Hall et al. 2017). In addition, fluorescence-based methods employing the use of 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG), a florigenic substrate of SA-β-galactosidase, are also currently available for the quantitative measurement of senescent cells via flow cytometry, fluorescence microscopy, or microfluidic chip detection (Debacq-Chainiaux et al. 2009; Evangelou and Gorgoulis 2017). Lipofuscin accumulation has emerged as another feature of senescent cells and is accordingly used for the development of a new method established on the biotin-conjugated Sudan black B analog that has been recognized as a consistent test system for monitoring senescent cells in various cells and tissue types (Fig. 5.1) (Evangelou and Gorgoulis 2017).

5.2.2 Senescence-Associated Metabolic Changes

Although senescent cells do not proliferate, they retain an elevated metabolic activity due to an increased need for energy and other constituents to maintain the senescent phenotype (Hernandez-Segura et al. 2018; Kwon et al. 2019; Nacarelli and Sell 2017). Thus, ongoing senescence catabolism in cells mainly depends on elevated glucose consumption to yield ATP (Birsoy et al. 2015). Consequently, an increase in glycolysis ensues in oncogenic or stress-induced senescence as well as in replicative senescence (Kwon et al. 2019; Nacarelli and Sell 2017). Additionally, mitochondrial dysfunction that is a characteristic of senescence impacts on ATP generation and the NAD+/NADH ratio, leading to growth arrest (Kwon et al. 2019; Passos et al. 2007). The catabolic signaling pathways such as AMPK and NFκB are also often changed in senescent cells (Kwon et al. 2019; Nacarelli and Sell 2017; Birsoy et al. 2015). Conversely, anabolism of senescent cells is mostly concentrated on protein and lipid synthesis because of the factors participating to SASP and lipids that are used to constitute new organelles containing membranes and to provoke an autophagy response (Kwon et al. 2019; Nacarelli and Sell 2017). The most frequently altered signaling pathways related to anabolic processes for the period of cellular senescence include GSK3, ATM, SREBP1, and mTOR pathway (Nacarelli and Sell 2017; Nacarelli et al. 2018). Additionally, an increase in glycogen synthesis has been reported in senescent human fibroblasts and various aging tissues (Kim et al. 2010; Seo et al. 2008).

5.2.3 Senescence-Associated Mitochondrial Dysfunction

Senescent cells encompass a number of modifications in mitochondrial mass, membrane potential, and mitochondrial morphological structure (Nacarelli and Sell 2017). Dysfunction of mitochondria is characterized by membrane potential changes, resulting in proton leakage and excessive production of ROS, which in turn leads to oxidative damage and senescence (Fig. 5.1) (Kwon et al. 2019; Passos

et al. 2007). Elevated levels of oxidative stress in cells' ongoing senescence have been associated with dysfunctional mitochondria. During senescence, the antioxidant defense mechanisms are also incapable of neutralizing the effects of excessive ROS production (Kordowitzki 2021; Mancini et al. 2021). Several studies have shown that telomeres are the most sensitive regions of the DNA affected by the adverse effects of ROS. Whereby ROS can result in DNA breaks inducing telomere dysfunction and premature senescence (Kordowitzki 2021; Wang et al. 2021; Duan et al. 2005). Conversely, agents reducing mitochondrial ROS, for example, nicotinamide and MitoQ, have been reported to prolong the replicative life span of cells in culture by hindering the telomere dysfunction (Chapman et al. 2019).

Further studies have shown that senescence and ROS are also closely related in terms of senescence signaling as ROS plays a significant role in downstream regulatory pathways of senescence. Excessive ROS production has been shown during replicative, oncogene, and stress-induced senescence.

Moreover, experimental activation of main signaling molecules regulating senescence, including p16, $p21^{CIP1}$, and p53, has been associated with elevated ROS (Passos et al. 2007; Passos et al. 2010; Ogrunc et al. 2014). These findings supported the notion that ROS may act as a signaling molecule to help the stabilization of senescence-induced growth arrest in cells undergoing senescence. Hence, it was suggested that the storage of ROS in senescent cells participates in the maintenance of DDR, instigating the growth arrest (Passos et al. 2010; Ogrunc et al. 2014; Parrinello et al. 2003; Nassrally et al. 2019; Stockl et al. 2006).

5.2.4 Main Effectors of Senescence: DNA Damage Responders and Cell Cycle Regulators

Senescent cells are eminent with their permanent growth arrest state. Eukaryotic cell cycle progression is maintained via the activation of cyclin-dependent kinase (CDK) complexes that coordinate and ensure the timely transition between cell cycle phases (Hernandez-Segura et al. 2018; Kastan and Bartek 2004). Cell cycle arrest occurring in the progression of cellular senescence is mostly mediated by activation of tumor suppressor pathways, namely, p53-$p21^{CIP1}$ and/or $p16^{INK4A}$-pRB (Fig. 5.1) (Gorgoulis et al. 2019; Bai et al. 2007; Kim et al. 2015; Yano et al. 2021; Zhu et al. 2002; Zheng et al. 2006). Both signaling pathways are highly complex and require upstream regulators and downstream effectors to fully function in the execution of senescence. In most of the somatic cells, various pro-senescent stress stimuli causing DNA breaks converge on activation of DNA damage response mainly involving ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) kinases to initiate senescence (Di Micco et al. 2008; von Zglinicki et al. 2005; Efeyan et al. 2009; Bartek and Lukas 2007). ATM and ATR kinases are recognized as sensor kinases and start a downstream phosphorylation cascade initiated with phosphorylation of histone H2AX located in DNA breaks. The phosphorylated form of H2AX, known as the γ-H2AX foci, is required for enrolling and anchoring the other DDR players. Activation of ATM and ATR kinases results in phosphorylation

of checkpoint kinases CHK1 or CHK2, respectively which results in stabilization of p53. Further, p53 transactivates the various genes, including the cyclin-dependent kinase complexes inhibitor $p21^{CIP1}$ (also known as *CDKN1A*) halting the cell in G1 or G2/M phases by inhibiting the activity of CDK2 or CDK1, respectively (Bartek 2011; Bartek et al. 2007a; Brazina et al. 2015; Falck et al. 2001).

Initiation of the mitogen-activated protein kinase p38/MAPK signaling by ATM/ATR also increases the expression of *CDKN2A* encoded CDK4/6 inhibitor $P16^{INK4A}$, acting upstream of the other tumor suppressor protein pRB and facilitating its hypophosphorylation together with $p21^{CIP1}$ (Bartek 2011; Bartek et al. 2007a). Hypophosphorylated Rb protein binds to E2F and thereby cell cycle's progression is halted and senescence is instigated. Irrespective of specific triggers, DDR signaling cascades converge on both p53-$p21^{CIP1}$ and $p16^{INK4A}$-Rb tumor suppressor pathways to activate senescence (Bartek and Lukas 2007; Bartek 2011; Bartek et al. 2007a; Brazina et al. 2015; Evangelou et al. 2013). Activation of the other product of *CDKN2A* gene *ARF* known as "tumor suppressor ARF" through epigenetic de-repression mediates the inhibition of MDM2 and thus prevents p53 degradation and promotes cellular senescence via p53-$p21^{CIP1}$axis (Brookes et al. 2004, 2002). Long-term overexpression of any of the key elements p53, pRB, $p16^{INK4A}$, and $p21^{CIP1}$ was competent to initiate senescence (Pantoja and Serrano 1999; Brookes et al. 2004; Lin et al. 1998). The senescence-associated cell cycle arrest was initially thought to be permanent and irreversible; however, growing evidence supports the reversibility of senescence by showing early-senescent or pre-senescent cells able to return the cell cycle under specific genetic or transcriptomic conditions (Beausejour et al. 2003; Katoh et al. 2021; Lee et al. 2016; Walters et al. 2016).

5.2.5 Senescence-Associated Epigenetic Regulations

Cells require intense epigenetic regulations and chromatin changes, resulting in senescence-associated heterochromatic foci (SAHF), a gene repressing mechanism employed to induce senescence, yet another peculiarity of senescence (Hernandez-Segura et al. 2018; Kosar et al. 2011). SAHF encompass distinct histone modifications including trimethylated Lys9 or Lys20 (H3K9me3, H3K20me3), and acetylated Lys27 (H3K27ac) on histone H3, as well as increased activity of chromatin repressors heterochromatin protein 1 gamma (HP1γ), high mobility group protein A (HMGA), histone variant macroH2A, histone co-chaperones HIRA, and ASF1A49–51.a (Kocylowski and Halazonetis 2011; Sanders et al. 2013; Zhang et al. 2007; Zhang et al. 2005; Zhang et al. 2014). Importantly, SAHF formation has been originally related to senescence-associated proliferation arrest due to the presence of downstream targets of E2F involved in cell cycle execution (Kocylowski and Halazonetis 2011; Zhang et al. 2014). SAHF can be observed as 4,6-diamidino-2-phenylindole (DAPI)-positive nuclear assemblies in cells undergoing senescence. However, SAHF is not accepted as a widespread marker of senescence due to its dependence on ATR activation and DNA replication stress as well as oncogene

activation (Kocylowski and Halazonetis 2011). SAHF execution of a DDR-resistant heterochromatin structure also hinders DDR signaling (Di Micco et al. 2011). Not surprisingly, therefore, histone deacetylase (HDAC) inhibitors induce chromatin loosening to enhance DDR signaling and consequently induce cell death via apoptosis (Di Micco et al. 2011; Abramova et al. 2011; Kochetkova et al. 2017).

5.2.6 Senescence-Associated Changes in Cell Survival Pathways

Senescent cells display upregulated survival signaling such as PI3K/, leading to upregulation of antiapoptotic proteins of the Bcl-2 family, including Bcl-2, Bcl-xl, Bcl-w, and $p21^{CIP1}$, which encourages the resistance against apoptosis (Hernandez-Segura et al. 2018; Di Micco et al. 2021). In physiological environments, various stress factors inducing oxidative damage or intense DNA damage can activate p53 via DDR, which in turn can promote apoptosis by initiating the transactivation of the pro-apoptotic participants of the Bcl-2 family Noxa and Bax (Yin 2000). Nevertheless, senescent cells display upregulated activity of $p21^{CIP1}$ protein promoting survival signaling and preventing induction of apoptosis (Yosef et al. 2017). Moreover, downregulated Bcl-w and Bcl-xl can lead to abolishment of senescent cells in the lung and epidermis as shown in vivo (Yosef et al. 2016). However, whether the sustained viability of senescent cells is due to the selection against apoptosis sensitivity or whether it is an internal outcome of the senescence process remains to be examined.

5.2.7 Senescence-Associated Secretory Phenotype

Senescence-associated secretory phenotype (SASP) delineates the other intriguing function of senescent cells engaging several pro-inflammatory factors comprising the cytokines, chemokines, growth factors, angiogenic elements, proteolytic enzymes, lipids, components of extracellular matrix, and matrix metalloproteinases (MMPs), and extracellular vesicles (Malaquin et al. 2019). SASP is composed of soluble proteins released into the extracellular milieu, cleaved trans-membrane proteins, and the factors transported in exosome-like vesicles (Maciel-Baron et al. 2016; Tanaka and Takahashi 2021).

Interestingly, SASP is not an essential characteristic of senescence and some types of cells experiencing senescence do not necessarily associate with inflammatory secretory phenotype as exemplified in human primary fibroblasts induced to senesce by ectopic expression of $p16^{INK4a}$ and cyclin-dependent kinase inhibitor, $p21^{CIP1}$ (Faget et al. 2019). The SASP can operate in a cell-autonomous (autocrine) or non-cell-autonomous (paracrine) manners, and takes the responsibility for supporting and disseminating senescence phenotypes and maintaining tissue homeostasis (Maciel-Baron et al. 2016).

SASP is strictly regulated in a complicated way encompassing diverse signaling molecules such as nuclear factor-kappa light-chain-enhancer of activated B cells

(NF-κB) CCAAT/enhancer-binding protein beta (C/EBPβ), mammalian target of rapamycin (mTOR), and NOTCH1. NF-κB and C/EBPβ are the two main transcription factors responsible for the transcriptional regulation of SASP proteins, including IL-1A, IL-6, and IL-8, that are employed in inflammation (Maciel-Baron et al. 2016; Salminen et al. 2012). The cytosolic DNA-sensing GMP-AMP synthase stimulator of interferon genes (cGAS-STING) pathway and the senescence-associated Alarm in high mobility group box 1 (HMGB1) protein are also involved in governing of SASP and secretion of pro-inflammatory (Davalos et al. 2013; Loo et al. 2020).

SASP is categorized into two secretory components, namely the inflammatory components, mainly regulated by IL1, and the TGFβ-related components, relying on NOTCH signaling (Acosta and Gil 2012; Hoare et al. 2016). The SASP can act as a double-edged sword and may exert both beneficial and deleterious effects that may be predisposed by various factors including its composition, intensity, and the microenvironment (Coppe et al. 2010a). Of note, the composition of SASP is considered rather heterogeneous and may vary among the cell types and may also change with the stimuli triggering senescence (telomere loosening, oncogene activation, oxidative stress, etc.) (Loo et al. 2020). The pro-inflammatory cytokines such as IL-1, IL-6, and IL-8 are the most common elements of SASPs and do not vary by senescence stimuli or cell types (Faget et al. 2019). The beneficial effects of SASP mainly depend on its paracrine functions contributing to maintenance of senescence implicated in the immune surveillance, tumor suppression, cell-to-cell communication, clearance of senescent cells, and wound healing (Malaquin et al. 2019). Remarkably, pro-inflammatory factors play a role in the promotion of tumor progression and thus resemble detrimental functions of SASP (Coppe et al. 2010b). It can obstruct tissue repair and regeneration and support organismal aging through the storage of senescent cells and attenuation of stem/progenitor cell components (Tanaka and Takahashi 2021)

5.3 Oncogene-Induced Senescence (OIS) and Tumor Suppression

5.3.1 Mechanisms of OIS

Eukaryotic cells have evolved genetically encoded cellular failsafe programs such as induction of apoptosis or senescence to overcome the potentially hazardous events that may lead to malign transformation (Lee and Schmitt 2019; Lowe et al. 2004)

In pre-neoplastic cells, once activated p53 mainly stimulates the cell cycle arrest over transactivation of $p21^{CIP1}$ and promotes the DNA repair response temporarily. However, when DNA damage persists, due to extensive damage p53 induces mitochondria-mediated apoptosis via transactivating numerous pro-apoptotic participants of the Bcl-2 family genes comprising Puma (p53 upregulated modulator of apoptosis), Bax, Bak (Bcl2 antagonist/killer) (Lowe et al. 2004; Galluzzi et al. 2018). Activation of senescence through p53-$p21^{CIP1}$ corporation inducing insistent cell cycle arrest establishes the alternate mechanism for elimination of the

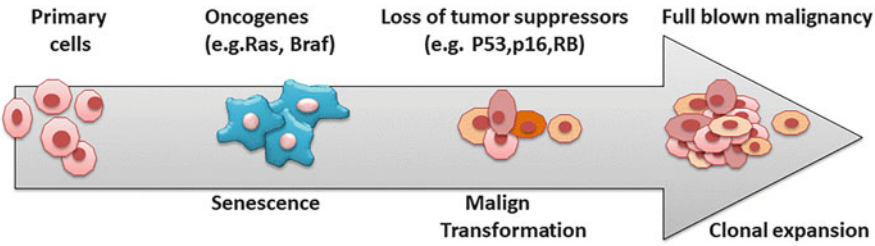

Fig. 5.2 OIS acts as an intrinsic tumor-suppressor mechanism. OIS is acutely induced in primary cells via activation of mitogenic oncogenes such as Ras/BRAF and can be bypassed by loss of tumor suppressors, e.g., P53, p16, and RB. Bypass of senescence promotes progression into the full malignancy

precancerous lesions (Collado et al. 2005; Collado and Serrano 2005). Although both processes share a common activating mechanism involving DDR pathway and p53, the decision of cells whether to activate senescence or apoptosis mainly depends on the characteristics of the activated oncogene, cell type, or environmental factors forcing the cell into malignant transformation (Lowe et al. 2004).

In mammalian cells, when excessive mitogenic signals are perceived through activated oncogenes, such as *RAS* or *BRAF,* accumulation of oncogene-induced ROS produced by NADPH oxidases may boost up the initial hyperproliferative phase accompanied with increased DNA replication that eventually results in replication stress and telomeric dysfunction, leading to DNA damage and induction of cellular senescence (Di Micco et al. 2011, 2021, 2006; Bartkova et al. 2010). Evidently, in hyperplastic cancerous regions in human tissues, accumulation of oncogene-induced dysfunctional telomere, and marks of activated DDR has been detected (Bartkova et al. 2005).

This phenomenon is clearly represented by the ectopic overexpression of the oncogenic *HRAS* (HRasG12v) in human diploid fibroblasts, proving that oncogene activation is a potent stimulator of cellular senescence triggering a hyperproliferative response associated with exhausted DNA replication machinery, ultimately leading to activation of DDR signaling and of senescence (Ogrunc et al. 2014; Di Micco et al. 2006; Serrano et al. 1997). This course is known as "oncogene-induced senescence" (OIS), mainly signified by halted cell proliferation and higher expression of $p16^{INK4a}$ and p53-$p21^{Cip1}$ (Collado and Serrano 2006, 2005) (Fig. 5.2).

Besides prolonged DDR activation, OIS display similar characteristics as of the other types of senescence including $p21^{CIP1}$-mediated cell cycle arrest and $p16^{INK4A}$ increased ROS levels and thus oxidative damage, upregulated survival proteins (including anti-apoptotic Bcl-2 family proteins), metabolic alterations, and accumulation of SA-β-gal and SASP as well as SAHF (Collado and Serrano 2006, 2005; Di Micco et al. 2006).

Hyperactive mutated members of the MAPK signaling pathway, including RAS, RAF, MEK, BRAF, and various other mitogenic response players such as PI3K, AKT, MYC, ERBB2, and p38MAPK exerting an oncogenic potential have been

reported to induce OIS (Astle et al. 2012; Braig et al. 2005; Damsky and Bosenberg 2017; Ferbeyre 2018). Likewise, loss of tumor suppressor functions of PTEN, NF1 can also initiate cellular senescence due to hyperproliferation and DDR activation (Jung et al. 2019; Larribere et al. 2015). Of note, activation of PI3K/Akt pathway promotes p53-dependent senescence without inducing hyperproliferating signals or excessive DNA damage, suggesting a different mechanism. OIS is induced as a telomere-independent mechanism incorporating the p53-$p21^{CIP1}$ and $p16^{INK4A}$-RB axis (Fig. 5.2) (Aoki and Fujishita 2017). Different studies have revealed that the accumulation of RB, the downstream partner of $p16^{INK4A}$, is particularly important in pursuance of senescence due to its influence on the suppression of E2F-target genes connected to DNA replication (Di Micco et al. 2021; Dimri et al. 1996). P53 emerges as another indisputable component of OIS as reports have indicated that loss of p53 or its regulator $p19^{ARF}$ triggers RAS-persuaded cancer cell invasion in mice, while its recurrence suppresses tumor growth along with signs of senescence (Serrano et al. 1997). Although p53 and $p16^{INK4A}$ are widely accepted as well-established key players of OIS, under certain circumstances depending on cell type, neither p53 nor $p16^{INK4A}$ is required for the induction or execution of OIS (Cipriano et al. 2011). For example, Ras-induced initiation of senescence does not involve $p16^{INK4A}$ or p53 in human mammary epithelial cells, reflecting the disagreement between cell types in the activation of OIS (Cipriano et al. 2011).

In fact, studies have reported other inconsistencies related to OIS. For example**,** loss of tumor suppressor protein PTEN has been described to instigate cell cycle arrest and thus senescence recognized as "PTEN loss-induced cellular senescence (PICS)," which was further shown to involve hyperproliferation and DDR for induction of cellular senescence in vivo (Jung et al. 2019; Chen et al. 2005). However, activation of PI3K−AKT oncogenic signaling has been reported to provoke a p53-dependent senescence response without substantial hyperproliferation and DNA damage accumulation unlike oncogenic *RAS* or *BRAF (*Astle et al. 2012*;* Aoki and Fujishita 2017*)*. These studies suggest that there may be different underlying mechanisms implicated in the induction of senescence driven by activated oncogenes or loss of tumor suppressors.

Recently, NOTCH signaling, which has been identified as a highly conserved key signaling pathway involved in cell development, differentiation, and also cancer, was implicated in the regulation of OIS (Bray 2016). NOTCH signaling is instigated by ligand-dependent proteolytic cleavage processes in that Notch intracellular domain (NICD) unbound from the plasma membrane and translocates into the nucleus in order to cooperate with the DNA binding protein CSL to trigger transcription (Bray 2016). Studies revealed that NOTCH1 is upregulated during OIS concomitant with the induction of transforming growth factor-beta (TGFβ) (Hoare et al. 2016; Ito et al. 2017). Remarkably, NOTCH1 activity seems to be elevated transiently only in the early stage of OIS as it returns to its basal levels in the later stages of OIS (Hoare et al. 2016). Most interestingly, overexpression of N1ICD (the active intracellular form of the Notch1 receptor) in human fibroblasts drives cell-autonomous senescence identified as "Notch-induced senescence" (NIS) or primary NIS, which considerably differs from the "Ras-induced" senescence by an altered

secretory phenotype and chromatin structure (Hoare et al. 2016; Ito et al. 2017). SAHF formation is excluded in NIS cells comprising reduced amounts of the pro-inflammatory cytokine IL-8 and stimulation of the well-known ligand of NOTCH JAG1 as well as TGFβ1 (Hoare et al. 2016). Considering NOTCH's key roles in important physiological and pathological processes, understanding its functional implication in the course of senescence is of major attention.

5.3.2 OIS and Tumor Suppression

Functional studies have been identified in the biological role of OIS as an explicitly tumor-suppressive mechanism, an early intrinsic cellular barrier against tumorigenesis, as shown in both human pre-neoplastic lesions and animal models (Collado et al. 2005; Collado and Serrano 2005, 2010). In numerous studies including premalignant lung and pancreatic tumors, it was demonstrated that conditional expression of oncogenic *KRASV12* leads to neoplastic transformation that is concomitant with senescence characteristics (Collado et al. 2005; Efeyan et al. 2009). In mouse lymphoid cells, ectopic expression of *NRAS* activated OIS, preventing full-blown malignancy (Fig. 5.2) (Braig et al. 2005). The role of OIS in preventing tumor suppression has been also shown in a mouse model of prostate cancer where the loss of *PTEN* is utilized as an inducer (Chen et al. 2005). Remarkably, one of the most fascinating evidence showing OIS as an intrinsic barrier against tumorigenesis comes from the study conducted on human melanocytic naevi both in vitro and in vivo settings (moles) (Dhomen et al. 2009; Michaloglou et al. 2008; Michaloglou et al. 2005). This study has shown that 80% of melanomas harbor *BRAFV600E* mutation and melanocytes expressing *BRAFV600E*- are arrested in cell cycle and display numerous characteristics of OIS, including increased expression of $p16^{INK4A}$ and SA-β-gal positivity (Fig. 5.2) (Michaloglou et al. 2008, 2005). The naevi are capable of transforming into malignant tumors upon interruption or reversal of BRAF-induced senescence, suggesting that they are benign lesions remaining stably arrested for years before the onset of melanoma carcinogenesis that also resembles the OIS at best in vivo (Michaloglou et al. 2008, 2005). The previous in vitro studies have shown that OIS can be circumvented by disabling RB and p53 tumor suppressors. Subsequently, induction of senescence cannot be accomplished if any of p53 and/or RB are abrogated in a cell prior to an oncogenic activation (Fig. 5.2) (Serrano et al. 1997).

Hence, OIS can serve as a break in response to excessive proliferation, providing an initial barrier, and eventually protecting cells against tumorigenesis. OIS has been widely studied, and there are various examples as shown in vivo (Collado and Serrano 2010; Kuilman et al. 2010).

Likewise, other types of senescence, cells undergoing OIS also retain a SASP that provides OIS cells to intermingle with each other (Acosta et al. 2013). Various studies have shown that OIS cells include SASP components such as IL-6 and IL-8 together with other inflammatory molecules, which in turn provoke a self-amplification action in an autocrine fashion via augmented expression of n NF-κB

and CCAAT/enhancer-binding protein beta (C/EBPβ) (Coppe et al. 2010b; Acosta et al. 2013; Chien et al. 2011; Sebastian et al. 2005). OIS cells also exert paracrine functions that are involved in cell to cell transmission and alteration of their extracellular milieu, ultimately inducing a senescence phenotype called "secondary senescence" in their proliferating neighboring cells through secreted SASP elements (Acosta et al. 2013). Studies conducted with omics technologies such as quantitative proteomics have further identified the TGFβ (transforming growth factor-beta), VEGF (vascular endothelial growth factor), and CCL2 (chemokine CC motif ligand 2) as the main players of paracrine senescence (Acosta et al. 2013; Coppe et al. 2008). Besides the secretion of IL-1, TGFβ has been also shown to mediate the relationship concerning oxidative stress-mediated DNA damage, and paracrine senescence, along with DDR signaling persuading senescence in bystander cells (Di Micco 2017). The paracrine activities of OIS are mainly driven by the SASP secretome that represents a multifaceted and dynamic character and in part regulated by NOTCH signaling (Acosta et al. 2013). While NOTCH-primed secondary senescence mainly depends on growth factors, TGF-β and the expression of fibrillary collagens, SASP-primed secondary senescence is determined by the secretion of pro-inflammatory cytokines, C/EBPβ manifestation, and SASP dissemination (Acosta et al. 2013). Nevertheless, secondary senescence is manifested by the contribution of NOTCH-primed and SASP-primed secretome profiles (Faget et al. 2019).

Paracrine senescence is evidently shown in experimental models of human and mouse cells displaying OIS. In fact, an important connection between the manifestation of SASP factors and the stimulation of paracrine senescence has been established by non-cell-autonomous actions of OIS in vivo, which may have comprehensive biological meanings in terms of tumorigenesis (Acosta et al. 2013). Cells undergoing OIS release SASP factors that have been shown to recruit immune cells for eradication of tumor cells concomitant with an unappreciated tumor-promoting activity modulated mainly by the physiological conditions (Kang et al. 2011; Yevsa et al. 2012). In various studies utilizing cancer models, including skin, prostate, and liver, SASP has been shown to enable movement of tumor cells or provoke an immunosuppressive milieu endorsing proliferation, angiogenesis, and metastasis potential of cells (Greten and Eggert 2017; Alimirah et al. 2020; Guccini et al. 2021). In conclusion, it is broadly recognized that OIS may function in dual ways in cancer progression that is mainly influenced by the genetic background of the tissue, the content of SASP, and the extent of senescence (Acosta et al. 2013). Thus, while OIS exerts a cancer-preventive effect in the early phase of tumorigenesis, over time it may change to a deleterious cancer-promoting precancerous stage that appears as an in vivo antagonistic pleiotropy phenomenon (Coppe et al. 2010b).

Hence, it is of significant biological interest to investigate the tumor-preventive process and its genetically programmed dynamics in advance to cells' evasion of senescence and expansion into full-blown malignancy.

5.4 Intersection of Hypoxia and Senescence

Although atmospheric oxygen levels are roughly 20%, the received levels of oxygen by organisms are different. The amount of oxygen in the body is significantly lower and highly variable within the different organs and tissues. The oxygen concentration is approximately 4% in human brain tissue, in skeletal muscle, or in the liver, while it is near 5 or 6% in lung alveoli, and reaches only 1% in the skin (Vaupel et al. 1990, 1989). If an oxygen deficiency occurs under certain conditions or pathology, mammalian cells retain a systemic and molecular adaptive response to compensate for the hypoxia (Pouyssegur and Lopez-Barneo 2016; Semenza 1999). This adaptive response can be induced in all cell types under lack of oxygen and mainly relies on the stabilization of hypoxia-inducible factors (HIFs), HIF1α, HIF2α, or HIF3α (Wang et al. 1995; Semenza 2012; Semenza 2014). Under hypoxic conditions, a dimerization occurs between HIFα and HIF1β (ARNT) subunits and subsequently the heterodimer complex translocate to the nucleus, thereby binding to hypoxia response elements (Wang et al. 2005) in DNA and inducing the transcriptional activation of the target genes. HIFα is mainly regulated in a post-translational manner by prolylhydroxylases (PHDs) that are highly susceptible to oxygen levels. PHDs hydroxylate HIFα from the proline residues and thereby target these proteins to the von Hippel–Lindau protein (VHL) complex, which mediates the ubiquitination and degradation in the proteasomal complex. Another factor, known as the factor inhibiting HIF1α (FIH), is also an oxygen-regulated protein that works at lower oxygen levels than that of PHDs, and mediates the hydroxylation of HIFα by asparagine residues. However, FIH-mediated inhibition is exclusive to HIF1α because HIF2α is resistant to FIH-mediated modifications (Semenza 2012, 2014, 2007).

Notably, HIF1α and HIF2α isoforms differ in function. HIF1α is ubiquitously expressed, whereas expression of HIF2α is restricted to some cell types, such as endothelial cells, hepatocytes, cardiomyocytes, or glial cells (Semenza 2012, 2014). Moreover, HIF1α has been mostly involved in the acute response to hypoxia, whereas HIF2α has been associated with the chronic response to hypoxia or hypoxic response occurs at geographical altitude. Also, a third HIFα subunit, namely HIF3α, is expressed in different tissues such as the thymus, lungs, brain, heart, and kidneys that comprise alternative splicing variants and differ from the two other isoforms by lacking the transactivator domain. Eventually, HIF3α can act as an inhibitor of HIF-dependent transcription particularly via the splicing variant IPAS that can bind and constitute a heterodimer complex with HIF1α incapable of activating the transcription of HRE comprising genes (Semenza 2012, 2014).

Both HIF1 and 2α share some mutual transcriptionally regulated targets including GLUT1 or VEGF, though both differ in some others like LDHA and PGK1, which are specifically regulated by HIF1α or, EPO, MMP9, and OCT4 that are the cases for HIF2α. These differences in target preferences are mainly determined by the cell type or the level of oxygen concentrations (Semenza 2012, 2014)

Hypoxia is one of the major characteristic features of solid tumors and has been associated with poor prognosis or poor response to chemotherapy (Schito and

Semenza 2016; Semenza 2003; Kilic et al. 2007). However, its importance in the regulation of senescence is not well recognized. Senescence can be controlled by a plethora of factors; among those, the level of oxygen in the tissues emerges as particularly important (Kilic Eren and Tabor 2014). Intriguingly, as mentioned earlier, in living organisms O_2 levels vary between the tissues and also present as significantly lower than that of the in vitro cell culturing conditions achieved under 20% of O_2 at atmospheric levels. Thus, most of our understanding of cellular senescence has been defined by studies obtained in hyperoxic conditions that itself can potentially induce senescence (von Zglinicki et al. 1995).

During the last years, various studies have demonstrated that hypoxia can revise the life span of cells by modulation of replicative, therapy, or oncogene-induced senescence, in human or mouse cells (Parrinello et al. 2003; Kilic Eren and Tabor 2014; Chen and Ames 1994; Chen et al. 1995; Yuan et al. 1995)

Low levels of oxygen prolonged the life span of human fibroblasts 20%, whereas bovine and mouse fibroblasts are 80% and 500%, respectively (Packer and Fuehr 1977; Saito et al. 1995). In hypoxia, mouse embryonic fibroblasts (MEF) pursue proliferation despite an intact wt $p19^{ARF}$/p53 pathway and expressed $p16^{INK4a}$, which is indispensable for evasion of immortalization (Parrinello et al. 2003; Welford et al. 2006; Betts et al. 2008). Hypoxia-mediated suppression of replicative senescence is mainly related to decreased amount of DNA damage or reactive oxygen species (ROS) in mouse cells. Accordingly, normoxia has been suggested to induce senescence in MEFs due to accumulated DNA damage whereby alterations of $p19^{ARF}$/p53 pathway enable these cells to become unresponsive to DNA damage and thus immortalization occurs (Parrinello et al. 2003; Welford et al. 2006). Furthermore, the prolonged life span has been related to various signaling molecules, in particular, the HIFα comes forward (Welford et al. 2006). The regulators of cell cycle $p21^{Cip1}$ and Myc have been identified as the two target genes of HIF1α and HIF2α, respectively (Tsai et al. 2011; Gordan et al. 2007). HIF1α has been also suggested to activate the hTERT (human telomere reverse transcriptase) that is associated with mitochondrial ROS (Bell et al. 2007). In another study, HIF-1α was shown to display a crucial role in retardation of senescence in MEFS via transactivation of MIF and inhibition of p53-governed pathways (Fig. 5.3) (Welford et al. 2006). Akin to replicative senescence, hypoxia decreased the levels and the amount of chemotherapy-induced senescence in cancer cells, depending on HIF-1α activity (Sullivan et al. 2008). Accordingly, targeting of HIF-1α in human breast and colon cancer cells averted senescence and, eventually, bypassed the resistance to chemotherapy under hypoxic conditions (Sullivan et al. 2008). Furthermore, HIF-1α was shown to be capable of suppressing drug-induced senescence under normoxic conditions (Rohwer et al. 2010).

Hence, these studies underline the implication of hypoxia and HIF-1α in the modulation of replicative as well as therapy-induced senescence.

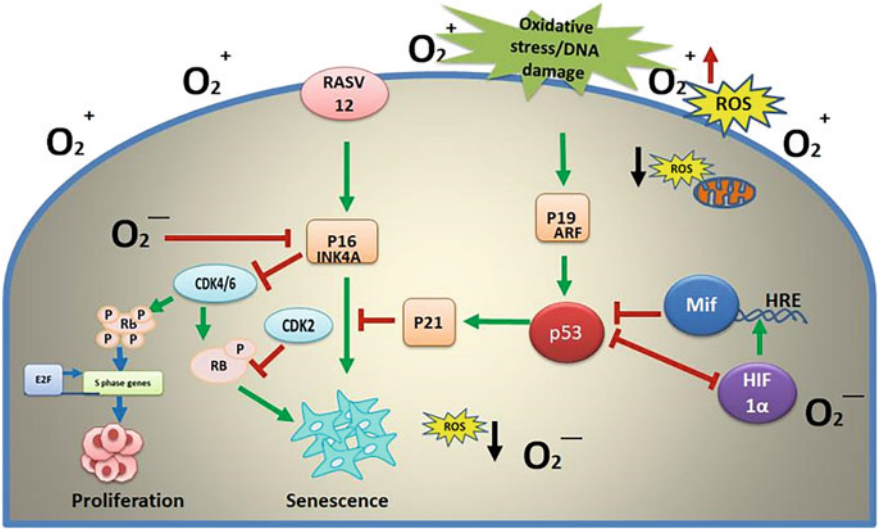

Fig. 5.3 Impact of hypoxia on senescence. Normoxia induces senescence in MEFs due to accumulated DNA damage via activation of p19ARF/p53 pathway. MIF as a target of HIF-1α directly binds and inhibits p53 and $p21^{CIP1}$. In hypoxia, Ras provoked senescence (RASV12) in human diploid fibroblasts (HDFs) involves HIF1α-independent $p16^{INKA}$, but dependent p53 and $p21^{CIP1}$ downregulation. Inactivation of either p16-Rb and/or p53-p21 tumor suppressor pathways is essential for inactivating OIS

5.4.1 Impact of Hypoxia on Regulation of Cell Cycle

It is frequently shown in various cell types that hypoxia itself can induce cell cycle arrest (Goda et al. 2003; Hammer et al. 2007). When oxygen levels drop from the atmospheric ranges (21%= of 159 mmHg) to the 7.6 mmHg, hypoxia induces an arrest at the G1/S phase of the cell cycle via HIF1α-induced activation of cell cycle regulators such as $p21^{CIP1}$ or $p27^{KIP}$ (Goda et al. 2003; Green et al. 2001). Additionally, HIF1α suppresses the activation of CSC25A phosphatase (Goda et al. 2003). Under extreme conditions of hypoxia (≤0,8mm Hg) or even in anoxia, cell proliferation arrest was achieved in an HIF1α-independent manner at different checkpoints (Freiberg et al. 2006). HIF1α-induced expression of downstream targets is accomplished at varying conditions, for example, expression of classical target genes of HIF1α such as VEGF, GLUT1, PGK1, etc., mainly relies on HIF-1α's binding to HRE within the specific promoter site. In contrast, expression of $p21^{CIP1}$ has been linked to a noncanonical way engaging the HIF-1alpha PAS domains binding and dislocation of Myc from $p21^{CIP1}$ promoter, which functions as a silencer (Koshiji and Huang 2004). Undoubtedly, the mechanisms involved in HIF1α's targeting effect on different genes may vary depending on the cell type or context. In previous reports, HIF-1α was shown to induce activation of TWIST in human mesenchymal stem cells (MSCs), which decreased the expression of $p21^{CIP1}$ by transposition of

E2A from the $p21^{CIP1}$ promoter, thereby allowing MSCs to bypass senescence in hypoxia (Tsai et al. 2011).

HIF-1α and HIF-2α may also have distinct effects on regulation of the cell cycle and cell proliferation as exemplified in clear cell renal carcinoma, where HIF-1α is involved in limitation of cell growth but HIF-2α initiation of tumor growth (Gordan et al. 2007, 2008). Thus, in hypoxia, various strategies are employed to regulate the gene expression that mainly differs with the level of oxygen, or with the nature of the involved transcription factor, or the target gene or the cell type. Although it is not yet completely understood, these differences may certainly impact on overall regulation of senescence in primary cells.

P53 is the central transcription factor activated upon various stresses such as DNA damage to activate the failsafe mechanisms such as cell cycle arrest, DNA repair, induction of apoptosis, or senescence. Hypoxia is among those stress factors inducing p53 activation (Chen 2016). This is despite the fact that the level of hypoxia desired to activate p53 is rather severe (almost anoxia) than that required for HIF1α. Previous reports indicate a direct interaction between HIF1α and p53 that likely occurs to enhance p53 stabilization and HIF1α degradation (Chen et al. 2003; Blagosklonny et al. 1998; Hammond et al. 2002; Hansson et al. 2002; Ravi et al. 2000). Presumably, retaining p53 stabilization by HIF1α would promote induction of senescence; however, secondary targets seem to take the action to cross-regulate p53 or HIF1α. In the case of p53, its target, PML, has been shown to repress the translation of HIF1α via the inhibition of mTOR (de Stanchina et al. 2004). Conversely, p53 and the interconnected responses including senescence are inhibited by the direct binding of MIF, which is a downstream target of HIF1α (de Stanchina et al. 2004; Ferbeyre et al. 2000; Jung et al. 2008; Petrenko et al. 2003). Collectively, these data substantiate that HIF1α and p53 can exert opposing activities in various contexts and also secondary effectors can be in charge.

5.4.2 Impact of Hypoxia in Metabolism

In hypoxia, cells display various adaptations, including the metabolic change from aerobic to anaerobic metabolism, so-called Warburg effect, to promote tumor survival under inappropriate conditions (Warburg et al. 1927). HIF-1α plays a central role in inducing the transcriptional activation of the vast majority of genes involved in the glycolysis such as the glucose transporters GLUT1 and GLUT3, or glycolytic enzymes PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK, PFKFB1–4, and PDK1 and MXI1 implicated in obstruction of the mitochondrial functions (Semenza 2007; Denko 2008).

Glycolysis serves as a source of ATP under hypoxic conditions and reduces the level of oxidative stress that results in increased life span. Evidently, overexpression of the glycolytic enzyme phosphoglucose isomerase (PGI) and phosphoglycerate mutase (PGM) genes has been associated with bypass of senescence (Denko 2008; Kondoh et al. 2005). Overall, these data suggest that hypoxia and HIF-1α-induced

activation of glycolysis and concomitant reduction of oxidative stress also contributes bypass of senescence.

5.4.3 Impact of Hypoxia in OIS

Given the significant effect of hypoxia on replicative and drug-induced senescence, explicating the role of hypoxic environment in the regulation of oncogene-induced senescence becomes extremely interesting as OIS constitutes the fundamental part of tumor suppression (Collado and Serrano 2006; Di Micco et al. 2021; Collado and Serrano 2005). OIS is identified as a genetically programmed failsafe mechanism withstanding as an essential initial block against malignant transformation by its tumor preventive act (Collado and Serrano 2006; Di Micco et al. 2021; Collado and Serrano 2005). Activation of p53-$p21^{CIP1}$ and/or $p16^{INK4A}$–pRb signaling is essential for OIS (Collado and Serrano 2006; 2005). Undoubtedly, in murine cells when p53 or its upstream partner $p14/p19^{ARF}$ is inactivated, H-RasV12-induced senescence is abrogated (Serrano et al. 1997). Conversely, in human cells inactivation of $p16^{INK4A}$ appears more crucial compared to p53 as the accomplishment of OIS entirely depends on activation $p16^{INK4A}$ (Brookes et al. 2002). In experimental models of OIS, in normoxic conditions, ectopic expression of oncogenic RAS (HRASV12) in primary fibroblasts induced senescence that is accompanied by increased intracellular in particular mitochondrial reactive oxygen species (Lee et al. 1999). However, under low oxygen conditions RASV12-expressing senescent cells can be rescued due to the decreased production of reactive oxygen species (Welford et al. 2006; Lee et al. 1999). Moreover, in hypoxic conditions (1% O_2), RasV12 is incapable of triggering the increase in the expression of $p21^{CIP1}$ and thus induction of senescence activation (Lee et al. 1999). Intriguingly, in a different study utilizing MEFs, it was shown that intracellular levels of ROS are increased in hypoxia and essentially required for hypoxic activation of HIF-1α, which in turn leads to ultimate extension of replicative life span (Welford et al. 2006). These conflicting data on ROS may be due to the differences in hypoxic conditions or cell types, but its involvement in the modulation of cellular senescence or the regulation of hypoxia cannot be underestimated. Of note, ROS has been reported to play a dual role by contributing simultaneously to two signaling pathways that have opposite functions in tumorigenesis, namely, RAS-RAF-MEK1/2-ERK1/2 and the p38 mitogen-activated protein kinases (MAPK) pathways (Hutter et al. 2002). RAS-RAF-MEK1/2-ERK1/2 signaling is extensively linked to oncogenesis, whereas the p38 MAPK pathway promotes cancer suppression implicating oncogene-induced senescence, inflammation-induced senescence, replicative senescence, contact inhibition, and DDR (Ogrunc et al. 2014; Hutter et al. 2002). Thus, these studies suggest that ROS may not be considered as an absolute tumor-promoting or -suppressing factor.

In line with others, our group has also shown that hypoxia prevents Ras-provoked senescence *(RASV12)* in human diploid fibroblasts (HDFs) involving HIF1α governed p53 and $p21^{CIP1}$ downregulation and reduced DDR (Kilic Eren and

Tabor 2014). As previously shown, HIF1α and p53 proteins may have direct interactions, mostly allowing HIF-1α-dependent p53 stabilization or p53-dependent HIF-1α degradation (Kilic Eren and Tabor 2014; Blagosklonny et al. 1998). Accordingly, downstream effectors of p53 and HIF-1α may also regulate each other reciprocally (Goda et al. 2003; Hammer et al. 2007). As confirmed in replicative senescence, MIF as a target of HIF-1α directly binds and inhibits p53 and $p21^{CIP1}$ (Fig. 5.3) (Welford et al. 2006).

In studies conducted under normoxic conditions, $p16^{INK4a}$ is widely accepted as an essential modulator of oncogene-induced senescence and found to be upregulated predominantly through its implication of the RB pathway (Collado and Serrano 2006; Collado et al. 2005; Collado and Serrano 2005). In contrast, studies accomplished in hypoxia presented intriguing data showing the expression of $p16^{INK4A}$ is downregulated in decreased oxygen levels (Kilic Eren and Tabor 2014). Our group reported that $p16^{INK4A}$ is downregulated in RASV12-expressing HDFs in an HIF1-α-independent manner in hypoxic conditions (Fig. 5.3) (Kilic Eren and Tabor 2014). Additionally, in another study hypoxia/anoxia (0.1% O_2) downregulated the expression of $p16^{INK4A}$, depending on constitutive activation of PI3K/Akt but not HIF1α (Box and Demetrick 2004). Thus, inactivation of the $p16^{INK4A}$-Rb and p53-p21 tumor suppressor pathways is essential for overcoming OIS. Of note, previous studies substantiated $p16^{INK4A}$ and p53 are mutated, and/or inactivated that in most human cancers either in the initial stage or during the progression of tumorigenesis (Sarkar et al. 2000; Rivlin et al. 2011). For example, senescence can be induced in noninvasive neoplastic papillary bladder cells via activation of the $p16^{INK4A}$-Rb pathway in vitro, but this is not possible in aggressive bladder carcinoma cells due to the inactivation of $p16^{INK4A}$-Rb and p53 signaling while cells developing into aggressive carcinoma (Sarkar et al. 2000; Romagosa et al. 2011).

DDR via ATM/ATR kinases is a key mediator of all types of senescence including OIS (Di Micco et al. 2011), However, whether or not it is implicated in the prevention of senescence in hypoxic conditions is still not completely elucidated. In general, particularly low levels of hypoxia (0.1% O_2) were shown to persuade DDR, in which both ATR and ATM kinases interceded signaling activated to mediate subsequent induction of p53-dependent apoptosis (Hammond et al. 2002; Hammond and Giaccia 2004). Several studies reported the effect of hypoxia on DDR and its relationship with cell cycle arrest or senescence (Kilic Eren and Tabor 2014; Hammond and Giaccia 2004). Our group previously reported that hypoxia decreased the phosphorylation levels of key mediators of DDR, including ATM, ATR, and Chk1 and Chk2 kinases in RASV12-expressing HDFs, suggesting a role in bypassing OIS under hypoxic conditions (Kilic Eren and Tabor 2014). Thus, the DDR barrier prevailing the induction of senescence upon oncogenic stress emerges as another mechanism that must have been successfully inactivated in cells that have fully undergone malignant transformation. Indeed, the defects in the DDR barrier, such as ATM and p53 inactivating mutations, have been frequently shown in human cancers (Bartek 2011; Bartek et al. 2007a, b). Remarkably, overexpression of HIF-1α has been demonstrated in premalignant, malignant, and metastatic lesions in the most common types of cancer (Zhong et al. 1999; Lu and Kang 2010;

Semenza 2000). In particular, the expression of HIF1α in premalignant tissues in the early stages of cancer formation even before the onset of histological initiation of angiogenesis or invasion is exceptionally intriguing (Zhong et al. 1999). From this point of view, it is plausible that the expression of HIF1α in premalignant tissues may create a selective pressure in cells and may emerge as an antagonistic mechanism contributing to bypass senescence to further promote progression into the full malignancy.

SASP is one of the hallmarks of senescence comprising the secretion of various inflammatory and immune-modulatory cytokines, growth factors, and cell surface molecules as mentioned earlier. It is evident that SASP is per se a multifaceted process employed in the promotion of complex processes, including malign transformation and paracrine and autocrine responses of senescence (Acosta et al. 2013). In previous studies, the SASP factors such as chemokine receptor CXCR2 and its ligands IL-8 and GROα have been identified as key mediators of senescence and knockdown of these factors leads to a bypass of senescence (Lee et al. 1999). Additionally, inflammatory cytokines IL-6 and IL-8 are found to be critical for oncogenic BRAF-induced senescence (Hutter et al. 2002). Interestingly, among those SASP factors IL-6, IL-8, CXCR-2 PAI1, and GROα are regulated by hypoxia as targets of HIF1α in different settings (Box and Demetrick 2004; Sarkar et al. 2000; Rivlin et al. 2011; Romagosa et al. 2011). In addition, recently, hypoxia has been shown to downregulate the detrimental pro-inflammatory SASP factors in cultured cells and tissues through AMPK-mediated mTOR suppression (Romagosa et al. 2011). Thus, hypoxia may regulate several senescent-promoting factors or pathways through HIF-1α in a context-dependent manner.

Loss of VHL, the primary regulator of HIF-α in aerobic condition, has been also associated with induction of cell cycle arrest and senescence involving pRB through HIF1α, p400, and $p27^{CIP1}$ in mouse fibroblasts (Ohh et al. 2000; Young et al. 2008), which can be reversed by the increased oxygen levels (Welford et al. 2010). Likewise, in renal epithelial cells where moderate hypoxic condition is present (~10-50mm Hg) in vivo, loss of VHL was found to be inadequate to induce senescence, whereas addition of oxidative stress to existing conditions was sufficient (Young et al. 2008; Welford et al. 2010). These data suggest that in vitro in atmospheric oxygen conditions the mechanisms that are sufficient to induce senescence may not be sufficient under low oxygen tension in vivo.

In conclusion, previous studies have shown that cells cultured under hypoxic conditions are capable of bypassing oncogene-induced senescence through the modulation of main factors such as HIF1α, p53, $p21^{CIP1}$, and $p16^{INK4A}$ despite the fact that the modulation may depend on the severity of the hypoxia, or the cell type (Welford and Giaccia 2011; Kilic Eren and Tabor 2014; Welford et al. 2006). Given that oncogene-induced-senescence is activated as an intrinsic tumor suppressor barrier in the cell, it is of great importance to a comprehensive understanding of the molecular mechanisms and identifies the mediators of the hypoxia or HIF-1α primed bypass of oncogene-induced senescence.

5.5 Conclusions and Future Perspectives

Mammalian tissues are heterogeneous in their oxygen-delivering capacity and internal oxygen levels, which can be further influenced by the intrinsic and extrinsic factors. Consequently, an evolutionarily conserved adaptation mechanism has been developed to adjust the lack of oxygen in the tissues.

Senescence is an essential part of life and emerges as crucial for both physiological and pathophysiological conditions. The contemporary senescence knowledge mainly relies on the experiments achieved through atmospheric oxygen levels. Obviously, varying levels of hypoxia exist in different tissues in living organisms and activation of HIF1 pathway can exert preventive effects on instigation of senescence triggered by various stimuli including oncogenic activation. In particular, in the context of OIS, it is to be expected that hypoxic areas will become more vulnerable to malignant transformation. Also, considering the well-known role of hypoxia on aggressive tumor behavior of and resistance to cancer therapy, modulation of tumor hypoxia or HIF1 may be beneficial to restore the tumor-suppressive function of senescence as well as to obtain better outcome from conventional treatment approaches. Further investigations directed to better understand the hypoxia-related mechanisms and factors involved in bypassing oncogene-induced senescence will certainly contribute to the development of novel and effective cancer treatment strategies..

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Hypoxia-Regulated Gene Expression and Metastasis

6

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Abstract

Mammalian cells have adopted mechanisms to regulate cellular oxygen levels for the maintenance of the cellular homeostatic balance. "Hypoxia" states the condition of insufficient oxygen supply at the tissue level. Commonly hypoxia is known as a characteristic feature of solid tumors playing an important role in supporting tumor progression and metastasis by promoting various processes such as angiogenesis or epithelial to mesenchymal transition (EMT). Hypoxia-inducible factors (HIFs) are the major transcription factors activated under hypoxic conditions and implicated in transcriptional activation and regulation of metastatic processes. In particular, HIF1α enables tumors to gain invasive and metastatic properties by regulating the major transcription factors leading to EMT. In this chapter, we reviewed the recent knowledge on hypoxia-regulated gene expressions including transcriptional factors, enzymes, extracellular matrix elements, and signaling molecules that are involved in the process of cancer metastasis.

Keywords

Hypoxia · EMT · HIFs · Metastasis · Gene expression

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6.1 Introduction

Eukaryotic organisms have evolved various mechanisms regulating oxygen tension to maintain cellular, tissue, and organ homeostatic balances. Mammalian cells require a physiological oxygen level (physioxia) of 4–9% to retain the necessary energy and aerobic metabolism. However, under various conditions, the oxygen level of human tissues or cells drops to the lower levels than physioxia, which is called "hypoxia." Hypoxia has an impact on the physiological process of embryogenesis or erythropoiesis, but also implicated in various pathological conditions such as heart attack, inflammatory diseases, and cancer (Shi et al. 2021; Wilson et al. 2020; Nakayama and Kataoka 2019).

Hypoxia is a characteristic of solid tumors, yet even hematological cancer types are affected by hypoxia (Chee et al. 2019). Each tumor body consists of a heterogeneous population of cells in its microenvironment (Tam et al. 2020). In a heterogeneous tumor population, the degree of hypoxia varies with the distance of the tumor from the tumor microvessels, with some areas being anoxic (0% oxygen) or even severely hypoxic (approximately 0.2% oxygen) (Shi et al. 2021).

Biological responses to hypoxia are quite extensive; adaptations of cancer cells to hypoxia involve a transition to anaerobic energy production via changes in gene expression. Hypoxia modulates processes such as lipid and glucose metabolism by increasing oxygen transport and delivery as well as glycolysis and glucose uptake, regulates apoptosis, and mediates a series of cellular responses, including invasion and metastasis of cancer cells. Consequently, readjustment of biological processes in hypoxia enables cancer cells to survive and adopt the hypoxic environment (Tam et al. 2020; Filippopoulou et al. 2020).

Metastasis is a multistep biological process starting with local invasion into the tumor-associated stroma and subsequently results in intravasation into the hematopoietic, lymphatic systems, or peritoneum. Cancer cells survive in the blood stream throughout this entire process and extravasate for its next step to pre-metastasis. Ultimately, all steps are accomplished by colonizing in distant organs to form metastatic niches and metastases. Cancer cells generally experience metabolic problems in terms of oxidative stress as well as nutrient and oxygen availability in the regions where they colonize by metastasizing (Wei et al. 2020).

For tumor cells proliferation, it is crucial to transport nutrients and oxygen to the cells creating the microenvironment. Thus, the vascularization around the tumor provides the cell required nutrients and the oxygen. This vascularization is generally irregular, does not have adequate function, and has a leaky structure, leading to insufficient vascular perfusion. Hypoxia-inducible factor (HIF) is the major transcription factor activated under hypoxia that provides transcriptional activation of various genes that are implicated in adaptation of the cellular responses to hypoxia in tumor microenvironment (Akanji et al. 2019; Zhang et al. 2021a).

6.2 Hypoxia-Inducible Factors

Hypoxia-inducible factor (HIF-1) is the main transcription factor activated under hypoxic conditions and also involved in the tumor microenvironment to mediate adaptive cellular responses (Lv et al. 2017). The HIF-1 protein consists of two subunits; while the α subunit is regulated by oxygen and stabilized only under hypoxia, the β subunit (HIF-1β or ARNT) is constitutively expressed (Pilevneli and Kilic-Eren 2021). Among the three types of HIF-α, HIF-1α is the most intensively studied hypoxia-inducible factor. HIF-1α and HIF-2α are sensitive to varied levels of hypoxia as they harbor different prolyl hydroxylase domains. In severe hypoxia (0–2%), HIF-1α is accumulated, while under moderate hypoxia (2–5%) HIF-2α exhibits more persistent expression. Functions of the other HIF-α variant HIF-3α are based on the layout of other HIF complexes (Tao et al. 2021).

In normoxic conditions, HIF1-α, which contains an oxygen-sensitive domain, namely prolyl hydroxylase domain protein 2 (PHD2), becomes hydroxylated by prolyl hydroxylases and thereby recognized by E3 ubiquitin ligase complex involving the von Hippel–Lindau protein for degradation. In hypoxia, however, the HIF-1α protein dimerizes with the HIF-1β subunit and becomes stabilized and allows to translocate to the nucleus to induce expression of various genes, implicated in angiogenesis and glycolysis, to promote cell survival and metastasis (Reczek and Chandel 2017).

HIFs and tumor hypoxia are involved in numerous hallmarks of cancer, such as genomic instability, immune evasion, cell proliferation, metabolism, apoptosis, invasion, vascularization, and metastasis. Implication of HIFs in regulation of cellular processes in cancer cells hypoxia also endorses resistance to chemotherapy and radiotherapy. HIFs' expressions have been clinically associated with the relapse and poor prognosis in cancer therapy as well. Furthermore, HIFs are recognized among the molecular targets that can be employed in the clinical field in order to improve the treatment of metastatic and treatment-resistant tumors (Wigerup et al. 2016).

6.3 Hypoxia-Induced Regulators of Metastasis

6.3.1 Epithelial to Mesenchymal Transition

Metastasis is the most important process complicating the treatment modalities and ultimately leading to death in cancers. Hypoxia has an important role in terms of the induction and regulation of the various steps of metastasis. The metastatic process in tumor cells consists of multiple steps starting with the epithelial to mesenchymal transition (EMT) and results in colonization (Tsai and Wu 2012). EMT plays a substantial role in diverse physiological processes, including embryo implantation, wound healing, and inflammation, but also it enables the transformation of epithelial cancer cells into mesenchymal cells, which mainly provides tumors' acquisition of invasive and metastatic properties. EMT is a reversible process in which epithelial

features of malignant tumors are lost for supporting the mesenchymal properties, including improved migratory potential and invasiveness. The process of transition comprises the downregulation of epithelial cell markers, such as E-cadherin, and subsequent upregulation of mesenchymal factors such as N-cadherin, vimentin, fibronectin, several matrix metalloproteases (MMPs), and integrins (β1 and β3) (Tsai and Wu 2012).

EMT causes cancer cells to acquire invasive and metastatic features mainly by activation of the specific transcriptional factors, cell surface proteins, and extracellular matrix (ECM) enzymes (Tam et al. 2020). Hypoxia is one of the major regulators of EMT implicated in the promotion of metastasis. Hypoxia impacts the process of EMT through several ways, including direction of signaling pathways implicated in EMT, control of the expression of EMT-associated TFs, and coordination of EMT-associated miRNA and lncRNA interplays. Hypoxia activates a number of signaling pathways implicated in induction of EMT, including TGF- β, NF-κB, Notch, and Wnt.

6.3.2 Signal Mediators of Hypoxia-Regulated EMT

As mentioned earlier, hypoxia-regulated EMT, metastasis, and invasion of tumor cells are governed by signaling pathways such as TGF-β, Wnt, Jagged/Notch19, PI3K/Akt, and AMPK (Tirpe et al. 2019; Saxena et al. 2018). Activation of AMPK under hypoxic conditions has a supporting role in EMT and metastasis (Saxena et al. 2018), whereas TGF-β signaling executes EMT-mediated progression, tumor immunity, and organ fibrosis (Lin et al. 2020). The TGFβ signaling pathway is activated in a variety of important developmental processes such as cell proliferation, cell differentiation, morphogenesis, tissue homeostasis, and regeneration. In developing cancers, TGFβ functions as a tumor suppressor in early steps of the disease. In later stages, TGFβ lose the growth inhibitory functions and may transform to an oncogenic protein that is recognized as an initial inducer of EMT (Hapke and Haake 2020). **TGF-β** increases cancer invasion and metastasis by inducing EMT. Under hypoxic conditions, HIF-1α expression in renal cell carcinoma increases TGF-β expression, and HIF-TGF-β interaction activates the EMT pathway (Mallikarjuna et al. 2019).

Notch signaling displays different functions in processes such as cell development and differentiation, cell proliferation, and cell death (Hori et al. 2013). Deregulation of Notch pathway is associated with different types of cancers, with either oncogenic or tumor suppressor properties in a context-dependent manner (Moon et al. 2021). Upon **Notch** receptors binding to its ligand, the activated Notch intracellular domain (ICD) is fragmented and translocates to the nucleus, where it transactivates a variety of target genes. In cancer cells, Notch1 activation was found to induce EMT (Zhang et al. 2017). Notch1's role in EMT is complicated, but mainly depends on its interactions with EMT transcription factors (TFs) SNAIL1 and SNAIL2, accompanied by TGFβ pathway activation (Sahlgren et al. 2008). Hypoxia signaling shares similarities with Notch signaling and suggests a functional

cooperation or interaction between the two of them. Hypoxia activates Notch signaling by alleviating Notch1 ICD and enhancing the expression of Notch ligand, Jagged2. Conversely, Notch ICD may function as a competitive inhibitor of FIH-1, which is likely to be responsible for the increase in HIF activity upon Notch activation. Hypoxia-induced Notch activation stimulates EMT in human cancer (Chen et al. 2010). In addition, hypoxic tumor cells entail Notch signaling for induction of EMT and implies that Notch is essential for hypoxia-induced EMT under different circumstances (Sahlgren et al. 2008). It stimulates HIF-1α-mediated proliferation, migration, and invasion via TLR4/MyD88 (Toll-like receptor 4/myeloid differentiation primary response 88) pathway in hypoxia-induced hepatocellular carcinoma (HCC). The TLR4/MyD88/NF-kB pathway participates the process by facilitating the proliferation, invasion, and migration of HCC cells, along with the stabilization of TLR4 by hypoxia-induced ubiquitin-specific peptidase 13 (USP13) (Gao et al. 2020). NOTCH leads to hypoxia-induced EMT with tumor invasion and migration via SLUG and SNAIL (Tian et al. 2015; Liu et al. 2018). NOTCH ligand Jagged2, urokinase-type plasminogen activator receptor (Dudonne et al. 2011), and cyclooxygenase-2 (COX-2), which is one of the hypoxia-induced biomolecules, contributes to the invasion and EMT of breast cancer cells (Liu et al. 2015).

NF-κB and **β-catenin** pathways mediate the hypoxia-induced EMT as well as the invasive and metastatic properties of the tumors in hepatocellular carcinoma cells (HCCs) (Guo et al. 2020). HIF-lα-induced activation of **histone lysine-specific demethylase 4B** (KDM4B, JMJD2B) is involved in epigenetic regulation and increase of invasion and metastasis in colorectal cancers (CRC) (Glaser et al. 2020). JMJD2B is also associated with breast cancer and lung metastasis (Luo et al. 2012). **Myc** is known to regulate the expression of several genes that control the variety of cellular processes, including cell cycle, cell growth, cell death, and differentiation (Dang 2013). In tumors, Myc is often constitutively expressed and promotes cell cycle progression; however, in adaptation to low levels of oxygen, Myc is negatively regulated by HIF-1α (Li et al. 2020).

uPAR is a GPI-anchored cell membrane receptor that mediates hypoxia-induced invasion (Nishi et al. 2016). uPAR is involved in invasion and metastasis as a member of the protease system. Hypoxia promotes cell invasion and EMT by upregulating uPAR expression and activating AKT and RAC1 downstream signaling pathways. uPAR is activated upon binding to its ligand urokinase plasminogen activator (uPA), thereby providing plasminogen activity. Plasminogen activator inhibitor-1 (PAI-1), one of the well-characterized endogenous inhibitors of uPA urokinase, also supports angiogenesis and tumor metastasis (Mahmood et al. 2018; Peterle et al. 2018). A 67-kDa laminin receptor (67LR)-mediated increase in uPA, MMP-9 (matrix metalloproteinase-9), and TIMP-1 (tissue inhibitor of matrix metalloproteinase protein) expressions in hypoxic conditions induces gastric cancer metastasis (Liu et al. 2010). Another receptor that plays a role in invasion and metastasis via HIF-1α is the RON tyrosine kinase receptor that affects the invasion and migration of tumor cells in the ECM and blood vessels (Kato et al. 2021). A different signal player controlled by hypoxia through a regulation of HIF1 or HIF2 is the expression level of IRS2. Studies on metastatic breast cancer cell lines have

shown that IRS2 plays a role in tumor survival and invasion in hypoxic conditions (Tsai and Wu 2012).

Ion channels and transporters are among those that play a significant role in the processes of proliferation, migration, invasion, and apoptosis of human tumor cells in hypoxia. **Chloride intracellular channel 1 (CLIC)** is an intracellular channel associated with p64 and participates in the invasion and metastasis of gastric cancer cells by regulating hypoxia and reoxygenation-induced intracellular ROS. **Sodium hydrogen antiporter 1 (NHE1)** is important in various biological processes of the cell, such as regulation of cell volume and intracellular pH. NHE1 expression level, responsible for regulating the pH, is found to be higher in gastric mucosa. In gastric cancers, NHE1 activation and upregulation under hypoxic conditions are implicated in tumor cell migration and invasion (Chen et al. 2021).

6.3.3 Transcription Factors of Hypoxia-Induced EMT

Hypoxia-induced EMT promotes the invasive and metastatic potential of cancer cells in particular by decreasing the epithelial-associated gene expression and increasing the mesenchymal-associated gene expression (Muz et al. 2015). Hence, HIF-1α plays a significant role in hypoxia-mediated promotion of metastatic potential by activating EMT-associated transcription factors (TF), including **SNAIL1**, **SNAIL2** (SLUG)**, TWIST1, E12/E47 and zinc finger E-box binding homeobox 1 (ZEB1),** and **2 (ZEB2)** (Peng et al. 2021). Among those, SNAIL1, SLUG, TWIST1, and ZEB1 contain HRE sequences in their promoters, suggesting a direct interaction with HIF-1α.

SNAIL1 is an indispensable factor for development and is involved in mesoderm formation. SNAIL1 upregulates the expression of crucial mesenchymal factors such as fibronectin (FN1) and matrix metalloproteinase 9 and downregulates the E-cadherin expression, thereby inducing EMT. SNAIL1 expression was found to correlate with HIF-1α expression and increased the invasiveness of liver and ovarian cancer cells (Hapke and Haake 2020).SNAIL1 and SLUG's joint action repressing the transcription of E-cadherin contributes to the induction of EMT. Expression of SLUG was found to be correlated with HIF-1α expression in head and neck squamous carcinoma, lung, and pancreatic cancer cells (Tam et al. 2020). TWIST1 is a basic helix–loop–helix transcription factor and also plays a crucial role in metastases. Previously, it was shown that hypoxia-induced EMT is associated with increased expressions of HIF-1α and Twist1. HIF-1α-induced increased expression of TWIST1 promotes invasion and metastasis in various types of cancers, including stomach, pancreas, breast, non-small-cell lung, nasopharyngeal, prostate, and uterine cancers under hypoxic conditions (Yang and Wu 2008). HIF-lα-induced TWIST expression has been shown to be responsible for promoting EMT in thyroid cancer cells as well (Yang et al. 2015)**. ZEB1** is another transcription factor that is known to support tumor invasion and metastasis by promoting EMT in cancer cells. HIF-1α and ZEB1 are both accepted as tumor-initiating factors, but studies also reported that ZEB1 is a downstream target of HIF-1α and upregulated in various cancers such as

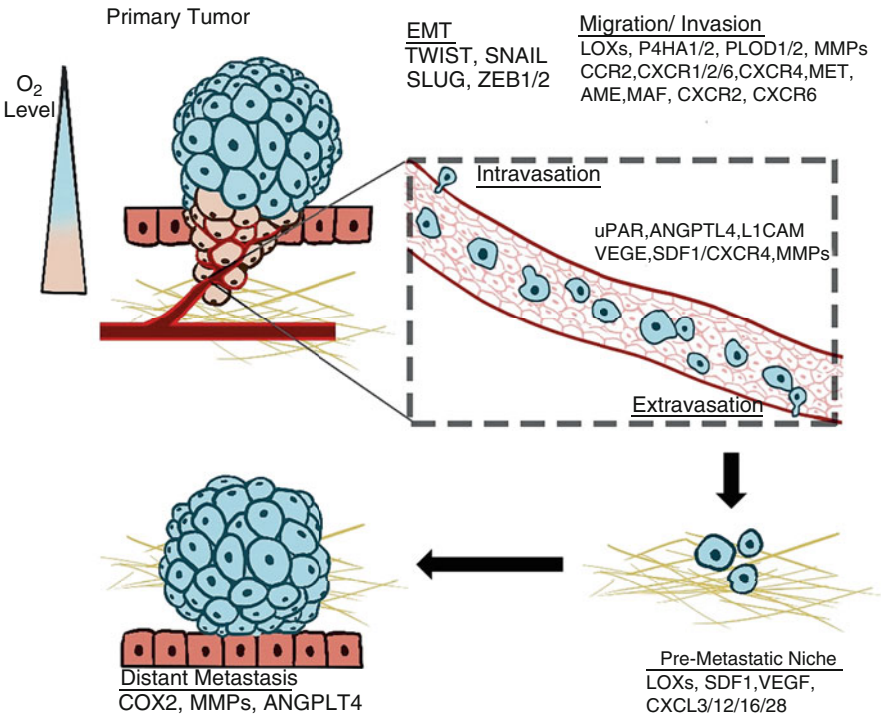

Fig. 6.1 Hypoxia controls multiple steps in cancer metastasis. Hypoxia-induced stages of tumor metastasis and associated metastatic players including epithelial–mesenchymal transition (EMT), invasion, intravasation, circulation, extravasation, pre-metastatic niche, and distant metastasis are shown

colorectal (Zhang et al. 2015), bladder cancer (Zhu et al. 2018), or ameloblastic carcinoma (AC) (Yoshimoto et al. 2019). In AC cells, in hypoxia ZEB1 and HIF-1α persuades the induction of TGF-β, which results in increased EMT (Yoshimoto et al. 2019). Inhibitor of differentiation/DNA binding 2 (ID2) is another factor regulated by HIF-lα, which causes progression of neuroblastoma cells (Lofstedt et al. 2004) (Fig. 6.1).

Zinc finger E-box binding homeobox 2 (ZEB2) is a transcriptional regulator that downregulates E-cadherin and other epithelial genes. HIF-1α and transforming growth factor ß (TGFß) regulate EMT-associated ZEB2 expression in tumors, and overexpression of ZEB2 promotes cancer metastasis (Fardi et al. 2019). In renal cell carcinoma (RCC), HIF-1α activates ZEB2 transcription that binds to the promoter of the E-cadherin-encoding gene and activated ZEB2 inhibits E-cadherin transcription, thereby activating EMT in cancer (Krishnamachary et al. 2006).

6.3.4 Hypoxia-Regulated Enzymes in Cancer Invasion and Metastasis

Lysyl Oxidase (LOX) is an HIF1α-dependent and hypoxia-induced extracellular matrix (ECM) remodeler protein secreted by tumor cells and creates a "pre-metastatic niche" in hypoxic tumors (Chan and Giaccia 2007). Breast cancer cells often secrete LOX enzyme into the circulation. After localization of LOX in the metastatic tissue in bone marrow-derived cells, a premetastatic niche is formed as a result of the collagen cross-linking of LOX enzymes in metastatic tissues (Liu et al. 2015). Hypoxia-induced carbonic anhydrase IX (**CAIX)** is an enzyme commonly found in tumors and activated in acidic conditions. CAIX promotes the invasion and metastasis ability of tumor cells by disrupting the basal membrane structure. In addition, CAIX inhibition has been shown to reduce tumor metastasis in breast tumors (Ward et al. 2015). **Hypoxia-induced migration inhibitor factor** (MIF) is another enzyme involved in COX-2 and PGE2 upregulation, in angiogenesis and tumor proliferation, as well as invasion and metastasis (Conroy et al. 2010). Hypoxia-induced **Supervillin** also promotes metastasis and invasion by activation of RhoA/ROCK and ERK/p38 signaling pathway. In addition, hypoxia-induced supervillin accelerates HCC metastasis by regulating the expression of EMT genes. The increase in hypoxia-induced supervillin expression causes an increase in HCC metastasis, leading to poor survival in patients (Chen et al. 2018). **Glutaminase 1** (GLS1) is known to be an enzyme that ensures the rapid proliferation of cancer cells. In hypoxic conditions, HIF1α increases mRNA and protein expression of GLS1. Hypoxia-induced GLS1 expression in colorectal cancer cells causes tumor growth, invasion, and metastasis (Xiang et al. 2019). **Growth and differentiation factor 15** (GDF15) is a member of the TGF-β superfamily. Circulating levels of GDF15 have been reported to be elevated in a variety of cancers, including pancreatic, colorectal, endometrial, and prostate cancers. Hypoxia-mediated ER stress and PERK-eIF2a activation in CRC cells promotes metastasis by regulating GDF15 expression, which is thought to be involved in EMT (Zheng et al. 2020). Increased HIF1α expression in breast cancers causes an increased risk of metastasis and cancer-related deaths. Recently, the contribution of HIF1α-induced disintegrin and metalloproteinase-12 (**ADAM12)** in increasing breast cancer metastasis was demonstrated. Activation of HIF1α-dependent ADAM12-mediated EGFR-FAK signaling leads to cell migration, invasion, and distant metastasis. HIF-1α-dependent ADAM12 signaling induces cell motility and invasion through the extracellular matrix under hypoxic conditions (Wang et al. 2021)**.**

It has been also shown that there is a significant correlation between HIF-1α and matrix metalloproteinases (**MMPs)** in various cancers. In ovarian cancers, a correlation between HIF-1α **MMP13** expression in metastatic lesions has been reported. In addition, the expression of MMP13 was found to be significantly higher in in A2780 ovarian cancer cells in hypoxic conditions than in the normoxic conditions. Of note, suppression of HIF-1α expression by siRNA suppresses the expression of MMP13 despite the hypoxia suggesting MMP13 as a HIF-1 α-dependent enzyme. Thus, HIF-1α is an important factor affecting the invasion and metastasis of ovarian cancer

by modulating MMP13 expression under hypoxic conditions (Zhang et al. 2019). Other studies have also reported that HIF-1α upregulates the expression of MMP-2 and MMP-9 (Liu et al. 2015).

COX-2 has been also associated with cancer invasion and distant metastasis. COX-2 participates in EMT regulation together with HIF-1α and plays an important role in metastasis. HIF-1α-mediated COX2 increases proliferation and metastasis of ovarian cancer (Ding et al. 2021)**.** HIF-1α increases COX-2 expression and promotes EMT in hepatocellular tumors, resulting in enhanced invasion of HepG2 cells under hypoxic conditions (Huang et al. 2016). Expressions of pro-collagen prolyl (P4HA1, P4HA2) and lysyl (PLOD1 and PLOD2) are known to be increased in breast cancer metastases. HIF-1α was found to be responsible for the increased expression levels of **PLOD1**, **PLOD2, P4HA1,** and **P4HA2** in breast cancer cells (Liu et al. 2015).

6.3.5 Hypoxia-Regulated Chemokines in Cancer Invasion and Metastasis

Chemokines are divided into four groups depending on the positioning and the number of the first two conserved cysteine motif: C, CC, CXC, and CX3C61. Hypoxia-regulated chemokines are associated with metastasis and cancer progression (Semenza 2016). Hypoxic condition in ovarian cancer was shown to induce the expression of the chemokine ligands **(CCL2/CXCL12)** and receptors **(CCR2/CXCR1-2-4)**. In prostate cancer cells, hypoxia mediates the expression of **CX3CR1** and **CXCR6**, which are involved in invasion and migration61. CX3CR1 expression is regulated by HIF-1α in primary and metastatic ovarian cancer cells. High levels of CX3CR1 expression responsive to CX3CL1 (Fractalkin/chemokine ligand 1) lead to progression and metastasis of ovarian cancer63. Chronic hypoxia affects the expression of **CXCL12**; however, the level of expression is different among various cancer types (Wong and Tran 2020; Fujikuni et al. 2014; Gilkes 2016; Xin et al. 2016; Theys et al. 2011; Chang et al. 2021). It has been reported that in hypoxia the expression of CXCL12 is decreased in melanoma and ileal carcinoids but increased in cervical cancer cells and is not changed in breast cancer69. Furthermore, chronic hypoxia increases the expression of **CXCR4** via HIF-1α in multiple myelomas. CXCR4 stimulates the migration of myeloma cancer cells via the CXCL12→CXCR4 pathway and allows them to metastasize by colonization in the bone marrow. Upregulation of CXCL12, primarily controlled by HIF-2 and partly by HIF-1, causes angiogenesis and osteoclastic bone resorption in the bone marrow. Increase in **CCR1** expression, together with the hypoxia-mediated increase in HIF-2, results in the emergence of multiple myeloma cells (Hao and Li 2020). **CCL3** (CC-motif chemokine ligand 3), a CCR1 ligand, reduces the response of multiple myeloma to CXCL12 and consequently leads to the migration of these cells from bone marrow. Increased expression of CXCR4 in chronic hypoxia promotes proliferation, invasion, and migration of cancer cells (Chang et al. 2021). Hypoxia is one of the mechanisms responsible for the high metastatic potential of diffuse-type gastric cancer to the peritoneum by controlling upregulation of CXCR4 via the

CXCL12/CXCR4 axis71. CXCR4, a chemokine receptor regulated by HIF, directs metastasis in breast cancer through binding to stromal-derived factor-1 (SDF-1α) in primary and metastatic tumor cells14. Increased expression of CXCL12 mediated by HIF signaling not only stimulates bone metastasis in osteoblast-lineage cells, but also stimulates breast cancer growth and spreads to other secondary organs72. Another chemokine ligand controlled by hypoxia and HIF-1 is **CXCL16**. Regulation of CXCL16 expression in chronic hypoxia is tightly controlled in tumor metastasis. Increased expression of CXCL16 in breast cancer cells in chronic hypoxia affects the migration of cancer cells by increasing the expression level of **CXCR6** due to HIF-1α. Various studies have shown that upregulation of CXCR6 increases migration in renal cell carcinoma, prostate cancer, and breast cancer. In addition, expression level of CXCR6 is found to be higher in metastatic tumors, including cervical cancer, Ewing's sarcoma, nasopharyngeal carcinoma, ovarian carcinoma, melanomas, and papillary thyroid cancer in comparison to human primary tumors (Nath et al. 2013) (Fig. 6.1).

6.3.6 Hypoxia-Regulated Adhesion Molecules in Cancer Invasion and Metastasis

Changes in the expression levels of various membrane proteins through hypoxia or HIF play major roles in cancer metastasis. In hypoxic breast cancer cells, HIF1-dependent upregulation of **L1 cell adhesion molecule (L1CAM/CD171)** and **angiopoietin-like 4 (ANGPTL4)** lead to vascularization-mediated metastasis of cells to the lungs. ANGPTL4 stimulates extravasation of breast cancer cells by binding to receptors on endothelial cells (EC) and thereby inhibits EC–EC interactions. In vivo studies have shown that inhibition of L1CAM, which is responsible for binding to vascular endothelial cells, prevents lung metastasis in breast cancer (Semenza 2016). ANGPTL4 and L1CAM adhesion molecules modulate intra- and extravasation (Wigerup et al. 2016). Expression levels of various integrin proteins are increased under hypoxic conditions in an HIF-dependent manner as in increased expression of α5β1-integrin, related to lymph nodes and lung metastasis of breast cancer cells by HIF-1 and HIF-2 (Yousefi et al. 2021). Cluster of differentiation membrane proteins **CD151**, **CD24,** and **CD147** are also involved in hypoxia-mediated cancer metastasis (Tsai and Wu 2012). Hypoxic conditions were shown to regulate cell adhesion molecules and metastasis via regulation of CD151 expression. In hypoxia, CD151 expression was downregulated in colorectal cells by HIF-1α activity (Wong and Tran 2020). The studies investigating P-selectin ligand CD24 showed that shRNA-mediated suppression of CD24 reduction in metastasis of breast cancer cells. CD24 also functions as the L1CAM interacting protein, and its overexpression in breast cancer has a potential role in HIF-1 regulation (Fujikuni et al. 2014; Gilkes 2016). **CD147/EMMPRIN**, a member of immunoglobulin family, mediates the secretion of MMP from cancer cells, endometrial cells, and fibroblasts, causing degradation of ECM and basement

membrane and promoting tumor proliferation, invasion, and metastasis (Xin et al. 2016).

E-cadherin is an important transmembrane protein that ensures epithelial cell–cell adhesion. E-cadherin loss has been found to play a major role in metastasis and invasion in renal cancers (Theys et al. 2011). In hepatocellular cancer cells (HCC) cells, HIF-1α binds to the SNAL1 promoter-activating EMT and decreasing expression of E cadherin, increasing expression of N-cadherin and vimentin, which results in instigation of metastasis (Guo et al. 2020). Overexpression of **desmoglein2** (DSG2), a desmosome-mediated intercellular adhesion molecule, has been found to promote tumor growth and promote metastasis. Hypoxia-mediated downregulation of DSG2 leads to an increase in the expression of epithelial–mesenchymal transition (EMT) genes that further encourage cells of the primary tumor to undergo intravasation (Chang et al. 2021)**. EFNA1** (Ephrin A1), a membrane protein anchored to the cell surface by GPI linkage expression, is induced by hypoxia through HIF-1α and HIF-2α, triggering HIF-dependent angiogenesis in tumors. EFNA1 also contributes to the regulation of HIF-2α by binding to the HRE on the HIF-2α promoter and thereby upregulating its expression level (Hao and Li 2020).

Hypoxia-mediated increased **galectin-3** expression also plays a role in breast cancer progression and metastasis. Overexpression of galectin-3 has been reported in hypoxic regions of primary tumors that is associated with metastasis and further plays a role in increasing tumor aggressiveness in vivo (de Oliveira et al. 2015). **MUC1** is an O-glycoprotein membrane-bound mucin that is upregulated directly by HIF-1α in hypoxic conditions and was shown to be responsible for initiating metastasis and angiogenesis. *MUC1* gene overexpression in hypoxia is associated with metastasis and poor prognosis in patients with pancreatic, colon, and breast cancer (Khodabakhsh et al. 2021; Nath et al. 2013; Thompson et al. 2006).

Semaphorins in transmembrane and GPI-linked glycoproteins (Sema3A, Sema3B, Sema3C, Sema3E, Sema3F, Sema4B, and Sema4D proteins) are involved in tumor growth, metastasis, and vascularity in relation to hypoxia. Investigations on prostate cancer delineated the importance of Sema3 family proteins *Sema3A, B, C, D, E,* and *F* genes comprise HRE sequences at their promoters. While Sema3C upregulation induces metastasis downregulation of Sema3A and 3E inhibits metastatic spread, suggesting that Sema3 family proteins play important roles in the progression of prostate cancer in hypoxia. A recent study showing that hypoxia induces Sema4B overexpression and inhibition of Sema4B causes increased invasion in lung carcinoma suggests that Sema4B is also important in suppression of growth and metastasis of NSCLC. Inhibited *Sema4* gene leads to increased invasion in lung carcinoma, whereas induction of Sema 4B by hypoxia causes overexpression and thus suppresses invasion in cancer cells. Sema4D has been shown to play a role in tumor growth and vascularization in an HIF-dependent manner in head and neck squamous cancer and epithelial ovarian cancer (Valentini et al. 2021; Lontos et al. 2018). HIF-1α-mediated expression of Sema4D under hypoxic conditions inhibits osteoblast differentiation in osteolytic bone metastasis of lung cancer, along with ADAM17 (metalloproteinase 17) upregulation (Chen et al. 2019a). **Caveolin-1**

(CAV-1), a scaffold protein, is known to exert both metastasis-promoting and tumor-suppressing activities (Sanhueza et al. 2020). Hypoxia promotes invasion and metastasis by inducing HIF-1α-mediated CAV1 expression in metastatic cancer cells (Castillo Bennett et al. 2018). In hepatocellular carcinoma, hypoxia-induced CAV1 mediates tumorigenesis and metastasis (Chen et al. 2019b). The increased expression of CAV1 calcium-binding protein supports S100P-mediated metastasis (Mao et al. 2016).

NEDD9 (neural precursor cell expressed developmentally downregulated protein 9) and HEF1 is overexpressed in gastric cancer under hypoxic conditions. It regulates cell migration and metastasis depending on MICAL1 (microtubule-associated monooxygenase, calponin, and LIM domain containing 1) and RAC1 (Zhao et al. 2019). NEDD9 is also overexpressed in hypoxic regions of human colorectal cancers. NEDD9 is among the targets of HIF-1α downstream genes and causes cancer cell migration together with SOX2(Wang et al. 2019) (Fig. 6.1).

6.3.7 Hypoxia-Regulated Other Players Associated with Cancer Metastasis

Hypoxia and HIFs-regulated secondary signal players involved in cancer metastasis comprise small GTPases, chemokines, kinases, cell membrane receptors, ion channels, and trasporters (Tsai and Wu 2012). **Ras-related C3 botulinum toxin substrate 1 (RAC1)** and **cell division control protein 42 homolog (CDC42)**, plasma membrane-associated small GTPases promote tumor migration, metastasis, and progression in hypoxic conditions (Yang and Wu 2008). Loss of RAC1 has been shown to reduce HIF1α expression (Maldonado and Dharmawardhane 2018; Tatrai et al. 2017). Transcription levels of another small GTPase **RhoA** and its main upstream effector serine/threonine kinase Rho-associated helix-helix kinase (ROCK) are increased in breast cancer by hypoxia-inducible factor in the hypoxic microenvironment (Wei et al. 2016). Both RHOA and ROCK1 are associated with migration, invasion, and microvesicle formation in breast cancer cells. RHOA and ROCK1 mRNA levels were found to be increased in the metastatic breast cancer cell lines compared to the nonmetastatic cell lines. In addition, elevated RhoA and ROCK 1 protein levels were observed in metastatic biopsy specimens and connected with breast cancer progression. **RIOK3 (RIO3 kinase)** is a serine/threonine kinase regulated in an HIF-1α-dependent manner and promotes cancer cell migration, invasion, and metastasis by rearranging the actin cytoskeleton within breast cancer cells (Wigerup et al. 2016). Functional isoform 1 of the **FAM13A (Sequence Similarity Family 13 Member A)** protein that contains Rho GTPase-activating protein (GAP) domain is implicated in non-small-cell lung cancer (NSCLC) proliferation, migration, invasion, and apoptosis under hypoxic conditions. Knockdown of FAM13A in NSCLC cells negatively regulates proliferation, migration, and invasion by altering actin cytoskeleton under both normoxic and hypoxic conditions (Ziolkowska-Suchanek et al. 2021). Besides hypoxia-mediated induction of

metastasis, there is also an HIF-dependent control of metastasis that is interceded by various proteins such as HACE1, Parkin, p53, and GLS2.

HACE1 (Ankyrin repeat-containing E3 ubiquitin-protein ligase and HECT domain) acts as a tumor suppressor and decreases HIF1α accumulation by mediating degradation of RAC1 GTPase (Turgu et al. 2021). **Parkin**, the E3 ubiquitin ligase, plays a role in the inhibition of metastasis by directly binding and thereby leading to the degradation of HIF-1α in breast cancer (Liu et al. 2017). **p53** plays a significant role in suppressing cancer metastasis and invasion (Zhang et al. 2020). As a consequence of hypoxia and ROS, the mitochondrial glutaminase GLS2 enzyme is activated in a p53-dependent manner. Regardless of its enzymatic activity, GLS2 inhibits RAC1 by directly binding to the molecule and thereby leads to inhibition of metastasis (Zhang et al. 2021b). Molecular hallmarks of hypoxia and HIFs-induced tumor metastasis are shown in Fig. 6.2.

6.4 Conclusion

Adaptation of cancer cells to hypoxia involves the understanding of a number of biological processes, namely the transition to anaerobic energy production through changes in gene expressions. In hypoxia, biological processes are readjusted and cancer cells require an adaptation to survive. Hypoxia and HIFs contribute to the metastatic process in different levels. In particular, hypoxia-induced EMT plays a key role in cancer metastasis and entails diverse signaling pathways and transcriptional factors. Additionally, hypoxia-induced enzymes, small GTPases, chemokines, kinases, cell membrane receptors, signal mediators, ion channels, and transporters are implicated in metastasis. Currently, hypoxia-associated metastatic players and their individual relationship to the various stages of metastasis are known. Further investigations are needed for a better understanding of the interrelationships of these factors in order to identify their potential as a therapeutic target and develop new treatment strategies.

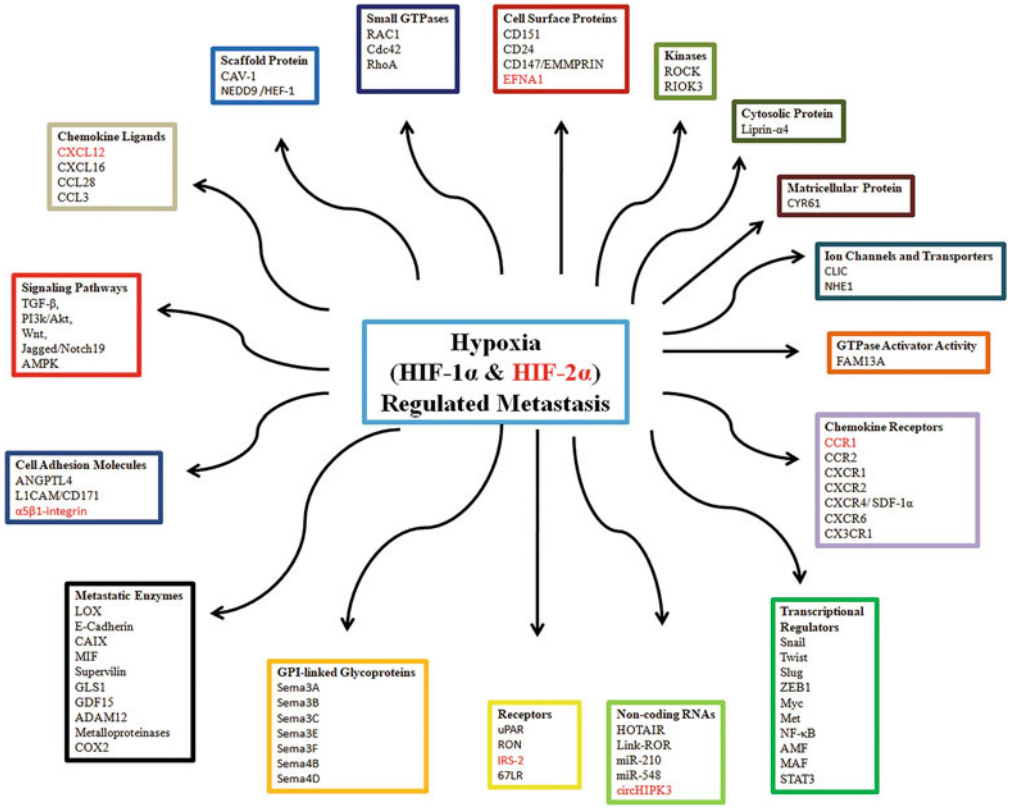

Fig. 6.2 Molecular hallmarks of hypoxia and HIFs-induced tumor metastasis. Hypoxia and HIFs regulate metastasis including transcriptional regulators, signaling pathways, metastatic enzymes, noncoding RNAs, etc.

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MicroRNA Signatures of Tumor Hypoxia

7

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Abstract

Micro RNAs (miRNAs) are regulatory RNA molecules that can act as oncogenes or tumor suppressors. Over the years, many studies have documented altered expression of miRNAs in different types of tumors in response to hypoxic conditions. The hypoxic microenvironment favors the selection of cancer stem-like cells that show features like indefinite self-renewal, chromosomal abnormalities, highly dysregulated differentiation, and tumor development. These factors favor the aggressiveness of tumors. So, the role of miRNAs in diagnosis, prognostication, and therapeutics of cancer is being explored by many researchers. In cancer therapeutics, different approaches like viral vectors, artificial biomolecules, locked nucleic acid, antisense anti-miR oligonucleotides, miRNA antagomirs, and miRNA sponges are being tried to revert the expression of miRNA back to its physiological state.

Keywords

Biomarkers · Cancer · Hypoxia · microRNA · Signature · Tumor

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7.1 Introduction

Around 70–80% of the human genes are transcribed to RNA. Out of this, only around 2% are coded to proteins. The remaining genome is made up of noncoding genes that get transcribed to noncoding RNA (ncRNA). ncRNAs comprise "long noncoding RNAs, microRNAs (miRNAs), PIWI-interacting RNAs, small interfering RNAs, and others." miRNAs are nonprotein-coding molecules containing about 24 nucleotides. They are not expressed to proteins. However, they are important as regulators of gene expression. These act as regulators in a number of cell processes, e.g., cell differentiation, proliferation, cell death, and development of cancer (Shen et al. 2013). miRNAs were initially discovered as small ncRNAs that regulate the timing of the development of larvae in Caenorhabditis elegans (Lee et al. 1993).

7.1.1 MicroRNA Biogenesis

The formation of miRNAs is a complex process and involves multiple steps. Long primary transcripts are processed by successive cleavage and maturation steps to form miRNAs. The major pathways of miRNA biogenesis are canonical pathway and noncanonical ones.

Canonical Pathway The formation of pri-miRNA by transcription of the noncoding region is the first step of miRNA synthesis. RNA polymerase II catalyzes this step. In the next step, the hairpin structure of pri-miRNA is cut by microprocessor complex that results in the formation of pre-miRNA. The microprocessor complex in turn is formed by the interaction of Drosha (RNase III enzyme) with the "Di George Syndrome Critical region 8" (DGCR8) gene. Further processing of pre-miRNA occurs after its transport to the cytoplasm by Exportin 5. There "RNase III Dicer" cuts the stem-loop structure of pre-miRNAs to form a double-stranded miRNA moiety, containing 18–24 nucleotides. "RNA-induced silencing complex" (RISC) is a molecular complex that is formed by Argonaute 2, Dicer, and other proteins. One strand of duplex miRNA remains incorporated into RISC. This strand is called guide or active strand. The other strand of the miRNA is subjected to degradation (Yoda et al. 2010). RISC binds to the target sequence on the mRNA in the 3' untranslated region. If miRNA base pairs perfectly with the mRNA target sequence, it promotes the degradation of mRNA. Imperfect pairing causes translational repression (Wightman et al. 2018). In various cancers, mutations are commonly found in genes coding for enzymes like Drosha, Dicer, Exportin-5, and Argonaute (Adams et al. 2014).

Noncanonical Pathway In this pathway, the proteins of canonical pathway like Dicer, Drosha are involved but combinations are different. There are two types of noncanonical pathways: Drosha/DGCR8-independent and Dicer-independent. miRtrons biogenesis is not dependent on Drosha/DGCR8 (Berezikov et al. 2007).

These pri-miRNAs are processed by splicing to form pre-miRNAs (Ruby et al. 2007). Pre-miRNAs are transported to the cytoplasm by Exportin-5. In cytoplasm, Dicer processes them to form mature miRNAs (Havens et al. 2012; Goymer 2007). The Dicer-independent pathway involves the cleaving of pri-miRNA to pre-miRNA by Drosha protein. Maturation of this miRNA is completed by loading it into Ago2 in the cytoplasm. For example, the maturation of miR-451 is not dependent on Dicer protein (Cheloufi et al. 2010). The biological roles of noncanonical miRNAs and their contribution to the development of cancer are not clearly understood.

7.1.2 Role of miRNA in Tumor Angiogenesis

The formation of new blood vessels, i.e., angiogenesis is important for physiological processes like the development of embryos and reproduction. It also plays an important part in pathological processes like the healing of wounds, tumor development, and progression. The various modes of development of new blood vessels in tumors are sprouting angiogenesis, intussusception, vasculogenesis, vascular mimicry, vessel co-option, or cancer stem-like cells differentiation into endothelial cells. The various factors regulating angiogenesis are growth factors, vascular genes, epigenetic mechanisms, and the expression of miRNAs. miRNAs regulating the various angiogenic mechanisms are called angiomiRNAs (angiomiRs) (Annese et al. 2020).

7.2 MicroRNAs

7.2.1 MicroRNAs in Cancer

Progressive DNA alterations play an important part in the development of cancers (Hanahan and Weinberg 2011). The initiation and growth of tumor are favored by angiogenesis, inflammatory mechanisms, and capacity of the tumor cells to evade immune response. Cancer is a disease of multifactorial etiology and involves both coding and noncoding parts of DNA. DNA mutations and epigenetic deregulation of the noncoding RNAs have been described in tumor progression. miRNAs have quickly evolved as a key molecular component. The role of noncoding genome in cancer development was first elaborated in chronic lymphocytic leukemia. The most common chromosomal abnormality in CLL (chromosome 13q14 deletion) was found to target miR-15 and miR-16 (Calin et al. 2005).

7.2.2 Biomarkers and Their Usefulness in Cancer Diagnosis

Biomarkers are very important for diagnosing and managing cancer patients. Each type of tumor has a specific microRNA signature that helps in its differentiation from the surrounding normal tissues. Based on these signatures, cancers can be

Table 7.1 Current clinical trials (open) using miRNA components in cancer diagnosis (ClinicalTrials.gov)

miRNA	Trial reference no.	Disease	Observation/intervention
Circulating	NCT04906330	Breast cancer	miRNAs released from the tumor cells into the bloodstream can be detected even when the tumor is undetectable by other methods
Circulating	NCT04965259	Liver cancer	Validating a panel of miRNAs for detecting hepatocellular carcinoma at an earlier stage in high-risk patients
miRNA-10b	NCT01849952	Glioma	Evaluating the expression levels of miRNA-10b in tumor, blood, and cerebrospinal fluid samples in patients with glioma
10 miRNAs	NCT04285476	Thyroid cancer	Validating a signature of 10 miRNAs to allow the stratification of the cytology of indeterminate type
Numerous	NCT04305366	Head and neck cancer	Investigating the presence of miRNA biomarkers in saliva, blood, fine needle aspirate, and tissue samples in patients with carcinoma of head and neck region and control group
miR_CPMRC	NCT04662996	Prostatic cancer	Studying the miRNA (MiR_CPMRC) expression to find the factors predicting the risk of resistance to treatment in metastasized castration-resistant prostate cancer
Circulating	NCT05146505	Ovarian cancer	Investigating miRNAs as probable biomarkers for diagnosing and assessing prognosis in high-grade serous ovarian cancer
miR200b	NCT03776630	Ovarian cancer and Endometrial cancer	Validation of the 5-miR index to assess the risk of metastasis to lymph nodes in ovarian cancer Prognostic value of the pre-/post-treatment changes in plasma concentration of miR200b with regard to progression-free survival

categorized into different prognostic groups. The circulating miRNAs are used as biomarkers for different cancers and this field is expanding rapidly. Currently, many observational studies and clinical trials are studying the importance of miRNA in diagnosing and assessing the prognosis of different cancer types. Some of these clinical trials are briefly summarized in Table 7.1.

7.2.3 Functions

miRNAs perform fine adjustments in the translation process according to cellular requirements and thus regulate gene expression. miRNAs strengthen the cellular processes by regulating the transcriptional processes (Ebert and Sharp 2012). miRNAs thus allow the cells to adapt to sudden, temporary changes in their microenvironment like in response to stress. e.g., in glioblastoma, a decrease in the level of miR-451 is found when glucose levels are low. This activates the "adenosine monophosphate-activated protein kinase" (AMPK) pathway leading to decreased cell proliferation and increased cell survival. The reverse occurs when energy levels are high. High miR-451 levels cause suppression of AMPK signaling and activate cell proliferation. Thus, miR-451 level in glioma correlated with the rapid growth of tumor and poor prognosis (Godlewski et al. 2010).

7.2.4 Therapy

Besides the role of miRNA as a biomarker, the miRNAs and anti-miRNA constructs are being investigated as potential therapeutics against cancer. Many clinical trials have evaluated the roles of OncomiRs or tumor suppressor miRNAs in regulating various cellular processes (Table 7.2). Currently, both nonviral (lipid-based, polymeric, nanoparticles) and viral miRNA delivery systems are being used. The miRNA-based therapies are a newly emerging field, and the adverse effects of these treatments need to be assessed.

7.3 miRNAs Responsible for Cancer Aggressiveness

Cancer aggressiveness term is used for highly invasive, end-stage cancer that is resistant to therapy and bears a poor prognosis. Depending on the target transcripts, miRNAs are classified into oncogenic (oncomiRs) and tumor suppressors (antioncomiRs). The cellular processes like proliferation, migration, invasion, and cell death are regulated by changes in miRNA expression. Besides this, regulation of miRNAs also affects angiogenesis, epithelial–mesenchymal transformation, sensitivity to radiotherapy and chemotherapy that favors tumor aggressiveness. Various studies focusing on the function/expression of miRNA in carcinoma prostate, carcinoma cervix, breast cancer, and glioblastoma have proven that miRNAs affect different aspects of tumorigenesis (Macharia et al. 2019). A study found the correlation of miR-10b expression in carcinoma breast patients with staging, survival, and size of tumor. Further, involvement of lymph nodes was associated with a higher miR-10b expression (Zhang et al. 2018a). So, it has been proposed that miRNAs may be used for the diagnosis and assessment of prognosis in many types of cancers.

Table 7.2 Clinical trials evaluating miRNA-based therapies in human cancers (Adapted from (Balacescu et al. 2019), ClinicalTrials.gov)

miRNA	Trial reference no.	Cancer type	Delivery system	Trial status
miR-16 mimic	NCT02369198	Non-small cell lung cancer Malignant pleural mesothelioma	EDVs (EnGeneIC Delivery Vehicle nanocell)	Phase I complete Likely to enter phase II
miR-34 mimic	NCT01829971	Small cell lung cancer Lymphoma Multiple myeloma Non-small cell lung cancer Melanoma Renal cell carcinoma Primary liver cancer	Lipid nanoparticles	Phase I terminated (serious adverse reactions related to immune system)
Anti-miR-255	NCT02580552	Chronic lymphocytic leukemia Cutaneous T-cell lymphoma Adult T-cell leukemia/ lymphoma Mycosis fungoides Diffuse large B-Cell lymphoma, ABC subtype	Locked Nucleic acid-modified antisense inhibitor	Phase I (active, recruitment not started)
Anti-miR-255	NCT03713320	Mycosis fungoides Cutaneous T-cell lymphoma	Locked nucleic acid (LNA)-modified antisense inhibitor	Phase II terminated (business reasons, no issues regarding efficacy/safety

7.4 miRNAs, Epigenetic Mechanisms, and Cancer Aggressiveness

Studies have found dysregulation of miRNA in a number of malignancies, yet the mechanisms underlying this phenomenon are not clear. miRNA dysregulation in cancer has been found to be due to abnormal DNA methylation and modifications in histone proteins mainly (Suzuki et al. 2013).

DNA is methylated by the enzyme DNA methyltransferase (DNMT). There are various types of DNMTs (DNMT1, DNMT3A, and DNMT3B). Out of these, DNMT1 is found to be most abundant in mammalian cells. In general, methyltransferases convert cytosines of CpG (cytosine-phosphate-guanine) dinucleotide sequences to 5-methylcytosine using S-adenosyl methionine as a methyl donor. Studies have reported both hyper and hypomethylation in different types of cancers. DNA hypomethylation is known to upregulate oncogenic miRNAs while tumor suppressors are downregulated by DNA hypermethylation, this facilitates the start and progression of cancer (Macharia et al. 2019).

Many types of histone modifications occur, acetylation and methylation being the common ones. Histone acetyltransferases (HAT) mediate lysine acetylation, which usually leads to the uncoiling of the chromatin and enhanced transcription. Removal of acetyl group mediated by histone deacetylases (HDAC) provokes chromatin condensation and leads to gene inactivation. The effect of methylation of lysine residues on gene expression is site-dependent and varies with the extent of methylation. Gene expression is activated by methylation at K36, H3K4, and K79 while at H3K9, H3K27, or H4K20 it leads to transcriptional repression.

The downregulation of miR-101, a tumor suppressor, is found in many tumors like glioblastoma. It regulates the histones H3K27me3, H3K9me3, H3K4me2, and H4K20me3 and reverses the methylation status of "cytoplasmic polyadenylation element-binding protein 1" (histone-modified hypomethylated gene) promoter (Li and Wu 2017). The breast cancer susceptibility gene (*BRCA1)* associates with HDAC2 and causes repression of miR-155 expression. The in vivo growth of tumor cell lines is accelerated by overexpression of miR-155 while its knockdown attenuates tumor growth *(*Chang et al. 2011*)*. miR-615 is epigenetically activated through the H3K9 acetylation and loss of DNA methylation in prostate cancer cells (Hulf et al. 2011).

7.5 Hypoxia Microenvironment

The tumor microenvironment (TME) is constituted of malignant cells, blood vessels, lymphatics, fibroblasts, immune cells, pericytes, adipocytes, and chemical and physical components (Balkwill et al., 2012). The interaction of TME with tumor cells is the predominant factor affecting clinical outcomes. During the development of tumor, cancer cells often have limited access to oxygen. Aberrant blood vessel formation and restricted blood supply in most of the solid tumors lead to hypoxic environment. This causes release of "hypoxia-inducible factors" (HIFs) that mediate cell response to hypoxia. The adaptation, selection, and propagation of cancer and stromal cells are dependent upon HIF signaling pathways, thus favoring the cancer progression (Petrova et al. 2018).

HIF helps in the transcription of various factors involved in normal homeostasis. Additionally, hypoxic tumor cells overexpress the factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), epidermal growth factor, transforming growth factor-beta (TGF-β), enzymes of the glycolytic

pathway, and anti-apoptotic factors that lead to increased cell survival, growth, and metastasis. (Vaupel and Harrison 2004).

HIFs (HIF1, HIF2, and HIF3) are dimeric proteins containing alpha and beta subunits. The alpha subunit is cytoplasmic and is regulated in response to oxygen levels, whereas beta subunit is nuclear and is constitutively expressed. HIF1α and HIF2α, in association with HIF-1β, facilitate the major HIF transcriptional activity. In normoxic conditions, the two conserved proline residues of alpha subunit get hydroxylated. Von Hippel–Lindau tumor suppressor binds to hydroxylated HIF1α and gets degraded by the proteasomal system. During hypoxic conditions, HIF-1α is not hydroxylated and thus is not degraded; instead, it forms a heterodimer after binding with HIF-1β subunit in the nucleus. This active form of HIF-1 binds to specific areas on genes called hypoxia response elements of the target genes and regulates transcription (Emami Nejad et al. 2021). Under different oxygen tension, HIF1α and HIF2α display different stabilization patterns with respect to different oxygen concentrations. HIF-1α is mainly active in severe hypoxic condition (1% O_2) while HIF-2α is strongly expressed in cancer areas with good vascular supply (5% O_2) (Holmquist-Mengelbier et al. 2006).

7.6 Hypoxia and Cancer Aggressiveness

Pathological hypoxia in tumors favors the survival of cells and tumor growth. Hypoxia leads to upregulation of expression of HIF-1α and HIF-2α and its signaling molecules. This promotes angiogenesis, makes the tumor chemo- and radioresistant, and increases cancer aggressiveness.

7.6.1 Blood Vessel Formation

During embryogenesis, blood vessel formation takes place by vasculogenesis involving precursor cells that get differentiated into endothelial cells. This is followed by angiogenesis, i.e., the process of development of new blood vessels from the already present vessels. Then, the process of maturation occurs when the vessels interact with smooth muscles and other cells of connective tissue. In pathological conditions like tumor progression, abnormal angiogenesis is noted where hyperproliferating cancer cells become hypoxic due to a gap in oxygen supply and demand. The transcription factors HIF-1α and HIF-2α take part in various steps of blood vessel formation. They cause the endothelial progenitors to differentiate to mature endothelial cells. Matrix metalloproteinase expression is also enhanced, which leads to the sprouting and splitting of the already existing vessels. Lastly, HIF-α also helps in vessel maturation by inducing PDGF and TGF-β.

The new vessels formed in tumors are often immature and leaky, thus unable to meet the oxygen and nutrient requirements of the growing tumor cells. As the tumor grows, oxygen demand is further escalated. This aggravates the hypoxia, which in turn acts as a stimulus for angiogenesis. This culminates in a vicious circle. As a

result, the tumor tissue has abnormal, excessive vasculature that is unable to meet its oxygen demand (Muz et al. 2015).

7.6.2 Metastasis

Highly permeable and heterogeneous vasculature promotes the spread of tumor by the movement of tumor cells to different parts of the body. Hypoxic cells have better abilities to metastasize. Hypoxia affects the invasiveness and metastatic behavior of cancer cells by "epithelial–mesenchymal transitions" (EMT). EMT is an important phenomenon whereby the epithelial cells acquire mesenchymal phenotype (Thiery and Sleeman 2006). The phenomenon of EMT is observed during the development of embryos, tissue regeneration, and in many malignancies. There is a decrease in adherence of cells to each other and thus cell motility increases. There is downregulation of the expression of epithelial-associated genes like β-catenin and E-cad, and an increase in expression of vimentin, SMA, N-cad, and CXCR4 (mesenchymal-like genes). Hypoxia increases the concentration of TGF-β, a master regulator, that increases EMT by upregulating the synthesis of transcription factors like Snail and Slug (Muz et al. 2015).

7.6.3 Radiation and Drug Resistance

Hypoxia has also been implicated as a factor responsible for resistance to chemotherapy and radiotherapy in tumors. Hypoxia induces resistance to chemotherapy by (i) causing arrest in cell cycle, (ii) inhibiting cell death, (iii) regulating autophagy and mitochondrial activity, (iv) decreasing drug delivery and cellular uptake through an increase in acidity, and (v) reducing cytotoxicity of a number of drugs due to lack of adequate oxygen concentration.

Similarly, the state of tissue oxygenation is one of the significant factors for the radiation to act on cancer cells. Normoxic cells are irreversibly damaged by the ionizing radiations as oxygen increases the interaction with free radicals. On the contrary, hypoxic conditions confer radioresistance by decreasing the interaction between oxygen and ionizing radiation. Radiosensitivity also depends on phase of the cell cycle. Tumor cells are least sensitive if they are exposed to ionizing radiation at end of the S-phase and intermediate sensitive in the G1 phase. Radiosensitivity is observed maximally in the late G2 and M phases. Therefore, low proliferating, quiescent, hypoxic regions of tumor do not respond to chemo- and radiotherapy while rapidly proliferating normoxic tumor cell are sensitive (Muz et al. 2015)

7.7 microRNAs and Hypoxia Microenvironment

miRNAs act as regulators of many genes including hypoxia-related genes (Macharia et al. 2019). During hypoxia, miR-210 decreases the expression of succinate dehydrogenase cytochrome b small subunit, which is a part of the mitochondrial electron transport chain. This increases the stability of HIF-1, which further results in increased survival of cancer cells (Rupaimoole and Slack 2017). miR-210 has also been implicated in downregulating the apoptosis-inducing factor mitochondria associated 3 (AIFM3), thus supporting cancer cells' survival (Wang et al. 2014) and ephrin A3, an inhibitor of angiogenesis, leading to enhanced angiogenesis (Fasanaro et al. 2008). The von Hippel–Lindau (pVHL) is a major regulator of cell response to hypoxia. Its expression is downregulated by miR-155. pVHL in turn negatively regulates the HIF-1. Thus, the downregulation of pVHL promotes angiogenesis and survival of cancer cells (Kong et al. 2014). The injection of plasmids carrying antisense HIF-1a and VHL into the tumors led to complete regression of large tumors. This therapy promotes cell death by decreasing HIF-1, VEGF, and angiogenesis. These findings indicate that combinational therapy using blockage of HIF-1α and enhancement of VHL may prove to be more effective in the management of cancer patients (Sun et al. 2003). Additionally, some other miRNAs e.g., miR-21-5p, also downregulate VHL, which increases the expression of VEGF-A (Zhang et al. 2014). Similarly, miR-7-5p targets O-linked N-acetylglucosamine transferases and decreases the expression of VEGF receptor 2 (Babae et al. 2014). On the contrary, miR-128 overexpression leads to downregulation of p70S6K1, which reduces the expression of VEGF and HIF, leading to decreased angiogenesis and tumor growth (Shi et al. 2012).

The hypoxic microenvironment affects the synthesis of plenty of miRNAs. For example, tumor hypoxia, through hypoxia-responsive transcription factors, downregulates the expression of Drosha, the enzyme responsible for miRNA processing (Rupaimoole and Slack 2017).

Similarly, miR-103/107, let-7, and miR-630 directly target the DICER 30 untranslated region and downregulates it. Tumor hypoxia is shown to pronounce this effect (Macharia et al. 2019).

7.8 The Stem cells, Cancer Aggressiveness, and Hypoxia

Cancer stem-like cells (CSCs) are a group of cells in the parent tumor that can initiate self-renewal of the tumor cells via differentiation (Chen et al. 2012). They are also called tumor-initiating/propagating cells. CSCs comprise 0.1–0.8% of total tumor cells. CSCs bear similarity to normal stem cells in some features like self-renewal, capacity to divide for long periods, promotion of tumor spread to distant body sites and expression of the cell surface markers of stem cells. But unlike normal stem cells, CSCs show some differences in terms of indefinite self-renewal, growth pattern, chromosomal abnormalities, highly dysregulated differentiation, and

tumor development. Their role has been shown in the origin, progression, spread, and relapse of a number of tumors (Singh et al. 2003; Xie et al. 2016).

Thus, CSCs contribute to tumor aggressiveness as they influence growth of tumors and also have a role in chemo- and radioresistance (Debeb et al. 2009; Li et al. 2008).

Epigenetic modifications are important for the generation of CSCs (Shukla and Meeran 2014; Tao et al. 2018)). The reprogramming causes downregulation of the genes associated with differentiated state, whereas genes specific to stem cells are upregulated (Shukla and Meeran 2014). The Wnt/b-catenin signaling is crucial for cell processes like apoptosis and renewal of stem cell state. The abnormalities of this pathway have been implicated in numerous cancers. Abnormal activation of Wnt/b-catenin signaling due to DNA methylation leads to the silencing of various Wnt inhibitors in breast tumor (Klarmann et al. 2008). In addition, activation of EMT can induce tumor-initiating properties in cells. The defining characteristic of EMT is the loss of adherens junction protein, E-cadherin. Transcription gene silencing of E cadherin occurs due to histone deacetylation. Methylation of the E-cadherin promoter causes the recruitment of histone deacetylases (HDAC) to the site. Deacetylation compacts the chromatin by increasing the ionic interactions among histones and DNA and thus represses transcription (Wang and Shang 2013).

Thus, as is clear from the examples given above, abnormal epigenetic modifications may convert normal stem cells into CSCs and these acquire stem-like phenotypes. This stem-like phenotype helps to support malignant growth.

In addition, different cancer stem cell state markers are being explored to find their importance in cancer development and prediction of prognosis. Cancer stem cell-state markers CD24, CD44, CD133, and aldehyde dehydrogenase 1 (ALDH1) have been associated with carcinoma ovary. The presence of these markers has been linked with worse prognosis (Tao et al. 2018). Similarly, the CSCs markers CD44 and ALDH1 predict poor prognosis in carcinoma breast patients (Kong et al. 2018).

The tumor suppressor protein $p16^{Ink4a}$ limits the G1-S progression and thus regulates cell cycle. The stem cells with reduced expression of $p16^{Ink4a}$ and increased expression of ALDH1 have been found in cervical cancer patients. The level of these markers correlates with poor response to radiotherapy (Fu et al. 2018). On the contrary, cervical cancer patients with a higher level of $p16^{Ink4a}$ showed a better prognosis (Lin et al. 2014). Some other examples of the association of cancer stem markers with the prognosis of different cancers are the negative correlation of CD133 and CD44 with survival rate in gall bladder cancer (Pietras et al. 2014), and overexpression of OCT-4 in cervical cancer tissue in comparison to the normal tissue (Yang et al. 2014). The expression of Nanog protein is found to vary with the stage of cervical cancer. Increased expression is also found in cervical dysplasia in comparison to the normal epithelium (Ye et al. 2008). miRNAs have been studied in the context of hypoxia and stem-like state in some common aggressive tumors. For example, miR-210 has been associated with hypoxia in cervical and breast cancers while miR-23b and miR-125b have been associated with stem-like state in cancer cervix (Macharia et al. 2019).

7.9 miRNAs, Hypoxia, Stem-Like State, and Their Role in Therapeutics

The basis of miRNA therapeutics in the treatment of cancer is the fact that such molecules can be designed that mimic or inhibit mature miRNA. Based on the roles of miRNAs studied so far, it seems pertinent to think that overexpressed miRNAs can be targeted in cancer. In addition, reexpressing those miRNAs, which are downregulated in cancer, also represents a valid and logical approach. Similarly, the cancer stem cells and hypoxia microenvironment may be targeted too.

During prolonged hypoxic conditions, a process of HIF switch occurs that is a "shift of signaling from HIF-1 to HIF-2 and then to HIF-3." This promotes angiogenesis and prolongs cell survival. If the cell is unable to decrease the HIF-1 level, it initiates apoptosis. This switch can be targeted in cancer therapeutics. The role of miRNAs in regulating HIF switch during low oxygen conditions has been well documented, thus making them important therapeutic targets (Serocki et al. 2018). Upregulation of miR-18a after exposure to hypoxia for 24 hours has been documented. This miRNA directly targets HIF1A mRNA. This suggests that miRNA can induce a HIF switch (Han et al. 2015). Additionally, a different HIF signal regulation has also been elucidated. Here miRNA-103/107 mediates the suppression of beta subunits, thus limiting HIF-1 activity and inhibiting angiogenesis (Deng et al. 2016). Although the role of HIF-3 is not very clear, it mainly inhibits the function of HIF-1. miR-147a reduces its level in human cervical cancer (HeLa) cells, leading to stabilization and accumulation of HIF-1 (Wang et al. 2016). HIF-1α is known to be present in cancer stem cells as well as in normal progenitors, which precludes its use as a therapeutic target. Unlike HIF-1α, HIF-2α is not found in non-glioma stem-like cells but increased in glioblastoma stem cells, making it a target in terms of chemotherapeutic (Li et al. 2009). Although many HIF inhibitors have been discovered, none of them is specific inhibitors of HIF-1 (Rodriguez et al. 2016). Efforts are also being focused on developing selective HIF-2 inhibitors. In 2021, an oral HIF-2α inhibitor has been approved for management of von Hippel–Lindau (VHL) disease in adults. Currently, it is indicated for those patients of VHL, who also have associated tumors of kidney, CNS, and pancreas. This HIF-2α inhibitor decreases the transcription and expression of HIF-2α target genes, leading to decreased tumor growth and angiogenesis (FDA 2022).

The development of therapies against cancer stem cells poses a significant challenge as both cancer and stem-like cells need to be targeted. Additionally, these therapies may affect the normal organ/tissue function by blocking the regeneration of noncancerous tissue (Huang and Rofstad 2017).

However, a different cell-based delivery system using miRNAs or their inhibitors can be tried. It will affect the CSCs by modifying their functions or by altering the expression of important proteins related to their regulation.

As explained earlier, epigenetic mechanisms might be an important target of therapeutics, as these regulations play an important role in the modulation of miRNA expression. Targeting components of epigenetic pathways can stop the progression of cancer and modify the properties of cancer stem cells (Macharia et al. 2019).

Some of these epigenetic modulators are already approved by FDA while many others are in different stages of clinical trials (Toh et al. 2017).

7.10 Exosomal MicroRNAs in Cancer

Exosomes are extracellular vesicles released from most of the cells in eukaryotic organisms and they are present in all body fluids. Their size varies between 30 and 100 nm. A lipid bilayer forms their outer boundary while a variety of biomolecules like nucleic acids, lipids, and proteins can be their constituents (Abak et al. 2018).

Exosomes regulate cell milieu by the selective release of toxic biomolecules present in cells and immune response stimulation (Desdín-Micó and Mittelbrunn 2017). Their role in tumor development has also been described as they can be transferred between adjacent tumor cells and also from tumor cells into the adjoining milieu (Bullock et al. 2015). Exosomes may increase and maintain tumor cell proliferation and survival. They do so by initiating angiogenesis, remodeling of extracellular matrix, and affecting immune cells function (Iero et al. 2008). Additionally, they have a role to play in imparting drug resistance among tumor cells (Zhao et al. 2015).

miRNAs are an important constituent of exosomes, and their dysfunctional role has been documented in the proliferation of different cancer. For example, it is observed that let-7 miRNAs are found in exosomes released from gastric tumor cells (Ohshima et al. 2010). Additionally, levels of exosomal miRNAs like let-7f, miR-21, etc., are increased in cancer subjects in comparison to the control group (Silva et al. 2011).

Exosomal miRNAs exert significant effects on the control of cancer growth, angiogenesis, and spread to distant sites and impart chemoresistance. They regulate proteins related to cell cycle or signaling pathways. In colorectal cancer (CRC), the transmission of miR-200b is observed between cancer cells and thus their growth is promoted (Zhang et al. 2018b). The growth of hepatocellular carcinoma cells is inhibited by inhibition of the transfer of oncogenic miR-21 into exosomes (Liang et al. 2018).

Exosomal miRNAs also affect EMT. In oral cavity cancer, induction of EMT process occurs due to vimentin and snail's enhanced expression and E-cadherin's reduced expression. These changes are initiated by exosomal miR-21 (Li et al. 2016). While in prostate cancer, inhibition of the activities of N-cadherin and vimentin by exosomal miR-1246 leads to the suppression of the EMT process (Bhagirath et al. 2018). In HCC cells, endothelial proteins are the target of exosomal miR-103, thus increasing blood vessel permeability and advancing the spread of tumor (Fang et al. 2018).

7.10.1 Exosomal miRNAs Affect Chemotherapeutic Resistance in Cancer Cells

Exosomal miRNAs affect chemosensitivity by manipulating cellular signaling pathways. In prostate tumor cells, exosomal miR-34a regulates Bcl-2, leading to increased sensitivity to docetaxel (Corcoran et al. 2014). Gemcitabine sensitivity in pancreatic ductal adenocarcinoma (PDAC) cells is reduced by the macrophage-derived exosomes (MDEs) (Binenbaum et al. 2018). The breast cancer cells that are resistant to tamoxifen are found to have elevated levels of exosomal miR-221/222. This could decrease the target gene expression of p27 and estrogen receptors, hence imparting resistance to tamoxifen (Wei et al. 2014).

7.10.2 Exosomal miRNAs Can Be Used for Diagnosis and Prognostication of Cancers

A lot of research is going on to explore exosomal miRNAs as potential biomarkers for diagnosis and assessment of prognosis of cancer. For example, it is seen that exosomal miRNAs like miR-181b-5p, and miR-361b-5p levels were elevated in subjects suffering from non-small cell lung carcinoma (NSCLC) (Jin et al. 2017). Hence, NSCLC may be detected early by using these miRNA biomarkers. Additionally, in NSCLC patients, exosomal miR-451a, miR-21, and miR-4257 were detected in higher concentrations in comparison to controls and predict poor prognosis (Kanaoka et al. 2018; Dejima et al. 2017).

In esophageal squamous cell carcinoma, exosomal miR-21 and miR-1246 are elevated and are linked to tumor aggressiveness and progression (Tanaka et al. 2013; Takeshita et al. 2013). In glioma, the level of CSF exosomal miR-21 is found to be increased compared to controls and is associated with tumor spread, relapse, and dismal prognosis (Shi et al. 2015).

Epithelial ovarian cancers with elevated exosomal miR-200c and miR-200b show increased growth and bad prognosis (Meng et al. 2016), while, in patients with HCC, a negative correlation of exosomal miR-638 with HCC progression is observed (Shi et al. 2018). Thus, exosomal miR-638 may be studied to explore its role in determining the prognosis of HCC.

Early diagnosis is of paramount importance in starting prompt treatment and thus improving the prognosis in patients living with cancer. Presently, serum biomarkers like alpha-fetoprotein, carcinoembryonic antigen, and prostate-specific antigen are used for detecting some specific tumor, but due to inadequate sensitivity and specificity, their role is limited (Lu et al. 2017). The role of exosomal miRNAs as useful biomarkers for cancer detection seems promising because of their better stability, noninvasive nature and widespread presence in different body fluids.

7.11 MicroRNAs as Potential Therapeutics Against Cancer

miRNA-based gene therapy is being explored as a new approach for cancer treatment owing to its ability in predicting tumor growth and its local and distant spread. The expression of miRNA back to physiological state can be achieved in two ways. If oncomiR is overexpressed, miRNA activity needs to be inhibited, while in the case of suppression of tumor suppressor miRNA, miRNA activity needs to be restored. (Abd-Aziz et al. 2020).

7.11.1 miRNA Inhibition Therapy

In this approach, inhibitors of miRNA are employed to suppress oncomiRs activity in tumor cells. This can be achieved by using various biomolecules such as locked nucleic acid (LNA), antisense anti-miR oligonucleotides (AMO), miRNA antagomirs, and miRNA sponges. miRNA inhibitors lead to the inactivation and removal of the miRNA from RISC (Shah et al. 2016).

AMOs are chemically altered oligo nucleotides. They restore normal translation by inhibiting the binding of miRNAs to specific mRNAs. For example, LNA, a modified AMO inhibits overexpressed miR-21 in glioblastomas (Griveau et al. 2013).

Antagomirs are ssRNA molecules containing 23 nucleotides. They are chemically altered and act as complementary to the targeted miRNAs, thus helping in increasing their stability and preventing it from the breakdown (Meister et al. 2013; Krützfeldt et al. 2005). For example, in tumor cells of mouse mammary glands, metastasis can be inhibited by using miR-10b antagomirs (Ma et al. 2010).

miRNA sponges act as competitive inhibitors of oncomiRs and contain multiple artificial miRNA binding sites (Ebert and Sharp 2010). The circular RNA (circRNA) found naturally in cells acts as endogenous miRNA sponges. With the help of a simple enzymatic ligation technique, a functional and artificial circRNA sponge can be produced. It regulates miRNAs by binding to different miRNA sites. It has been shown that the circRNA can be transfected into miRNA21, leading to inhibition of the proliferation of gastric tumor cells (Liu et al. 2018).

7.11.2 miRNA Restoration Therapy

In this approach, downregulated tumor suppressor miRNAs are restored, leading to apoptosis induction or inhibition of the proliferation of tumor cells. Viral vectors that express miRNAs or artificial biomolecules that mimic miRNAs are employed. miRNA mimics act by restoring the normal functioning of endogenous miRNAs. The chemically altered miRNAs are incorporated into RISC, leading to the target mRNA inhibition (Shah et al. 2016). For example, miR-15, an miRNA mimic, reduced growth in prostatic cancer cell lines by inducing cell death (Bonci et al. 2008).

In another strategy, miRNA expression vectors are used to induce tumor suppression by increasing the expression of the miRNAs. Some examples of vectors are adenoviral, lentiviral, and retroviral vectors. For example, in liver cancers, miR-26 expression is lost. Recombinant adenovirus loaded with the miR-26 injected through intravenous route results in suppression of tumorigenicity by increasing tumor apoptosis and inhibiting cellular proliferation (Kota et al. 2009).

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piRNA-Based Cancer Therapy in Hypoxic Tumor

8

Suman Kumar Ray and Sukhes Mukherjee

Abstract

Tumor aggressiveness is encouraged by hypoxia, which also lowers patient survival. Numerous individuals with hypoxic tumors had poor outcomes, which shows that additional factors may affect how the tumors react to hypoxia. Hypoxia is frequently found in solid tumors and is known to influence aggressive tumor activity, chemotherapy resistance, and radiation resistance, all of which lead to a bad prognosis for the cancer patient. PIWI-interacting RNAs (piRNAs) control how tumor cells react to hypoxia, but little is known about how piRNAs function in hypoxic tumors. PiRNAs are brand-new, tiny, noncoding RNA molecules with 24 and 31 nucleotides length. They frequently interact with proteins from the PIWI protein family to regulate the epigenetic regulation of gene expression, which is essential for understanding cancer genetics. Numerous studies have demonstrated that abnormal piRNA expression is a hallmark of various tumor forms, although their precise tumorigenic roles are still unknown. Patients' cancer type-specific piRNA signatures differ from one another. The malignancy renal cell carcinoma, defined by constitutive activation of hypoxia-related signaling brought on by a common mutation or deletion of the von Hippel–Lindau factor, is found to have highly uniform piRNA profiles across patients (VHL). According to prior reports, piRNAs and PIWI proteins may be crucial in cancer development, prognosis, and management. However, it has yet to be determined how these compounds might be relevant in therapeutic settings.

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The utilization of piRNAs and PIWI proteins as cancer treatments and diagnostic and prognostic biomarkers is also considered prospective options. In this chapter, we cover recent research on the biogenetic mechanisms, roles, and emerging roles of piRNAs in hypoxia cancer, offering fresh perspectives on the possible uses of piRNAs and PIWI proteins in the detection and clinical management of hypoxic cancer.

Keywords

piRNA · PIWI · Hypoxia · Cancer · Cancer diagnosis · Clinical treatment

8.1 Introduction

Several solid tumors form areas that are hypoxic or poorly oxygenated. Hypoxia-inducible factor-1 (HIF-1) and HIF-2, heterodimeric transcription factors, activate more than 90 genes involved in anaerobic glycolysis, pH control, angiogenesis, cellular migration, and metastasis (Giatromanolaki et al. 2001; Forsythe et al. 1996). There are several ways to identify hypoxic tumors, and patients with hypoxic tumors typically have worse outcomes than patients with normoxic tumors (Wilson and Hay 2011; Simi et al. 2006; Hung et al. 2009). Patients with hypoxic tumors usually have an aggressive tumor phenotype and decreased patient survival. In addition, hypoxic tumor cells increase the risk of metastatic disease and reduce the effectiveness of radiation therapy and most forms of chemotherapy. However, there is a range of poor outcomes in patients with hypoxic tumors, and identifying those patients who would have the worst effect is a significant unmet need in the field of oncology. The impact of hypoxia on RNA expression is extensive. PiRNAs are a subclass of small noncoding RNAs (sncRNAs) that have recently been realized to be important in cancer biology. However, their effects on other small noncoding RNAs (sncRNAs), such as PIWI-interacting RNAs (piRNAs), are unknown. In humans, more than 30,000 piRNAs have been identified (Ostheimer et al. 2014; Ku and Lin 2014). Their ability to direct related chromatin-silencing machinery to transposon-encoding DNA regions in the genome (Huang et al. 2013a) best describes them.

A subset of piRNAs may also be able to control protein-coding genes through DNA methylation, which, if the regulatory targets are cancer-relevant, may impact the development of cancer (Watanabe et al. 2011; Jacobs et al. 2016). Humans have four different PIWI protein isoforms, including PIWIL1 (HIWI), PIWIL2 (HILI), PIWIL3, and PIWIL4 (HIWI2); rodents have three different isoforms, including PIWIL1 (MIWI) PIWIL2 (MILI), and PIWIL4 (MIWI2); and Drosophila has three different isoforms, including PIWI, Aub, and PiRNAs that are essential in controlling gene expression and acting as transposon silencers (Dharap et al. 2011; Yan et al. 2011; Rizzo et al. 2014; Liu et al. 2018). piRNA expressions that are abnormally high have been found in various illnesses, particularly neoplasms. PiRNAs are intriguing new therapeutic targets for onco-medicine and promising biomarkers for early diagnosis. Germline cells have high levels of piRNA

expression, which controls genomic stability by identifying DNA target sequences and aids in the recruitment of the required machinery to cause transposable element epigenetic silencing (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006).

Recent data suggest that they are expressed, functionally active, and connected to epigenetic pathways of cancer development in somatic tissue (Esteller 2008; Rouget et al. 2010; Esteller 2011; Fu et al. 2014; Ha et al. 2014a; Barckmann et al. 2015; Gebert et al. 2015; Moyano and Stefani 2015; Ng et al. 2016a). Nevertheless, little is known about piRNA expression mechanisms in somatic cells or solid malignancies. Patients with renal cell carcinoma (RCC) have indicated that the solid tumor microenvironment may have a role in controlling piRNA expression and perhaps contributing to the diverse piRNA expression seen between patients, according to Martinez and his colleagues (2015) (Martinez et al. 2015a). Since von Hippel–Lindau factor (VHL) loss-of-function mutations frequently occur in RCC tumors, constitutive, oxygen-independent stabilization of HIF-1(Maxwell et al. 1999) and HIF-mediated upregulation of hypoxia-associated gene products, Maxwell and his team (1999) discovered that piRNA expression was remarkably consistent between RCC tumors.

8.2 Biogenesis of piRNAs and Generation of Mature piRNAs

Genetic areas called piRNA clusters, which can be classified as uni-strand or dual-strand clusters, where a significant majority of piRNA precursors are created. Dual-strand clusters produce precursors that map to both genomic strands, unlike uni-strand collections, which make precursors that only map to one strand. Additionally, some piRNA precursors may be originated from individual transposons or the 30 UTR of protein-coding genes (Robine et al. 2009; Saito et al. 2009). Uni-strand cluster transcription is comparable to canonical mRNA transcription. The transcription-associated histone 3 lysine 4 demethylation (H3K4me2) mark is present at promoters in uni-strand clusters. Additionally, piRNA precursors are 30 terminated, and 50 methyl-guanosine capped (Mohn et al. 2014; Lim and Kai 2015). On the other hand, dual-strand clusters lack distinct RNA polymerase II (RNA Pol II) promoter signatures, such as H3K4me3 and RNA Pol II peaks, and non-polyadenylated piRNA precursors are generated (Le Thomas et al. 2013; Chen et al. 2016).

Several proteins, including RNA Pol II, the Rhino-Cutoff-Deadlock complex (RDC complex), Moonshiner, TATA-box binding protein-related factor (TRF2), and the three prime repair exonuclease, are involved in the transcription of dual-strand clusters into piRNA precursors (TREX). piRNA precursors are moved out of the nucleus after transcription is complete. The RNA helicase Armitage first resolves the secondary structures (Armi). After deserialization, the mitochondria-associated endonuclease Zucchini cleaves piRNA precursors to produce pre-piRNAs with a 50 monophosphate (Zuc). Then, a 30–50 exonuclease called Nibbler trims (Nbr) the pre-piRNAs at the 30 ends after loading them onto PIWI proteins. Primary piRNAs are created through a process known as primary piRNA biogenesis, known as

primary piRNAs. The production of piRNAs is boosted by the participation of Ago3 and Aub proteins and is primed by initial piRNAs.

8.3 piRNAs: Novel Functions in Cancer Expression and Selectively Deregulation by Hypoxic Tumors

Martinez and his group (2015) used our previously published bespoke small RNA sequencing analysis pipeline (Martinez et al. 2015a) to assess the expression levels of 23,440 human piRNAs on a per-tumor basis. PiRNAs were only included in analyses if they had a median expression of 10 (reads per kilobase million) RPKM in at least one of the groups (hypoxic and/or normoxic) and a minimum of twofold change in median RPKM expression values. It was done to identify the most significant changes in piRNA expression between hypoxic and nonhypoxic groups. Comparisons can be made using the nonparametric Mann–Whitney U test, with the Benjamini–Hochberg technique being used to compensate for multiple testing's false discovery rates (Lee and Lee 2018).

8.4 piRNAs Maintain Genomic Integrity by Silencing Transposable Elements

Transposable elements (TEs) can cause genetic variety and instability, making them prime candidates for increased cancer-causing potential in humans (Cordaux and Batzer 2009; Chenais 1835; Suntsova et al. 2015). The establishment of a repressive chromatin state can be caused by the position of the PIWI-piRNA complex in the nucleus. It has been discovered that the ectopic expression of piRNAs increases heterochromatin-1 (HP1) and H3K9me3, which, when bound, results in a heterochromatin state (Suntsova et al. 2015). Restoring tumor suppressor p53 and associated piRNAs may be a promising approach for treating cancer (Levine et al. 2016) in light of the carcinogenic impact of LINE-1 and other repetitive elements.

8.5 piRNAs Contribute to Tumorigenesis Through Regulation of DNA Methylation

Another epigenetic process with a functional connection to piRNAs is DNA methylation. Recent research suggests that piRNAs and associated PIWI proteins can encourage retrotransposons' de novo DNA methylation(Aravin et al. 2007; Kuramochi-Miyagawa et al. 2008; Kojima-Kita et al. 2016). Additionally, piRNAs can control DNA methylation on non-transposon loci as well as in the context of transposons. It is noteworthy that the mutation rs1326306 G > T related to piR-021285 discovered by Fu, and his colleagues (2015) was found to be substantially connected with breast cancer (Fu et al. 2015). They discovered methylation variations in several genes involved in cancer development by comparing the

genome-wide methylation profiles in MCF7 cells transfected with either the wild type or mutant piR-021285. The mechanisms behind its oncogenic action also revealed that the suppression of piR-823 resulted in a notable decrease in the production of DNMT3A and DNMT3B, which decreased global DNA methylation and caused the methylationsilencedp16INK4A tumor suppressor gene to become active once again.

8.6 Post-Transcriptional Regulation of Gene Expression by piRNAs

DNA methylation and chromatin silencing are just a few of the things piRNAs can do. Numerous studies have shown that piRNAs can suppress the expression of their target genes like miRNAs (Ha et al. 2014b; Martinez et al. 2015b; Zhang et al. 2015). In contrast to CDSs and 5′-UTRs, expressed piRNAs have been concentrated in the human testis' 3′-untranslated regions (UTRs). The mRNAs targeted by piRNA in the case of MIWI mutant mice were overexpressed due to their inability to be cleaved in the absence of MIWI. Indeed, several studies have shown that piRNAs can work as a surveillance system to destroy retrotransposon-associated mRNAs to avoid the ubiquitous dissemination of transposon sequences (Watanabe et al. 2006).

8.7 piRNAs Have Tumorigenic or Suppressive Roles in Cancer Development

Research shows that piRNAs have a role in cancer development as either tumor suppressors or oncogenes. Additionally, they might aid in the growth and spread of cancer cells. For instance, in patients with non-small cell lung carcinoma, the upregulation of piR-651 was discovered to be related to cancer progression (NSCLC). Notably, piR-651 overexpression dramatically increased tumor development and metastasis in the A549 lung cancer cell line. Additionally, piR-651 overexpression causes cell cycle arrest by upregulating the production of CDK4 and cyclin D1. It implies that piR-651 may function as an oncogene in this cancer (Li et al. 2016; Yao et al. 2016). Another well-known oncogenic piRNA, PiR-Hep1, aids in the invasion, migration, and proliferation of liver cancer cells (Law et al. 2013).

8.8 piRNAs in the Maintenance of Cancer Stemness and Chemoresistance

The dogma in the piRNA field is being challenged by a growing body of recent studies that show the PIWI pathway is also connected to the preservation of cancer stemness. Zhang and his colleagues (2013) extracted CD44 (+)/CD24 (-) tumor cells (cancer stem cells [CSC]) from 1086 clinical specimens for breast cancer and found

Table 8.1 The clinical application of piRNAs in cancer (Weng et al. 2019)

Target/marker		Types of cancer	Name of the piRNA
Therapeutic target		Breast cancer	piR-008114 piR-019676 piR-000552 piR-020548 piR-008113 piR-016735 piR-020450 piR-017033 piR-020365 piR-019675 piR-019914 piR-015249 piR-009294 piR-021032 piR-009051 piR-000753 piR-008112 piR-020814 piR-001318 piR-006426 piR-017184 piR-020829 piR-019912 piR-018780 piR-018849
		Prostate cancer	piR-651 piR-823
		Colorectal carcinoma	piR-823
		Lung cancer	piR-651 piR-55,490
		Pancreatic cancer	piR-017061
		Multiple myeloma	piR-823
Marker	Diagnostic marker	Breast cancer	piR-021285
		Gastric cancer	piR-651 piR-823
		Lung cancer	piR-L-163
	Prognostic markers	Kidney cancer	piR-30,924 piR-38,756 piR-32,051 piR-39,894 piR-43,607
		Colorectal carcinoma	piR-1245
		Liver cancer	piR-Hep1

that these cells expressed more PIWIL2 and piR-932 than control cells did (Zhang et al. 2013). Furthermore, the authors hypothesized that by encouraging abnormal methylation of the Latxin gene the piR-932 PIWIL2 complex might support the persistence of breast cancer stem cells. One of the defining traits of cancer stem cells is chemoresistance. Greater tumorigenicity, tumor sphere formation, and increased chemoresistance in vivo were all caused by the overexpression of HIWI (PIWI proteins in humans) in cervical cancer cells, together with the activation of many stem-associated genes (Liu et al. 2014a). Targeting the PIWI pathway may be a promising technique for chemotherapy and other clinical applications of cancer since PIWI proteins and their associated piRNAs play a vital role in maintaining the stem cell characteristics of cancer cells (Table 8.1).

8.9 The Role of piRNAs in Hypoxic Cancer

In human malignancies, the roles of PIWI proteins and piRNAs have begun to be revealed (Liu 2016). There is mounting evidence that many tumor cells express PIWI proteins in mice and humans, including PIWIL2-like proteins, HIWI, and PIWIL2 (Esteller 2011; Siddiqi and Matushansky 2012). Additionally, piRNAs were found in these cells (Esteller 2011). Human cancers such as gastric, bladder, breast, colorectal, and lung cancer have been documented to express piRNAs aberrantly. These data suggest that the piRNA pathway may be involved in the emergence of cancer. Although the possible involvement of piRNAs in cancer is still being explored, little is known about the functional role that particular piRNAs play in human cancer. These findings underline how critical it is to comprehend the precise function of the piRNA pathway during carcinogenesis and present brand-new therapeutic options. Table 8.2 and Fig. 8.1 show piRNA expression concerning the various cancer types.

Table 8.2 piRNA expression in different types of cancer (Chalbatani et al. 2018; Wu et al. 2020; Liu et al. 2019)

piRNA expression	Name of the piRNA	Type of cancer
Up	piR−36743, piR−36026, piR−31106, piR-021285, piR-932	Breast cancer
	piR-Hep1	Liver cancer
	piR-1245, piR-54265	Colorectal carcinoma
	piR-52207, piR-33733	Ovarian cancer
	piR-32287, piR-32512 piR-36095 piR-38581, piR-52205, piR-52206, piR-57816	Neurological cancer
	piR-32051, piR-39894, piR-43607	Kidney cancer
	piR-34871, piR-52200	Lung cancer
Down	piR−34736, piR−36249, piR−35407, piR−36318, piR−34377, piR-36712	Breast cancer
	piR−55490, piR-L−163, piR-35127 piR-46545	Lung cancer
	piRABC, piR-60152	Bladder cancer
	piR-57125	Kidney cancer
	piR−015551	Colorectal carcinoma
	PiR−823	Gastric cancer
	piR-598	Neurological cancer
	FR140858	HNSCC
	piR-39980	Osteosarcoma
	piR-30188, piR-8041, piR-DQ593109	Glioblastoma
	piR-39980	Fibrosarcoma

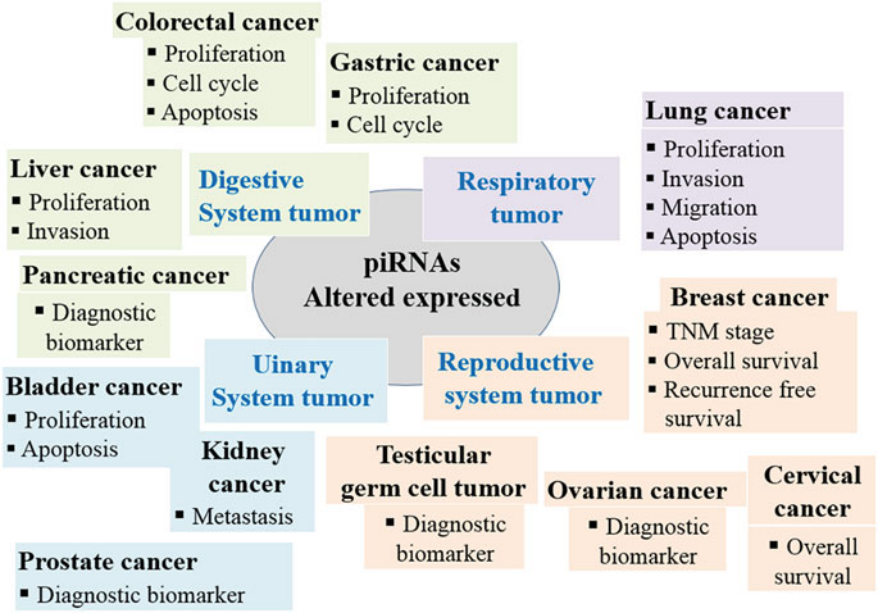

Fig. 8.1 Role of piRNAs in different types of cancer

8.10 Gastric Cancer

Gastric cancer is the second-largest contributor to cancer-related fatalities worldwide (Pan et al. 2013; Shomali et al. 2017) and the fifth most common cancer worldwide (Torre et al. 2012; Jiang et al. 2017). There was no correlation between the expression levels of piR-823 and its clinical–pathological characteristics in gastric cancer tissue (Cheng et al. 2012a). Due to piR-651's overexpression as an oncogene in gastric cancer, the TNM (tumor node metastasis) stage showed a positive connection. Additionally, a piR-651 inhibitor could reduce cell development in the G2/phase, proving that piRNAs are essential for carcinogenesis (Cheng et al. 2011). Additionally, it was noted that piR-651 and piR-823 had reduced levels of CTCs (circulating tumor cells) in the peripheral blood of patients with gastric cancer compared to normal controls (Cui et al. 2011).

However, piR-823 and piR-651 were more sensitive than commonly used biomarkers for gastric cancer, such as CA19-9 (carbohydrate antigen 19-9) and CEA (serum carcinoembryonic antigen). It is because piRNAs are short fragments that are less likely to be degraded, and levels of piR-651 and piR-823 in blood samples are generally stable. Additionally, these piRNAs can pass through cell membranes and are, therefore, easily detected. These results imply that piRNAs may represent novel therapeutic targets for treating gastric cancer (Li et al. 2014).

8.11 Bladder Cancer

The most prevalent malignancy of the urinary system and the ninth most common cancer overall are bladder cancers (Park et al. 2014; Ploeg et al. 2009). The researchers profiled three pairs of bladder cancer samples and their surrounding normal tissues using the ArrayAtarHG19 piRNA array, which is for human piRNAs. They discovered that the crucial piRNA piRABC (also known as DQ594040) was downregulated in bladder cancer (Chu et al. 2015). PiRABC demonstrated extremely high differential expression levels between healthy tissues and bladder cancer. Tumor Necrosis Factor Superfamily Member 4 (TNFSF4) and piRABC may interact, leading researchers to speculate that piRABC may encourage bladder cancer cell death by upregulating TNFSF4 (Pardini and Naccarati 2018).

8.12 Breast Cancer

Deep sequencing was done on four matched nontumor tissues and four breast cancer tissues to identify differentially expressed piRNAs. Four piRNAs (piR-20365, piR-20582, piR-20485, and piR-4987) were later upregulated in 50 breast cancer samples by RT-PCR. Patients' clinical pathology characteristics were noted, including lymph node status, tumor size, estrogen receptor (ER) status, and Her2 status. Additionally, lymph node metastasis correlated favorably with piR-4987 upregulation (Huang et al. 2013b).

A study demonstrated that the piR-932/PIWIL2 complex might favorably influence the process of breast cancer stem cells, which promotes EMT, by encouraging the methylation of Latex (epithelial–mesenchymal transition). PIWIL2 and piR-932 have been proposed as potential targets for preventing the spread of breast cancer (Zhang et al. 2013). Similar findings were made by another study, which discovered that piR-021285 plays a role in methylation at several known breast cancer-related genes, specifically attenuated 5′ untranslated regions (UTR)/first exon methylation at the pro-invasive ARHGAP11A gene and invasiveness in an in vitro cell line model (Han et al. 2017).

8.13 Lung Cancer

Lung cancer is the most common cause of cancer-related death worldwide and is broadly classified as non-small cell lung cancer (about 85% of cases) and small cell lung cancer (about 15% of cases) (Blandin Knight et al. 2017). Furthermore, they demonstrated that piR-L-163 could regulate phosphorylated ERM and play a crucial part in protein activation by directly binding to and doing so (Mei et al. 2015). Targeting piR-L-138 could be a possible technique to overcome chemoresistance in patients with lung squamous cell carcinoma (LSCC) as the researchers discovered that it was elevated by cisplatin (CDDP)-based chemotherapy both in vivo and in vitro (McClatchey and Fehon 2009). The top-downregulated piR-L-163 in

NSCLC cells prevented cell migration and invasion by maintaining the activity of phosphorylated ezrin-radixin-moesin (p-ERM), which links transmembrane proteins like EBP50 and filamentous actin (F-actin). In a different investigation, piR-55490 was discovered to inhibit lung cancer cell proliferation by interacting with the 30 UTR of the mTOR mRNA (Peng et al. 2016). In NSCLC A549 and HCC827 cell lines, elevated piR-651 increased cell proliferation, migration, and invasion while suppressing cell death (Zhang et al. 2018). However, further research is required to understand its mechanism. Additionally, compared to primary cancer cells, metastatic lung adenocarcinoma cells drastically decreased the expression of piR-57125 (Daugaard et al. 2017).

8.14 Liver Cancer

According to predictions, liver cancer will rank as the sixth most frequently diagnosed cancer worldwide and the fourth most prevalent cause of cancer mortality. According to research by Law et al., piR-Hep1 is elevated in nearly half of HCC tumors (46.6%) compared to the neighboring nontumor liver. Cirrhotic nodules (CNs), low-grade dysplastic nodules (LGDNs), high-grade dysplastic nodules (HGDNs), early hepatocellular carcinoma (eHCC), and advanced HCC (pHCC) are the different stages of the development of liver cancer (Ng et al. 2016b). Numerous piRNAs served as markers for each stage.

PiR-LLi-24894 was only expressed in CNs, while piR-LLi-30552, hsa-piR-020498, and hsa-piR-013306 were largely expressed in HGDNs, eHCCs, and pHCCs, and were exclusively accumulated in HCC. PiR-823 also encouraged the formation of extracellular matrix components such as collagen type I alpha 1 and a-smooth muscle actin (a-SMA), leading to cirrhosis (Tang et al. 2018). By triggering the signal transducer and activator of transcription 3 (Stat3)/Bcl-xL signaling pathway, piR-Hep1 may have a role in reducing cell death(Law et al. 2013).

8.15 Stomach Cancer

The third most common cancer-related cause of death worldwide and the fifth most frequently diagnosed malignancy (Bray et al. 2018), gastric cancer is still a severe disease. PiR-823 was found to be markedly downregulated in gastric cancer tissues. PiR-823 mimics reduced cell proliferation, and a xenograft nude mice model demonstrated that piR-823 prevented tumor growth (Cheng et al. 2012b). Additionally, peripheral blood showed decreased expression of piR-651 and piR-823 in patients with gastric cancer. There have been reports of an increase in piR-32105, piR-58099, and piR-59056 in gastric cancer tissues (Cui et al. 2011). It is commonly recognized that surgery or endoscopic treatment can cure stomach cancer in its early stages, but the prognosis for advanced gastric cancer is uncertain. While immunotherapy, like anti-PD-1/PD-L1 therapy, is a potential treatment for cancer, patients

with advanced gastric cancer are not exceptionally responsive to immunotherapy alone or when paired with chemotherapy in first-line treatment.

8.16 Colorectal Cancer

The second most frequent disease in women and men worldwide is colorectal cancer (Law et al. 2013; Tang et al. 2018). According to several experts, PIWI has been linked to the onset of colorectal cancer (Weng et al. 2018; Mai et al. 2018a). According to Weng and his team's (2018) hypothesis, piRNA-823 was one of the piRNAs that aided in developing colorectal cancer. These genes included the activating transcription factor 3 (ATF3), the BTG anti-proliferation factor 3 (BTG1), the dual specificity phosphatase 1 (DUSP1), the fas cell surface death receptor (FAS), the nuclear factor kB (NF-kB) inhibitor an (NFkBIa), the uridine phosphorylase 1 (UPP1), the sestrin 2 (SESN2), and the tumor protein p53 inducible nuclear Additionally, piR-54265 hampered treatment, and individuals with more serum piR-54265 levels responded to chemotherapy noticeably less (Mai et al. 2018a). By encouraging its phosphorylation at Ser326, piR-823 has been shown to stimulate the production of heat shock transcription factor 1 (HSF1) and promote colorectal carcinogenesis (Yin et al. 2017). Additionally downregulated in CRC patients were piR-5937, piR-001311, piR-004153, piR-017723, piR-017724, piR-020365, piR-28876, piR-32105, piR-58099, and piR-59056 (Vychytilova-Faltejskova et al. 2018; Qu et al. 2019; Ng et al. 2016c).

8.17 PIWIs May Be Used for Cancer Diagnosis and Prognosis

Most of the existing research on PIWIs and carcinogenesis and cancer progression is derived from reports of clinical–pathological findings. PIWIs may be employed as biomarkers for clinical diagnosis and prognosis relating to poor outcomes, according to these studies. However, examining all four human PIWI homologs, most of this work concentrated on PIWIL1 and PIWIL2. According to research, HIWI expression gradually rises in normal gastric tissues, atrophic gastritis, intestinal metaplasia, and gastric malignancies, indicating that HIWI may have a role in the emergence of gastric cancer (Liu et al. 2006). HIWI was demonstrated to promote chemoresistance in cervical cancer and was proposed as a cancer stem cell marker (Liu et al. 2014b). Elevated HIWI mRNA and protein levels in pancreatic ductal adenocarcinoma have no overall effect on patient survival.

However, patients who had aberrant HIWI mRNA expression had a substantially higher probability of dying from a malignancy (Grochola et al. 2008). HIWI expression significantly increased with the progression of tumor grades in gliomas, demonstrating the relationship between higher positive HIWI and worse outcomes (Sun et al. 2010). In contrast, PIWIL1 has not been linked to the clinical– pathological characteristics of endometrioid cancer (Liu et al. 2010). Human testicular seminomas, prostate cancer, breast cancer, gastrointestinal cancer, ovarian cancer,

and endometrial cancer were all shown to have increased PIWIl2 expression, as were mouse breast cancer, rhabdomyosarcoma, and medulloblastoma (Lee et al. 2010). It is interesting to note that human testicular non-seminoma cancers did not exhibit elevated PIWIl2 expression, and the equivalent PIWIL2 isoform is PL2L60A, not PL2L80A (Gainetdinov et al. 2014). PIWIl2 was primarily expressed in cancer stem cells, 81% of in situ carcinomas, and 90% of invasive carcinomas in breast cancer (Lee et al. 2010). Additionally, he and his coworkers (2010) noted that PIWIl2 was expressed at different stages of cervical cancer (He et al. 2010). EIF2C1 and PIWIL2 may serve as potential colon cancer biomarkers with early diagnostic value, according to another study that found that their elevated expressions were substantially related to the disease (Li et al. 2010). Curiously found that overexpression of PIWIl2 caused cisplatin resistance in human ovarian cancer cell lines, indicating that PIWIL2 was a marker for cisplatin resistance in cancer chemotherapy (Wang et al. 2011).

Clinical patients with breast cancer revealed significant expression levels of PIWIL2 and PIWIL4, but not PIWIL1 or PIWIL3 (Hashim et al. 2014). Elevated PIWIL1 and PIWIL2 expressions were associated with worse overall survival, and PIWIL1 was suggested to be an independent prognostic factor (Wang et al. 2012). In gastric cancer, the expression of PIWIL1-4 was significantly correlated with the T-stage, lymph node metastasis, and clinical TNM (cTNM). Stage III epithelial ovarian cancer patients' primary and metastatic tumors showed significantly increased PIWIL1-4 expression (Chen et al. 2014). Some piRNAs have been linked to the characteristics of malignancies so far, including human piR-Hep1, piR-823, piR-651, piR-4987, piR-20365, piR20485, piR-20582, and piRABC (Tan et al. 2015). By controlling de novo DNA methylation and angiogenesis in multiple myeloma, piR-823 was discovered to support carcinogenesis (Yan et al. 2015). By enhancing the methylation process of Lactein in breast cancer stem cells, it has been proposed that piR-932 binds PIWIL2 (Zhang et al. 2013). However, more research is needed to understand how piRNAs in association with PIWI proteins contribute to carcinogenesis, invasion, and metastasis.

8.18 piRNAs as Biomarkers in Cancer Potential Clinical Applications of piRNAs as Cancer Biomarkers

Determining the various expression patterns of piRNAs that are particular to different cancer types might enable the development of new cancer biomarkers, given the significance of piRNAs and their associated proteins in many cellular processes. The prognosis for cancer is improved by early detection and treatment. RNA sequencing has shown that human blood contains piRNAs in addition to miRNAs and other types of noncoding RNAs (Huang et al. 2013c; Freedman et al. 2016). Since piRNAs are similar in length to miRNAs, they can easily pass through cell membranes and enter the bloodstream (Mei et al. 2013). Additionally, they are exceedingly stable and resistant to destruction by ribonucleases in bodily fluids (Mitchell et al. 2008).

Therefore, the piRNAs found in circulating tumor cells (CTCs) are intriguing novel complimentary tumor indicators for cancer.

It was observed that GC patients have lower levels of piR-651 and piR-823 in their peripheral blood than healthy controls. Liu and his team (2019) found that piR-651 and piR-823 are more sensitive compared with the favorable detection rates of serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels, suggesting that these piRNAs are more sensitive for gastric cancer screening than the frequently used biomarkers (Liu et al. 2019). Although piR-5937 and piR-28876 had an excellent diagnostic value even for individuals in clinical stage I, their expression in the serum of CRC patients dropped dramatically with the advanced clinical stage. Serum piR-54265 levels also serve as a therapeutic measure that predicts how well chemotherapy treats CRC patients. Patients with low serum piR-54265 levels exhibited a more excellent response to chemotherapy than those with high levels (Mai et al. 2018b). Compared to serum from healthy persons at diagnosis, piR-651, which is downregulated, originates from circulating rather than tumor cells. The patients' downregulation may result from variations in the peripheral blood populations linked to the existence of lymphoma (Cordeiro et al. 2016). Numerous studies have identified a piRNA signature in clear cell renal carcinoma (ccRCC) that may act as a prognostic indicator. Cordeiro et al. (2016) found three piRNAs (piR-30,924, piR-57,125, and piR-38,756) that were substantially linked with tumor recurrence and overall survival by employing piRNA microarray and subsequently validating candidate piRNAs in a larger cohort (Cordeiro et al. 2016).

8.19 New Therapeutic Approaches Using piRNAs

It is an exciting application to use synthesized piRNAs as weapons to inhibit the manufacture of cancer-related proteins by attaching to mRNAs since it may avoid the need for processing by enzymes like Dicer. In addition to being used as a post-translational strategy in combinatory therapy for various hypoxia cancers, PIWI antibodies may have a therapeutic effect on the proliferation of hypoxic cancer. Because the suspected regulators of mRNA PIWI expression, at least in the form of piRNAs, are already known, piRNA sequences should be employed for post-transcriptional silencing. At first impression, it could be preferable to prevent the development of a dangerous component rather than combat the undesirable consequences of a molecule already in operation. It is feasible to use specialized synthetic piRNAs designed to bind to PIWI proteins and exert genomic silence on PIWI genes at a transcriptional level, which differs from miRNAs and piRNA post-transcriptional mRNA inhibition. In a reverse way, this tactic is comparable to the "ping-pong" method of piRNA synthesis (Ross et al. 2014). For example, PIWI antibodies could be used to deliver drugs to hypoxic cancer cells, a delivery strategy already used with other antibodies. It would reduce the side effects of conventional cytotoxic medicines and perhaps improve the response to hypoxic cancer therapy.

8.20 Database for piRNAs and Functional Predictions

Lakshmi and his colleagues first created the piRNABank as a website resource on classified and clustered piRNAs in 2008 (Sai Lakshmi and Agrawal 2008). This database details the piRNAs found in humans, mouse, rats, and drosophila. The website (http://pirnabank.ibab.ac.in/) gathers all potential piRNA clusters and displays piRNAs and their related genomic elements, such as genes and repeat regions, on a genome-wide map. Chinese researchers Wang et al. and Zhang et al. have developed a more potent tool for piRNA functional studies called piRNA database-piRBase (Wang et al. 2018; Zhang et al. 2014). This database combined 264 datasets from 21 different taxa, and more than 173 million piRNAs were gathered. Additionally, it includes possible data on piRNA targets and piRNAs linked to diseases. Additionally, documented piRNA targets and epigenetic data are gathered. As a result, these databases (http://www.regulatoryrna.org/database/piRNA/) incorporate post-transcriptional regulation and epigenetic control data to facilitate the functional study of piRNA.

8.21 Future Directions in piRNA Research in Hypoxic Oncology

According to the studies we evaluated, PIWIs may be crucial in regulating the development of some cancer hallmarks in hypoxic cancer cells. Various pieces of evidence proved the prognostic and therapeutic potential of PIWIs. It is necessary to conduct additional research to explain this discrepancy. Further research is needed to determine whether PIWI scan is an essential marker for clinical diagnosis and prognosis because there is currently little clinical data available. There is undoubtedly a need for more information regarding the specific molecular mechanisms through which PIWIs contribute to carcinogenesis and the growth of hypoxic tumors. PiRNAs may be used as possible tumor biomarkers, a hot area of research, as several recent studies have revealed that several piRNAs are significantly expressed in blood samples. Additionally, several piRNAs' expression was linked to pathogenic factors or clinical outcomes. Regarding potential limitations, most investigations involved retrospective clinical material, indicating that prospective, multicenter clinical trials should confirm these findings. There is still much to learn about the roles of piRNAs and the proteins that interact with them in cancer, and this subject could provide a wealth of helpful information. Future usage of thorough and high-throughput methods could uncover more useful piRNA biomarkers and their interacting proteins unique to various types or stages of cancer. piRNAs and the proteins interacting with them will draw a lot of attention if their functions and mechanisms are investigated since they would be excellent biomarkers for diagnosis and novel targets for skillful therapeutic modulation.

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Conflict of Interest No.

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Hypoxia and the Metastatic Cascade

9

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Abstract

Metastasis is a leading cause of mortality in cancer patients. The journey of a detached primary tumour cell to a secondary homing site is a multi-step process with several intercellular interactions. In this process, a metastatic cell overcomes many barriers and adapts to the changing microenvironment. Our understanding of motility, invasion and epithelial to mesenchymal transition has led to the identification of targetable signalling networks and regulatory pathways. Emerging research has also identified genetic heterogeneity and variable microenvironment in the primary tumour cell as different from its metastatic progeny, leading to differential immune response. Therapeutic regimes with both standard chemotherapy and, in some cases, targeted therapy have only marginally improved the overall survival in metastatic cancer patients. It is for this reason that the current research strives to understand metastatic cancer, its origin and the role of the tumour microenvironment in genesis and persistence of metastasis. The hypoxic tumour microenvironment is known to arise in such growing primary tumours, where the vasculature is unable to keep pace with the high rate of proliferation, forming hypoxic niche within the tumours. Hypoxia, therefore, will always precede metastasis, thus implicating that the former plays a larger role in the development of the aggressive phenotype. Some of the leading research of our times is trying to understand latency of cancer cells, immune-metastatic cell interaction and epigenetic changes that promote metastasis and chemoresistance. With exciting possibilities of immunotherapeutic and other

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intervention strategies, a comprehensive understanding of the role of hypoxia in promoting metastasis will be useful. In this review, we dissect how hypoxia, hypoxia-inducible factors and other associated molecules dynamically modulate various stages of metastasis.

Keywords

Hypoxia · Metastasis · HIF · Invasion · Tumour-microenvironment · EMT · Signalling · Immune evasion · Epigenetics · Anti-cancer therapy · Chemoresistance · CTCs

9.1 Life of a Cancer Cell in the Hypoxic Tumour Microenvironment and Beyond

Metastasizing cells appear to be a life of their own. Surviving and adapting just as we do, sometimes under adverse circumstances, often alone. While fully aware of the dreadful consequences associated with this disease, one cannot help but marvel at the skillful resilience and adaptations of this entity.

Metastasis is defined as the sequential event in which cancer cells break off from their site of origin and colonise new sites. This propensity of cells to be motile is frequently seen in normal cells too, where cells migrate in response to chemical attractants such as that observed in morphogenesis during embryo development and movement of immune cells to site of infection. A normal cell, therefore, has all the molecular components that can be triggered to initiate these events, and a cancer cell is endowed to be even more receptive to triggers from its neighbouring tumour microenvironment (TME). Over the years, several new paradigms on cancer progression and molecular underpinnings of cancer metastasis have been extensively delineated. With these studies, researchers have overcome the challenge of detection limits, where the small fraction of disseminated circulating tumour cells has been identified in the backdrop of millions of RBCs and WBCs in blood (Hong and Zu 2013; Bankó et al. 2019). The entire field of circulating tumour cells emerged with the effort to look for early dissemination of cancer cells with increasing sensitivity and also specific for certain tumour-associated mutations.

9.1.1 Manifestation of Metastasis

Metastatic disease spread is progressive and fatal. Most cancer-related deaths are said to occur due to metastasis, and in a majority of patients, the disease is detectable only in autopsy. In a significant number of patients with metastatic disease (not related to skin), a primary tumour has not been identified – tumours of unknown

primary source. While it is generally reported that 90% of cancer-related deaths are due to metastasis, these numbers may vary. A study from the data of cancer registry at Norway reported 67% mortality as being caused by cancer over a period of 10 years (Dillekås et al. 2019). Chemotherapy-induced metastasis has also been reported wherein cytotoxic chemotherapy inflicts tissue damage, hypoxia and cancer cell apoptosis, enforcing the release of proinflammatory cytokines and chemokines locally as well as systemically, leading to immunosuppression and acquisition of metastatic properties (Karagiannis et al. 2019).

Cancer metastasis is considered to be a secondary event and a delayed step in tumour progression associated with tumours of diameter more than 2 cm (Koscielny et al. 1984). Initially it was assumed that removal of such tumours should dramatically improve the prognosis and survival; however this was not always observed to be true (Bedenne et al. 2007). Recurrence was a frequent event in certain breast cancers which were equal or less than 1 cm (Gonzalez-Angulo et al. 2007). Further, many of the breast cancer tumours at T1 and T2 (Zimmer and Steeg 2015) including some with clinically undetectable tumours (Mauri et al. 2005) already exhibited metastasis. This led researchers to believe that metastasis may be an early, clinically undetectable event in the development of cancer. Thereon, researchers have looked at smaller tumours and their molecular profiles carefully. Critically, investigations on the role of the tumour microenvironment around the primary tumour cell, particularly hypoxia, gained momentum.

9.1.2 Cellular Characteristics of a Metastatic Cancer Cell

Cancer cells acquire the potential to migrate, exploit the fluid nature of the circulatory and lymphatic system to move greater distance and utilise the presence of conducive external growth factors at distant sites to foster their second homes. The malignant cells of a growing tumour undergo epithelial to mesenchymal transition (EMT), a cell redevelopment program, in which the cell loses its epithelial characteristics and gains mesenchymal properties (Thiery 2002). Acquisition of these attributes enable cells to invade other tissues and colonise them. A tumour cell with its newly acquired skill-set interacts with a neighbouring vessel. Crosstalk between these EMT transitioned tumour cells and endothelial cells of the blood vessels allow the upskilled tumour cells to breach the basement membrane of these blood vessels and intravasate into them. Subsequently, this metastatic cell shall overcome challenges from immune cells and unfamiliar environments to establish itself elsewhere. Only a few cancer cells are successful in acquiring these properties, and even fewer survive the harsh vascular environment. The migrating cells that succeed reach distant tissues and become harbingers of micrometastases. Once established, micrometastases undergo proliferation leading to macrometastases, and subsequently secondary tumour colonies emerge. The series of steps that the cells undergo is known as metastatic cascade. Once the cells exit their site of origin, they may either be destroyed in blood or sustain for long enough to get lodged in capillary beds of other tissues. If sustained, the cells would then extravasate into a

new niche for the formation of a potential tumour colony (Fidler and Paget 2003). However, the colonisation in this niche is dependent on the fate of the cell.

Tumour metastasis is the major cause of mortality associated with cancers, leading up to 90% of cancer patient deaths. The uncontrolled cell growth causes various microenvironment stresses such as inflammation, hypoxia and deprivation of nutrients. The hypoxic microenvironment thus developed helps in advancement of the tumour into phases of metastasis such as local invasion, EMT, intravasation, extravasation, formation of micrometastasis and ultimately colonisation via macrometastasis (Majidpoor and Mortezaee 2021). Several new paradigms have emerged on the evolution of cancers that put forth comprehensive discourse on when and how these metastatic cells arise. However, we do know that the metastatic cancer cell fate is indelibly linked to cancer evolution.

9.1.3 Fate of a Cancer Cell

There are many theories proposed for delineating cancer evolution. The understanding of cancer biology has grown by leaps and bounds from the initial reductionist view on cancer cells (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011) to that of heterotypic cell biology that regard tumour as a complex tissue. A series of mutations paired with proliferation of dominant cell types results in tumour growth and development that gives rise to a favourable tumour microenvironment by virtue of different growth signals and intercellular interactions. The latter view suggests that a tumorous mass comprises proliferating cancer cells; cancer stem cells along with tumour-associated immune cells together make a tumour self-sufficient with self-renewal properties, enabling it to invade niches foreign to the cancer cells (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). The understanding of the interaction of cancer cells with the tumour microenvironment is still developing, and many interesting aspects have emerged including that of cancer stem cell niche.

Displacement and spread of cancer cells away from the primary tumour was observed and termed as metastasis in 1829 by Joseph-Claude-Anthelme Récamier, a surgical oncologist. The identification of epithelial cells in blood circulation came a few decades later in 1869 by Thomas Ashworth and in the lymphatic system and lymph nodes in 1881 by William Hampstead (Akpe et al. 2020; Galvis et al. 2021). Different theories have attempted to explain the emergence of migrating cancer that eventually finds a second home. Rudolf Virchow's chronic irritation theory suggested that the new site of growth is due to an inflammatory stimulus (Balkwill and Mantovani 2001). Ewing's mechanical hypothesis postulates that the pattern of circulation determines the secondary tumour location and the lungs are most prone as it is the first organ encountered in circulation. Contrary to this, Stephen Paget's seed and soil hypothesis, deduced from autopsies of 735 breast cancer patients with high incidence of metastases in the liver, ovary and bone, suggests that a cancer is a 'seed' that circulates in the body and upon finding a microenvironment conducive for its development, the circulating cancer cell lodges itself in the fertile 'soil' (Paget

and Paget 1989). A large meta-data study on 179,581 patients has shown that anatomical/mechanical constraints can contribute to site determination and the two theories are not always mutually exclusive (Riihimäki et al. 2018). These circulating tumour cells face new microenvironments with different growth signals and regulatory factors, evolving and adapting through sequential mutations and selection. However, upon encountering a hostile environment at site, the cells can undergo dormancy, or its complete eradication may occur at any point of tumour development and metastasis. Microfluidic chips are widely used to validate the metastatic potential of cells and in turn also help in identifying potential homing sites for cancers in vivo (Aleman et al. 2019). This versatile approach has been utilised to constitute an in vivo-like microenvironment, including low oxygen, to study differentiation of stem cells (Chen et al. 2013) as well as simulate the extravasation and micrometastasis of breast cancer cells to bones (Bersini et al. 2014). It has further been used to demonstrate the ability of mesenchymal stromal cells (MSCs) to undergo homing in presence of hepatoma cells, enhanced under influence of TGF-β and suppression through physical constraints of the 3D microstructures (Yang et al. 2017).

9.1.4 The Dormant Cancer Cell and Plastic Cancer Stem Cell

We now know that cancer cells can remain dormant in both the primary site and the metastatic secondary site. The dormant cells can reawaken, proliferate and cause tumour relapse. They are clinically undetectable and contribute to the therapeutic resistance. They are actively undergoing 'editing', accumulating genetic and epigenetic changes that could improve their adaptation in the environment. Quiescent cells have been shown to evade immunity by forming an immunosuppressive niche (Baldominos et al. 2022). In their quiescent state, the cancer cells dynamically interact with the tumour microenvironment, modulating their characteristics until they can lead to tumour outgrowth and dissemination. After treatment, some cancer cells may become quiescent or latent and form clinically undetectable minimal residual disease. Increasing evidence shows that this repertoire of cells that can switch between dormant and proliferative states, enabling greater metastatic potential, is cancer stem cells (Reya et al. 2001; Giancotti 2013). They also exhibit chemoresistance and actively engage with the microenvironment. Parallels have been drawn between the state of cancer cell dormancy and the quiescent cancer stem cell with experimental evidence of being regulated by common signalling pathways such as mTOR, Wnt and Notch (Reya et al. 2001; Schewe and Aguirre-Ghiso 2008; Oskarsson et al. 2011; O'Connell et al. 2011). It has been possible to populate cancer stem cells from dormant cancer cell populations by invoking certain signalling pathways (Ranganathan et al. 2006; Gao et al. 2012). Regardless, the two terms cannot be used interchangeably because not all dormant cancer cells are cancer stem cells and vice versa. Importantly, the role of hypoxia and tumour microenvironment in cellular adaptations undertaken by the quiescent cancer stem cells cannot be undermined.

Studies have been conducted to determine whether hypoxia emerges as an independent prognostic factor for patient outcome and disease progression. Several studies have demonstrated that patients with hypoxic tumours have an increased risk of metastasis and mortality. One such study on 247 patients of prostate cancer measured tumour hypoxia before radiotherapy and reported that the median O_2 was 10mmHg (~1.3% O_2) which was 10-12 times lower than muscle tissue O_2 (Milosevic et al. 2012). Taking prostate-specific antigen as a marker, they determined that degree of hypoxia in the tumours before radiotherapy was a predictor of quicker biochemically measured relapse (Milosevic et al. 2012). Similarly in breast cancers, a compilation of 125 studies of pre-treatment oxygenation status of solid tumours showed that the mean partial pressure of oxygen (pO_2) in breast tumours ranges from 2.5 to 28 mm of mercury (Hg), with a median value of 10 mm Hg, as compared with 65 mm Hg in normal human breast tissue (Vaupel et al. 2007). In pancreatic cancers, which are highly metastatic and usually unresectable, tracer molecules such as 18F-fluoroazomycin arabinoside (Metran-Nascente et al. 2016) as well as electrodes (Koong et al. 2000) have been used to measure hypoxia. In a pancreatic ductal adenocarcinoma mice model, the highly metastatic pancreatic ductal adenocarcinoma subpopulation is enriched for hypoxia-induced genes (Chiou et al. 2017).

9.1.5 Role of Hypoxia in Determining the Fate of a Cancer Cell

Hypoxia forms an important part of the tumour microenvironment and cancer stem cell niche. The unhindered growth of cancer cells and absence of adequate vascular support gives rise to oxygen-starved cells and tissues. The oxygen starvation is commonly known as hypoxia. While genetic defects and cellular damage by reactive oxygen species (ROS) remain overarchingly dominant, hypoxia works at the gene-environment interface. Under high oxygen tension, hypoxia-inducible factors (HIFs) are constantly inactivated and prepared for ubiquitination by prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH). The stabilisation and activity of HIFs is an inevitable response under the prevailing reduced oxygen tension. HIFs are heterodimeric transcription factors, consisting of constitutively expressed β-subunit and oxygen tension-regulated α-subunit. The action of HIF transcription factors is dependent upon its binding affinity to specific sites in the promoter region of hypoxia-responsive genes. Hypoxia, through HIF-dependent signalling, affects myriads of life processes such as vascularisation, metabolism, proliferation, apoptosis, invasion, and metastasis (Semenza 2013; Schito and Semenza 2016; Kaelin and Ratcliffe 2008; Pugh and Ratcliffe 2003).

The first encounter of a cancer cell with a hypoxic microenvironment is likely to occur after the primary cell population has expanded at rates faster than the vasculature to form areas of low perfusion. A cancer cell in a proliferating population is likely to go through many rounds of ischemia (low oxygen) and reperfusion (normal tissue oxygen). A cancer cell facing transient acute hypoxia affects the metabolic processes that involve oxygen and are likely to activate autophagy. In such cells,

proliferation and autophagy may co-exist. Ensuing rapid genetic, molecular and biochemical changes promote cells with increased heterogeneity and proliferative and adaptive capabilities. HIF-1α is known to be the key transcription factor that is stabilised in acute hypoxia (Hu et al. 2003). Importantly, HIF-1α null cells show markedly reduced growth and proliferation (Guimarães-Camboa et al. 2015).

A growing tumour will also have cells exposed to chronic hypoxia as a result of an imbalance of oxygen supply and demand in areas at a distance greater than 70-150um from the blood vessels (Cairns et al. 2001; Cairns and Hill 2004). The cells in this region are likely to stabilise the isoform HIF-2α best known for its role in maintaining the vasculature and vascular remodelling (Kapitsinou et al. 2016; Skuli et al. 2012). As a transcription factor, it promotes expression of many genes that help cancer cells to adapt to the changing microenvironment, different from those regulated by HIF-1α. In particular, HIF-2α has been implicated to be at an oncogenic axis, while both HIF-1α and HIF-2α participate in cancer stem cell maintenance. The quiescent cancer cells, therefore, are likely to interact with the hypoxic tumour microenvironment and invoke HIF to strategise a cellular adaptation program that will eventually lead to metastasis and chemoresistance. These cancer stem cells, under the influence of hypoxia and HIF, exhibit epithelial to mesenchymal transformation, invasion and metastasis.

An angiogenic shift marks the adaptation of cancer cells to low oxygen that results in dysregulation of genes associated with chaotic vasculature consisting mostly of truncated and stubbed vessels. The anomalous vasculature along with the secretion of abnormal signalling molecules leads to localised hypoxic niches or pockets of hypoxia further affecting angiogenesis in normal embryogenesis as well as pathophysiological context as in solid tumours. HIF-2α is highly expressed in the vascular endothelial cells and upregulates the expression of an array of target genes that can influence vascular remodelling as well as angiogenesis. The vascular endothelial growth factor, VEGF, is a major protein required in normal cells for generating new vasculatures such as that observed in foetal tissue, corpus luteum and placenta. In abnormal growth such as cancer, VEGF and other related proteins such as Angiopoietin, Ang2 are responsive to the tumour microenvironment and particularly hypoxia. It is reported that similar to cancer dormancy, quiescent cells also promote angiogenic dormancy. These cells are not proliferative and exist as a clinically undetectable population.

Every cancer type has a preferential site or destination of secondary metastasis. These have been mentioned in Table 9.1, along with the respective change in hypoxia of tumour tissue compared to normal. Interestingly, few recent reports on breast cancer have shown that hypoxia and HIFs can influence the choice in the site of metastasis. Initial studies showed that HIF-1α expression in breast cancer cells promotes lung dissemination in genetic models (Liao et al. 2007). Hypoxia-induced angiogenesis and angiogenic genetic signature were shown to promote both primary tumour growth and lung metastasis but was not essential for bone metastasis (Lu and Kang 2010). HIF-1α and TGFB together were reported to promote bone metastasis (Dunn et al. 2009). Recently published study (Todd et al. 2021) generated a spontaneous murine mammary carcinoma model where mammary-specific deletion

Table 9.1 A comparison of normal and tumour tissue hypoxia in various sites and their preferred sites of metastasis

Tissue	Cancer	Tissue physoxia (median % O2)	Related tumour tissue (median % O2)	Fold change in median % O2 (approx)	Preferred site of metastasis
Rectal mucosa	Rectal carcinoma	3.9	1.8	–2.1	Thorax, liver
Brain	Brain tumour	4.6	1.7	–2.7	Lung, cervical lymph nodes
Liver	Liver cancer	4.0–7.3	0.8	–(5.0 – 9.1)	Lungs, bones
Cervix (nullipara)	Cervical cancer	5.5	1.2	–4.5	Skin
Lung	Non small-cell lung cancer	5.6	2.2	–2.5	Bone, brain
Ovary	Ovarian cancer	7.1	0.3	–23.7	Liver, lymph node, lung
Prostate	Prostate cancer	3.9	0.3	–13	Bone, lung, lymph nodes
Pancreas	Pancreatic tumour	7.5	0.3	–25	Liver
Breast	Breast cancer	8.5	1.5	–5.7	Liver, bone, lung, brain
Kidney coretex	Rental cancer	9.5	1.3	–7.3	Humerus, pancreas

Adapted and modified from Deng et al. (2018), Muz et al. (2015), Hammond et al. (2014a)

of HIF-1α, HIF-2α or von Hippel-Lindau factor (Vhl) was done using the Cre-Lox system. Their work shows a bias in the preferred site of metastasis in HIFs and associated molecules, VHL. While HIF-1α or HIF-2α deletion in the primary tumour decreased metastatic tumour burden in the bone marrow, Vhl deletion increased it. Surprisingly, there was a marked increase of the metastatic burden in the lung with only HIF-1α and not HIF-2α or Vhl. These reports, although early, suggest the exciting possibility of redirecting the site of metastasis to a preferred destination by modulating the tumour microenvironment through specific targeting of HIF and HIF regulatory molecules.

9.2 Hypoxia Orchestrates the Metastatic Cascade

The process of metastasis initiates before cells attain the capability to migrate to a secondary site. Primary cells with metastatic potential acquire an invasive phenotype which begins with the resistance to anoikis; polarisation and elongation of migrating cell(s); the protrusion of cells in the form of lamellipodia, pseudopodia or invadopodia; and its attachment to the extracellular matrix (ECM) components. Further, adhesion and de-adhesion of the leading edge and the cell body respectively generates a contraction force that propels the entire cell forward. In this section, the contribution of hypoxia in various steps and events of metastatic disease manifestation are described.

9.2.1 The Invasive Phenotype and EMT

EMT is one of the major steps in metastasis characterised by the loss of adherent properties of epithelial cells and acquiring new mesenchymal features (Kalluri and

Weinberg 2009). Activation of the EMT pathway can prime the epithelial cells to detach, gain motility as well as transform the transcriptome in such a way that it favours its invasion through the lymphatic and the circulatory system. Cell adhesions are facilitated by intracellular proteins called cadherins. Preponderance of adherent junctions declines as a result of loss or downregulation of E-cadherin and upregulation of N-cadherin. This transition of E-cadherin to N-cadherin is a molecular characteristic of EMT. On the other hand, mesenchymal proteins like actin, fibronectin and vimentin rearrange the cytoskeleton to stimulate cell motility. Several gene families play an important role in invasion – of which matrix metalloproteinases (MMPs), urokinase plasminogen activator/receptor (uPAR), integrins and cathepsins are some of the prominent ones. These proteins function to modify the surrounding microenvironment, making it more suitable for cells to move around and out of their niche. The MMPs function to alter the extracellular matrix by degrading the components of the ECM and the basement membrane (Quintero-Fabián et al. 2019). uPAR converts plasminogen to plasmin, leading to a series of proteolytic cascades that result in ECM degradation (Kwaan and Lindholm 2019). Cathepsins, a family of proteases, are often overexpressed on the cancer cell surface from where they attack and digest E-cadherin, dissolving the adherent junctions (Mijanović et al. 2019). These ECM disrupting enzymes, tightly controlled in normal development, are significantly dysregulated in cancers with poor outcome. Apart from the loss of E-cadherin, the gain of molecular markers such as N-cadherin, vimentin and fibronectin are also evident in EMT (Loh et al. 2019). The changes in gene expression while undergoing EMT is the result of signalling cascade(s) regulated by transcriptional signatures consisting of genes such as SNAIL, SLUG, ZEB and TWIST (Comijn et al. 2001; Batlle et al. 2000; Hajra et al. 2002). The EMT transcription factors act downstream of key molecular pathways such as JAK-STAT, NF-κB, Notch and TGFβ and regulate metastatic progression (Gonzalez and Medici 2014).

9.2.2 Local Invasion of Cancer Cells

In primary solid tumour microenvironment, hypoxia enhances the motility and invasiveness of cancer cells by encouraging EMT. Hypoxia via HIF-dependent mechanisms regulates the transcription of Snail, Zeb1, Twist and Tcf3 in cancer cells which further helps in reducing the expression of E-cadherin and subdues the epithelial phenotype of cancer cells (Lu and Kang 2019). HIF-1 also promotes EMT by enhanced signalling of tyrosine kinase receptors pathway (Rankin et al. 2014), TGF-β pathway (Mingyuan et al. 2018) and the Wnt/ β-catenin signalling pathway (Shi et al. 2022). In gliomas, a significantly worsened prognosis is associated with the highly malignant glioblastoma in which the primary tumour aggressively invades surrounding normal brain tissue (Lah et al. 2020). Hypoxia is implicated as a major contributor to such aggressive tumours (Monteiro et al. 2017), often leading to differential expression of signature proteins that in turn regulate critical pathways of invasion (Abou Khouzam et al. 2021; Zou et al. 2019; Yang et al. 2021a; Bhushan

et al. 2021). In breast cancer cells, there is an interplay between HIF-1α, Notch and GPER which activates the HIF-1α-NOTCH-SNAIL pathway to present an enhanced EMT phenotype (De Francesco et al. 2018). hFAT1 is reported to upregulate HIFs and promote glioma invasion and stemness (Srivastava et al. 2018; Irshad et al. 2021) while being under the transcription regulation of NFkB (Srivastava et al. 2020). Several studies on different cancers with hypoxia-mediated molecules that promote EMT are known (Tam et al. 2020). As a potent ECM remodeler, hypoxia via HIF-1 and HIF-2 can induce the transcription of integrin subunits α5 and β1 (Ju et al. 2017) and integrin α6 (Brooks et al. 2016). Hypoxia can also modulate important proteins such as fibronectin receptor, syndecan-4, in a HIF-independent manner (Koike et al. 2004).

The migrating cells require collagen deposition and biogenesis. The collagen architecture is thick and linear in tumours and curly/smooth and relatively thin, in normal tissue (Fang et al. 2014). The role of collagen in different phases of metastasis is evident while exposing extracellular matrix, stimulating adhesion to the endothelium as well as mediating cancer cell aggregation in blood vessels (Spivey et al. 2012). Procollagen-proline dioxygenase (also known as Prolyl 4-hydroxylase) is an essential enzyme for collagen deposition vital in tumour fibrosis and maintenance of stiffness, and procollagen-lysine 2-oxoglutarate 5-dioxygenases (PLOD1 and PLOD2) are required for cross-linking of collagen fibres (Qi and Xu 2018; Wolf et al. 2009). Reports suggest that HIF-1 induces the expression of domain protein, prolyl 4-hydroxylase α-subunit isoform 1 and 2 (P4HA1 and P4HA2), and procollagen-lysine 2-oxoglutarate 5-dioxygenase 1 and 2 (Gilkes et al. 2013) thus playing a key role in the biogenesis of collagen. In fact, recent studies showed that HIF-mediated expression of PLOD1 contributes to a malignant form of glioblastoma (Wang et al. 2021a), while stabilisation of HIF-1 by P4H involvement in stabilisation of HIF-1α subunit induces chemoresistance in triple-negative breast cancer (TNBC) (Xiong et al. 2018).

9.2.3 Intravasation into Blood Vessels

How does it feel, how does it feel?
To be on your own, with no direction home
A complete unknown, like a rolling stone
~ Bob Dylan (June, 1965)
Nobel prize in Literature, 2016

The detached cancer cell finally crosses the endothelial layer of the lymphatic or the blood and ventures out on its own or in a group in a process termed cellular intravasation. This step, crucial for distant metastases, is affected by the tumour microenvironment in addition to the molecular signals, proteases and the tissue vasculature. Hypoxia via HIF-1-dependent mechanism is associated with an increased expression of type IV collagen-degrading enzymes – MMPs (MMP2, MMP9 and MMP14) (Gilkes et al. 2014; Turunen et al. 2017; Petrella et al. 2005).

Apart from degradation of collagen by matrix metalloproteinases, HIF-1 also induces the expression of a membrane receptor, PLAUR (urokinase plasminogen activator surface receptor), which degrades the extracellular matrix components (ECM). The altered expression of PLAUR changes the interactions between integrins and ECM which further helps in tumour invasion (Shyu et al. 2007).

The cancer cells in the lumen of lymphatic or blood vessels (now called circulating tumour cells, CTCs) will move to a new location. These CTCs may travel alone or in clusters in the blood and lymphatic vessels (Aceto et al. 2014). The CTC clusters have been shown to have greater success in micrometastases compared to the individual cell, possibly because of more pronounced cancer stem cell properties, such as CD44 expression in breast cancer cell-derived CTCs in mouse models (Liu et al. 2019). Circulating tumour cells (CTCs) have been established as the early indicators of metastasis and relapse. In the absence of a clinically detectable secondary metastasis, detection of CTCs becomes of utmost importance. Present in negligible numbers, CTCs carrying the specific mutations associated with a cancer cell have driven a new field of research and biomedical engineering. From methods of isolation of CTCs (Bankó et al. 2019) to facilitating detection on sensors of new and improved nanomaterials (Sun et al. 2021; Pallares et al. 2019), the urgency to detect poor cancer progression outcome early and with ease has seen a multidisciplinary approach like never before. Some of the work from our lab has attempted to utilise the multidrug-resistant p-glycoprotein as a bait to capture the CTCs in leukaemia with fair success (Gulati et al. 2018a, b, 2019). CTCs are representative of the tumour cell heterogeneity and allow oncologists a peep into the biology of the tumour as well as prediction of the genetics of the metastatic lesion. They have been shown to reflect hypoxia-induced heterogeneity and represent hypoxia-enriched gene signatures. The study of CTCs has exposed dramatic intra-patient and inter-patient heterogeneity and their evolution over time (Micalizzi et al. 2017).

9.2.4 Moving to a New Home: Extravasation from Blood Vessel to Secondary Site

Extravasation is a remarkable stage in the journey of the migratory cancer cell. The rogue cell escapes the circulatory or lymphatic system and searches for a new address to finally establish a home and proliferate in a challenging microenvironment quite different from its site of origin. In order to escape, the ECM is remodelled which aids the cells in crossing the basement membrane barrier. HIF-1 stimulates the expression of angiopoietin-like 4 (ANGPTL4), a protein that helps in disruption of intercellular endothelial cell (EC) interaction, and L1 cell adhesion molecule (L1CAM) helps in adherence of breast cancer cells to the EC monolayers (Zhang et al. 2021a). The elevated expression of ANGPTL4 and L1CAM facilitates the hypervascular metastasis of breast cancer cells to the lungs (Zhang et al. 2012). HIF-1-mediated expression of ANGPTL4 has also been shown to promote cancer cell growth, resistance to anoikis and progression of scirrhous gastric cancer (SGC) (Baba et al. 2017). Hypoxia also promotes the EFNA3 protein accumulation by

engaging the previously unknown long noncoding RNAs from EFNA3 locus. By promoting the overexpression of ENFA3, hypoxia contributes to the extravasation process and metastatic potential of breast cancer cells to surrounding tissues. Hypoxia thus promotes metastasis by helping cancer cells to extravasate to new locations and form micrometastatic lesions.

9.2.5 Pre-metastatic Niche Formation

Reversal of the EMT process, the transitioning from mesenchymal to epithelial phenotype of the recently migrated cells, precedes the formation of pre-metastatic niche at the homing site. This process is called colonisation. Establishing themselves as a secondary growth is challenging as the cells program themselves to adapt and survive in unfamiliar tissue microenvironments. Though unfamiliar, the 'soil' is not always unwilling to accommodate the 'seed'; therefore despite the absence of familial growth factors as in the primary tumour site, the metastasising cells attempt to colonise. There can be many outcomes associated with this event. Without the familiar molecular and physiological support, the metastasising cells may not survive at all in the host tissue. However, when they do survive for a small period of time, they may form small clumps, known as micrometastases, and further disseminate widely throughout the body of a cancer patient, leading to poor overall and disease-free survival outcomes. Finally, these micrometastases can increase in size to form larger lesions, macrometastases.

During this stage, the bone marrow-derived cells, such as tumour-associated macrophages (TAMs) and tumour-associated neutrophils (TANs), are recruited to the pre-metastatic niches. These cells are a major source of ECM remodelling proteases at the metastasis site that leads to angiogenesis. Hypoxia via HIF-1α mediates the recruitment of neutrophils (Du et al. 2008), the major source of MMP9 inducing angiogenesis (Deryugina et al. 2014), whereas TAMs employed in hypoxic conditions provide proteins leading to upregulated synthesis and assembly of collagen (type I, IV and XIV) for deposition, cross-linking and linearisation of the collagen fibres near invasive cancerous cells (Afik et al. 2016). Furthermore, these cells also constitute collagen cross-linking enzyme, lysyl oxidase (LOX), which stimulates metastasis in breast cancer. LOX-mediated cross-linking of collagen stimulates ECM stiffness and activates the integrin-dependent invasive phenotype in breast cancer (Levental et al. 2009). Expressions of LOX and LOX-like family proteins (LOXL1 and LOXL2) are greatly influenced by HIF-1α (Erler et al. 2006).

Studies have shown that hypoxic primary tumour cells release exosomes and microvesicles (MVs) which support the formation of a conducive pre-metastatic niche (King et al. 2012; Jafari et al. 2020; Peinado et al. 2011). For the recruitment of bone marrow-derived cells, these transport vesicles provide vascular permeability and fulfil the requirement of biomolecules like mRNAs, miRNAs, proteins, lipids, etc. HIF induces the expression of a small GTPase molecule RAB22A, important for the formation of budding vesicles for intracellular trafficking; thus hypoxia regulates

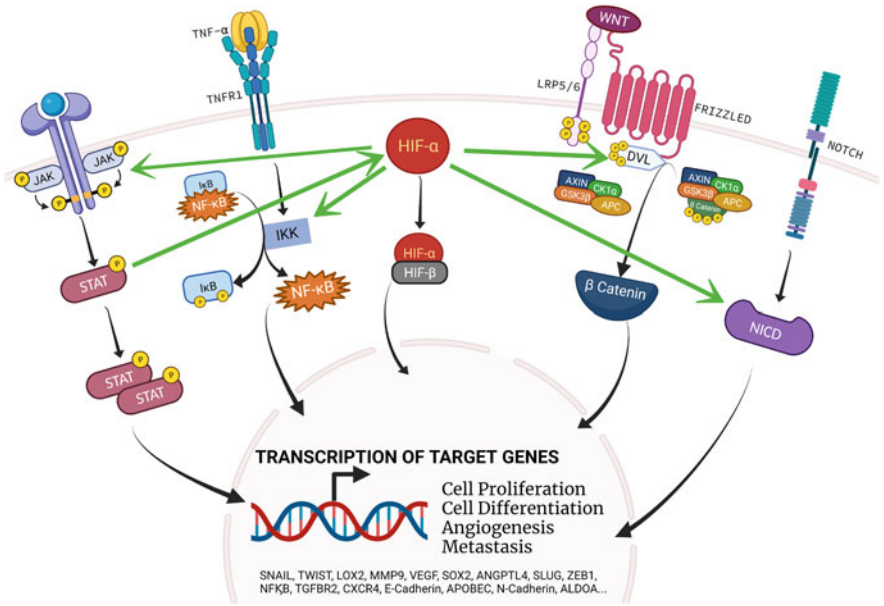

Fig. 9.1 Hypoxia-inducible factor-mediated signalling pathways of metastasis. Stabilisation of HIF proteins in the hypoxic tumour microenvironment results in transcriptional activation of genes of key signalling pathways of JAK/STAT, NFκB, Wnt/ β-catenin and Notch. These signalling pathways affect various cellular functions which are crucial for sustaining cancer progression and metastasis

the biogenesis of exosomes and MVs (Wang et al. 2014). Interestingly, the transcriptome and proteome analysis of exosomes isolated from glioblastoma multiforme (GBM) patients reveal the presence of HIF-regulated mRNAs and proteins (like MMPs, LOX, PDGF, IL-8, etc.) (Kucharzewska et al. 2013). Thus, hypoxia not only induces the exosomes biogenesis but also manipulates the exosome cargo loading to promote the cancer progression. Interestingly, these pre-metastatic niche promoting exosomes also determine a bias towards host sites and organotropism (Syn et al. 2016). Figure 9.1 depicts some of the key signalling networks which contribute to hypoxia-mediated promotion of metastasis.

9.2.6 Metabolic Reprogramming During Metastasis

A century ago, the German biochemist Otto Warburg first explained the connection between tumour progression and dysfunctional metabolism (Warburg et al. 1927). He also explained how hypoxia cells favourably convert glucose into lactose instead of its oxidation in mitochondria despite abundance of glucose. Metabolic rewiring and the metastatic cascade are highly intertwined processes, and together, they promote multiple steps of cancer metastasis (Wei et al. 2020). During metastasis,

both the cancer cells of the primary tumour and the cells in the metastatic niche face multiple metabolic challenges, particularly nutrient and O_2 deprivation, amid high metabolic requirements. The cells undergo metabolic reprogramming to increase cellular adaptation. Hypoxic tumour microenvironment greatly influences cellular metabolism by modulating cellular processes like glycolysis and lipid metabolism. Most of the glycolytic enzymes and glycolysis proteins are regulated by HIF-1 leading to higher uptake of glucose in tumour cells than normal cells. The following glycolytic protein isoforms are known to be transcriptionally regulated by HIF-1α and overexpressed in malignant cells compared to non-malignant cells – adenylate kinase-3; aldolase-A,C (ALDA,C); carbonic anhydrase-9; enolase-1 (ENO1); glyceraldehyde phosphate dehydrogenase (GAPDH); hexokinase 1,2 (HK1,2); phosphofructokinase L (PFKL); phosphoglycerate kinase 1 (PGK1); and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate-3 (PFKFB3) (Semenza 2010; Ke and Costa 2006). HIF-1 directly regulates the expression of glucose transporters (GLUT1 and GLUT3) (Liu et al. 2009; Calvert et al. 2006) and upregulates the lactose secretion by promoting the expression of LDHA (Cui et al. 2017). The hallmark of hypoxia-induced metabolic reprogramming is a shift in ATP production from OXPHOS to glycolysis (Colwell et al. 2017). Tumours with isocitrate dehydrogenase (IDH1) mutations acquire gain-of-function and convert alpha-ketoglutarate to the oncometabolite 2-hydroxyglutarate (Sasaki et al. 2012). Similarly hypoxia is also known to induce the production of the L-enantiomer of 2-HG, independent of IDH1/2 mutations that performs similar functions (Intlekofer et al. 2015). Similar to 2-HG, increased L-2HG in ccRCC suppresses the methylcytosine dioxygenase (TET) and KDM6A, leading to hypermethylation of DNA and histone (Shelar et al. 2018). Therefore it appears that in hypoxia, the frequently mutated IDH1 cells undergo epigenetic reprogramming to cancer stem cell state, driven by the altered metabolic activities. Additionally, other metabolites such as succinate, fumarate and glutamine are implicated to play a role in EMT, while metabolism of fatty acids and fructose supports MET.

9.2.7 Chemoresistance and Poor Prognosis

The quest for a magic bullet against cancer has seen several hopeful candidates but none that have truly succeeded. The effort of researchers and oncologists is to move towards targeted therapy powered by personalised medicine, far different from the common cure that is envisaged with every discovery. Recently, immunotherapy that attempts to block the immune evasion tactics of the cancer cell have been awarded and found clinical success, though in limited cancers. The standard of care continues to be surgical interventions, radiotherapy and chemotherapy. Among other strategies such as surgery, immunotherapy, combination therapy, targeted therapy and others, chemotherapy is the most accepted therapy owing to the vast data and widespread use, with no clear replacements. Some targeted therapies have dramatically improved progression-free outcome such as those using trastuzumab (against

HER2 in breast cancer) and imatinib or Gleevec (targeting BCR-ABL tyrosine kinase in chronic myelogenous leukaemia).

Drug resistance is the principal impediment in response to chemotherapy. Most of the known chemotherapies rely on oxygen-dependent generation of reactive oxygen species, and in the hypoxic interiors of most solid tumours, their action is limited. Due to the poor vasculature and inaccessible niches, the interiors of the solid tumours do not get sufficient drug. Sub-lethal dose of drugs, together with hypoxia-induced upregulation of channels that evict drugs, leads to chemoresistance. The problem is compounded by spiralling tumour burden, highly variable growth kinetics, genetic heterogeneity, fallible physical barriers, fallow immune system and the hypoxic microenvironment (Vasan et al. 2019). Though drug resistance is governed by a large number of factors, our focus in this section will be upon 'chemoresistance' as a result of the interaction between hypoxic microenvironment and metastasis.

The tumour microenvironment (TME) is of paramount importance in the initiation and progression of cancer. Consisting of many cellular and non-cellular components, it acts as a bedrock for the induction of proliferation, angiogenesis, inhibition of apoptosis, suppression of the immune system and evasion from immune surveillance (Deepak et al. 2020). Hypoxia promotes the selection of apoptosis-resistant cell clones and induction of tumour metastasis (Li et al. 2018), along with all the aforementioned processes. Further, the continuous communication, in this hypoxic TME, between cancer cells and the surrounding stromal cells also encourages the progression of cancer to metastatic stages of disease through epithelial to mesenchymal transition. The accumulation of mutation acts as a precursor for chemoresistance; though the mutations are mostly random, their subsequent snowballing gives rise to clonal expansion, genetic diversification and clonal selection. These iterative processes affect the tissue ecosystems leading to expansion of resistant variants.

Hypoxia reduces drug-induced susceptibility to apoptosis by chemotherapeutic agents, by reducing ROS formation and modulating mitochondrial function (Kim et al. 2021; Ge et al. 2018). These drug-resistant clones may adopt the properties of mesenchymal cells to circulate, invade and metastasise. As described earlier, the key molecular contributors in the signalling pathway for the regulation of metastasis are widely known. Hypoxic stress and therefore HIF signalling pathway drive the tumour aggressiveness by affecting the steps within the metastatic cascade and creating conducive pre-metastatic niche conditions. According to a study by Schietke et al., hypoxia acts upon and reduces the expression of E-cadherin and thus promotes EMT, by inducing the LOX (Lysyl oxidase) and LOXL2 (Lox-like 2); LOX and LOXL2 are implicated as direct target of HIF-1 (Schietke et al. 2010). Hypoxia, through the master-regulator HIF-1α, remodels the vessels with increased expression of matrix metalloproteinases MMP1 and MMP2 that helps intravasation. In ovarian cancer cells, hypoxia increases the exosome secretion by upregulating Rab27a and downregulating Rab7, LAMP1/2 and NEU-1 and promotes metastasis and thus chemoresistance (Dorayappan et al. 2018). As the ambit of cancer research is expanding, researchers are exploring the role of noncoding genes as a mediator of

chemoresistance in cancer cells. Induction of lncrna MALAT1 is involved in the promotion of migration and invasion in A549 lung adenocarcinoma cells (Hu et al. 2018). In colorectal cancer, chemoresistance is shown to be one of the two effects meted out by hypoxia-responsive lncRNA NORAD (Zhang et al. 2018).

9.2.8 Immune Evasion by Programming

Hypoxia paves the way for tumour cells to evade immune surveillance by activating transcription of certain genes through HIFs (You et al. 2021). The infiltration of CD8+ cytotoxic T cells (adaptive) and NK cells (innate) makes up the primary antitumoural immunity. However, transcriptional activation by HIF allows tumour cells to activate genes that impair the functioning of the immune system in the tumour microenvironment and prevent flooding of immune cells (Vaupel and Multhoff 2018). Quiescent cancer cells (QCCs) are suspended in the G_0 phase, but possess the ability to re-enter cell cycle and assist in tumour growth. Since QCCs have the capacity to evade the immune system and revert themselves to a mitotic phase, these play a major role in cancer recurrence and metastasis. They form an "immunosuppressive niche" (Baldominos et al. 2022). Notably, QCCs are different from dormant tumour cells, in the sense that QCCs are present in the active primary tumour, whereas dormant cells achieve their dormancy after disseminating from the tumour site of origin. The immunosuppressive niches initiated by QCC include exhausted T cells, QCC, hypoxic tumour cells and suppressive fibroblasts. Despite being in close proximity to T cells, QCCs are able to evade them by downregulating the surface antigen presentation of MHC Class I at the transcript and protein level (Agudo et al. 2018). In another possible way, tumour cells escape entrapment from NK cells by altering the expression of surface ligands for NK cell receptors or by altering expression of receptors on NK cells themselves. NK cells have certain inhibiting and activating receptors, which include NCR, NKp30, NKp44, NKp46 and NKG2D. TGFβ is known to downregulate the expression of NKp30 and NKG2D on NK cells, which suppresses their cytolytic activity, thereby indirectly contributing to evasive mechanisms (Lee et al. 2004). Apart from these direct interactions between tumour cells and immune cells, escaping the immune system of the body is also mediated by tumour-associated macrophages (TAMs), which lie outside of the tumour microenvironment. Multiple studies suggest that TAMs are major promoters of metastasis and assist cancer cells in all the stages of metastasis (Komohara et al. 2014; Ruffell and Coussens 2015). TAMs produce various proteolytic enzymes and immune checkpoint inhibitors, thereby debilitating T cells from exhibiting any immunologic properties near a TME. In hypoxia, activation of HIF-1α induces expression of PD-L1 on macrophages, which binds to its receptor PD1 on T cells, thus deactivating their immune effector properties (Henze and Mazzone 2016). Tumour cells, therefore, escape immune surveillance and undergo further proliferation and metastasis, most of which is mediated by hypoxia-inducible factors.

9.2.9 Epigenetic Changes Regulating Metastasis

Epigenetic alterations, together with genetic aberrations and biochemical changes, can drive tumour development and progression (Timp and Feinberg 2013). Interdependence between hypoxia and proteins that modulate epigenome thus promotes metastasis. Experiments suggest that epigenetic mechanisms promote HIF-α stabilisation; and hypoxia, through HIF-dependent mechanism, leads to epigenetic changes in critical signalling molecules, further fostering the metastatic potential of the tumour. Figure 9.2 shows some of the genes regulated by hypoxia-mediated epigenetic reprogramming. Jumonji C (JmjC) domain-containing oxygenases are histone demethylases that are known to introduce changes to global methylation of histones and work as a downstream effector of HIF-α (Beyer et al. 2008). Decreased JMJD1A is associated with reduced N-cadherin and Twist and increased E-cadherin, all making the non-invasive epithelial phenotype (Yamada et al. 2012; Krieg et al. 2010). Inhibition of HDAC1 by Scriptaid synergised with Cisplatin in hypoxia is known to reduce metastasis, effectively leading to cell death of hypoxic lung cancer cells (Pradhan et al. 2016). DNA hypermethylation leads to silencing of BNIP3 which is involved in hypoxia-related cell death, which indicates that the cell bypass apoptosis in hypoxia by suppressing BNIP3 (Okami et al. 2004). DNA hypomethylation is maintained in hypoxic glioma cells via increase in ten-eleven translocase (TET) expression, as well as decreased expression of DNA methyltransferases. TET1 and TET3 are known to bind to Oct4 and Nanog in their regulatory regions and induce pluripotency associated with glioma stem cells and invasion (Prasad et al. 2017). Histone deacetylase HDAC1 induces angiogenesis by suppressing the tumour suppressor p53 and VHL under the influence of low oxygen tension. HDAC1 also increases the expression of VEGF (Kim et al. 2001). The increased expression of JMJD1A is linked to increase in expression of genes linked to invasion (Krieg et al. 2010). All these reports are supportive of the fact that epigenetics in conjunction with hypoxia is playing an important role in procreating conditions suitable for metastasis to occur.

9.3 Case Study: Hypoxia in Breast Cancer Metastasis

A large body of work has implicated that reduced O2 availability exerts a significant effect in the progression of breast cancer. More than 50% of breast cancers foster regions of low oxygen, particularly the triple negative breast cancers (TNBC). The probability of metastasis increases especially in patients with elevated HIF expression in primary tumour biopsies. Orthotopic mouse models with null HIF-1α and HIF-2α have revealed a marked decrease in the metastasis to the lymph nodes (Schito et al. 2012). HIF-1α knockdown also resulted in decreased bone metastasis (Dunn et al. 2009) and lung metastasis (Zhao et al. 2014) in mouse models. Recent work by Godet et al. (2019) mechanistically evaluated the potential of post-hypoxic tumour cells to individually study all steps in the metastatic cascade separately (Godet et al. 2019). They demonstrated that hypoxia-exposed breast cancer cells

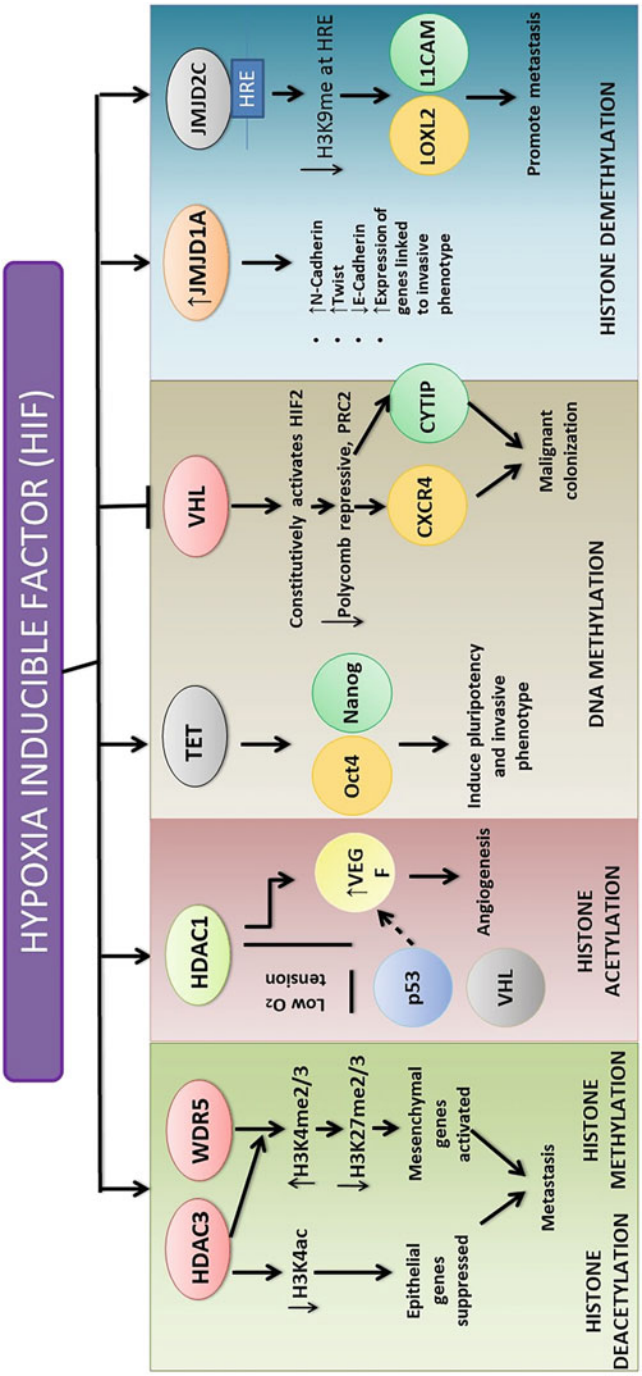

Fig. 9.2 HIF signalling co-opts epigenetic mechanisms to promote metastasis. By regulating various downstream molecules, hypoxia-mediated changes in the epigenome by directly modifying epigenetic modulators such as TET demethylase, Jumonji domain-containing histone demethylases (JMJDs) and histone deacetylases (HDACs), HIF can modulate downstream genes that finally promote metastasis. HIF can also cause epigenetic changes to downregulate its own suppressor, VHL, thereby promoting other HIF-regulated events such as upregulation of CXCR4 and CYTIP through aberrant DNA methylation and histone demethylation, respectively

(BCCs) have an enhanced ability to invade/intravasate and to survive in the bloodstream, but they are not more efficient at extravasating or proliferating at the metastatic site compared with their oxygenated counterparts. Their work revealed that cells exposed to hypoxia have increased metastatic potential and a ROS-resistant phenotype providing a survival advantage to the metastasising cell. Hypoxia induces the HIF-dependent expression of placental growth factor (PGF) by BCCs, which binds to VEGF receptor 1 on mesenchymal stromal cells (MSCs) and promotes their recruitment to primary breast tumours (Chaturvedi et al. 2013). They also showed that chemokine CXCL10 secreted by MSCs binds to its receptor CXCR3 on the BCCs; this triggers the release of chemokine CXCL16 by the BCCs which in turn can bind to the receptor CXCR6. PGF, CXCR3 and CXCL16 released by BCCs are known to be induced by hypoxia in a HIF-dependent manner. Increased expression of Na+-driven bicarbonate transporters (NDBTs) in response to hypoxia was observed in TNBC (McIntyre et al. 2016; Parks and Pouyssegur 2015), and recently S0859, an inhibitor targeting (NDBTs), has shown promise in curbing metastasis in vivo (Carroll et al. 2022). Epithelial cells are generally stiff and rigid; however, for motility, these cells will have to become pliable and undergo epithelial and mesenchymal transformation (EMT) (Lindsey and Langhans 2014). Hypoxia is known to activate extracellular matrix (ECM) remodelling in BCCs which results in increased expression of collagen and ECM stiffening that assists in cell motility. Hypoxia is sufficient to activate RhoA-mediated FAK signalling, which is the basis for BCC motility (Gilkes et al. 2014). Hypoxia also leads to an increase in the levels of ADAM12 in a HIF-dependent manner, leading to increased ectodomain shedding of HB-EGF, activation of EGFR signalling to focal adhesion kinase (FAK) and increased cell motility and basement membrane invasion, making ADAM12 a promising drug target (Wang et al. 2021b). Recent work on desmoglein2, an intercellular adhesion factor, showed that its downregulation in hypoxic tumours allowed single tumour cell dissemination, while DSG2-expressing tumours generated more CTC clusters (Chang et al. 2021). Multiple studies have attempted to recreate the hypoxia-induced molecular changes by verifying hypoxia gene signatures in different subtypes of breast cancers (Ye et al. 2018; Zhang et al. 2021b; Sun et al. 2020). Similar signatures have also been established for hypoxia-mediated noncoding RNAs (particularly, miRNA and lncRNA) (Kulshreshtha et al. 2007; Dhawan et al. 2018; Macharia et al. 2019). Figure 9.3 illustrates the journey of a primary breast cancer cell to the site of secondary lung metastases.

9.4 Advances in Hypoxia-Related Drug Development Research Against Metastatic Cancers

Technological advances in research and imaging have facilitated our understanding of cancer. Despite our current understanding, the treatment remains a challenge, and metastasis continues to be the main cause of mortality and failure of disease-free survival outcome. Most patients coming to the clinic for treatment have advanced

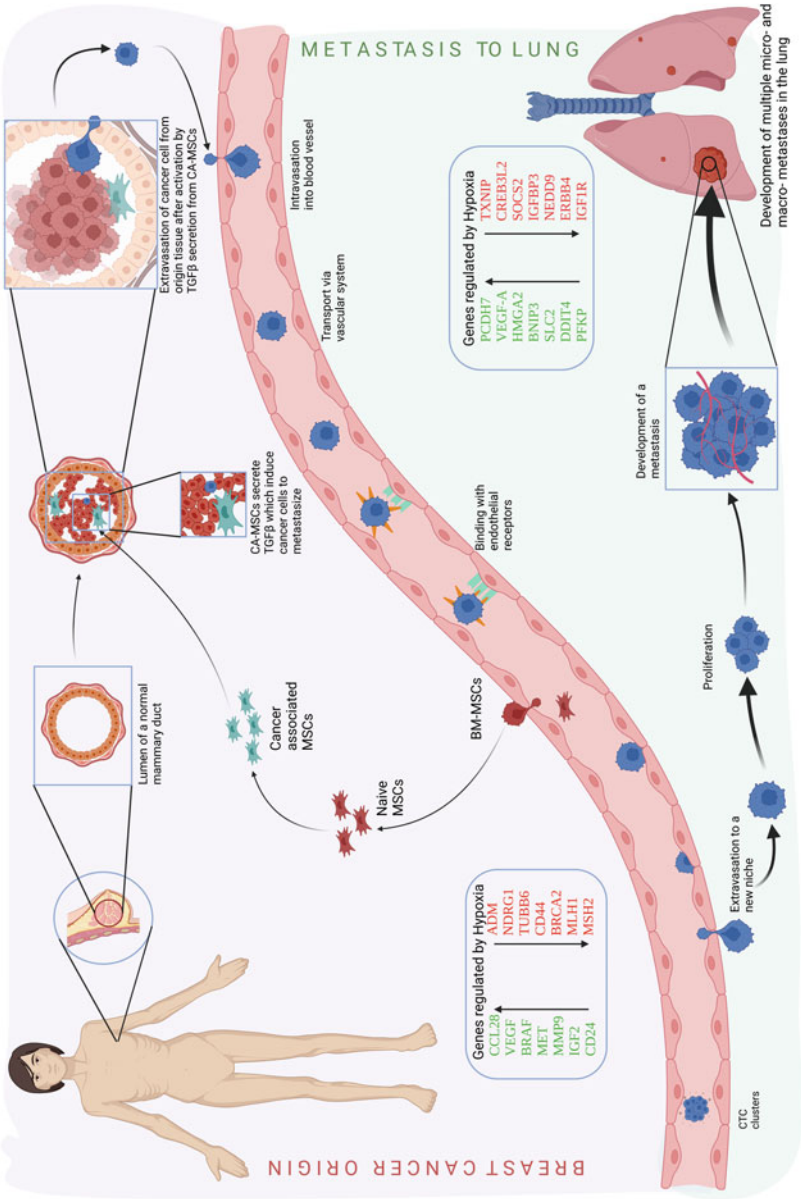

Fig. 9.3 The metastatic cascade of a primary breast cancer cell to site of secondary lung metastases. A growing tumour with hypoxic interiors at the primary site acquires metastatic potential as a consequence of its adaptations. Mesenchymal stromal cells (MSCs) from the bone marrow (BM), upon entry in the tumour

stage cancer. Therefore, a significant body of work is required towards development of drugs that can target metastatic progression.

9.4.1 Treatment Challenges in Metastatic Cancers

Eradicating the metastatic cancer cells is challenging. Contrary to localised tumours, it is a systemic disease requiring a targeted therapeutic approach. Metastatic cancers have a low response to conventional treatment approaches and a high mortality rate. When primary tumours acquire phenotypic plasticity, they become resistant to treatment and metastasise (Bhutia et al. 2016). Hypoxia plays an important role in the cancer evolution and development of the resistant clone. Even a small population of cancer cells that resist therapy may lead to an aggressive disease. Various factors interfere with drug delivery to the targeted site such as solubility, absorption, metabolism and clearance of drugs. These factors further complicate the therapeutic approach to this disease (Ganesh and Massagué 2021).

The important implications of a successful drug treatment are slower tumour growth, survival benefit and decreased chance of relapse. Although there are certain side effects of chemotherapeutic treatment, treatment relies on trade-off between benefit and side effects. Most of the potential drugs fail in the different phases of clinical trials, leaving only a few choices ahead. It is for this reason that drug development is moving towards precision medicine and targeted therapies, hopeful of translation from bench to bedside.

Some of the challenges are (1) detecting metastatic burden in the course of the disease and treatment, (2) finding a way to predict which patients are likely to have metastasis, (3) understanding the biology of the metastatic program and (4) finding ways to target metastasis.

9.4.2 Recent Advances in Technology

Tools such as deep learning image analysis algorithms, which can determine the presence of cancer cells in tissues, and amalgamate them into clinically established pipelines are working towards improvement of sensitivity and reproducibility of pathological metastasis staging (Ehteshami Bejnordi et al. 2017; Bi 2019).

Detection of CTCs continues to be developed as a marker for early minimal residual disease markers as well as tumour biomarker profiling strategy. The low number of CTCs has hampered quick advancement in this field. In turn, ctDNA-based assays have shown more promise and have led to a plethora of sequencing-based analysis that have given insight into the disseminated tumour biology.

Fig. 9.3 (continued) tissue, become cancer-associated MSCs, which induce luminal breast tumour cells to metastasise. These circulating tumour cells extravasate in a new niche and proliferate to form metastases of varying degrees (micrometastases and macrometastases). Genes upregulated and downregulated in response to hypoxia are highlighted in red and green, respectively

Table 9.2 Some specific chemical-based drugs targeting hypoxia-inducible factors

Chemotherapeutic drug	Target	Mechanism	Reference
PT-2385	HIF-2α	It is a selective HIF-2α antagonist, binds to its PAS-B domain. Used in combination with doxorubicin to treat hepatocellular carcinoma	Courtney et al. (2018)
PX-478	HIF-1α	The first know novel HIF-1α inhibitor. It possesses potent anti-tumor activities by inhibiting HIF-1α. It can cross the blood-brain barrier.	Lee and Kim (2011)
LW-6	HIF-1	It downregulates HIF-1α protein expression but not HIF-1β expression	Sato et al. (2015)
Belzutifan (PT2977)	HIF-2α	Selectively inhibits HIF-2α. It is a potential treatment clear cell renal cell carcinoma (ccRCC)	Jonasch et al. (2021)

Continuously updated 'omics' databases and its attached annotations are another collection of scientific tools to bioinformatically analyse the relevant information such as drug response. Growth factors, cell surface antigens, cell death, receptors or signal transduction pathways which regulate cell cycle progression, angiogenesis and metastasis are promising targets (Lee et al. 2018). There is cogent evidence presenting AKT, a serine threonine kinase involved in key critical pathways like apoptosis and angiogenesis, as one of the novel targets for breast cancer and ovarian cancer treatment (Shariati and Meric-Bernstam 2019). The clinical management of paediatric low-grade glioma is dependent upon the mutations in several genes, such as BRAF and MAP2K1 (de Blank et al. 2020); human epidermal growth factor 2 (HER2) overexpression has been implicated in gastric cancer recently, and trastuzumab, a recombinant humanised IgG1 monoclonal antibody targeting HER2 by antibody-dependent cytotoxicity, has been tested (Patel and Cecchini 2020). Many of these molecules are known to be overexpressed in hypoxia. The novel targets identified in in vitro studies are earmarked for use in clinical settings, with stringent pre-clinical and clinical phase trials. Some direct inhibitors of the hypoxia-inducible factor have also been used in clinical trials and research (Table 9.2). Similarly ongoing research hypoxia-based gene signatures also reveal an exciting possibility of finding new drug targets.

9.4.3 Recent Advancements in Drug Development in Context of Hypoxia-Driven Metastasis

Strategies around pursuit of understanding cancer cell metastasis and hypoxia mechanisms will provide great insight for developing a new line of treatment. There are many revolutionary ideas being explored by researchers; however, in this chapter we will review some possibilities that either target hypoxia or some

that have found promise in targeting hypoxia-regulated genes important for metastasis.

9.4.3.1 Nanomaterial

Nanoparticles improve the stability, solubility, half-life, and concentration of drugs. Many nanomaterials have been developed in recent times, and these have opened exciting new possibilities with biomedical applications including bioimaging, drug delivery to target tissues and therapeutic delivery (Senapati et al. 2018). A nanoparticle typically consists of core material, surface modifications, and a therapeutic payload that may be a drug, nucleic acid, protein, antibody, antibody-drug conjugate, or combination of multiple agents for cancer therapy. Nanoparticles accumulate in tissues thereby reducing the dose and toxicity of the drug being delivered which in turn lowers the chances of cancer patients developing drug resistance (Ediriwickrema and Saltzman 2015). Diminishing oxygen dependence for hypoxic tumour therapy has also been postulated as a therapeutic option in recent years, e.g., by taking advantage of penetration of therapeutic gas or generation of toxic substances in situ in hypoxic tumour (Zou et al. 2021). Oxygen nanobubbles have been used to suppress expression of hypoxia-inducible factor-1α (HIF-1α) (Song et al. 2020) albeit with some caveats (Cavalli et al. 2016). Similarly, oxygen could be generated in tumours by using catalase-loaded hydrogel, and this could help overcome the immunosuppressive tumour microenvironment (Meng et al. 2019). However, these strategies need to be further explored before they can be applied clinically (Ruan et al. 2021).

9.4.3.2 Antibodies

Monoclonal antibodies (MAb) are commonly used in antibody therapy to target cancer cells. Antibodies can target cancer cells using various mechanisms including inhibition of immune checkpoints, signalling disruption, neutralising target receptors, downregulation of receptors, antibody-dependent cell-mediated cytotoxicity and complement-dependent cell-mediated cytotoxicity (Hafeez et al. 2020). MAbs that are most commonly used for targeting cancer cells include avelumab (anti-PD-L1), cemiplimab (anti-PD-1), ipilimumab (targeting CTLA-4), nivolumab (anti-PD-1), etc. (Yang et al. 2021b). Recently, two new antibodies, CA9hu-1 and CA9hu-2, have been developed against carbonic anhydrase IX (CA IX) to specifically target hypoxic cells (Zatovicova et al. 2022). Monoclonal antibodies can be conjugated with cytotoxic drugs, radioisotopes and immunotoxins. Researchers are exploring methods to increase their effectiveness.

9.4.3.3 Antibody Drug Conjugate

During the conjugation process, it is necessary to preserve the cytotoxic property of the drug and the specificity of the monoclonal antibody. An antibody-drug conjugate (ADCs) remains stable until it reaches the target site. The conjugates are biodegradable and non-immunogenic. Once the conjugate carrier and target cell interact, the drug is released. A few of the ADCs that are available include brentuximab vedotin (targets CD30), gemtuzumab ozogamicin (targets CD33), moxetumomab pasudotox

(targets CD22), trastuzumab deruxtecan (targets HER2), etc. (Dahlgren and Lennernäs 2020). A study revealed that HER2 signalling results in elevated expression of HIF-1α and HIF-2α, which plays a key role in hypoxia in cancer (Laughner et al. 2001). HER2 treatment with ADCs can downregulate the HIF-1 and HIF-2 expression levels, thereby targeting cancer cells in a hypoxia-sensitive manner. The major disadvantage of this type of therapy is the amount of drug delivered to the target site. As each antibody is conjugated with a single drug molecule, its potency is reduced in most cases. Presently, several antibody-drug conjugates are available, but either the specificity of the antibody or the cytotoxicity of the drug is compromised.

9.4.3.4 Prodrugs

Hypoxic tumours have radio- and chemoresistant niche within, posing great challenges for effective drug delivery and outcome. Hypoxia-activated prodrugs, also known as HAPs or bioreductive drugs, are designed and used to target hypoxia. Some well-known HAPs and their descriptions are listed in Table 9.3. Under hypoxic conditions, HAPs can be reduced with the help of specific reductases into cytotoxic compounds that can target the hypoxic cancer cells precisely with little or no effect on surrounding normal tissues (Wilson and Hay 2011). Hetero-aromatic N-oxides, quinones, nitroaromatics and aliphatic N-oxides are currently among the classes developed (Li et al. 2021).

9.4.3.5 Drugs Targeting Hypoxia

Targeting molecular mechanisms such as HIF and unfolded protein response (UPR) aids hypoxia and tumour cell adaptation to hypoxia. HIF transcription factor activates various downstream genes that help tumour cells survive. Various drugs have been designed to specifically target HIFs to counter the hypoxia, including flavopiridol, rapamycin, KC7F2, digoxin, EZN-2968, etc. (Shirai et al. 2021). Due to severe hypoxia in TME, there is an increased concentration of unfolded proteins in the endoplasmic reticulum (ER) resulting in protein synthesis reduction due to the loss of mRNA translation. Abetment of a few members of eukaryotic initiation factors (eIFs) also contributes to the mRNA translation loss (Hammond et al. 2014b). Salicylaldimine, ONC201, bortezomib, eeyarestatin I, epigallocatechin gallate, etc. are a few drugs that target these UPRs (Ojha and Amaravadi 2017).

9.4.3.6 Biomedical Devices

With the help of biomaterial chips, implantable devices are being designed that directly deliver drugs to the targeted site. Chips impregnated with drug molecules can also be used in conjunction with chemotherapy and gene therapy. Different countries have approved implantable devices for the treatment of malignant cancer after successful clinical studies (Pial et al. 2022).

Another technique for targeted delivery of drugs uses infrared radiation. Near-infrared fluorescence (NIRF) imaging agents can be used for imaging and targeting cancer cells. NIRF conjugated with cytotoxic drugs can be successfully delivered to the target site using imaging at NIR wavelength (700–10,000 nm). These agents are specific to cancer cells, and their uptake is mediated by tumour hypoxia. Activated

Table 9.3 List of some hypoxia-activated prodrugs (HAPs) that have completed Phase II trials

HAPs	Target	Function	Reference
Evofosfamide (TH-302) (Phase 2 as per the latest study of 2021)	DNA (cross-linking) and HIF-1α	This prodrug releases Bromo-iso-phosphoramide (Br-IPM), a DNA cross-linking agent that exerts cytotoxicity. It also causes cell cycle arrest and downregulation of HIF-1α.	Duan et al. (2008), Brenner et al. (2021), Salem et al. (2018)
Banoxantrone (AQ4N) (Preclinical as per the latest study of 2018)	Topoisomerase II	Reduced to AQ4 form, which binds to DNA and exerts a cytotoxic effect.	Patterson (1993), Papadopoulos et al. (2008), Salem et al. (2018)
Tirapazamine (Phase 3 as per the latest study of 2021)	Purines and Pyrimidine in DNA (double-strand break)	Reduced drug can generate oxidative radicals, which can cause oxidative damage in hypoxic regions. It also causes cell cycle interruption and apoptosis.	Laderoute et al. (1988); Abi-Jaoudeh et al. (2021), Salem et al. (2018)
Apaziquone (EO9) (Phase 1 as per the latest study of 2018)	DNA (single-strand break)	In hypoxic cells, it is reduced to semiquinone, which generates superoxides.	Kanimozhi and Prasad (2015), Hendricksen et al. (2012), Salem et al. (2018)
PR-104 (Phase 2 as of the latest study of 2018)	DNA (interstrand cross-linking)	Reduced to PR-104H and PR-104M forms cross-link in DNA strands and are cytotoxic.	Singleton et al. (2009), Konopleva et al. (2015), Salem et al. (2018)
SN30000 (CEN-209) (Early phase trials as of the latest study in 2017)	DNA (double-strand breaks)	It is a modified analogue of Tirapazamine with similar pharmacological effects. It also inhibits cell proliferation and causes cell cycle arrest.	Hunter et al. (2012), Chitneni et al. (2013), Hay et al. (2017)

hypoxia-inducible factor 1α (HIF-1α) or organic anion-transporting polypeptides (OATP) signalling axis is the primary contributor. When OATP is present, NIRF dye can easily cross the BBB/BTB and enter cancer cells even in the absence of hypoxia. In addition to crossing the blood-brain barrier, these dyes target the cancer site without harming healthy cells. In mice models, the NIRF signal intensity has also been shown to quantify tumour burden. A study is currently underway to evaluate its use in male cancer patients (Wu et al. 2015).

9.4.4 Promising Strategies for Drug Development

A new study or clinical trial is conducted every day that either seeks to detect cancer earlier or to develop treatments to combat metastasis and hypoxia. The need for interdisciplinary approaches has become paramount in treating this disease. This section discusses the upcoming and promising methods, approaches and treatments

for metastatic cancer and hypoxia. METPlatform is a new drug screening platform designed to identify metastatic drug vulnerabilities in vivo and ex vivo. This method involves testing candidate drugs before treatment using organotypic cultures derived from patients. This study also helps predict patient response to a specific treatment, making it one of the highly desirable methods for designing personalised medicine (Zhu et al. 2022). Bacteria are single-cell living organisms that can proliferate and translocate. It is possible to biosynthesise bacteria that can transport therapeutic substances directly to cancer cells. CAP, which coats bacteria and protects them from the human immune system, can be biosynthesised to improve therapeutic load delivery in vivo (Cress et al. 2014). In situ trafficking and high drug dosages can be used to increase therapeutic efficacy and safety. Thus far, this method has only been tested successfully on mice. Further studies are currently underway on human models (Harimoto et al. 2022). Hypoxia aids the tumour cells in evading immune cells by promoting immune escape and inhibiting the antitumour effector cells. These immune cells are affected by a mild thermal effect. Application of mild thermal therapy (hyperthermia) to the tumour microenvironment increases the vascular perfusion, thereby increasing the oxygen concentration at the tumour site. Reduction of hypoxia in TME provides an opportunity to treat the tumour with chemotherapy, radiotherapy or immunotherapy which is currently being explored in some clinical studies (Lee et al. 2010).

9.5 Concluding Remarks

We are only just beginning to understand the extent of heterogeneity exhibited by the tumour cells including the circulating tumour cells. It is apparent that the tumour microenvironment which includes hypoxic regions play a very important role in determining the outcome of a developing cancer. Hypoxia modulates the interaction of the primary tumour cells with its surroundings by mobilising immune cells. Cellular adaptation in the hypoxic niche equips the cells with metastatic and immuno-evasive capabilities that allow it to migrate and metastasise at one or more secondary sites. Modelling tumour hypoxia using in vitro, in vivo and patient-derived cells have their advantages and utility for advancing our basic knowledge. Enriching cancer stem cells in these model systems will allow us to assemble molecular pathways of reprogramming and pluripotency. Together, artificial intelligence, imaging genomics and spatial and molecular profiling are poised to give greater insights and breakthrough discoveries in the use of hypoxia as an early predictive marker of relapse and metastasis. Emerging possibilities include prediction of metastatic sites and modulation of hypoxia-regulated site specificity of metastasis. Epigenetic modulators that can reverse hypoxia-induced gene signatures and antibody/inhibitors against hypoxia-regulated molecular targets show promise in therapeutic value. Collective and multidisciplinary efforts are required to improve quality of life and survival outcome in metastatic disease burden.

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Hypoxia and Extracellular Matrix-Major Drivers of Tumor Metastasis

10

Prasad Neerati and Durga Polati

Abstract

The most common source of cancer-related mortality is metastatic malignancy, which involves dynamic interplay between cancerous cells and associated surroundings. Hypoxia is a powerful microenvironmental element that encourages metastatic spread. In a variety of tumor forms, hypoxia and the expression of the hypoxia-inducible transcription factors HIF-1 and HIF-2 are attributed to greater distant metastasis and poor survival. Furthermore, HIF signaling in cancer cells affects a number of phases in the metastatic process. The two-way interaction between resident cells and the extracellular matrix (ECM) through cell-matrix interactions and ECM remodeling shapes the tissues dynamically. Tumors use ECM remodeling to produce a microenvironment that encourages tumorigenesis and spread. To allow for metastatic progression, these tumor-driven alterations promote tumor growth, boost tumor cell migration, and remodel the ECM in distant organs. For designing therapeutic therapies for patients, a deeper knowledge of the underlying mechanisms of tumorigenic ECM remodeling is critical. In this chapter, we focused on how the hypoxic tumor microenvironment promotes metastatic progression, what are the possible hypoxia-regulated biomarkers and therapeutic targets, as well as ECM modifications that could be used in initiatives to prevent and treat metastatic illness and how the phytochemicals targeting HIFs and ECM remodeling.

Keywords

Metastasis · ECM · Hypoxia · Tumor · Phytochemicals

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10.1 Introduction

The uncontrolled proliferation of cancer cells is the primary defect that leads to the development of cancer. Cancer cells proliferate, divide uncontrollably, and infiltrate normal tissues organs and eventually spreading throughout the body, instead of responding adequately to the signals that control normal cell behavior. Cancer cells demonstrate a broad loss of growth control as a result of accumulated aberrations in various cell regulatory systems, which reflects in several features of cell activity that distinguish cancer cells from their healthy counterparts.

All the malignant tumors experience the status of nonphysiological oxygen tension and lead to tumor hypoxia. If the tumor hypoxia advanced, it results in abortive vascularization with cellular mobility and metastasis which in turn induces resistance to therapy by altering cellular metabolism and the cell signaling complex network like HIF1α, PI3K, MAPK, and NFkB pathways in cancer cells. New therapeutic techniques will emerge by targeting hypoxia tumor cells and the hypoxic microenvironment (Muz et al. 2015). Tumor hypoxia can be reduced with dietary strategies like restricted caloric, low-carbohydrate intake and acute exercise by enhanced blood flow to the solid tumors (Kirkham et al. 2021). Hypoxia reduces the therapeutic potential of the cancer treatment, observed in a case study of glioblastoma multiform (GBM), a malignant brain tumor, which requires surgery followed by radiation therapy and chemotherapy, where in hypoxia acts as cofactor for the reduced potential of both radiotherapy and chemotherapy. So, this is essential to identify the early and/or late acquisition in hypoxia sites to express the hypoxia marker HIF-1α to identify hypoxic areas in GBM lesions to guide the proper therapeutic scheme to treat GBM patients (Gangemi et al. 2019). Chronic stress induces oxidative stress and hypoxia in cancerous cells and promotes the tumor progression, migration, and invasion of metastatic cancer cells (Huang et al. 2019). In the solid tumor cells, the level of oxygen may be less than 1% and affects the cell populations like macrophage activity and functionality in head and neck cancer (Ishizu et al. 2021). In hypoxic tumors, the increased expression of HIF-1α, Glut-1, VEGF, and Ki-67 responsible for production of tumor cells, highly resistant to radiotherapy, causes poor prognosis in cancer patients (Kim et al. 2020). Chemotherapy brings the status of reduced blood supply in solid tumors that leads to hypoxic microenvironment and causing solid tumor resistance to chemotherapy. Hypoxia can allow tumor cells to escape the hypoxic environment by neovascularization and metastasis.

Hypoxic circumstances will influence metabolism in the core portions of solid tumors, activating glycolytic pathways in a metabolic change known as the cancer metabolic shift. Following this metabolic shift, an acidic pH is formed in the extracellular environment, but the pH inside tumor cells remains neutral. Acidity has been found to encourage cell migration by degrading the extracellular matrix (ECM). H^+ diffuses from the TME to surrounding tissues, contributing to ECM degradation and aiding invasion, whereas invasion does not occur in areas with normal pH.

HIF factors (hypoxia-induced factors) that play a role in metastasis include LOX (Lysyl oxidase). It is driven on by hypoxia and results in changes in the extracellular matrix (ECM). LOX crosslinks collagen and elastin in the ECM, and it plays an important role in tissue growth and remodeling in healthy tissue. When released by tumor cells, LOX works as a pro-metastatic factor, encouraging ECM remodeling and cancer cell invasion. The expression of the LOX gene is linked to disease progression and metastasis.

CTGF (connective tissue growth factor) is a hypoxia-induced extracellular matrix protein. Tumor development and metastasis are connected to expression levels of CTGF (Lu and Kang 2010).

Tissues are dynamically shaped by bidirectional communication between resident cells and the extracellular matrix (ECM) through cell-matrix interactions and ECM remodeling. Tumors leverage ECM remodeling to create a microenvironment that promotes tumorigenesis and metastasis. The tumor-driven changes support tumor growth, increase migration of tumor cells, and remodel the ECM in distant organs to allow for metastatic progression. A better understanding of the underlying mechanisms of tumorigenic ECM remodeling is crucial for developing therapeutic treatments for patients (Winkler et al. 2020). In the normal organs, the microenvironment consists of vasculature, extracellular matrix (ECM), and stromal and immune cells. Solid tumors are complex organ-like structures, and the tumor microenvironment (TME) comprises the tumor mass component excessive along with the other microenvironment of the normal organs. ECM of solid tumors is different from that of ECM of normal organs, and if not controlled it affects abruptly the cellular transport, metabolic reactions, intratumoral signals, cellular oxygen interactions, and immunogenic reactions too. The regulatory control of tumoral ECM will influence the tumor growth, malignancy, and the metastasis, and also its response toward the therapy (Henke et al. 2020). In solid tumors, ECM is perturbed, and the tumor matrix promotes growth, survival, and invasion of the cancer and drive to metastasis. Due to this, it results in the failure to the therapy by tumor fibroblast and immune response modifications. Tumor matrix promotes the tumor cell invasion into the stroma and migration into the vasculature and resulting intravasation and vascular dissemination and finally leads to metastatic outgrowth. Basically, the resident cells generally secrete minerals, proteoglycans, and fibrous proteins which composes the ECM with interlocking mesh of water.

10.2 Hypoxia

It is a condition, in which the tissue is deprived of oxygen, ensuing in altered biological function. Tissue hypoxia is a common characteristic, particularly in aggressive solid tumors. As tumor cells develop aberrantly, they lose the ability to satisfy the oxygen demand from the preexisting vasculature and become more vulnerable to hypoxia. This typically occurs when the tumor is larger than 1 mm in diameter. Tumor cells may develop compensatory mechanisms in response to hypoxia, which have a negative impact on the success of curative treatment,

Table 10.1 Oxygenation levels at malignancies and respective tissues

Type of malignancy	Median % O_2	Tissue/organ	Median % O_2
Glial tumors	1.7 (Vaupel et al. 2007)	Brain	4.6 (Dings et al. 1998; Hoffman et al. 1996)
Lung cancer	2.2 (Le et al. 2006)	Lung	5.6 (Le et al. 2006)
Rectal adenocarcinoma	1.8 (Kallinowski et al. 1995)	Rectal tissue	3.9 (Kallinowski et al. 1995)
Breast adenocarcinoma	1.5 (Vaupel et al. 1991)	Breast tissue	8.5 (Vaupel et al. 1991)
Renal cell cancer	1.3 (Lawrentschuk et al. 2005)	Renal cortex	9.5 (Muller et al. 1998)
Cervical squamous cancer	1.2 (Vaupel et al. 2007)	Uterine cervix	5.5 (Höckel et al. 1991)
Pancreatic adenocarcinoma	0.3 (Koong et al. 2000)	Pancreatic tissue	7.5 (Koong et al. 2000)
Hepatocellular carcinoma	0.8 (Leary et al. 2002)	Liver	7.3 (Leary et al. 2002)

regardless of the type of treatment used (Vaupel and Harrison 2004). Due to differences in blood supply and tolerance to hypoxia, there is variability in the oxygenation/physoxia of diverse normal tissues (4–10% O_2). Renal cortex, liver, breast, and pancreatic tissues are the most oxygenated organs, whereas the brain, lung, and intestinal mucosa are the least oxygenated organs (Table 10.1). In cancers, oxygenation levels are substantially lower than in normal tissues; even in the most vascularized tumors, the O_2 rate is only 2% (lung cancer); but it is much lower in most cases, especially in pancreatic cancers, where it is the lowest as 0.3% (Muz et al. 2015).

10.2.1 Causes of Hypoxia in Tumor Microenvironment

Hypoxia can be caused by a variety of factors, including poor perfusion, diffusion, or anemia. Perfusion-related (acute) hypoxia is because of the lack of adequate blood flow in tissue. Newly growing tumors with a diameter greater than 1 mm should have their own vascular network, and tumor angiogenesis factors influence the formation of new microvessels (Folkman 1990). The blood vessels in neovasculature are highly different from those present in normal tissue. They exhibit extensive range of structural and functional abnormalities, including leakiness due to irregular endothelial lining, loss of wall contractile ability, lack of expression of pharmacological/physiological receptors, arteriovenous shunts, blunt ends, as well as changes in endothelial lining architecture and basement membranes. These anomalies may result in poor perfusion, reducing the delivery of oxygen and nutrients to tumor cells and increasing the risk of cancer (Dvorak et al. 1999). Diffusion-related (chronic) hypoxia occurs as a result of the rapid expansion of tumor, which causes an increase

in diffusion distances and results in insufficient O_2 transport for cells that are far away from the nutrient vasculature (>70 m). The oxygen diffusion limit is 100–200 m, which suggests that cells should be within this radius for proper oxygenation (Al Tameemi et al. 2019). Anemic hypoxia occurs when the oxygen-carrying capacity of blood is declined as a result of tumor or therapy-induced anemia. The experimental studies has revealed that O_2 delivery to tumors is considerably reduced, when hemoglobin levels fall below 10–12 g/dl, and hypoxia is aggravated, especially when low O_2 deliver capability coincides with a low perfusion rate. The production of carboxyhemoglobin in heavy smokers can result in similar conditions to those seen in anemic hypoxia (Vaupel et al. 2001).

10.2.2 Cellular Adaptations to Hypoxic States

The biological processes like HIF-mediated adaptation to hypoxia, epigenetic gene transcription regulation, extracellular matrix formation, and the cellular metabolism reprogramming are dependent on 2-oxoglutarate-dependent dioxygenases (2OGDDs), a super family enzyme (Losman et al. 2020). Hypoxia is one of the major contributory factors for the both progression of tumors and increased resistance to chemotherapy; this can be targeted by the hypoxia-activated prodrugs (HAPs); those are activating in low level of oxygen (Mckenna et al. 2018). Tumor hypoxia drives in to a condition an immunosuppressive tumor microenvironment (TME) via different pathways like hypoxia-inducible factor (HIF)-dependent upregulation of Programmed Death Ligand 1 (PD-L1). The hypoxia and immunosuppressive TME are independent negative factors for the treatment and prognosis for bladder cancer, and hypoxia upregulates PD L1 (Smith et al. 2021). Hypoxic microenvironment may develop multiple myeloma (MM), a major histocompatibility complex class II, DP alpha 1 (HLA-DPA1) as a hub gene identified as related to hypoxia in MM and HLA-DPA1 downregulated expression is linked to poor outcome in patients (Yang et al. 2020a). TME-like cancer-associated fibroblast regulates cancer tumorigenesis and metastasis, and hypoxia promotes CRC progression; a novel axis circEIF3K/miR-214/PD-L1 mediates hypoxia-induced CRC progression via CAF; this provides the rationale for developing new target for the treatment of CRC (Yang et al. 2021). RhoA/ROCK and Rac/PAK signaling pathways play the crucial roles in hypoxia-induced vasculogenic mimicry (VM) via Ser72 and Ser56 Vimentin phosphorylation in hepatocellular carcinoma (Zhang et al. 2020).

10.2.3 Hypoxia and HIF Signaling

A group of deoxygenases known as PHDs (prolyl hydroxylases: PHD1, PHD2, and PHD3) and factor inhibiting HIF (FIH) act as sensors and detect low oxygen levels, located within the cell. PHDs subsequently activate transcription regulators known as HIFs (hypoxia-inducible factors), which cause changes in the cellular gene

expression profile (Semenza and Wang 1992). The transcription factors of the HIF family are heterodimeric proteins. HIF1 is made up of two subunits with HIF-1α and HIF-1β. In the presence of prolyl hydroxylase domain proteins (PHD1-3), HIF-1α undergoes hydroxylation on 402 and/or 564 proline residues at physoxia. The hydroxylated HIF1α reacts with the tumor suppressor protein von Hippel-Lindau (pVHL), which then joins with an E3-ubiquitin-ligase complex to polyubiquitinate HIF-1α and leads to proteasomal destruction. In the presence of oxygen, the HIF-1α is short-lived, with a half-life of less than 5 min, due to ubiquitin-dependent proteasomal degradation. So, HIF-1α subunit is constantly generated and metabolized in normoxia. In a hypoxic environment, the oxygen-dependent PHDs are deactivated; HIF-1α is stabilized, escapes ubiquitination and degradation, accumulates, and eventually translocates to nucleus. It forms a hetero dimer with the HIF1β subunit, which is constitutively expressed. The newly formed HIF-1 heterodimer will then attract transcriptional coactivators (p300/CBP), which will bind to hypoxia response elements (HREs) in multiple gene promoters and initiate a transcriptional program that will lead to cellular adaptation to hypoxia (Fig. 10.1).

HIF-2 is a member of the same family and has a comparable structure. HIF-2 is a heterodimer of the HIF-2α subunit linked to the HIF-1β subunit, similar to HIF-1α. HIF-2α, an oxygen-dependent subunit, is regulated in a similar way to HIF-1α. HIF-1 and HIF-2 affect various sets of target genes and appear to be regulated differently depending on the amount and duration of hypoxia (Jiang et al. 2021), and many enzymes regulates HIF-1 (Table 10.2).

10.2.4 The Role of Hypoxia in Cancer Development

The adaption of several genetic or epigenetic mutations is needed for cancer development due to resulting from genetic instability in the dividing cell. Cell cycle arrest, resistance to DNA repair and growth inhibition, evasion of immune surveillance, apoptosis, unlimited replication potential, angiogenesis, invasion, and metastasis are all examples of instability caused by defects in the mechanisms that control the cell cycle and normal cell differentiation (Hanahan and Weinberg 2000).

Metastasis is a multistep process in which malignant cells migrate and invade adjacent tissue, intravasate and extravasate blood and lymphatics, and infiltrate target organs while having the ability to grow and sustain in the primary tumor environment (Obenauf and Massagué 2015). Furthermore the genetic and epigenetic events that happen during malignant tumor growth and stromal cells within the TME significantly affect tumor progression and metastasis through various mechanisms (Hanahan and Coussens 2012).

10.2.5 Hypoxic Signaling Promotes Metastasis

Hypoxia and the hypoxia-inducible transcription factors (HIF-1 and HIF-2) promote metastasis that contributes to an unfavorable outcome in patients. In metastasis, HIF

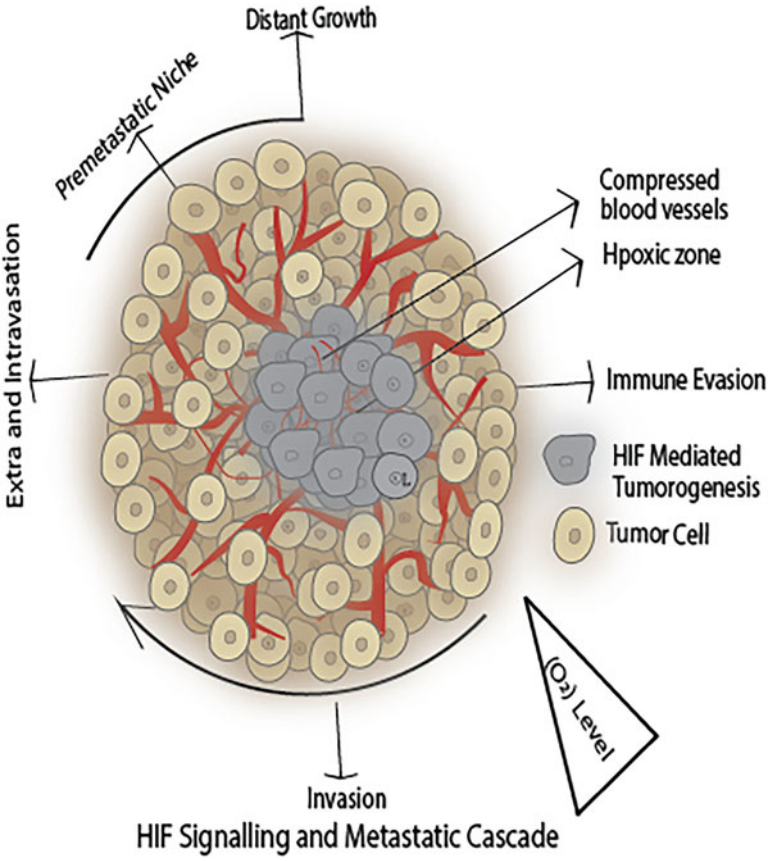

Fig. 10.1 HIF signaling-mediated metastasis. Due to abnormal tumor cell growth, the vasculature become compressed, failing to supply oxygen and nutrient, and hypoxic zone is established. Hypoxia-inducible factors (HIF-1 and HIF-2) in tumor and stromal cells are stabilized in the tumor microenvironment, resulting in the activation of target gene programs that aid metastasis by various mechanisms. The main steps in ECM remodeling and the metastatic cascade. (**a**) In situ carcinoma. The intact basement membrane prevents tumor cells from migrating and penetrating into the surrounding tissue. Integrins can physically join tumor cells and stroma cells across the basement membrane. Curly ECM is found in the interstitial matrix. (**b**) Non-proteolytic, force-mediated ECM remodeling (1) and proteolytic ECM degradation by proteases released by cancer cells and activated stroma cells (2) are two ways to disrupt the basement membrane. (**c**) Intravasation: tumor cells are liberated from the primary tumor microenvironment and then traverse the interstitial connective tissue before breaking the vascular basement membrane to obtain access to the circulation. Angiogenesis and strong MMP activity at the original tumor site damage the vasculature, allowing tumor cells to enter the circulation and intravasate. (**d**) CTCs evade immune surveillance in the circulation: CTCs come into contact with immune cells via direct cell-cell contact. Immune-mediated elimination occurs as a result of these interactions. CTC survival in the circulation is aided by changes in MHC molecules. (**e**) Extravasation: MMP activity increases, resulting in a leaky vasculature that allows CTCs to extravasate into the surrounding tissue. (**f**) Growth factors, MMPs, LOX, ECM proteins like fibronectin, and exosomes, all derived from the initial tumor, promote angiogenesis and create a premetastatic microenvironment at a distant site to prime the new tissue for metastasis

Table 10.2 Major enzymes regulating HIF-1

Casein kinase 1 (CKI)	Phosphorylation within PAS-B (Ser-247)destabilizes the HIF-1 α-ARNT complex and reduces transactivation (Kalousi et al. 2010)
Prolyl hydroxylase domain (PHDs)	Hydroxylation within ODDD (Pro-402) and TAD-N (Pro-564) leads to reduced stability of HIF-1α (Fong and Takeda 2008)
Factor inhibiting HIF-α (FIH)	Hydroxylation within TAD-C (Asn-803) causes faulty transcriptional activation by HIF-1α (Koivunen et al. 2004)
Glycogen synthase kinase 3 beta (GSK3β)	Phosphorylation within ODDD(Ser-551, -555, -588) results in diminished stability of HIF-1α (Flügel et al. 2007)
Extracellular signal-regulated kinase ERK1	Phosphorylation within C-terminal domain (Ser-641, -643) masks a nuclear export signal and results in nuclear accumulation and enhanced activity of HIF-1α (Buscà et al. 2016)
Protein kinase A (PKA)	During intermittent hypoxia, PKA functions like ERK115 (Bullen et al. 2016)
p300/CBP-associated factor (PCAF)	Acetylation within C-terminal domain (Lys-674) leads to increased HIF-1α levels and binding of p300 (Zhou et al. 2019)
p300	Acetyl-transferase activity of p300 within C-terminal domain (Lys-709) block subiquitination-induced degradation of HIF-1α (Zhou et al. 2019)
Casein kinase (CKII)	Hypoxia-induced phosphorylation within TAD-C (Thr-796) decreases affinity (Mottet et al. 2005)

and its signaling pathways play a crucial role (Yang et al. 2008), and there are different mechanisms, by which hypoxic signaling promotes metastatic progression (Fig.10.2).

10.2.5.1 Evasion of Immunity

Tumors, including primary and metastatic, can resist immune attack. In hypoxic settings, tumor cells block antitumor immune responses through a variety of ways. Hypoxia increases resistance to death by cytotoxic CD8+ T cells (CTLs). When an antigen is detected, CTLs release cytotoxic granules containing the granzymes and perforin, both of which promote apoptosis in cancer cells (Harty et al. 2000). HIF signaling induces antiapoptotic responses and autophagy in cancer cells, culminating to resistance to lysis. Hypoxic tumor cells may trigger apoptosis in CTLs via potentiating the expression of PD-L1 on cancer cells. HIF signaling is often found to suppress CTL function by upregulating CD39/CD73 enzymes in tumor cells that enhance the deposition of extracellular adenosine, a powerful regulator of lymphocyte cytolytic function (Hatfield et al. 2019). NK cell-mediated destruction is inhibited by hypoxic tumor cells through autophagy induction, comparable to CTLs (Baginska et al. 2013). Furthermore, hypoxic tumor cells avoid phagocytosis by inducing the expression of CD47, a cell surface protein that interacts with signal regulatory protein alpha (SRPα). Moreover, hypoxic tumor cells escape macrophage-mediated phagocytosis through the upregulation of CD47, a cell surface protein that interacts with signal regulatory protein alpha (SRPα) coating on macrophage surfaces to prevent phagocytosis (Zhang et al. 2015). Oxygen-deprived

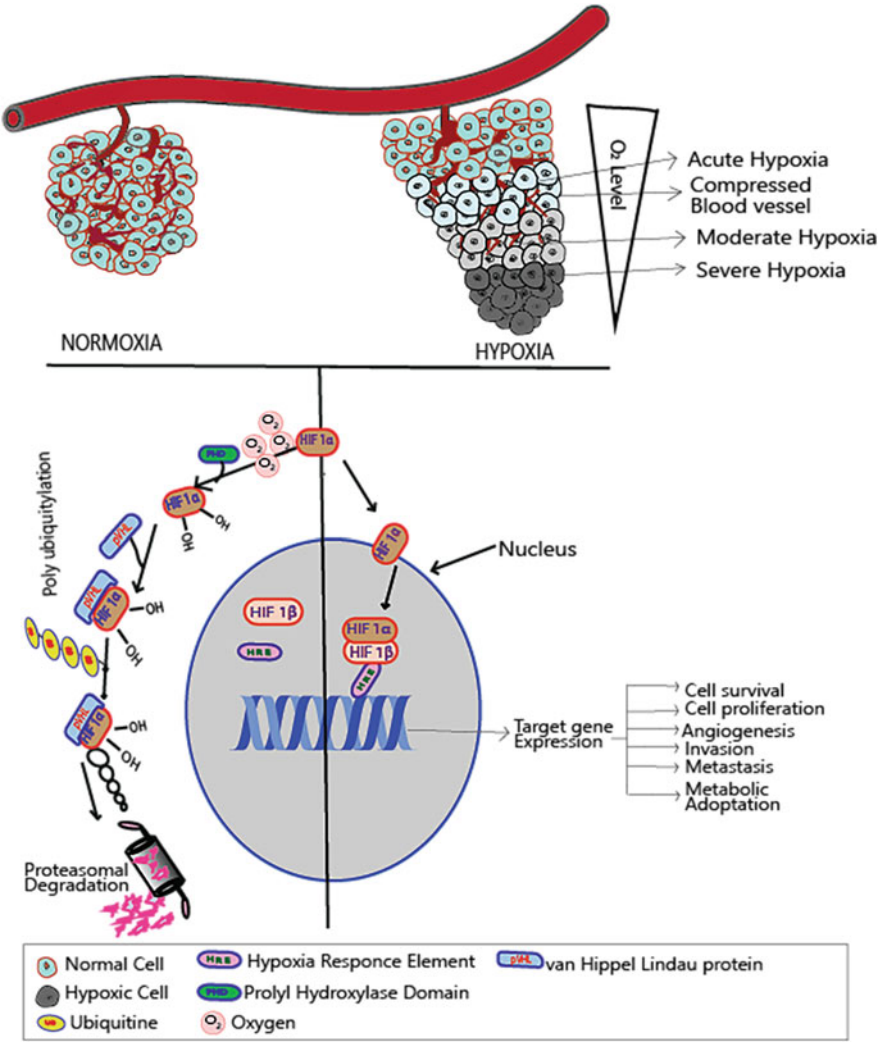

Fig. 10.2 HIF signaling. Hypoxia-inducible factor (HIF)-1α is a protein containing three residues which are priorities for regulatory hydroxylation. P402 and P564 are hydroxylated by prolyl hydroxylase domain (PHD) enzymes (notice that PHD3 can only hydroxylate P564), while N803 is hydroxylated by HIF factor inhibitors (FIH). P402 is found in the N-terminal O_2-dependent degradation domain, while P564 is found in the C-terminal domain. The von Hippel-Lindau tumor suppressor (pVHL) E3 ligase complex identifies prolyl hydroxylated HIF-1α, leading to degradation in normoxia. Prolyl and asparaginyl hydroxylation are both sensitive to hypoxia in different ways. Through the action of the N-terminal activation domain (NAD), inhibition of prolyl hydroxylation alone is sufficient to allow HIF-1 to escape pVHL E3-dependent proteolytic degradation and establish an active transcriptional complex with HIF-β. HIF-1α asparaginyl hydroxylation is also suppressed in more severe hypoxia, allowing p300/CBP co-activators to be recruited to its C-terminal transactivation domain (CAD) and boosting transcription of a select set of HIF-1 target genes. (HRE stands for hypoxia-response element)

cancer cells release chemokines and cytokines to drive immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) to the TME, in addition to directly suppressing T cell and macrophage antitumor responses (Palazon et al. 2014). Through the production of CCL28, transforming growth factor-beta (TGF–β), and vascular endothelial growth factor [VEGF], hypoxic tumor cells recruit Tregs expressing CCR10 (CC chemokine receptor type 10) and neuropilin-1 (NP-1) into the tumor microenvironment. Tregs increase immunological tolerance and angiogenesis in the tumor microenvironment, which aids metastatic tumor growth. Hypoxic tumor cells, too, recruit myeloid cells into the TME by secreting chemokines and cytokines such as CCL5, C-X-C motif chemokine 12 (CXCL12 or SDF-1), VEGF, and endothelins [ET-1 and ET-2]. Hypoxic signaling in MDSCs and macrophages promotes an immunosuppressive state and stimulates angiogenesis, which contributes directly to tumor growth. Hypoxia also suppresses tumor-infiltrating lymphocytes' effector actions, owing to the deposition of extracellular adenosine. Adenosine causes a rise in cyclic adenosine 3′,5′-monophosphate in T cells via A2A receptors, which suppresses T cell proliferation, expansion, and cytokine release (Kumar and Gabrilovich 2014). These findings show how HIF signaling in tumor cells, MDSCs, and tumor-associated macrophages contributes to the establishment of an immunosuppressive tumor microenvironment in a variety of ways.

10.2.5.2 Invasion

The local invasion of tumor cells from the primary tumor into the neighboring tissue parenchyma is one of the initial steps in the metastatic cascade. Tumor cells drive local invasion through a variety of methods. To improve cell mobility, they modify the expression of cell-cell and cell-ECM adhesion molecules (Hanahan and Weinberg 2011). They also upregulate the expression and secretion of extracellular proteases that breakdown the ECM and release growth factors that promote tumor invasion and growth. Additionally, invasive tumor cells recruit macrophages, fibroblasts, and mesenchymal stem cells (MSCs) to produce promigratory factors or deposit collagen networks for invasion (Obenauf and Massagué 2015).

HIF signaling can induce epithelial plasticity and a migratory phenotype through the direct and indirect regulation of the epithelial-mesenchymal transcription (EMT) factors Snail, Slug, Twist, and Zeb1 (de Bock et al. 2011). Invasion of the surrounding ECM can also be enhanced by HIF signaling through the upregulation and secretion of proteolytic enzymes, such as matrix metalloproteinases (MMPs), cathepsins, lysyl oxidases, and prolyl-4-hydroxylses (P4H), to support the initial stages of metastasis by matrix remodeling (Semenza 2016). Recently, hypoxia was shown to increase the secretion of tumor-derived procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD2), a HIF target that hydroxylates lysine residues on collagen to promote the formation of mature collagen crosslinks and facilitate metastasis in multiple tumor models. In addition to the direct effects on tumor cells, HIF signaling in tumor cells induces the expression of chemokines and cytokines that recruit macrophages and MSCs into the TME to support tumor cell invasion, migration, and metastasis (Chaturvedi et al. 2014).

10.2.5.3 Intravasation and Extravasation

HIF regulates tumor cell intravasation and extravasation from the vasculature. HIF activity in tumor cells results in the release of factors that modulate endothelial cell-endothelial cell and endothelial cell-tumor cell interactions. The upregulation of angiopoietin-like 4 (ANGPTL4) by HIF disrupts endothelial cell-endothelial cell interactions and allows for easy passage of tumor cells through blood vessels. Simultaneously, HIF strengthens tumor cell-endothelial cell interactions through the activation of L1 cell adhesion molecule (L1CAM) (Zhang et al. 2012). Another mechanism by which HIF promotes tumor cell intravasation and extravasation is through the activation of genes that control vascular permeability. Hypoxic induction of vascular endothelial growth factor (VEGF), angiopoietin 2, MMPs, and UPAR cooperatively act to destabilize the vascular wall and allow tumor cell entry (de Bock et al. 2011). In the endothelium, HIF signaling increases cell adhesion, coagulation, and endothelial permeability (Evans et al. 2012). Recent studies demonstrated that lymphatic endothelial cells within premetastatic niches are conditioned by triple-negative breast cancer cells to promote extravasation by recruiting tumor cells to these sites through CCL5-dependent mechanisms and by activating the expression of proangiogenic factors, such as VEGF. While the role of HIF-1 within lymphatic vessels remains to be determined, these studies suggest that HIF-1 contributes, at least in part, to metastatic spread by modulating lymphatics.

10.2.5.4 The Premetastatic Niche and HIF Signaling

The ability of disseminated tumor cells to colonize, survive, and proliferate within the distant tissue microenvironment determines the late phases of metastasis. Experiments in mice have established during the last decade that the establishment of a tumor-promoting premetastatic niche in secondary organs is required for the extravasation and proliferation of metastatic tumor cells at a distant site. The discovery that tumor-secreted factors play a role in establishing the premetastatic niche has aroused interest in understanding the mechanisms that govern tissue-specific metastasis for potential therapeutic treatment. The impact of TME variables including hypoxia in the regulation of these secreted factors is also a hot topic of research. HIF signaling in the primary tumor aids in the generation of secreted components that aid in the establishment of premetastatic niches. HIF signaling increases the expression and secretion of lysyl oxidase (LOX) and LOX-like proteins in breast cancer cells (LOXL2 and 4). These proteins alter the lung's collagen matrix to attract bone marrow-derived cells (BMDCs), which prepare the lung for metastatic colonization. The BMDCs enhance metastasis by generating chemokines that attract tumor cells to the lungs through processes such as tumor cell extravasation stimulation.

10.2.5.5 HIF Signaling, Cellular Development, and Life at a Distant Area

Successful metastatic colonization necessitates disseminated tumor cells adapting to the environment of the distant tissue, which may differ significantly from that of the main tumor-bearing tissue. HIF stimulates angiogenesis, which increases late stages of metastasis at a distant site. Angiogenesis is required for metastasis growth, as it

would be for original tumors. VEGF-A is a proangiogenic factor made by tumor cells that promotes endothelial cell recruitment and proliferation, as well as pericyte proliferation and migration. VEGF-A is a well-known HIF target, and HIF signaling induces its production in both primary tumors and metastasis. Hypoxia and HIF signaling promote multiple steps within the metastatic cascade. Therefore, a variety of HIF inhibitors and other agents that target the hypoxic signaling pathway are in preclinical and clinical development for cancer (Onnis et al. 2009). Given that HIF signaling impacts the behavior of tumor and stromal cells in the TME, it will be important to evaluate the efficacy of these agents in immune-competent preclinical models and in clinical studies to understand the impact of HIF inhibition on the TME and tumor progression. Additionally, the evaluation of HIF inhibitors in combination with antiangiogenic or immunotherapies that target other components of the TME may help to overcome resistance to targeted therapy.

10.3 ECM

The ECM is a comprehensive and multicompartment that regulates cell processes such as proliferation, migration, invasion, and differentiation by acting as an interfacial growing medium, providing essential structure, controlling inter-cellular communication, and presenting growth factors to their receptors. ECM molecules can organize in a variety of meshwork structures to give tissue with a variety of textures and functions. For example, the ECM can form an impermeable basement barrier that separates epithelial cells (or endothelial cells in the case of blood vessels) from connective tissue (or interstitial matrices), which contains cells (fibroblasts, immune cells) intercalated inside fibrous ECM. Cancer cells in tumors can break through the basement membrane and infiltrate connective tissue, which can act as pro- or antitumoral tissue depending on its composition (Hastings et al. 2019).

10.3.1 Components of the Extracellular Matrix (ECM)

Glycoproteins

1. Proteoglycan
   Proteoglycans consist of core protein with one or more attached glycosamino glycan chain(s), representing a special class of glycoproteins that are heavily glycosylated approximately more than 95%.
2. Fibronectin
   This is another glycoprotein; by this, the cells are attached to ECM by fibronectin and to the one end of fibronectin are attached to the plasmamembrane.
3. Crosslinked fibers of collagen
   Collagen is the most common glycoprotein in the ECM, and it forms a woven network with outside cells, and as they are strong fibers, they run throughout this network. Collagen has great tensile strength and contributes to the stability of tissues and organs and maintains their structural integrity.

Collagen: A Base of ECM Architecture

The most important component of the ECM is collagen which has 28 subunits, and most commonly occurs in human tissues (Myllyharju and Kivirikko 2004; Mouw et al. 2014a), each of this is composed with left-handed helical α chains twisting to form a right-handed triple helical domain (Shoulders and Raines 2009). All members of the collagen superfamily has a same common structural similarity by consisting a number of and/or at least one triple helical domain of Gly-X-Y, where X is proline and Y may be hydroxyproline (Ricard-Blum and Ruggiero 2005). There are different types of collagen which are involved in the building of the supramolecular structures that becomes the basic architecture of the ECM. Apart from the collagen-mediated structure, ECM requires the support of other proteins to get the chemical and physical properties. These other proteins are proteoglycans, laminins, fibronectin, etc., and these can also aid in anchoring between the cells and to the ECM.

Proteoglycan Role on the ECM

The extracellular matrix is made up primarily of proteoglycans. The matrix accounts for more than 90% of the dry weight of cartilage. Proteoglycans are proteins that have been substituted by glycosaminoglycans (GAGs), which are linear polysaccharides made up of a repeating disaccharide, usually an acetylated amino sugar alternated with uronic acid. The majority of proteoglycans are found outside of cells. The core proteins and GAG chain subtypes of proteoglycans, including chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS), and heparan sulfate (HS), are all exceedingly varied (HS). The negative charge on GAGs they secrete water, cations makes them to space fill and lubricating properties (Iozzo and Schaefer 2015). The transmembrane proteoglycans may present in pericellular and extracellular spaces; some of them are syndicans and have an intracellular domain, transmembrane domain, and ectodomain; these may act as co receptors. GAGs like heparan sulfates are found attached to the ectodomain, and by the action of matrix metalloproteinases (MMPs), it can perform many biological actions (Leonova and Galzitskaya 2013; Fares et al. 2017).

There are the two proteoglycans found in the extracellular space, namely, hyalectans and SLRPs. Four distinct genes are encoded in hyalectans, viz., aggrecan, versican, neurocan, and brevican. The abundance of aggrecan is in bone cartilage and brain, while neurocan and brevican are found in CNS, whereas versican is found in almost all tissues and organs of ECM, and all these are acting like molecular bridges between the cells and ECM. On the other hand, versican can bind to the substrates of integrins like collagen type I and fibronectin, leading to cellular adhesion defects (Wu et al. 2005; Yamagata et al. 1986). SLRPs are the largest family of proteoglycans that have a short protein core and are expressed in the ECM during various tissue developmental stages, like in directing organ size and shape, embryonic development, and homeostasis (Iozzo and Karamanos 2010; Kalamajski and Oldberg 2010). Thus, proteoglycans perform different functions in the ECM and became as integral in maintaining the healthy ECM; otherwise, it becomes unfunctional ECM and even may collapse its structural integrity. According to the results of extensive research, the ECM can alter hallmarks of cancer. ECM can

influence a number of biological processes, including tumorigenesis, apoptosis resistance, angiogenesis stimulation, and cancer cell migration and invasion, all of which contribute to cancer progression (Hanahan and Weinberg 2000).

10.3.2 Remodeling Mechanisms of ECM in TME

Different remodeling mechanisms occur during changes in the ECM, which can be grouped into four basic processes: (1) ECM deposition, which alters the abundance and composition of ECM components, affecting biochemical and mechanical ECM properties; (2) posttranslational chemical modification, which alters the biochemical properties and structural characteristics of the ECM; and (3) proteolytic degradation, which releases bioactive ECM fragments and ECM-bound factors and may be required for the release of cellular constraints, such as migratory barriers (Mouw et al. 2014b).

10.3.3 The ECM Modifications During Metastasis

The prevention and removal of cancer metastasis, which is responsible for the majority of cancer-related fatalities, is critical for cancer therapy. The importance of ECM remodeling during each stage of metastasis development, from surviving in circulation to creating premetastatic and metastatic niches, has been the subject of extensive investigation over the last decade. The events, which occur during ECF remodeling, enable tumor cell invasion and metastasis (Fig.10.3).

10.3.3.1 Invasion of Cancer Cells Through Basement Membranes

The basement membrane (BM) is a thin, firm ECM sheet that is essential for optimal tissue formation and function (Yurchenco 2011). Epithelia, endothelia, fat, nerve, and heart cells are separated from their supporting connective tissues by this membrane. The origins of the BM may be traced back to the emergence of multicellularity, and BM proteins are found in a wide range of multicellular animals (Fabris et al. 2018). Laminin, which offers cell signaling inputs, and collagen IV, which is assumed to serve as the BM's major structural backbone, are the two most prevalent components. Nidogens, proteoglycans, and growth factors are among the other components. The BM is important for cell signaling, structural integrity, and barrier protection against cells and very big molecules because it is rich in biochemical and mechanical stimuli. Invasion by BM can take in two forms: one is chemical and the other is physical.

BM Breakdown Is Influenced by Proteases

Cells use proteases, which are enzymes that specifically dissolve peptide links between amino acids, to chemically destroy ECMs like BM. Early research discovered that cancer cells can degrade collagen IV and that enhanced degradation was linked to a higher risk of metastatic spread (Liotta et al. 1980). Loss of BM can be

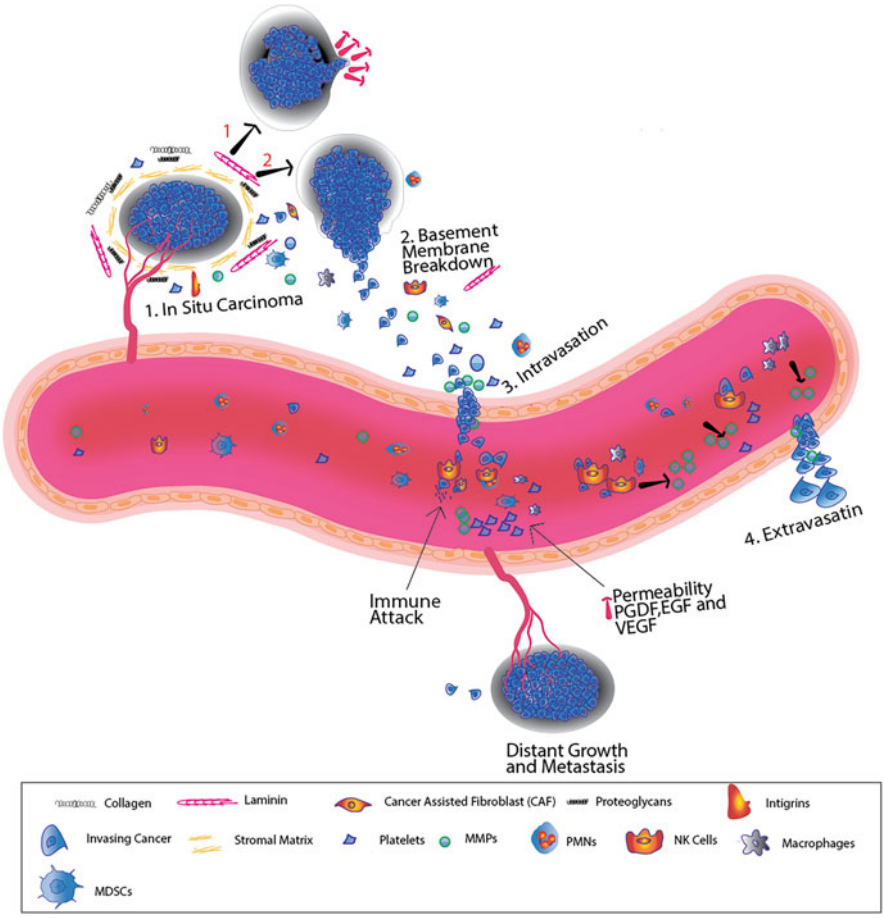

Fig. 10.3 The main steps in ECM remodeling and the metastatic cascade. (**a**) In situ carcinoma. The intact basement membrane prevents tumor cells from migrating and penetrating into the surrounding tissue. Integrins can physically join tumor cells and stroma cells across the basement membrane. Curly ECM is found in the interstitial matrix. (**b**) Non-proteolytic, force-mediated ECM remodeling (1) and proteolytic ECM degradation by proteases released by cancer cells and activated stroma cells (2) are two ways to disrupt the basement membrane. (**c**) Intravasation: tumor cells are liberated from the primary tumor microenvironment and then traverse the interstitial connective tissue before breaking the vascular basement membrane to obtain access to the circulation. Angiogenesis and strong MMP activity at the original tumor site damage the vasculature, allowing tumor cells to enter the circulation and intravasate. (**d**) CTCs evade immune surveillance in the circulation: CTCs come into contact with immune cells via direct cell-cell contact. Immune-mediated elimination occurs as a result of these interactions. CTC survival in the circulation is aided by changes in MHC molecules. (**e**) Extravasation: MMP activity increases, resulting in a leaky vasculature that allows CTCs to extravasate into the surrounding tissue. (**f**) Growth factors, MMPs, LOX, ECM proteins like fibronectin, and exosomes, all derived from the initial tumor, promote angiogenesis and create a premetastatic microenvironment at a distant site to prime the new tissue for metastasis

found at the site of change from a benign ductal carcinoma in situ to an invasive ductal carcinoma, according to histological data (Carraro et al. 2014). MMPs are required for tumor cell migration across collagen I-rich stromal matrix with extensive covalent crosslinks (Sabeh et al. 2004). The cleaved BM not only reduces the barrier function of the BM, but it also promotes cell migration through signals emitted by the cleaved BM (Koshikawa et al. 2004).

Force-Driven BM Invasion The role of protease-independent BM invasion has been controversial but has been implicated in immune cells and, more recently, carcinoma cells. Immune cells frequently transverse vascular BM to enter blood circulation during inflammation. Leukocytes, neutrophils, and monocytes all preferentially migrated through areas of venular BM that exhibited lower (<60%) protein deposition of laminin, collagen IV, and nidogen (Wang et al. 2006). While monocytes were highly deformable and squeezed through existing openings in the BM, neutrophil migration led to remodeling and subsequent enlargement of these low-protein sites. Dendritic cells have been shown to migrate through preexisting openings in the lymphatic BM by widening these small gaps. Being widened by cells, these gaps returned to a baseline slightly larger than the original gap size, indicative of mechanical plasticity of the BM. Protease-independent BM invasion in immune cells suggested the possibility of a protease-independent strategy to BM invasion by carcinoma cells. Mechanistically, invadopodia exert both protrusive and contractile forces during repeated extension and retraction cycles to deform their surroundings and permanently open up micronized channels in the matrix (Kraning-Rush et al. 2012; Gaiko-Shcherbak et al. 2021).

10.3.3.2 ECM Remodeling in Circulation

As previously mentioned, different ECM remodeling activities aid tumor cell motility and invasion, allowing them to eventually reach circulation. Surprisingly, single-cell RNA sequencing of pancreatic cancer cells revealed that circulating tumor cells (CTCs) in the blood upregulate expression of common stroma-derived ECM proteins like collagens (Col1a2, Col3a1), TIMP-2, the proteoglycan decorin (Dcn), the glycoprotein osteonectin (Sparc), and fibronectin (Ting et al. 2014). It is yet unknown whether CTCs actively secrete ECM and what role these ECM components may have in CTC support. CTC-secreted ECM may improve autocrine survival signaling, protect CTCs from immune cell clearance, and promote the formation of CTC clusters for efficient metastatic colonization, similar to platelets surrounding CTCs. CTCs, when caught by neutrophil extracellular traps (NETs), promote metastasis through interaction with integrin-1, which is expressed by both CTCs and NETs, according to a recent study. The ECM components released by CTCs may thus act as bridging molecules between NETs and CTCs (Nieswandt et al. 2006).

10.3.3.3 ECM Remodeling in Premetastatic Niche

Organ-specific microenvironments exist at potential metastasis sites, which are considerably different from the originating tumor. Even before the development of metastatic tumor cells, the main tumor causes the formation of a favorable milieu at

the distant tissue, which involves ECM remodeling, establishing a so-called premetastatic niche. The composition and remodeling mechanisms of the ECM at the metastatic site differ from those of the initial tumor and healthy tissue (Weigelt et al. 2005). Increased collagen deposition is the most prevalent ECM modification in the initial tumor environment. The premetastatic niche is formed mostly by fibronectin, as well as glycoproteins and proteoglycans such as tenascin C, osteopontin, and versican (Liu and Cao 2016). Primary tumor-derived substances stimulate premetastatic stromal cells to release new ECM molecules or remodel and change the ECM directly. The electrostatic charge and folding conformation of fibronectin and collagens, as well as their cell-adhesive properties, are affected by citrullination, which is the conversion of arginine residues into the noncoding amino acid citrulline. Peptidyl-arginine deiminases (PADs) are enzymes that are released into the extracellular space on neutrophil extracellular traps during inflammation and possibly also during premetastatic niche development. Colorectal cancer cells, on the other hand, release PAD4, which causes citrullination of collagen I in the liver and increases the adherence of disseminated tumor cells to liver tissue (Yuzhalin et al. 2018). Tumor-derived factors either establish an osteolytic (bone-degrading) or osteoplastic (bone-forming) premetastatic milieu in the bone. The more prevalent osteolytic metastasis occurs frequently in breast tumors and other cancer types, where bone homoeostasis is controlled by molecules such as IL-6, EGF-like growth factors, TGF-β, NF-B ligand (RANKL), and tumor-derived LOX2 (Lynch et al. 2005). Furthermore, ECM breakdown is required for the release of these soluble factors, such as EGF-like growth factors and TGF-β, which is mediated by MMP-1 and ADAMTS in the primary tumor. Collagen I, the most abundant ECM component in the bone, is degraded during osteolytic bone metastases. Collagens can also undergo non-enzymatic isomerization on their own. Collagen that has recently been synthesized is not isomerized; therefore it is increased in bone metastasis to compensate for the fast ECM turnover. As a result, instead of the isomerized form (-CTX-I), the non-isomerized C-telopeptide of collagen I (-CTX-I) can be employed as a biomarker for bone metastases from breast and prostate malignancies (Leeming et al. 2006).

10.3.3.4 ECM Remodeling in Metastatic Niche

CTCs must extravasate from the bloodstream into the new tissue to produce metastases, a process that is based on ECM remodeling. Endothelial cells in colorectal cancer patients' livers deposit and construct fibrillar fibronectin, which permits CTCs to bind to the arterial lumen, induce integrin-dependent focal adhesion, and finally extravasate into the liver tissue (Barbazán et al. 2017).

Both stromal and metastatic cells contribute to the formation of a metastatic niche and actively modify the ECM to favor metastatic development after colonization. Surprisingly, ECM remodeling of the metastatic niche differs between sites for the same tumor, showing tissue tropism. At the brain, lung, and other sites, stromal and cancer cells deposit unique combinations of matrisomal and ECM-associated proteins (Hebert et al. 2020). Glycoproteins such as osteopontin, periostin, and tenascin C are deposited at the metastatic site by infiltrating either cancer cells or

stromal cells and are critical for tumor cell colonization by triggering stemness-like signaling pathways (Oskarsson et al. 2011). Tenascin C is produced by breast cancer cells in the lungs, and it activates Notch and Wnt signaling. Wnt signaling in metastatic breast cancer cells is also increased by periostin deposition by activated lung fibroblasts. The c-Jun transcription factor is required for osteopontin and tenascin C expression. Chemotherapeutic therapy, interestingly, can stimulate the c-Jun N-terminal kinase (JNK) pathway, resulting in the deposition of these glycoproteins and fueling metastatic progression (Insua-Rodríguez et al. 2018). CTCs have mesenchymal properties, whereas initial and metastatic cancer cells have more epithelial phenotypes. By generating metastasis, versican deposition by bone marrow-derived cells supports it (Naba et al. 2016). Changes in the ECM may also be required for the reactivation of dispersed yet latent tumor cells. Matrikines have recently been linked to the reawakening of latent tumor cells and the promotion of metastasis. Extracellular neutrophil traps attach to extracellular laminin in an inflammatory environment and cause laminin degradation due to the presence of laminin-degrading proteases such as neutrophil elastase and MMP-9. A unique laminin epitope is exposed as a result of this proteolytic action. This matrikine activates an integrin-mediated signaling pathway, resulting in the reawakening of latent cancer cells at metastatic sites and their subsequent proliferation (Albrengues et al. 2018).

10.4 Phytochemicals Targeting HIFs and ECM in TME

Surgical excision or radiation treatment for massive, accumulated cancer biomass, followed by chemotherapy with chemotherapeutic drugs, is one of the currently available cancer treatment methods. The main issues with chemotherapy include drug resistance, cytotoxicity, and cancer recurrence (Choudhari et al. 2020). To overcome the limitations of ongoing therapies, new anticancer drugs must be discovered. As a result, there is a need to focus on medicines that are less toxic and cost-effective and have a lower risk of cancer recurrence. It is evident that phytochemicals are used in the majority of treatments that have fewer negative effects. Hypoxia and HIFs play significant role in cancer progression. HIF-1α, strongly expressed in malignancy compared to normal cell, is responsible for poor prognosis. Several cellular elements can be able to regulate the expression of HIFs in tissue hypoxic conditions and even in nonhypoxic conditions, thereby regulating cancer progression and survival. Extensive research has been done on the role of HIF1α inhibition, a promising tool for developing new anticancer agents. Inhibition of HIF-1α can upregulate the expression of HIF-2α as a compensatory mechanism and vice versa. Furthermore, HIF1α and HIF-2α divergently modulate target genes; they can differently affect the production of downstream targets. So, simultaneous inhibition of both HIF-1α and HIF-2α is needed for potential anticancer activity. Various phytochemicals and their derivatives have been proven for potent anticancer activity due to inhibition of HIF1α (Yun et al. 2021). Up to now, researchers have found that phytochemicals can mainly downregulate HIF-1α in the following ways:

Table 10.3 Phytochemicals targeting HIF-1α

Phytochemical	Mechanism of HIF-1 α inhibition
Curcumin	Curcumin can inhibit the mRNA and protein expressions of HIF-1α by reducing the DNA-binding potential of HIF-1α to hypoxia response element under hypoxic conditions (Yang et al. 2020b)
Isoquercitrin	By suppressing NF-κB activation, reduces HIF-1α mRNA levels (Fayed et al. 2019)
Apigenin	Reduce the expression of HIFα (Zhu et al. 2019)
Emodin	Inhibits HIF-1α and NF-κB signaling pathways (Lei et al. 2014)
Obtusifolin	Decrease in the mRNA and protein levels of HIF-1α (Wang et al. 2019)
Celastrol	Decrease PPARβ and HIF-1α and elevate p53 in HepG2 cells (Zhang et al. 2010)
Oridonin	Inhibiting epithelial-mesenchymal transition (EMT) and the HIF-1α/VEGF signaling pathway (Semenza 2003)
Y6, a derivative of EGCG	Downmodulates HIF-1 α (Liao et al. 2020)
Luteolin	Inhibits hypoxia-induced VEGF and HIF-1α expressions (Lin et al. 2013)
Vitexin	Inhibits HIF-1α expressions (Choi et al. 2006)
Silibinin	Reduces HIF-1α expressions (Deep et al. 2017)
Resveratrol	Reduces HIF-1α and VEGF expression (Zhang et al. 2005)
Baicalein	Inhibits HIF-1α stability (Hwang et al. 2008)
Deguelin	Blocks HIF-1α and angiogenesis (Gomez-Casal et al. 2015)
Dictamnine	Downregulates HIF-1α levels under hypoxia (Wang et al. 2018)
Nuciferine	Downmodulates the level of HIF-1αunder normoxic conditions (Liu et al. 2020)
Tetrandrine	Downregulates HIF-1αunder normoxic conditions (Chen et al. 2018)
Thymoquinone	Represses HIF-1 α expression under hypoxia (Lee et al. 2019)
Ursolic acid	Expression of HIF-1α is reduced (Shan et al. 2016)
Britannin	Interrupts mTOR signaling and inhibits MYC proto-oncogene (MYC), as well as HIF-1αexpression (Zhang et al. 2021)
Cephalomannine	A reduction of several HIF target genes (Oullah et al. 2021)
Ilexgenin A	Inhibits HIF-1α expression (Zhang et al. 2019)
Theasaponin E1	Depresses HIF-1α levels (Li et al. 2021)
Pristimerin	Downregulation of AKT activity and HIF-1α expression (Lee et al. 2016)
Panaxadiol	Suppresses HIF-1 α expression via the PI3K/AKT pathway under hypoxia (Wang et al. 2020)
Betulinic acid	Inhibits HIF-1 α accumulation by activating proteasome (Guimarães et al. 2017)
Micheliolide	Facilitates HIF-1α degradation (Kong et al. 2020)

(1) block HIF-1α/p300 interactions; (2) decrease the expression of HIF-1α mRNA; and (3) reduce stability of HIF-1α protein. Some phytocompounds targeting HIF1α are listed in Table 10.3.

Invasion of cancer cell induced by matrix metalloproteinase-9 (MMP-9) is one of pivotal steps in cancer metastasis. MMPs are zinc-dependent endopeptidases that are responsible for extracellular matrix (ECM) destruction and remodeling during

organogenesis, wound healing, angiogenesis, apoptosis, cell proliferation, and cancer progression. MMPs (excluding MP-11) are released as inactive zymogens that are activated by other MMPs or serine proteases outside the cell (Nagase et al. 2006). The tumor invasion is aided by MMP-induced ECM breakdown. Several clinically accepted MMP inhibitors have the ability to modulate MMP; however their use is limited due to side effects. MMPs can be suppressed during cancer metastasis by interfering with extracellular factors and signal transduction pathways, which can be done by limiting their activation or activity at the transcriptional level (i.e., NF-kB or AP-1). Herbal remedies have received a lot of attention in the healthcare system during the last few decades. Because of their safety and potency, phytochemicals derived from plants are a great source for innovative medication development. These secondary metabolites have the ability to modulate a variety of pathogenic pathways linked to a variety of diseases. The potential role of phytochemicals in the treatment of breast cancer is due to direct reduction of MMP activation or signaling pathway regulation (Khan et al. 2021). List of some phytochemicals with their MMP inhibitory action are given in Table 10.4.

10.5 Conclusion

Advanced insight into the role of the tumor microenvironment (TME) in cancer progression has opened up new avenues for cancer treatment targeting the extensive signaling pathways. The failures of currently available medications have highlighted the need for new drugs that can target several critical components of cancer growth (e.g., angiogenesis, uncontrolled cell division, and metastasis). Extensive knowledge about the role of TME in cancer progression and development is crucial for designing advanced cancer treatment techniques. Hypoxia-inducible factor 1 (HIF-1) induces the expression of genes involved in angiogenesis, cell survival, glucose metabolism, and invasion, among other aspects of cancer biology. Multiple events in the metastatic cascade are aided by hypoxia and HIF signaling. HIF-1 inhibition has been shown in numerous studies to have anticancer properties, as well as the ability to improve anticancer treatment efficacy by interfering with HIF-1-mediated signaling. The extracellular matrix's remodeling is also critical for cancer cells to acquire the phenotypic EMT that allows them to invade. Increased expression and activation of matrix metalloproteinase (MMPs) in the tumor microenvironment, which can destroy the extracellular matrix and basement membrane, support this hypothesis. MMP activity causes cancer cells to spread to new locations in this fashion. In tertiary chemoprevention, many phytochemicals inhibit MMP expression and act as suppressing agents.

Medicinal plants are still a valuable resource for finding and developing new pharmacological leads. One of the key advantages of medicinal plant-based drug discovery is the availability of ethnopharmacological data, which allows researchers to narrow down the vast number of potential leads to the most promising ones. To thoroughly exploit the potential of phytochemicals, a novel approach to integrated drug discovery is required, in which ethnopharmacological knowledge is supported

Table 10.4 Phytochemicals and their mechanisms of MMP inhibitory action

Phytochemical	MMP inhibitory mechanism
Berberine	Downregulation of MMP-2/−9 expression Attenuation of TNFα induced expression ofMMP-9 Reduces mRNA expression of MMP-9 via regulating Akt signaling pathway (Kim et al. 2008)
Evodiamine	Inhibits cell migration and invasion via downregulation of MMP-9 (Du et al. 2013)
Piperine	Inhibits PMA-induced MMP-9 expression through downregulation of PKCα/ extracellular signal-regulated kinase (ERK) 1/2 and reduction of NF-κB/AP-1 activation (Lai et al. 2012)
Sanguinarine	Reduces mRNA expression of MMP-2/-9 (Choi et al. 2009)
Quercetin	Downregulates Akt/MMP-9 pathway and suppresses PKCd/ERK/AP-1-dependent matrix metalloproteinase-9 activation (Lin et al. 2008)
Kaempferol	By reducing MMP-2 and MMP-9 expressions and subdual of AP-1 and MAPK signaling (Kim et al. 2016)
Delphinidin	By diminishing MMP-9 activity in ER+ MCF-7 cells by blocking NF-κβvia MAPK signaling pathways (Im et al. 2014)
Genistein	Downregulation of all MMP genes (Kousidou et al. 2005)
Kutkin	Downregulation of gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1 and MMP-13), decreased expression of MMPs at mRNA and protein level (Rathee et al. 2013)
Oleandrin	Suppresses MMP-9 activity and octamer binding transcription factor 3/4 (OCT3/4) through inhibition of phosphor-signal transducer and activator of transcription (STAT)-3 (Ko et al. 2018)
Ursolic acid	Downmodulation of MMP-2 and uPA expression is associated with inhibition of JNK-Akt and reduction of NF-Kβ p65 in nucleus (Yeh et al. 2010)
Tanshinone IIA	Suppresses MMP-7 and epithelium-specific (ETS) transcription factors involved in tumorigenesis (Wang et al. 2005)
Carnosol	Suppresses MMP-9 expression through inhibition of extracellular signal-regulated kinase (ERK) 1/2, AKT, and JNK signaling pathway (Huang et al. 2005)
Antroquinonol	Suppresses ERK-AP-1 and AKT-NF-kB-dependent MMP-9 activity (Lee et al. 2015)

by broad interdisciplinary forces involving medicinal chemistry, pharmacology, biochemistry, molecular and cellular biology, as well as natural product chemistry. In addition, developments in analytical technology and computational approaches, as well as the development of self-teaching artificial intelligence systems, will make it easier to find new phytochemical lead entities for pharmacological testing.

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Role of Hypoxia in Cancer Therapy: Introduction

11

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Abstract

Hypoxia and the hypoxia-inducible factors (HIFs) regulate various characteristic features of cancers such as genetic instability, dedifferentiation, metabolic alterations, neovascularization, metastasis, and drug resistance. Therefore, targeting the hypoxic phenotype is a good approach to eradicate malignant cells. Theoretically, HIF inhibition could overcome the resistance to chemotherapy. So, HIF inhibitors are useful targets in the treatment of cancer. Researchers are trying to get specific HIF-1 inhibitors for successful clinical exploitation. HIFs mediate the response, primarily by acting as transcription factors, which are also good targets for treatment.

Keywords

Hypoxia · HIFs · Metastasis · Malignant cells · Cancer therapy

11.1 Introduction

As the malignant cells proliferate uncontrollably, the cells far away (more than 200 m) from the blood vessels are affected by hypoxia. Invasive oxygen electrodes provide a direct measurement of oxygen tension in the tumor tissue. Although hypoxia is not clearly defined, about 8–10 mm Hg (~1%) is taken as the critical

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pO_2 (Hockel and Vaupel 2001). Hypoxia in the cancer tissue is an independent prognostic factor for cancer mortality. Hypoxia will select tumor cells to more resistant phenotypes (Roma-Rodrigues et al. 2019). Two recent reviews will give more light on this subject (Jing et al. 2019a; Nejad et al. 2021).

11.2 Hypoxia-Inducible Factor

Hypoxia-inducible factor (HIF) regulates the genes involved in the progression of cancer cells. HIF expression is associated with poor prognoses (Huang et al. 2017). HIF is a transcription factor, composed of two subunits, alpha and beta. The alpha unit is oxygen-sensitive, while the beta subunit is constitutive in nature (Semenza 2003). In mammals, there are three alpha isoforms (HIF-1α, HIF-2α, and HIF-3α). HIF-1 and HIF-2 have unique target specificities. HIF-1 induces the expression of glycolytic genes, while HIF-2 targets other genes (Wigerup and Påhlman 2016). The HIF-1α is overexpressed in various cancers, including breast, colon, gastric, lung, skin, prostate, and renal carcinomas compared to their normal tissues (Simiantonaki et al. 2008). HIF also contributes to chemotherapy resistance.

11.3 Tumor Angiogenesis

The upregulation of angiogenesis is a characteristic feature of hypoxia. Cells grown under hypoxic conditions activate transcription of vascular endothelial growth factor (VEGF), platelet-derived growth factor-β (PDGF-β), and angiopoietin-2, all of them leading to endothelial cell proliferation (Kelly et al. 2003).

Monoclonal antibodies that target VEGF (bevacizumab) or small-molecule inhibitors that target VEGF receptors have achieved clinical benefits for advanced cancer.

11.4 Metabolic Derangement

In 1924, Otto Warburg observed that many types of cancer cells prefer glycolysis rather than oxidative phosphorylation. Glycolysis generates only two ATP molecules per glucose molecule compared to 32 ATP molecules per glucose molecule during the TCA cycle. Under oxygen-deprived conditions, normal cells also convert pyruvate into lactate, a process known as "anaerobic glycolysis." However, cancer cells convert pyruvate into lactate even when oxygen is available, so their metabolism is often referred to as "aerobic glycolysis" or "the Warburg effect." In that sense, cancer cells prefer a pathway that produces less ATP. One explanation is that cancer cells do have enough nutrient supply, so they do not require maximal ATP production. Another reason is that the HIF-1 directly targets the gene encoding pyruvate dehydrogenase kinase 1 (*PDK1*). The increase in glycolysis for ATP

generation in cancer cells is frequently associated with resistance to doxorubicin and ara-c (Kim et al. 2006).

11.5 Tumor Immune Response

Hypoxia leads to immunosuppression and tumor resistance (Palazon et al. 2012). Tumor hypoxia and HIFs can attract suppressor cells and tumor-associated macrophages with immunosuppressive functions. Hypoxia also suppresses infiltrating cytotoxic T-lymphocyte activity in a HIF-1α-dependent manner.

11.6 Tumor Metastasis

Hypoxia and HIFs are involved in many different steps of the metastatic process. Epithelial-to-mesenchymal transition (EMT) is an initial step in which tumor cells lose expression of the intercellular adhesion molecule E-cadherin (encoded by *CDH1*) and acquire a motile phenotype. Hypoxia and HIFs can also induce tumor cell invasion through various mechanisms including upregulation of cathepsin D, urokinase-type plasminogen activator receptor, and matrix metalloproteinases. Activation of HIF-1 and -2 is associated with loss of E-cadherin, so as to increase invasion and metastasis. The HIF also upregulated proteins implicated in matrix remodeling, such as lysyl oxidase (LOX) and metalloproteases. In addition, HIF activates genes involved in metastasis and invasion, such as the *c-met* proto-oncogene and the chemokine receptor CXCR4 (Chan and Giaccia 2007).

11.7 Chemoresistance

In the majority of patients, the cause for treatment failure is the resistance to cancer therapy. Hypoxia induces drug resistance in a wide range of neoplastic cells, including mouse embryonic fibroblasts. When HIF-1 is inactivated, the effect of carboplatin and etoposide on cell proliferation is significantly enhanced. HIF-1α can be used as a marker of the survival rate of cancers. Hypoxia is a crucial mediator of chemoresistance. HIF overexpression in clinical samples is associated with therapeutic resistance or decreased survival following IR or chemotherapy (Lin and Koong 2018).

11.8 Hypoxia and Drug Resistance

In response to hypoxia, the HIF-1 activates the multidrug resistance 1 (*MDR1*) gene. The MDR1 gene encodes the drug efflux pump, P-gp, which decreases the intracellular concentration of various chemotherapeutic drugs (Tameemi et al. 2019).

11.9 Hypoxia and New Treatment Modalities

Hypoxia is very characteristic of solid tumors, and hypoxia mediates metastasis and resistance to chemotherapy. Therefore, hypoxia is the most attractive therapeutic target. Hypoxia-activated prodrugs, specific targeting of HIFs, or targeting pathways important in hypoxic cells such as the mTOR and UPR pathways have been tried (Semenza 2012).

11.10 Hypoxia-Activated Prodrugs

Prodrugs are activated in hypoxic tissue and then selectively kill hypoxic tumor cells have been tried. For example, tirapazamine has been extensively tested in clinical trials, but results are disappointing (DiSilvestro et al. 2014). The prodrug apaziquone (EO9), a mitomycin C derivative, showed efficacy in preclinical studies, but clinical trials were negative. The phase II clinical trial on the TH-302 combined with gemcitabine for pancreatic cancer is encouraging.

11.11 Drugs Targeting Hypoxic Signaling

Targeting HIF directly and targeting of downstream HIF signaling pathways have also been tried. Thus, monoclonal antibodies targeting VEGF (bevacizumab) or small-molecule inhibitors targeting the VEGF receptor have shown clinical benefits in advanced cancers. Translation of HIF-α mRNA is controlled by the PI3K/AKT/mTOR pathway. Thus, mTOR inhibition decreases HIF-1α and HIF-2α levels under hypoxic conditions (Mohlin 2015).

11.12 Topoisomerase 1 Inhibitors

Irinotecan and topotecan are topoisomerase-I inhibitors. Topotecan inhibits HIF-1α translation (Bertozzi et al. 2014). EZN-2968 is a synthetic antisense oligonucleotide, complementary to the mRNA coding sequence of human HIF-1α. The EZN-2968 binding leads to HIF-1α mRNA downregulation in a dose-dependent manner (Jing et al. 2019b).

11.13 Heat Shock Protein Inhibitors

Heat shock proteins (Hsp) are cellular chaperones. Hsp90 inhibitor geldanamycin (GA) can induce proteasomal degradation of HIF-1α under hypoxic conditions. The GA analogs 17-AAG (tanespimycin) and 17-DMAG (alvespimycin) and EC154 have been evaluated in phase I and phase II trials. Alteration in protein ubiquitylation

is commonly associated with cancer. Ubiquitylation can be opposed by deubiquitinases (DUBs), which have emerged as promising drug targets. There is a reciprocal regulation of DUBs by hypoxia, and therefore DUB-specific drugs may be useful in cancer therapy (Mennerich et al. 2019).

11.14 Inhibitors of HIF Transcriptional Activity

The HIF transcription is dependent on co-activators such as p300/CBP. Therefore, transcriptional inhibition has been tried as a therapeutic intervention. *Chetomin*, a fungus metabolite, could inhibit binding of HIF to p300, and in vivo anti-tumor effects were also demonstrated.

11.15 Proteasome Inhibitors

The proteasome inhibitor bortezomib blocked hypoxia-induced VEGF accumulation by inhibiting HIF-1α transcriptional activity. However, in a phase II trial, bortezomib was inactive in patients with metastatic colorectal cancer (Marignol et al. 2013).

Such targeted strategies include hypoxia-activated prodrugs, inhibition of HIF dimerization, decreasing transcriptional activity, siRNA treatment, as well as suppressing the PI3K/AKT pathway (Zhang et al. 2021). A combination of immunotherapy and HIF inhibition could be a better therapeutic approach.

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Hypoxic Regulation of Telomerase Gene Expression in Cancer

12

Suman Kumar Ray and Sukhes Mukherjee

Abstract

Non-cancerous and cancerous cells make different amounts of the enzyme telomerase, which lets cells grow and divide without limits. Even though telomerase is overexpressed in some cancers, most primary cells in humans don't have it. So, before telomerase is turned back on, there is a crucial change from a telomerase-negative state to a telomerase-positive state. This is when a lot of cancer forms. The telomere shortening that happens with cell division may either prevent or promote cancer by activating checkpoints and impacting chromosomal integrity during this transition. Telomerase probably plays a key role in the growth and maintenance of tumors in adults with malignancies because it keeps telomeres stable and helps cancer cells grow indefinitely. Basal telomerase activity depends on the expression of the human telomerase reverse transcriptase gene (hTERT) and the human telomerase transcript (hTR), and an increase in telomerase gene expression is linked to the growth of cancers. Therefore, it is conceivable that elements of the tumor's environment and the signaling pathways involved in tumor growth may have an impact on the regulation of the telomerase gene. Recent research has shown that hypoxia can increase telomerase activity in ways that haven't been found yet. Most solid tumors have hypoxic areas. Researchers have found the process that controls how much hTERT is made when there is not enough oxygen. In addition to increasing endogenous hTERT expression, hypoxia also activates the hTERT promoter. When antisense oligonucleotides are

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given to hypoxia-inducible factor 1 (HIF-1), it shows that HIF-1 is directly responsible for how hypoxia raises hTERT. Since the promoter of hTERT contains putative HIF-1-binding sites, researchers have discovered the function of HIF-1 in the control of hTERT and telomerase in tumor hypoxia. The telomeric repeat amplification protocol (TRAP) experiment showed that telomerase was activated in cervical cancer cells in response to hypoxia. Telomerase must be turned on by the transcription factor HIF-1, and new research shows that hypoxia turns on the transcription of hTERT. They also point to new ways for cancers to turn on telomerase and have implications for the molecular mechanisms behind hypoxia-induced tumor growth and HIF-1-based gene therapy for cancer.

Keywords

Telomerase · Hypoxia · hTERT · HIF · Cancer · Gene expression

12.1 Introduction

Telomeres, which are nucleoprotein caps, guard the ends of linear eukaryotic chromosomes (O'Sullivan and Karlseder 2010). Nucleotide repeats make up the intricate network of proteins that bind them and maintain the formation of a looped and protected chromosomal end (TTAGGG in mammals) (Srinivas et al. 2020). This tightly wrapped telomere structure (t-loop) not only shields the chromosome end from cellular machinery that would mistake it for a broken chromosome and activate the checkpoint, but it also prevents access to recombination enzymes, which can actually join chromosomes together. According to this method, maintaining the telomere structure is crucial for maintaining the stability of the chromosomes as well as the survival and growth of cells. Telomeres increasingly shorten as cells divide in the absence of telomerase, the enzyme that synthesizes telomere repeats, since DNA polymerase is unable to completely replicate the extreme ends of chromosomes (Calado and Young 2009). Due to the intricate biology that controls telomerase, including gene transcription and enzyme activity, it presents not just one but numerous possible therapeutic targets. If we know more about the different ways that telomerase can be controlled, we could reach many more goals. Primary human fibroblasts are impacted by progressive telomere shortening caused by proliferation in culture in two fundamentally distinct ways (Shammas 2011). First, fibroblasts undergo replicative senescence, which is a permanent growth halt and altered morphological condition necessitating intact p53 and Rb (retinoblastoma susceptibility gene product) tumor suppressor pathways. When both p53 and Rb are rendered inactive by viral oncoproteins or antisense methods, cells can avoid senescence and instead enter a state of crisis after an extended period of proliferation. A crisis is defined by widespread apoptosis and chromosomal instability as the full consequences of critical telomere shortening and chromosomal end-to-end fusions become apparent (Counter et al. 1992). By ectopically producing telomerase reverse transcriptase (TERT), the protein catalytic subunit of telomerase, human fibroblasts

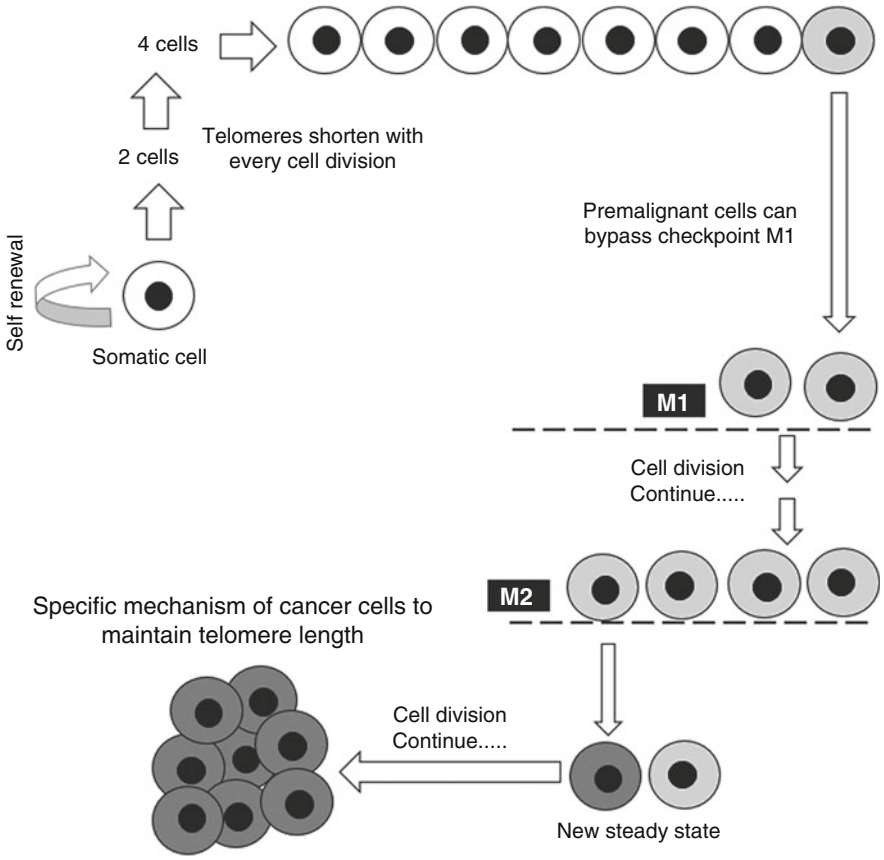

Fig. 12.1 Role of telomeres and telomerase in aging and cancer

can be given immortal proliferative properties. This can prolong telomeres, boost telomerase activity, and prevent senescence and crises (Stewart and Weinberg 2002). Role of telomeres and telomerase in aging and cancer are shown in Fig. 12.1.

It's noteworthy that computer-aided homology searches have discovered potential HIF-1 binding sites on the gene promoter of the catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT) (Lewis and Tollefsbol 2016). These results allow us to infer that HIF-1 is involved in the regulation of hTERT and telomerase. When tumors achieve their steady-state volume, the quantity of dividing malignant cells outnumbers the local supply of oxygen and nutrients, exposing them to a hypoxic environment. In order to continue development, tumor cells must adapt to hypoxic stress by altering their metabolic processes and inducing neovascularization, which delivers the additional blood needed to support cellular proliferation. These adaptive responses give tumor cells the ability to expand constantly, giving them a selective growth advantage that encourages the emergence of more aggressive phenotypes. This notion is supported by the finding that tumor

hypoxia is associated with a poor prognosis in a number of human malignancies (Birner et al. 2000; Wicks and Semenza 2022; Cangelosi et al. 2020).

A key regulator of the cellular response to oxygen deprivation is the transcription factor hypoxia-inducible factor 1 (HIF-1) (Semenza 1999). HIF-1 controls the transcriptional response to O_2 deprivation by binding to hypoxia response elements (HREs) in the promoters or enhancers of genes involved in glycolysis, glucose transport, erythropoiesis, and angiogenesis (Bunn and Poyton 1996; Wenger 1997). Hypoxia has been shown to increase the expression of the hTERT gene, suggesting that telomerase activation may also serve as a defense against the genetic stress caused by hypoxia (Chi et al. 2006; Minamino et al. 2001). Telomerase activity is a key part of cell growth, but it has been shown that low oxygen levels increase the expression of the hTERT gene. But nothing is known about the molecular mechanisms by which hypoxia promotes telomerase. Although it has recently been demonstrated that hypoxia can increase telomerase activity, it is still unclear how exactly this happens. The consensus hypoxia response element (HRE) sites present in both telomerase gene promoter sequences suggest that telomerase transcription may be regulated by hypoxia-inducible factor-1 directly interacting with these sites (HIF-1). HIF-1 is a transcription factor that interacts with and regulates promoters that contain the HRE motif. The HIF-1a subunit is susceptible to prolyl hydroxylation in normoxia, which results in this subunit being ubiquitinated and ultimately destroyed by the proteasome (Bárdos and Ashcroft 2004). In contrast, the HIF-1b subunit is generated constitutively. Role of HIF-1 in the development of hypoxia-induced cancer is shown in Fig. 12.2.

12.2 Telomeres in Hypoxic Cancer

Researchers have shown that the vast majority of cells in hyperplastic regions of three different and common pre-malignant human cancers—melanocytic nevi, colonic adenomas, and breast ductal hyperplasias—but not cells of their malignant cancer counterparts, displayed hallmarks of telomere dysfunction-induced senescence (TDIS), testing the hypothesis that replicative senescence evolved as a tumor-suppressing mechanism in humans (Suram et al. 2012). Although TDIS is currently considered to be an important tumor-suppressive mechanism in mammals, it is still unknown whether it actually acts as a stable and thus irreversible barrier to cancer progression (Patel et al. 2016). Recent findings from our group demonstrated that TDIS is an unstable barrier that cells can pass over after a protracted period of inactivity. TDIS is caused by the expression of HRasG12V and BRafV600E. Derepression of the hTERT promoter and MAP kinase-mediated stabilization of c-Myc expression, which together promote re-activation of hTERT expression, are two steps that contribute to escape from OIS (Yuan et al. 2019). In fact, telomerase reactivation has been found in human melanocytic skin and breast lesions at the earliest stages of cancer development, showing that telomerase reactivation aids in the progression of cancer in people. Although HIF-1a degradation is reduced in hypoxic conditions, this results in a rapid rise in protein levels and the creation of the

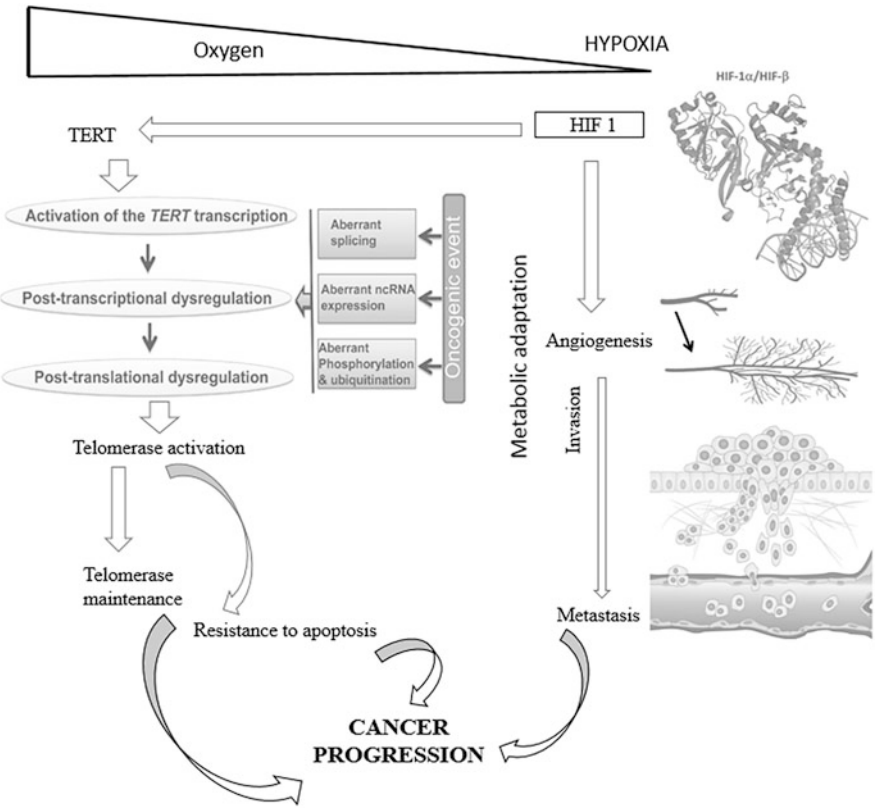

Fig. 12.2 HIF-1 functions in hypoxia-induced cancer development. Through increased angiogenesis and metabolic adaptation, HIF-1 produced by tumor hypoxia aids in the progression of the disease. It can also act as a transcription factor to increase hTERT mRNA expression. This turns on telomerase and may help cells grow by restoring telomeres and stabilizing the genome or by making them less likely to die by apoptosis (Yatabe et al. 2004)

beneficial heterodimer (Ziello et al. 2007). Using the hTERT gene promoter as their focal point, Yatabe and his colleagues (2004) recently demonstrated that hypoxia boosted hTERT expression and transcriptional activity in choriocarcinoma and cervical carcinoma cell lines (Yatabe et al. 2004). Both hTR and hTERT are described as being regulated by hypoxia in the current study's descriptions of telomerase gene regulation. Telomerase is active in hypoxic conditions, according to past studies that support this notion (Minamino et al. 2001). This shows that the mechanism used to protect against the genetic stress caused by hypoxia is telomerase activity. At the molecular level, it is still not known exactly how hypoxia turns on telomerase. Figure 12.3 depicts the role of telomeric gene expression for the development of cancer hypoxia.

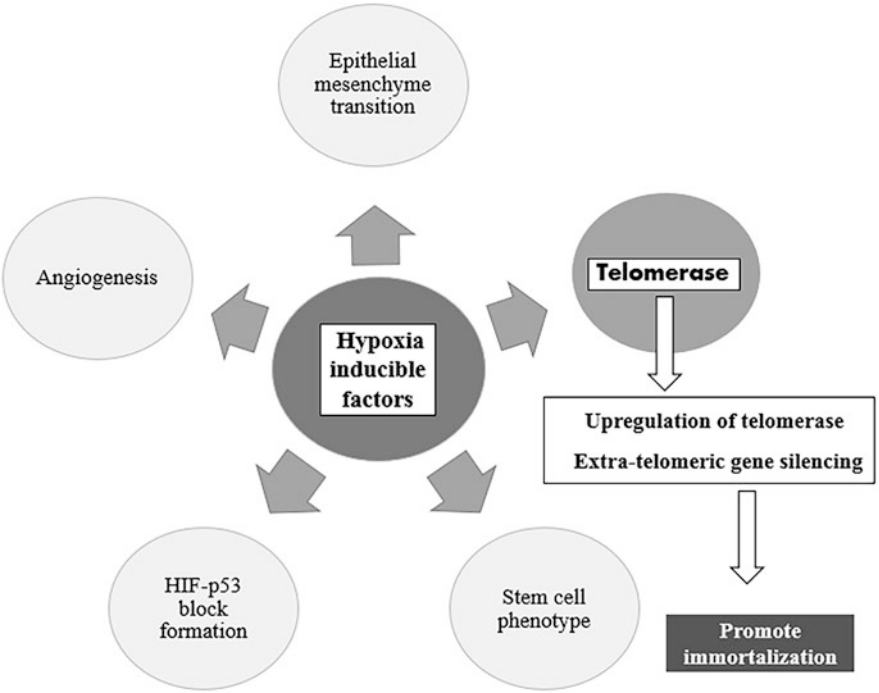

Fig. 12.3 Overexpression of telomerase and hypoxia-inducible factor in cancer

12.3 Connection Between Hypoxia and the Stimulation of Telomerase Activity

Recent research has revealed that HIF-1 is involved in the biology of tumors (Mukherjee and Ray 2022). A key regulator of the body's reaction to low oxygen levels is HIF-1. In nude mouse xenograft assays, analysis of isogenic cell lines reveals that overexpressing HIF-1 reduces tumor latency and increases vascular density, volume, and permeability, whereas underexpressing HIF-1 increases tumor latency and decreases vascular density (Jiang et al. 1996; Maxwell et al. 1997; Carmeliet et al. 1998). HIF-1a transfection renders cancer cells resistant to apoptosis and increases in vivo tumorigenicity. Cancer cells that express HIF-1a constitutively are more resistant to the apoptosis that is brought on by hypoxia than cancer cells that do not express HIF-1a constitutively (Akakura et al. 2001). These findings (Birner et al. 2000; Schindl et al. 2002; Sivridis et al. 2002) that HIF-1a is overexpressed in cervical cancer tissues (89%) (Birner et al. 2000) are consistent with reports that HIF-1 upregulation in several types of tumors is linked to treatment failure or a poor prognosis.

Normal cervical stratified epithelia exhibited limited HIF-1 expression, but malignant sections only exhibited considerable staining, indicating that HIF-1 has

a specific role in tumor biology. A typical cervix's parabasal cells have been discovered to have poor telomerase activity, which is remarkable and consistent with the location of HIF-1a expression. Weak HIF-1a expression in the cervical mucosa has unknown repercussions. Telomerase correlates with hTERT expression in cancer development, cell immortalization, and proliferation (Leão et al. 2018). Furthermore, hypoxia promotes cell division (Druker et al. 2021). There are at least two possible explanations for the link between hypoxia and the promotion of telomerase activity. First, hypoxic-induced telomere degradation has the potential to result in a DNA damage response. In response to this damage, HIF-1 may induce telomerase to repair the damaged chromosomal ends. As previously mentioned, the hypoxia-induced activation of telomerase can also result in an antiapoptotic reaction (Lipinska et al. 2017). The mechanisms through which HIF-1 causes a poor prognosis or resistance to apoptosis are unclear.

The metabolic tolerance to hypoxia is facilitated by glucose transporters and glycolytic enzymes (Semenza 2000). VEGF and NOS2 promote angiogenesis, whereas IGF-2 promotes cell survival and proliferation (Semenza 2000). Telomerase activation via hTERT transactivation is another potential mechanism by which HIF1 overexpression is associated with aggressive phenotypes or a poor prognosis for malignancies. The HIF-1 consensus-binding sequences (50-ACGTG-30) that overlap the E-box (CACGTG) seem to be in charge of the transactivation of hTERT caused by low oxygen levels. According to the ChIP study, hypoxia significantly boosted HIF-1's binding activity. These E-boxes are known to bind to the nuclear factors c-Myc, Max, and Mad (Kyo et al. 2000; Xu et al. 2001). It is therefore feasible that these factors' competition will have an effect on HIF-1 binding. Recent studies have found that hypoxia either inhibits c-Myc expression or encourages its deterioration (Li et al. 2020). The most recent ChIP results provide credence to these conclusions. HIF-1 binding to the hTERT promoter was greatly elevated under hypoxia, whereas c-Myc binding was decreased. Based on these results, it seems likely that HIF-1 and cMyc bind to the hTERT promoter and that HIF-1 preferentially binds to the promoter when there isn't enough oxygen.

12.4 Expression of hTERT Is Stimulated by Hypoxia, and HIF1 Interacts with Putative HREs in the hTERT Promoter

Hypoxia has been demonstrated to increase telomerase activity and HIF-1 expression. Nishi and his coworkers (2004) have investigated how hypoxia affects the telomerase activity in JEG-3 choriocarcinoma cancer cells (Nishi et al. 2004). They found that exposure to low oxygen levels caused a significant increase in telomerase activity. Since transcriptional regulation of hTERT is the main mechanism of telomerase activation, Nishi and his group (2004) also studied the effect of hypoxia on hTERT mRNA and protein expression (Nishi et al. 2004). It is feasible to increase the expression of HIF-1 and hTERT by giving the cells 1% oxygen. Compared to normoxia, HIF-1 overexpression boosted the hTERT promoter's activity by a factor of three to four (Nishi et al. 2004). The putative HREs produced by the hTERT

promoter interact with HIF-1 directly. A DNA-protein complex is produced when either TERT/HRE1 or TERT/HRE2 is treated with HIF-1-programmed rabbit reticulocyte lysate. According to Kyo and his colleagues (2008), increased HIF-1 activity on the hTERT promoter is what causes hypoxia to result in hTERT expression (Kyo et al. 2008).

12.5 In Telomerase-Deficient Animal Model, Telomere-Based Crises Increase Tumorigenesis

Telomerase is a heterodimer of TERT and telomerase RNA component (TERC), an essential RNA required for telomere formation and reverse transcription of the telomere sequence. A healthy mTERC−/−mouse is created when mouse TERC (mTERC) is inactivated through homologous recombination in embryonic stem cells. These mice lack telomerase activity but have phenotypically typical long telomeres (Blasco et al. 1997). Telomeres in mice are much longer than those in humans (40–60 kb versus 5–15 kb). When mTERC−/−mice were crossed, telomeres shrank and severely shortened in generations four through six. The mTERC−/−animals demonstrated proliferative problems and an increase in apoptosis in organ systems with high turnover, as well as infertility in these subsequent generations (Lee et al. 1998). When telomeres are uncapped, the tumor suppressor p53 is active and reacts to various cellular stresses like DNA damage, oncogene activation, and hypoxia. These limitations in cell multiplication and survival therefore occur. In late-generation mTERC−/−mice after loss of p53, the apoptotic response to telomere disruption was decreased, and cell survival was improved (DePinho 1999).

The health of the p53 checkpoint plays a critical role in regulating the cellular response to telomere disruption. Using late-generation telomerase-deficient mice, the influence of telomere attrition on cancer can be investigated in vivo. Based on observations of senescence/crisis responses in primary human cells and the high tumor resistance of late-generation mTERC−/−mice with intact p53 pathways, it is hypothesized that telomere shortening would prevent cancer.

In contrast to models with intact p53-dependent DNA damage response mechanisms, telomere uncapping significantly speeds up the pace of tumor growth in late-generation mTERC−/−p53−/−or p53+/− animals (Artandi et al. 2000). Telomere disruption in p53+/−mice accelerates carcinogenesis and significantly broadens the spectrum of tumor types. Late-generation mTERC−/−p53+/−mice generally succumb to epithelial malignancies, such as breast, skin, and gastrointestinal tract carcinomas, in contrast to p53+/−animals with intact telomeres, which primarily develop lymphomas and sarcomas. Breast tissue and other epithelial compartments may change on their own as a result of a telomere-base crisis or telomere shortening that isn't halted by p53 activation.

12.6 Copy Number Changes, Translocations, and Telomere Dysfunction

To determine the mechanism by which uncontrolled telomere uncapping causes murine breast malignancies, the chromosomal organization of breast carcinomas from late-generation mTERC−/−p53+/−mice was studied. A spectral karyotype study revealed that telomere shortening promotes translocations between nonhomologous chromosomes. Contrary to conventional balancing translocations, nonreciprocal translocations (NRTs) modify the copy number of the chromosomal regions involved. According to comparative genome hybridization (CGH) investigations, wide areas of amplification or deletion, which significantly impact gene copy number, are present in breast tumors and other carcinomas from late-generation mTERC−/−p53+/−mice (O'Hagan et al. 2002). These regions probably contain significant tumor suppressor genes and oncogenes. The chromosomal end is exposed to the recombination machinery when the telomere breaks off.

12.7 Telomere Dynamics and Genomic Changes in Hypoxic Breast Cancer

Similar genomic alterations are seen in human breast tumors and breast malignancies brought on by telomere dysfunction (Tanaka et al. 2012). Human breast tumors and other carcinomas have a NRT as well as changes in gene copy number. CGH-identified amplification sites have been discovered to include oncogenes, both well-known and novel. Chromosome instability occurs very early in the course of human breast cancer development (Bakhoum and Cantley 2018). It is unclear how the breast cancer genome becomes unstable early in the process of carcinogenesis before becoming stable again during the DCIS stage. Early-stage cancers that divide before telomerase turns on are thought to get around the p53 checkpoint. This leads to severe telomere shortening and cycles of chromosomal fusion-bridge-breakage, which promotes chromosomal instability and the growth of cancer. This theory is supported by the fact that invasive breast cancers' telomeres are stable at a reduced length after shortening during carcinogenesis (Odagiri et al. 1994). The majority of human breast cancers may have chromosomal rearrangements, but p53 mutations are not always present in these cancers. Only approximately 30% of patient breast cancers are actually p53 mutant, suggesting that p53 wild-type cancer cells can experience changes in gene copy number. These results suggest that mutations in other p53 pathway components or in other pathways that control DNA damage checkpoints may increase the likelihood of chromosomal rearrangements. It will take more research to find out what role telomere dysfunction plays in the development of chromosomal rearrangements in human breast cancer, as well as what other processes might be involved in making the breast cancer genome less stable.

12.8 Telomerase Reactivation Plays Two Unique Roles in Hypoxia-Induced Cancer

As a result, telomerase reactivation and the relative stability of the cancer genome occur simultaneously (Ding et al. 2012). If telomere dysfunction results in genomic rearrangements that promote cancer development, telomerase reactivation later in the course of carcinogenesis is likely to accelerate tumor growth (Maciejowski and de Lange 2017). Telomere maintenance must be reestablished in order to maintain telomeres, "lock in" the genetic alterations needed for cancer, and allow for significant clonal proliferation. Is it possible for telomerase reactivation to perform functions other than restarting telomere maintenance? Trials on this issue have been conducted with varying degrees of success. Even though the regulatory and checkpoint systems didn't change, reactivating telomerase made primary human fibroblasts and retinal pigment epithelial cells multiply faster (Morales et al. 1999; Jiang et al. 1999). Transgenic models of TERT overexpression in wild-type mice with long telomeres suggest that telomerase can promote neoplastic growth even in the absence of severely short telomeres. Intact TERT expression resulted in long-lasting spontaneous breast tumors in a variety of animal tissues (Artandi et al. 2000). TERT expression in mouse keratinocytes makes them more susceptible to the carcinogenic effects of chemical carcinogens (Gonzalez-Suarez et al. 2001). Based on these results, it looks like telomerase may have two jobs: one is to keep telomeres short and stop telomere dysfunction from affecting cell growth and survival, and the other is to help cells grow no matter how long their telomeres are.

12.9 Effect of Increased HIF-1a Expression on Telomerase Gene Promoter Expression

The component is the limiting subunit of the HIF-1 transcription factor, which is susceptible to proteasomal degradation under usual oxidative circumstances and stabilizes in hypoxia (Salceda and Caro 1997; Hashimoto and Shibasaki 2015; Jing et al. 2019). To determine the effect of hypoxia on telomerase gene promoter activity in vitro, luciferase reporter constructs controlled by either the hTR or hTERT promoter regions were transiently cotransfected into the ovarian cancer cell line A2780 (Anderson et al. 2006). As a positive control for the hypoxic response, Anderson and his coworkers (2006) co-transfected the HIF-1a expression vector with a luciferase reporter construct controlled by the hypoxia-responsive vascular endothelial growth factor (VEGF) promoter (Anderson et al. 2006). Further, Western blot analysis of HIF-1a expression levels demonstrated an increase in HIF-1a levels with titration of the expression vector, providing further proof of the hypoxic response. Both the increased expression of telomerase gene promoters and the overexpression of HIF-1a suggest that hypoxia controls the expression of telomerase genes in a tumor (Anderson et al. 2006).

12.10 Effect of Hypoxia on the Telomerase Genes' Endogenous Expression

To determine if hypoxia regulates the expression of the endogenous telomerase gene, semiquantitative PCR was applied to A2780 ovarian cancer cell extracts incubated under typical oxidative or hypoxic conditions. Semiquantitative PCR for VEGF was used as a positive control for the hypoxic response. B-actin was used as a loading control since -actin has no effect on the expression of b-actin but increases the expression of GAPDH (Graven et al. 1994). To investigate if alternative splicing might be involved in the control of hTERT expression under hypoxia, researchers utilized splice variant PCR with primers intended to identify the four primary splice forms of hTERT. According to research (Anderson et al. 2006), the ratio of splice variants changes in favor of the active form of the transcript as a result of post-transcriptional control of the hTERT gene expression being stimulated by hypoxia.

12.11 Identification of HIF-1 Binding to the Promoters of the Telomerase Genes

Both of the telomerase gene promoters contain HRE sites, which are known to be required for HIF-1-induced transcriptional activation of hypoxia-responsive genes. Using chromatin immunoprecipitation (ChIP) testing for the limiting HIF-1a subunit, researchers have examined how HIF-1 interacts with specific areas in the hTR and hTERT promoters in vivo (Anderson et al. 2006). Researchers used A2780 ovarian cancer cells in normoxic or hypoxic environments. Semiquantitative PCR for the hTR promoter reveals an increase in HIF-1a association after hypoxia therapy. An increase after hypoxia characterizes the interaction pattern of the hTERT promoter. The hypoxia-responsive VEGF gene promoter, which is known to bind HIF-1 through the consensus HRE sequence (Forsythe et al. 1996), is also linked to an increase in HIF-1a association.

12.12 Under Hypoxic Circumstances: The Telomerase Genes and the Transcriptional Complex

It is known that HIF-1 binds to the promoters of numerous different genes, where it recruits coactivators like the p300/CREB binding protein (CBP) complex and the fundamental transcriptional machinery to help it activate transcription (Ruas et al. 2010; Freedman et al. 2002; Kallio et al. 1998). The transcriptional complex is stabilized by TFIIB, an important component of the fundamental transcriptional machinery. There was practically no TFIIB at the hTR promoter before hypoxia treatment. The relationship between the transcriptional coactivator p300 and the expression of genes that are stimulated by hypoxia is well established (Greer et al. 2012). According to data already available for other genes, p300 is associated with the promoters of both the hTR and the hTERT genes. Because the presence of

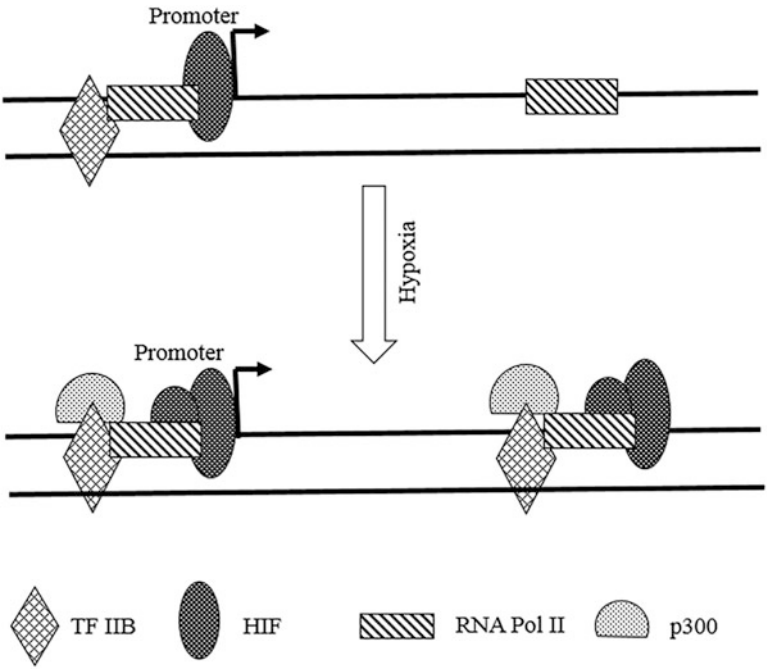

Fig. 12.4 A hypothetical hypoxia-related transcriptional regulatory mechanism for hTERT. The hypoxia therapy results in recruitment of the transcriptional coactivator p300, an increase in HIF 1 association, and the basal transcriptional machinery components RNA polymerase II and TFIIB. Under hypoxia, this transcriptional complex is still linked to the gene and is also linked to exon 12 of the hTERT gene, which is located 38 kb downstream of the promoter (Anderson et al. 2006)

transcriptional machinery components within gene coding regions correlates with active gene transcription (Zhang et al. 2003), it implies that hTERT is actively transcribed under hypoxic conditions and that the transcriptional complex remains bound to the gene as transcription progresses (Fig. 12.4). To compare these alterations in the recruitment of the essential transcriptional machinery at the promoters of the telomerase gene to those associated with a gene that is already known to respond to hypoxia, VEGF promoter semiquantitative PCR was employed.

12.13 Telomerase as a Hypoxic Cancer Target: Challenges and Opportunities

Adaptive drug resistance, limitations of preclinical models, and a lack of high-resolution structures of human telomerase have all hindered the development of effective therapeutic medicines, despite the fact that telomerase has many favorable qualities as a hypoxic cancer target (Guterres and Villanueva 2020). Because they rely on the slow loss of telomeres with each cell division, therapies based on

suppressing telomerase reverse transcriptase activity take a long time to manifest their anticancer effects. This may render them inappropriate for use as first-line therapy and raise the risk of the emergence and spread of resistant clones. Furthermore, telomerase expression in stem and progenitor cells, including hematopoietic lineages, raises the possibility that side effects of telomerase-directed therapies could develop. Still, telomerase is a good target for hypoxic cancer because it is almost always present, it is very selective for cancer cells, and it can give cancer cells the ability to keep dividing forever (Table 12.1).

12.14 Anticancer Strategies Targeting Telomerase in Hypoxic Cancer

Targeting telomerase can be done in a number of ways, such as by adding nucleoside analogues to newly extended telomeres, stabilizing G-quadruplexes, focusing on the TERT gene, using immunotherapies that recognize TERT tumor-associated antigens, using small molecule inhibitors or oligonucleotides that directly bind telomerase and stop it from extending telomeres, or using indirect methods to interfere with (Table 12.2).

12.15 Conclusion and Future Aspect

Recent research suggests that telomerase can be activated in hypoxia without hTERT transactivation. As demonstrated by Seimiya et al. in their study published in *Science (*Seimiya et al. 1999*)*, MAP kinase signaling is responsible for the hypoxia-induced telomerase activation in colon and ovarian cancer cells. In these cells, there was no elevation of hTERT mRNA expression following exposure to hypoxia. It was demonstrated using information from Minamino and his colleagues (2001) (Minamino et al. 2001) demonstrated that hypoxia largely activated telomerase through phosphorylation of the hTERT protein in vascular smooth muscle cells. Thus, telomerase activation by hypoxia appears to occur in diverse ways in distinct cell types. This is consistent with the finding that the degree of HIF-1 overexpression varies considerably between different types of tumors. Examples of cancers that frequently express HIF-1 at high levels include cervical malignancies (Fu et al. 2015; Wigerup et al. 2016; Lyng and Malinen 2017). The current review support the hypothesis that tumor hypoxia-induced HIF-1 promotes telomerase activation via hTERT overexpression and may contribute to tumor development. The latest research opens the door to a potential new telomerase activation mechanism in cancers and provides insight into the tumor cells' adaptive responses to hypoxic environments. These discoveries may also have important clinical ramifications for the molecular basis of a novel cancer therapy that specifically targets HIF-1 (Powis and Kirkpatrick 2004; Onnis et al. 2009; Masoud and Li 2015). Breast cancer can be developed in vivo in telomerase-deficient animals with chromosomal instability associated with uncontrolled telomere dysfunction, suggesting that telomere-based

Table 12.1 Telomerase as a cancer target for therapy and major opportunity

Different approaches	Major findings	References
Preclinical modeling of telomerase-directed therapies	• Often rely upon the availability of mouse models that closely recapitulate human pathology • However, mice have several shortcomings as models of human telomere biology • Established inbred mouse strains have far longer telomeres (5–10 times) than humans • Telomerase activation does not present a comparable barrier to replicative immortality in mice as it does in humans	Guterres and Villanueva (2020), Hemann and Greider (2000)
Structural models of telomerase in cancer hypoxia	• Structure and composition of the human telomerase holoenzyme were poorly characterized, hampering drug design and mechanistic analysis • A consequence of the low cellular abundance of telomerase hindering purification and crystallization of active telomerase • Improvements to structural resolution should facilitate the design of more effective small molecule inhibitors targeting human telomerase	Guterres and Villanueva (2020)
Activation of adaptive mechanisms of telomere maintenance	• Telomeres are maintained by telomerase-independent homologous recombination mechanisms known as alternative lengthening of telomeres (ALT) in ~10% of cancers that lack telomerase expression • ALT is uncommon in epithelial malignancies, but highly prevalent in cancers of a mesenchymal origin such as certain sarcomas • Although the emergence of ALT as a resistance mechanism to genetic ablation of telomerase activity was identified in rare (~2.5 × 10–7) clones following TERC knockout from telomerase-positive human cells	Guterres and Villanueva (2020), Heaphy et al. (2011), Dilley and Greenberg (2015), Henson et al. (2005), Min et al. (2017)

Table 12.2 Telomere-based anticancer strategies and major findings

Anticancer strategies	Major findings	References
Targeting TERT gene expression	• TERT expression is regulated by an atypical GC-rich promoter that harbors multiple binding sites for SP1 and c-Myc transcription factors, but lacks TATA and CAAT boxes • Repressive chromatin remodeling and epigenetic modifications silence TERT expression in non-transformed cells • In contrast, the vast majority of cancers acquire replicative immortality through telomerase re-expression • Genome editing reveals that TERT promoter mutations are sufficient to prevent TERT silencing and maintain telomere length upon differentiation of human embryonic stem cells without concomitant oncogenic mutations	Atkinson et al. (2005), Cong and Bacchetti (2000), Chiba et al. (2015)
Immunotherapies	• Endogenous TERT peptides produced by cancer cells can be recognized by major histocompatibility complex (MHC) class I or II molecules and trigger adaptive immune responses • Telomerase-directed immunotherapies include vaccines, adoptive cell transfer, and arguably oncolytic virotherapy • The elevated telomerase expression characteristic of cancer has been exploited by oncolytic virotherapies that target telomerase-expressing cells • Telomelysin is an oncolytic adenovirus designed to selectively replicate in cancer cells via E1 gene expression under the control of the hTERT promoter	Guterres and Villanueva (2020)
Direct inhibitors		Asai et al. (2003)

(continued)

Table 12.2 (continued)

Anticancer strategies		Major findings	References
	Oligonucleotide inhibitors	• Despite the extensive history of telomerase as a cancer target, only a single direct telomerase inhibitor, imetelstat, has progressed to clinical trials • Imetelstat is a lipidated 13-mer thiophosphoramidate oligonucleotide complementary to the TERC template region, which competitively inhibits telomerase activity, suppressing cancer cell viability in vitro and tumor growth in mouse xenograft models	
	Small molecule inhibitors	• While oligonucleotide and immunotherapeutic approaches to targeting telomerase have progressed furthest in clinical development, small molecule inhibitors such as BIBR1532 have generated promising preclinical results • BIBR1532 is a non-competitive small molecule inhibitor of telomerase that mediates progressive telomere shortening in cancer cells and replicative senescence following extended treatment	Damm et al. (2001)
Indirect inhibitors	G-quadruplex stabilizers	• Telomeric G-quadruplexes are resolved by DNA helicases prior to telomere extension • Thus, it is essential to establish which conformations of Gquadruplex stably form at telomeres in vivo in order to identify more telomere-specific Gquadruplex ligands • It remains to be seen whether the vast number of G-quadruplexes in the genome and structural similarity between	Drosopoulos et al. (2015), Moye et al. (2015)

(continued)

Table 12.2 (continued)

Anticancer strategies		Major findings	References
		G-quadruplexes at telomeres and nontelomeric sites will ultimately preclude sufficient selectivity of G-quadruplex ligands for telomeres	
	Nucleoside analogues	• Nucleoside analogues such as 6-thio-2′- deoxyguanosine (6-thio-dG) or 5-fluoro-2′-deoxyuridine (5-FdU) triphosphate rapidly induce telomere dysfunction and cell death • These nucleoside analogues act as telomerase-dependent "uncapping agents" that impede the binding of the shelterin complex to telomeric DNA and activate a DDR	Mender et al. (2015), Zeng et al. (2018)
Regulation of telomerase localization and catalysis		• The telomerase holoenzyme complex incorporates an RNA scaffold protein, TCAB1, which controls nuclear trafficking of telomerase and stimulates telomerase catalysis by regulating conformation of the TERC CR4/5 RNA domain • Loss of TCAB1 disrupts telomerase localization and impairs telomere extension • TCAB1 depletion suppresses growth of xenografted tumors, indicating that TCAB1 may be a potential anticancer target	Chen et al. (2018), Stern et al. (2012), Sun et al. (2014)

crises in people may likewise speed up the development of epithelial tumors (O'Hagan et al. 2002; Meeker and Argani 2004; Kazemi Noureini et al. 2018). This process may be responsible for the widespread chromosomal aberrations and copy number changes seen in human breast tumors. Restoring telomere function, reducing chromosomal instability, and promoting tumor maturation are all benefits of telomerase reactivation (Cleal et al. 2018; Romaniuk et al. 2019). According to recent research, telomerase probably has other roles in promoting carcinogenesis (Jafri et al. 2016). The use of drugs to stop telomerase from working is still a possible way to treat cancer in people.

Currently, research into telomerase positive cell immunotherapy and approaches using a suicide gene promoter targeted at hTERT-expressing cells is ongoing, and in

certain cases, early-stage clinical trials are taking place (Ellingsen et al. 2021). The advantage of these methods is that they do not require the lag phase that the traditional method of telomerase inhibition does. These treatments could, however, also prove to be more harmful to healthy somatic cells that express telomerase. Even while there is still a substantial amount of basic research to be done, it is hopeful that official preclinical research into telomerase inhibitors, oncolytic viruses built on the hTERTp gene, and hTERT immunotherapy has already started. Even if the vast majority of studies are positive, the true value of these and other medications based on telomere biology (Fan et al. 2021; Vaiserman and Krasnienkov 2021) won't be known until after clinical trials are finished and the drugs have been used on a variety of hypoxic cancer patients. Overall, telomerase is still a desirable target for cancer therapy despite major obstacles. Studies should concentrate on creating better inhibitors in conjunction with higher-resolution structural models of human telomerase if medicines are to be clinically effective (Robinson and Schiemann 2022; Shay and Keith 2008). The capacity of most cancer cells to quickly adapt to pharmacological challenges and the heterogeneity of hypoxic tumors (Muz et al. 2015) mean that successful tactics targeting telomerase will likely need to be paired with either targeted medicines or immunotherapies to produce the best anti-tumor effects.

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Conflict of interest No.

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CRISPR/Cas9-Editing-Based Modeling of Tumor Hypoxia

13

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Abstract

One of the critical characteristics of solid tumors is hypoxia, which has been linked to a poor prognosis for cancer patients. Solid tumors frequently exhibit tumor hypoxia, primarily caused by an insufficient and diverse vascular network. Hypoxia, brought on by inadequate blood flow and oxygen supply in tumors, reduces the sensitivity of tumor cells to anticancer therapy. The major cellular stressor, hypoxia, influences various molecular pathways, and as we learn more about the underlying molecular mechanisms, the clinical use of hypoxic modifiers may increase. Hundreds of genes are transcribed by hypoxia-inducible factors (HIFs), which enable cells to adapt to hypoxic settings. HIF-1 upregulation, linked to higher patient mortality in several cancer types, can be brought on by intratumoral hypoxia and genetic changes. On the growth of tumors, HIF-1 activity inhibition has a profound impact. The effectiveness of new HIF-1 inhibitors is being sought after, and their effectiveness as anticancer therapies is being investigated. The HIF-1 transcription factor complex binds to hypoxia-responsive element (HRE) sequences found in the promoters of target genes to mediate the expression of genes that are particular to hypoxia. The use of therapeutic cargo like CRISPR/Cas9 to inhibit HRE-driven gene expression is currently being extensively investigated to develop cancer-specific targeted

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therapeutics. The CRISPR/Cas9 technique has provided a window into previously unsolvable issues in our knowledge of cancer genetics, the noncoding genome, and heterogeneity in hypoxic tumors. It also offers new insights into therapeutic vulnerabilities. Combining CRISPR/Cas9-mediated HIF-1 knockdown with other antitumor strategies may make HIF-1 a viable therapeutic knockout target for treating several solid tumor types. A promising approach for altering the tumor microenvironment and preventing the growth and metastasis of hypoxic tumors is the downregulation of HIFs using CRISPR/Cas9, improving the therapeutic benefits of chemotherapy.

Keywords

Anticancer therapy · CRISPR/Cas9 · Gene editing · HIFs · Solid tumors · Tumor hypoxia · Tumor microenvironment

13.1 Introduction

A low oxygen tension level characterizes the pathophysiological characteristic of hypoxia. Once threshold oxygen levels are breached, hypoxia can exist in tissues, including tumors, impairing cellular or organ activities (Walsh et al. 2014). Particularly for malignant solid tumors, tumors' sensitivity to hypoxia is made worse by their abnormal growth. No matter the type of treatment used, the compensatory mechanisms that tumors use due to hypoxia negatively impact the delivery of curative care. Hypoxia sets off a series of actions encouraging tumor growth, strengthening immunity against the tumor, and promoting tumor angiogenesis (Abou Khouzam et al. 2021). The pathological angiogenesis that develops as the tumor grows results in an aberrant tumor vasculature that is randomly formed and leaky. As a result, the abnormally vascularized tumor microenvironment (TME) triggers immune suppression and upholds a persistent hypoxic condition (Lamplugh and Fan 2021). By restoring the vascular integrity of the tumor, the blood flow should improve, hypoxia should be relieved, and antitumor immunity should be reshaped.

Activating the transcription of genes required to avoid hypoxic (low oxygen level) situations is accomplished by hypoxia-inducible factors (HIFs) (Akanji et al. 2019). HIFs are essential for carcinogenesis. Despite clinical studies connecting elevated levels of HIF-1 with aggressive cancer growth and a poor patient prognosis, HIF-1 has been verified as a prospective target for innovative cancer therapies. Furthermore, preventing HIF-1 activity slowed the spread of cancer. As a result, HIF-1 is a promising target for cancer treatment (Mukherjee and Ray 2022). Given that cancer cells are known to be hypoxic, this might be expected. Cancer cells use the HIF-1 protein to activate several metabolic pathways to survive in the hypoxic microenvironment.

Table 13.1 HIF overexpression and its implications on various cancer cell/tumor types

Cancer cell/tumor type	HIF overexpression	Association
Breast LN, positive	HIF-1	Increased mortality
Breast cancer	HIF-1	Apoptosis inhibition
Cervical cancer	HIF-1	Increased mortality
Colorectal cancer	HIF-1 and HIF-2	Increased mortality
	HIF-1	Overexpression of drug efflux proteins
		Autophagy induction
GI stromal tumor of the stomach	HIF-1 and HIF-2	Increased mortality
Gastric	HIF-1	Increased mortality
		Apoptosis inhibition
Renal	HIF-1	Increased mortality
Lung adenocarcinoma	HIF-1	Overexpression of drug efflux proteins
Lung NSCLC	HIF-1	Increased mortality and also decreased mortality
Oropharyngeal SCC	HIF-1	Increased radiation resistance
Ovarian	HIF-1 and HIF-2	Increased mortality with p53
	HIF-1	Overexpression of drug efflux proteins
Esophageal, early stage	HIF-1	Resistance to photodynamic therapy

Furthermore, new anticancer therapy approaches can be developed using the HIF-1 path, as evidenced by cellular and molecular insights. Given the complicated relationships between HIFs, cancer growth, and carcinogenesis, the biological significance of HIFs cannot be overstated. HIF heterodimers, which include vascular endothelial growth factor (VEGF), bind to hypoxia-response elements (HREs) in the promoters of target genes after they are activated (Olenyuk et al. 2004). In the hypoxic microenvironment, there is a unique chance to send these proteins from outside the body to areas of the tumor where the most aggressive and drug-resistant cancer cells tend to live.

The clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein-9 (Cas9), a novel RNA domain-containing endonuclease-based genome engineering technology, has recently been demonstrated to be a powerful technique in the treatment of various cancers due to its multifunctional properties, including high specificity, accuracy, and time-reducing and cost-effective strategies with minimal off-target effects (Ray and Mukherjee 2021). Hypoxia-inducible factor-1 (HIF-1) downregulation using CRISPR/Cas9 is a potential strategy for altering the tumor microenvironment and preventing tumor spread (Li et al. 2019). Table 13.1 displays the effects of HIF overexpression on several cancer cells. The versatile CRISPR/Cas9 system may target and change specific DNA sequences in the genome, including HIFs. The versatility and simplicity of CRISPR have made it possible to quickly make practically any desired modification with more efficiency and at a cheaper cost than previous technologies. However, the CRISPR/Cas9 system's in vivo administration is still tricky. The development of CRISPR/Cas9

genomic editing has revolutionized how we control the genome and make it easier to study the biology of hypoxic tumor cells both in vitro and in vivo (Lino et al. 2018; Wilson and Gilbert 2018). Using the CRISPR/Cas system, we can edit the genome's sequence to downregulate or fix a mutation, dull an overactive gene or both (Fuziwara et al. 2022), and modify the gene expression of HIFs.

13.2 Tumor Hypoxia

Prokaryotic and eukaryotic organisms both require oxygen to survive, so they must be able to detect changes in oxygen levels and react to them. Oxygen-sensing mechanisms have been created to preserve tissue and cell homeostasis as well as to enable adaptation to the persistently low oxygen environments present in situations such as cancer (Maltepe and Saugstad 2009; Tretter et al. 2020). Most malignant tumors exhibit hypoxia, a non-physiological degree of oxygen tension (Ray and Mukherjee 2022). Having progressed but dysfunctional vascularization and acquiring the epithelial-to-mesenchymal transition phenotype, which results in cell motility and metastasis, are all effects of tumor hypoxia (Muz et al. 2015). Through the induction of cell quiescence, hypoxia modifies the metabolism of cancer cells and leads to therapeutic resistance. Hypoxia causes cancer cells to turn on a complex network of cell signaling pathways, such as HIF, PI3K, MAPK, and NF-B. These pathways work together to create positive and negative feedback loops that make the effects of hypoxia stronger or weaker (Jun et al. 2017).

Numerous genes are transcribed by hypoxia-inducible factors, enabling cells to adjust to hypoxic conditions (Fig. 13.1). It is understood that HIF controls the transcription of hundreds of genes (Dengler et al. 2014). HIF is a heterodimer composed of an aryl hydrocarbon nuclear receptor translocator (ARNT), also known as a hypoxia-activated component, and a constitutively expressed subunit (Mandl and Depping 2014; Wood et al. 1996). The subunit comes in three isoforms: HIF-1, HIF-2, and HIF-3. While research on HIF-3 isoforms is not as common, HIF-1 and HIF-2 have received greater attention (Jun et al. 2017; Hashimoto and Shibasaki 2015). Generally speaking, HIF-2 regulates genes comparable to HIF-1, but HIF-3 functions as a negative regulator of these genes. Human malignancies (Wigerup et al. 2016; Moreno Roig et al. 2018) of the bladder, brain, cervix, colon, endometrial, lung, oropharynx, pancreas, skin, and stomach are more deadly when HIF-1 overexpression in tumor biopsies is present. Many HIF target genes provide a mechanistic explanation for the many effects of intratumoral hypoxia on cancer development, as well as the relationship between HIF-1 overexpression and unfavorable outcomes for cancer patients that have been noted (Moreno Roig et al. 2018; Soni and Padwad 2017). HIF-1's potential target genes are beginning to emerge, which may contribute to the development of tumors (Masoud and Li 2015; Weidemann and Johnson 2008). One crucial issue is that different cancer types have different subsets of the HIF-1 target genes that react to hypoxia.

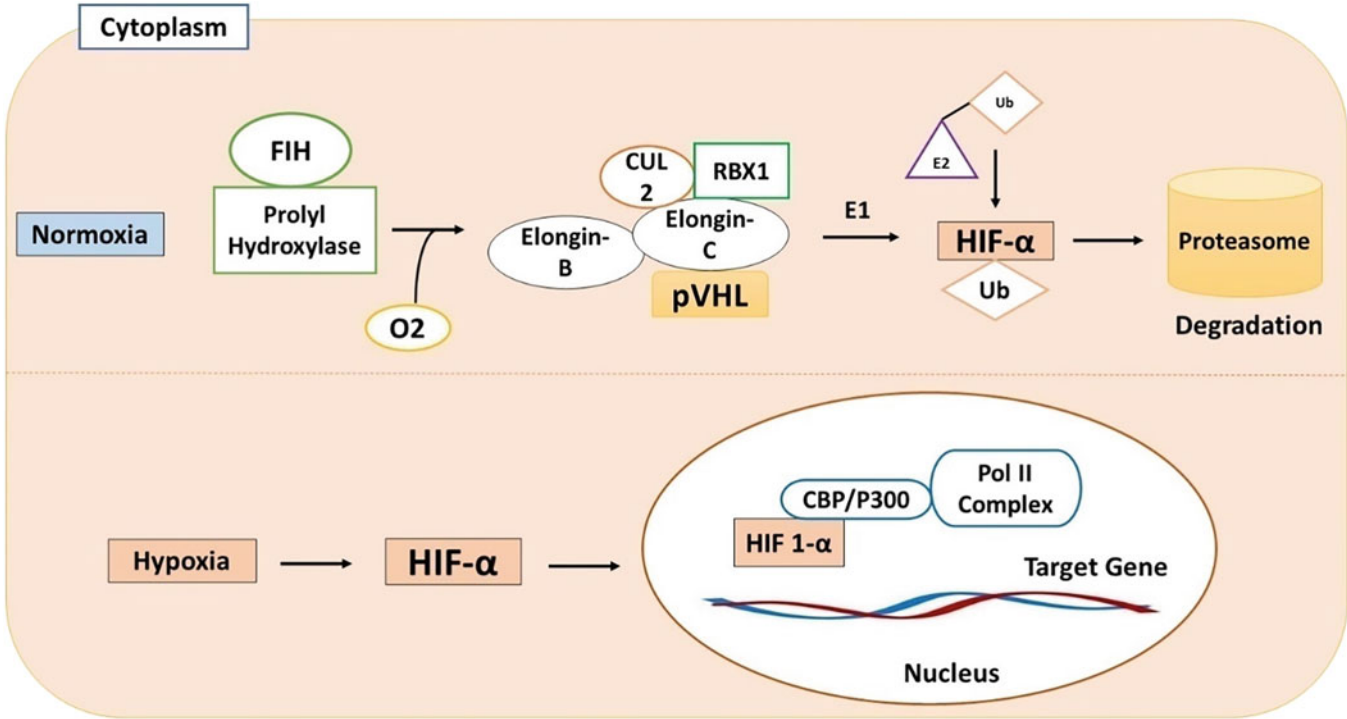

Fig. 13.1 HIF pathway in normoxia and hypoxia: HIF-1 is post-translationally modified by prolyl hydroxylase in the presence of oxygen (O_2) to interact with the von Hippel-Lindau (VHL) complex. Iron chelation prevents prolyl hydroxylase from functioning since it has an iron moiety. VHL is a component of a more extensive complex component consisting of elongin-B, elongin-C, CUL2, RBX1, and an enzyme that conjugates ubiquitin (E2). This complex is involved in the ubiquitylation (Ub) of HIF-1 together with an enzyme that activates ubiquitin (E1). Proteasome inhibitors can prevent the breakdown of HIF-1, which the Ub modification targets. However, HIF-1 cannot change HIF-1 without oxygen. Hence the protein is unaltered. Stabilized HIF-1 is transported to the nucleus, where it interacts with cofactors such as the DNA polymerase II (Pol II) complex, CBP/p300, and aryl hydrocarbon receptor nuclear translocator (ARNT) to bind to hypoxia-responsive elements (HREs) and activate transcription of target genes

13.3 CRISPR/Cas9 Technology: A Gene-Editing Tool

A collection of scientific techniques known as gene editing allows for modifying an organism's DNA. At specific sites in the genome, these technologies enable the addition, removal, or modification of genetic material. There are several methods for genome editing that have been developed. When compared to traditional methods (such as zinc finger nucleases and transcription activator-like effector nucleases), clustered regularly interspaced short palindromic repeats (CRISPR-associated nuclease 9, CRISPR-Cas9) have several advantages, including cost-effectiveness, flexibility, and ease of use (Kato-Inui et al. 2018; Gaj et al. 2016; Akram et al. 2022; Zhang et al. 2020). The most practical technique for gene editing remains CRISPR-Cas9, despite some drawbacks like effective delivery and safety. Furthermore, the CRISPR-Cas9 system has the potential to be used for genome editing, making it a novel therapeutic method for the treatment of diseases brought on by genome alterations, including cancer (Cox et al. 2015; Karimian et al. 2019; Shojaei Baghini et al. 2022).

CRISPR may have had the most influence on cancer research in pooled genomic screens. CRISPR knockout (KO) screens have become the "go-to" tool for examining gene function in cancer due to their simplicity in design, ease of cloning, effectiveness, and continual development of better sgRNA libraries (Shojaei Baghini et al. 2021; Hiranniramol et al. 2020). Positive selection CRISPR screenings continue to hone our understanding of how genes and pathways contribute to cancer in cell lines, organoids, and animals (Xing and Meng 2020; Hazafa et al. 2020). Although CRISPR has made pooled genetic screens possible in more challenging situations, there are countless examples of successful screening investigations in cell lines. For instance, in human colon organoids treated with the TGF-R inhibitor A83-01, Michels and his colleagues (2020) screened a targeted array of tumor suppressors to identify genes that limit tumorigenic expansion (Michels et al. 2020). Researchers have found putative regulators of non-small-cell lung cancer (NSCLC) metastasis using ex vivo transduction of a genome-wide library and subsequent engraftment in recipient mice (Sanghvi et al. 2019; Testa et al. 2018). Although it is difficult to transmit viral or plasmid-based vectors directly to organs in situ, it is conceivable to keep a representation of complicated libraries in vivo. Chow and his colleagues (2018) administered a genome-wide AAV sgRNA library to the brains of inducible Cas9-expressing mice to identify a subset of cancer drivers in the resulting glioblastomas. Table 13.2 is a summary of all the CRISPR/Cas9 clinical trials that are being done to treat different kinds of tumors.

13.4 Hypoxia-Specific Expression of CRISPR-Cas9

The Cas9 protein is a repurposed double-stranded DNA (dsDNA) nuclease that can be programmed to cut any genomic region and is employed for mammalian gene editing (Lino et al. 2018; Davis et al. 2022). By permanently destroying genes necessary for tumor cell viability, CRISPR-Cas9 presents an exceptional potential

Table 13.2 The ongoing CRISPR/Cas9 clinical trials investigations in different tumor types

Target	Study phase	Cancer type	Clinical trial identification
CRISPR/Cas9 inactivates the CISH gene	Phase II	Metastatic gastrointestinal epithelial cancer	NCT03538613
NY-ESO-1-redirected CRISPR edited T-cells (NYCE T-cells)	Phase I	Multiple myeloma melanoma synovial sarcoma myxoid/round cell liposarcoma	NCT03399448
PD-1 knockout	Phase I	Metastatic non-small cell lung cancer	NCT02793856
PD1 knockout	Phase II	Advanced esophageal cancer	NCT03081715
PD-1 and TCR gene knocked out	Phase II	Mesothelin positive multiple solid tumors	NCT03545815
PD-1 knockout	Phase II	Advanced stage Epstein-Barr virus (EBV)-associated malignancies	NCT03044743
CD19 and CD20 or CD22 CAR-T cell immunotherapy	Phase II	Relapsed or refractory leukemia and lymphoma	NCT03398967
CD19 and CAR-T cells (UCART019)	Phase II	Relapsed or refractory CD19+ leukemia and lymphoma	NCT03166878
HPV16-E6/E7 or HPV18 E6/E7 knockout	Phase I	Human papillomavirus-related malignant neoplasm	NCT03057912
TCR and B2M knockout	Phase II	CD19 + leukemia and lymphoma	NCT03166878
Fix NF1 mutation allele	–	Tumors of the central nervous system	NCT03332030
TCR and PD-1 knockout	Phase I	Multiple myeloma	NCT03399448
TCRα, TCRβ, B2M	–	Multiple myeloma	NCT04244656
TCRα, TCRβ	–	Lymphoma-cell lymphoma	NCT04035434
PDCD1	–	Stage IV gastric carcinoma, stage IV nasopharyngeal carcinoma, T-cell lymphoma stage IV, Stage IV adult Hodgkin lymphoma, stage IV diffuse large B-cell lymphoma	NCT03044743
PDCD1	Phase I	Metastatic non-small cell lung cancer	NCT02793856
PDCD1	Phase II	Metastatic renal cell carcinoma	NCT02867332
PDCD1	Phase II	Hormone refractory prostate cancer	NCT02867345
PDCD1	Phase I	Invasive bladder cancer stage IV	NCT02863913
PDCD1	Phase I	Esophageal cancer	NCT03081715
TCRα, TCRβ, PDCD1	–	Solid tumor, adult	NCT03545815
PDCD1	–	B-cell leukemia, B-cell lymphoma	NCT03398967

(continued)

Table 13.2 (continued)

Target	Study phase	Cancer type	Clinical trial identification
TCRα, TCRβ, B2M	Phase II	B-cell leukemia, B-cell lymphoma	NCT03166878
HPK1	Phase I	Leukemia, lymphoma	NCT04037566

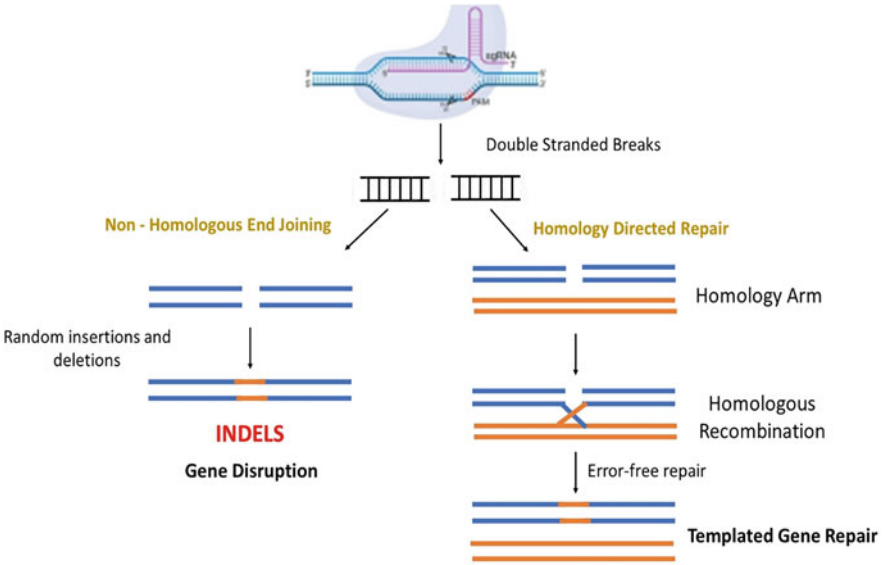

Fig. 13.2 CRISPR/Cas9-mediated gene editing. Either the NHEJ pathway or HDR is used to repair DNA damage. The NHEJ process results in error-prone repair because random insertions and deletions (indels) are inserted on the cut side and ligated. During repair in the HDR pathway, the damaged DNA follows the pattern of the homologous chromosomal DNA. It leads to a restoration that is free of mistakes

to treat cancer. Davis and his colleagues (2022) transfected cells with the Cas9 nuclease expressed from either the CBh or the 5HRE promoter to evaluate the viability and effectiveness of a hypoxia-regulated Cas9-expression system (Davis et al. 2022). However, when cells were treated under hypoxic circumstances, and gRNAs were expressed in the presence of the HRE-driven Cas9, a considerable decline in cell viability was seen (Thomas et al. 2021; Schwinn et al. 2018). Researchers have verified that Cas9 cleavage only occurs when the HRE promoter is used to induce the nuclease's expression in hypoxic settings (Lam and Truong 2021). Cancer cells exposed to hypoxia can specifically have their viability reduced by the HRE-promoter-driven Cas9 mechanism. Caki1 clear-cell renal carcinoma cells introduce inactivating mutations into the VHL protein gene using the CRISPR/Cas9 DNA editing system (Fig. 13.2) (Artemov et al. 2018; Wolf et al. 2020).

Because of the mutational change in the reading frame, its functionality has been altered.

13.5 Genome-Wide CRISPR/Cas9 Screening and Progression of Hypoxic Cancer

One of the key hallmarks of solid tumors is hypoxia. Tumor cells can adapt to a hypoxic microenvironment by activating HIFs, especially HIF-1a and HIF-2a (Ziello et al. 2007; Corrado and Fontana 2020). A robust method for evaluating gene function and searching for genes implicated in cancer cell proliferation and metastasis is the genome-scale CRISPR-Cas9 knockout (GeCKO) library (Shalem et al. 2014; Joung et al. 2017). CRISPR screening has advanced hypoxia cancer research significantly in recent years. However, few studies (Hazafa et al. 2020; Afolabi et al. 2021) have employed CRISPR systems to screen essential genes like HIFs that regulate immune evasion or tumor treatment resistance. In a study, Teng and his team (2021) discovered that hypoxia upregulates HIF-1 bound to the CBX8 promoter region and controls the transcriptional level of CBX8 expression. By CRISPR screening, Yang and his team (Yang et al. 2019) have discovered a set of genes linked to the development of pancreatic cancer by CRISPR. According to a combined analysis of clinical samples from patients with pancreatic cancer (Li et al. 2021; Shi et al. 2021), the expression of CBX8 is higher in pancreatic cancer tissue, and increased expression of CBX8 is associated with a poor clinical prognosis (Teng et al. 2021). Under low oxygen conditions, HIF-1 controls the transcription of CBX8, and CBX8 promotes the growth of pancreatic cancer cells by targeting IRS1, which turns on the PI3K/AKT pathway.

13.6 Knockdown of Hypoxia-Inducible Genes by Tumor Target Delivery of CRISPR/Cas9 System

Despite significant advancements in understanding the pathophysiology and spread of various hypoxic malignancies, this issue still poses one of the biggest threats to human health. The primary reasons for a poor prognosis in cancer are invasion and metastasis, which are correlated with epithelial-mesenchymal transition (EMT), which aids in the early-stage dispersion of hypoxic cancer cells (Ribatti et al. 2020; Roche 2018). In light of these difficulties, EMT inhibition might be a workable strategy to lower metastasis and increase the antitumor effects of chemotherapy. Fast tumor growth and limited vascular supply were factors in the development of the hypoxic tumor microenvironment. HIF-1, a component of HIF-1, is essential for tumor cell growth, death, and EMT (Jun et al. 2017; Lv et al. 2016). As a result, HIF-1 has emerged as a promising target for the management of tumor microenvironments and the therapy of cancer. The downstream molecules of HIF-1, such as VEGF, CXCR-4, MMP-9, and TWIST, may have significant

anti-proliferation and anti-metastatic effects on hypoxia-inducible tumor cells by inhibiting the production of HIF-1 (Li et al. 2018; Tam et al. 2020).

Genome editing has shown tremendous promise as a cancer therapeutic over the past few decades and has grown to be an essential tool in genetic engineering. The CRISPR/Cas system, which consists of a short guide RNA (sgRNA) and the Cas9 nuclease, is a hot topic in gene engineering (Xu and Li 2020). The Cas9 nuclease introduces a DNA double-stranded break after sgRNA binds to site-specific DNA sequences. It generates base pairs in the CRISPR/Cas9 system (Alagoz and Kherad 2020). CRISPR/Cas9 can be used for downregulating HIFs thanks to the thoughtful design of sgRNA. There are numerous ways to prepare Cas9 nuclease and sgRNA, including plasmids, proteins, RNAs, or viral vectors. However, because protein and RNA degrade quickly, delivering them to living organisms is still tricky. However, due to the high molecular weight of Cas9-expressing DNA or Cas9 protein and the requirement for nucleus entry, CRISPR/Cas9 also faces more difficulties than RNAi (Lino et al. 2018). The key to the widespread deployment of this cutting-edge technology is effective ex vivo and in vivo administration systems. Studies are focusing more and more on making different materials that will improve the effectiveness of CRISPR/Cas9 systems for systemic delivery (Luther et al. 2018).

Due to limited vascular supply, a typical hallmark of pancreatic cancer that is detrimental to the survival of tumor cells is the hypoxic microenvironment. The expression of specific genes and the metabolism of tumor cells are altered by factors like HIF-1, which increase the likelihood of metastasis (Huang et al. 2017). EMT may result from the activation of E-cadherin transcriptional repressors in hypoxic circumstances. HIF-1 overexpression controls several downstream molecules, including VEGF and MMP-9, which are connected to angiogenesis, EMT, and the proliferation of tumor cells (Wigerup et al. 2016; Petrova et al. 2018). It is also associated with abnormal p53 accumulation in cancer. Clinical samples have further demonstrated the relationship between HIF-1 and the VEGF and MMP-9 that are produced downstream. It has been suggested that stopping HIF-1 makes gemcitabine work better against cancer.

13.7 CRISPR/Cas9-Mediated Hypoxia-Inducible Factor-1α Knockout Enhances the Antitumor Effect

Although gene therapy and immunotherapy have made tremendous strides in treating solid tumors, their application in patients is currently limited due to their high-cost and labor-intensive nature. Therefore, adopting cutting-edge technology like CRISPR/Cas9 to treat hypoxia cancer may be a practical and inexpensive option. Translational medicine may use precise gene knockout to alter HIFs, and the CRISPR/Cas9 system is one of the best tools for this. With the help of a small guide RNA (sgRNA), which is made up of a target complementary CRISPR RNA (crRNA) and an additional trans-activating crRNA (tracrRNA) (Ran et al. 2013; Karvelis et al. 2013), the system can successfully target desired genomic locations. Technologically, tailored gRNAs and CRISPR/Cas9 can be expressed in a particular

cell by a delivery vector like a lentiviral vector. Base pairing between the crRNA sequence and the target DNA sequence allows the CRISPR/Cas9 endonuclease to target a particular genomic location, resulting in a double-stranded DNA break and a targeted gene alteration that disrupts the target gene. The CRISPR/Cas9 system seems like an excellent way to treat hypoxic cancer, both in the lab and in people, when there are genetic abnormalities and an unusually high expression of essential oncogenic proteins.

Hypoxia-induced oxygen-dependent transcription factor HIF1 is crucial for the maintenance of cancer stem cells as well as the aggressiveness, enhanced angiogenesis, and chemotherapy resistance of hypoxic tumors (Zhang et al. 2021). By boosting the expression of matrix metalloproteinase 2 (MMP2) and MMP9, HIF-1 precisely activates the hypoxia pathways, causing the overexpression of VEGF, activating the epithelial-mesenchymal transition (EMT), tumor invasiveness, and metastasis (Barillari 2020; Hapke and Haake 2020). According to research, HIF-1 is elevated in hepatocellular carcinoma and linked to portal vein metastases and hepatic capsular invasiveness. Furthermore, HIF1 regulates the production of the multidrug resistance protein (MDR), and its protein product P-glycoprotein (P-gp) can transport chemotherapeutic medicines out of cells (Badowska-Kozakiewicz et al. 2017). Therefore, inhibiting HIF1 may be a therapeutic strategy for treating many hypoxia-related cancers, including hepatocellular carcinoma. Liu and his team (2018) investigated the role of the human HIF-1 gene in the human liver cancer cell line SMMC-7721 using a lentivirus-mediated CRISPR/Cas9 system (Liu et al. 2018). Under low oxygen levels caused by CoCl2, the HIF1 disruption caused by the lentivirus decreased cell growth, migration, and invasion and induced apoptosis.

13.8 HIF-1α-Knockout via CRISPR/Cas9 Suppresses HIF-1α Expression and Impairs Cell Invasion and Migration

Cellular invasion is significantly influenced by hypoxia and HIF-1, which serve as its primary regulators. According to mounting data, a reservoir of cells resembling those of healthy stem cells may persist within tumors and be capable of promoting carcinogenesis. Numerous hypoxic tumors have been reported to contain these cells, also known as cancer stem cells (CSCs) or tumor-initiating cells. The ability of hypoxia to keep stem cells in their undifferentiated condition has been demonstrated. Méndez and his colleagues (Méndez et al. 2010) have shut down the expression of HIF-1 and assessed the migration and invasion capacity of these glioma cells to understand better the role that HIF-1 plays in glioma cell migration in vitro and in vivo. A gene expression profile study was conducted to find potential genetic pathways responsible for the decreased migration in vitro, decreased invasiveness in vivo, and decreased capacity to form tumor spheres in cells knocked down for HIF-1.

To identify sgRNAs specifically targeting the gene loci of HIF-1α, researchers have designed sgRNAs to target the exon of HIF-1α (Naeem et al. 2020). The efficiency of HIF-1αknockout is well established in the experimental mice models.

Immunohistochemical examination by Ding and his colleagues (2006) revealed that HIF-1α is highly expressed in the hypoxic hepatocellular carcinoma and CRISPR/Cas9 efficiently disrupted the expression levels of the HIF-1αgene and its targets, including VEGF, in liver cancer cells and xenograft tumor tissues (Ding et al. 2006). In vitro study can be possible using hypoxia mimetic cells by infecting the experimental cells with the lentiviruses and $CoCl_2$ (He et al. 2015). The disruption of HIF 1α with the CRISPR/Cas9 system inhibits hypoxic cancer cell migration and invasion, particularly under the hypoxic microenvironment.

13.9 CRISPR/Cas9-Based HIF-1α Disruption Suppresses Cell Proliferation and Induces Cell Apoptosis

Hypoxia can trigger apoptosis (Greijer and van der Wall 2004). Cells either undergo apoptosis (cells that die) or adapt to hypoxia and survive, depending on how severe the hypoxia is. A cell cannot undergo energy-dependent apoptosis in a hypoxic environment devoid of nutrients and instead becomes necrotic. The balance of apoptosis regulatory proteins is delicate. The occurrence of hypoxia in solid tumors is frequent. After repeated episodes of hypoxia, cells become accustomed to this environmental stress, leading to selection for resistance to hypoxia-induced apoptosis. These resistant tumors may be less responsive to therapy and have a more aggressive phenotype. Stopping growth is a typical cellular response to hypoxia (Muz et al. 2015; Vaupel 2008).

The activation of the HIF-1 transcription factor is a crucial aspect of the hypoxic response. Molecular oxygen is now used by mammalian cells to produce energy. Different oxygen concentrations can cause changes in a cell's metabolic state and growth rate, affecting how the cell reacts to the environment. Recent research suggests that hypoxia can affect cell proliferation in two ways: through programmed cell death and growth arrest (Hubbi and Semenza 2015; Pucci et al. 2000). In addition, hypoxia can cause apoptosis in transformed cells by activating the p53 pathway (Leszczynska et al. 2015). It can be a powerful mechanism for selecting p53 mutants in hypoxic tumor cell populations.

On the other hand, non-transformed hypoxic cells can experience cell cycle arrest at the G1/S interface without suffering any long-term viability changes (Goda et al. 2003; Chen 2016). Further research found through in vitro experiments cell cycle progression under hypoxia can be evaluated in vitro. Researchers have discovered that as the cell cycle advances, there is a simultaneous decrease in the proportion of cells in the G0/G1 phase and an increase in the G2/M phase (Bertoli et al. 2013). In addition, the CRISPR/Cas9 system's disruption of HIF 1 prevents hypoxic tumor cells from proliferating and triggers cell apoptosis. This problem may be caused by how CRISPR/Cas9 changes the cell cycle, so it would be helpful to know the cell cycle progression profile of any HIFs of hypoxic cancer caused by CRISPR/Cas9 treatment (Hazafa et al. 2020; Geisinger and Stearns 2021).

13.10 Hypoxia-Responsive Gene Editing to Reduce Tumor Thermal Tolerance for Mild Photothermal Therapy

CRISPR-Cas9, an emerging biological technology, is crucial in treating diseases such as hypoxia cancers and gene editing. The CRISPR-Cas9 system has been delivered using various techniques, such as electroporation, microinjection, viral vectors, and other nonviral vectors. Viral delivery methods among in vivo delivery systems likely result in unintended immunogenicity and carcinogenesis. Despite the enormous nonviral solutions developed to address biosafety concerns, it is still challenging to implement the on-demand release of CRISPR-Cas9 that is activated explicitly by the hypoxic tumor microenvironment (Leszczynska et al. 2015). Rapid cell division and microvessel destruction at the tumor site diminish oxygen delivery due to increased oxygen consumption and restricted blood flow. In most solid tumor microenvironments, hypoxia is a common symptom of an imbalance between inadequate oxygen supply and rising metabolic demand (Abou Khouzam et al. 2021; Eales et al. 2016). The trait encourages the development of more robust platforms for effective cancer treatment methods.

On the one hand, photothermal therapy (PTT) and hyperthermia, which uses light to produce localized heat, have attracted growing interest in tumor therapy due to their noninvasive and spatiotemporally programmable methods modes (Ferroni et al. 2019). On the other hand, a customized CRISPR/Cas9 system delivery technique responds to hypoxia to further realize mild hyperthermia. Using azobenzene-4, 4′-dicarboxylic acid, a hypoxia-responsive azobenzene linker, the CRISPR/Cas9 system is covalently cross-linked on Au nanorods at normal oxygen partial pressure (p-AZO). As a result, the N-N double bond of p-AZO can be reduced, and aniline derivative is produced due to the imbalance of cellular redox states in the hypoxic microenvironment of tumor cells (Chun et al. 2021; Hielscher and Gerecht 2015). To precisely knock down the HSP90 gene, the Cas9/sgRNA ribonucleic protein complex is released from AuNRs, which significantly lowers the heat tolerance of tumor cells (Dong et al. 2016). To further investigate the controllable release of Cas9, Briolay and his team (2021) used liver microsomes in a hypoxic environment. They showed that hypoxia caused Cas9 protein to be released, a good thing for gene editing targeting tumors and precision medicine (Briolay et al. 2021).

13.11 Genome-Wide CRISPR/Cas9 Deletion Screen for Tumor Cell Viability in Hypoxia

Hypoxia and other tumor-related microenvironmental circumstances can significantly impact mitochondrial function, triggering metabolic adaptations that support tumor cell survival and spread. For example, mitochondrial activity is crucial for maintaining tumor cell proliferation by producing ATP via OXPHOS and synthesizing precursors for biomass accumulation, such as amino acids, lipids, and nucleotides; mitochondrial activity is crucial for maintaining tumor cell proliferation (Vander Heiden et al. 2009; Liberti and Locasale 2016). Because of this,

mitochondria are essential for ensuring that cells and tissues get enough oxygen, and it has been found that they control when intracellular hypoxia starts to happen.

Hypoxia, which contributes to the course of the disease and is linked to treatment resistance and a poor prognosis in patients with solid malignancies, is a defining hallmark of the tumor microenvironment. Metazoan cells can change to keep cellular homeostasis and stay alive. They have developed several pathways, like the HIF pathway, that can sense and respond to changes in oxygen levels. With the help of CRISPR/Cas9 gene-editing technology, scientists can now study how cells respond to stimuli across the whole genome. Researchers used CRISPR/Cas9 deletion screening in different environments (normoxia-glucose, hypoxia-glucose, and normoxia-galactose) to determine how tumor cells depend on nuclear-encoded mitochondrial genes and non-mitochondrial genes for survival when oxygen or glucose is plentiful or scarce (Thomas et al. 2021).

The fundamental response of tumor cells to hypoxia is the loss of mitochondrial genes, including OXPHOS genes like succinate dehydrogenase subunit C (SDHC), which enhances the proliferation of U2OS cells as well as HeLa and MCF7 cells and downregulates the expression of OXPHOS proteins (Pustylnikov et al. 2018; Luo et al. 2020). In addition, respiration in renal cancer cells has been slowed down because of how the HIF pathway affects the activities of MYC and PGC1, which control the creation of new mitochondria (Li et al. 2020). Aside from the genes involved in OXPHOS, it is interesting that the CRISPR/Cas9 deletion screen is more common in hypoxia-glucose than in normoxia-glucose.

13.12 CRISPR/Cas9-Mediated Altered Expression of HIF-1α Enhances the Antitumor Effect

A therapeutic knockout target for the characteristics of hepatocellular cancer, HIF1, has the potential to be effective. In an orthotopic hepatocellular carcinoma model, CRISPR/Cas9-mediated genome editing demonstrated an anticancer effect in addition to extending survival (Shojaei Baghini et al. 2022). Furthermore, it is well-known that angiogenesis plays a crucial role in the initiation, development, advancement, metastasis, and recurrence of hepatocellular carcinoma. HIF-1 is the main factor that controls angiogenesis in low-oxygen environments. It is also good to tell how well someone with hepatocellular carcinoma will do. The CRISPR/Cas9 protein (Choi et al. 2016) and an HIF1-specific sgRNA were delivered by the lentiviral vector (LV-H721) and may be helpful for highly effective HIF1 modification in experimental liver tumor cells. HIF1 deletion decreases cell proliferation in hypoxic environments and is associated with enhanced cell death in hypoxic cancer, showing that the CRISPR/Cas9 technology effectively targets HIF1. Highly effective tumor genome engineering was accomplished by merely combining the expression of CRISPR/Cas9 and sgRNAs (Fuziwara et al. 2022). However, the absence of effective delivery mechanisms and the toxicity of the Cas9 nuclease restrict the use of the lentiviral CRISPR/Cas9 system in clinical situations. Notably, lentivirus infection and the CRISPR/Cas9 mechanism that mediates the deletion of an

inevitable gene result in off-target events and irreversible insertional inactivation of genes. Therefore, it would be ideal for creating a delivery vector and a CRISPR/Cas9 system that primarily targets the genomes of cancer cells.

13.13 Future Outlook

Although the qualitative and quantitative variations in the hypoxia response by both cell types are unknown, one of the main physiological differences between tumor cells and normal cells is their capacity to survive under hypoxic conditions. Hypoxia stimulates a complex transcriptome, including signaling pathways downstream of HIF-1 and other signaling pathways, in both tumor and normal cells. Most solid tumors exhibit hypoxia due to changes in the local vascular blood flow that cause oxygen deprivation. This event offers a rare chance to attack only the hypoxic tumor microenvironment while sparing the surrounding normoxic tissue. Additionally, it has been demonstrated that selectively altering tumor cells that express HIF1 can increase survival and slow the spread of metastatic disease, supporting the idea that targeting these cells is a promising therapeutic approach. Additionally, other genes and pathways have been discovered that merit additional research into their potential as therapeutic targets for tumor cells that are both normoxic and hypoxic.

The CRISPR-Cas9-based technologies discussed here have great promise for eradicating some of the most aggressive and resistant therapy cells found in hypoxic tumors. They merit additional investigation for improved tumor selectivity and delivery. The combination of CRISPR-based technologies with single-cell multi-omics methodologies opens up a vast array of potential applications for examining HIF gene function and tumor heterogeneity. The combination of spatial transcriptomics with pooled CRISPR libraries will be a powerful method for studying the effects of gene disruption on the relationships between hypoxic tumor microenvironments. The HIF pathway facilitates the mechanism for cellular oxygen homeostasis and its response to a low oxygen situation.

A crucial factor in cancer metastasis is the regulation, or dysregulation, of the HIF pathway, which is associated with a poor prognosis for cancer. HIF is a desirable target for chemotherapy against malignant cells because of its role in cancer development. Perhaps using HIF inhibitors in conjunction with conventional therapy will be therapeutically beneficial. Using a tumor-targeted lipid-based CRISPR/Cas9 delivery method may allow HIF-1, a crucial regulator of the hypoxic tumor microenvironment, to be suppressed. In vivo downregulation of HIF-1 by the CRISPR/Cas9 system in combination with chemotherapy could be used to treat diseases that have spread to other body parts.

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Tumor-on-a-Chip: Microfluidic Models of Hypoxic Tumor Microenvironment

14

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Abstract

Cancer is ranked as the deadliest prime health issue, albeit with mammoth efforts to panacea the ailment. To subdue the vast constraints in tumor therapy, one requires a superior cognizance of the tumor microenvironment (TME) including more sophisticated means to screen potential anticancer therapies. Hypoxia or low blood oxygen is a critical component of the TME and is significantly linked to tumor immune response, angiogenesis, metabolism, and cell proliferation. Thereby, detecting tumor hypoxia by employing cutting-edge tools, and emerging tumor-on-a-chip (ToC), is an upthrust area of research. Here, we assessed the state-of-the-art of the ToC tech, which incorporates tissue engineering, biomaterial research, microfluidics, and microfabrication in addition to profound novel contingency for fabrication and application of functional 3D in vitro tumor models for oncology research, immunotherapeutic, and screening. ToC microdevices, in particular, are competent for microscopic analysis of the interplay between tumors, immune, and cells in the TME, which are not

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amendable with simple tissue cultures or animal models. The difficulties in developing ToC tech for the next generation are also addressed.

Keywords

Tumor-on-chip · Microfluidic devices · Tumor microenvironment · Hypoxia · Microfabrication

14.1 Introduction

Cancer due to its high morbidity and mortality rate remains one of the world's leading health issues after cardiovascular disease, accounting for 18.1 million cases and approximately 10 million deaths in 2020, amid the plethora of research and substantial medication discovery efforts made over the past few decades to combat the malady (Ranjan et al. 2020; Sung et al. 2021; Parihar et al. 2021a, 2022c, e). The global burden of malignancies is presumed to increase more adversely, making it a research platform in the clinical arena (Thun et al. 2010). Patients diagnosed with cancer needs extensive and continuous monitoring. The current medical setup lags in sustaining incessant round-the-clock monitoring competencies and paves an overwhelming challenge for the healthcare department with an utmost burden on the country's capital expenditure. This is due in part to the hefty expense of developing a novel antineoplastic therapeutic, in addition to the necessity for an enhanced understanding of the proliferation of cells and the tumor microenvironment (TME), besides the functions of tumor vasculature, tumor-stromal heterogeneity, various immune effectors, suppressor responses, and inflammation (Labani-Motlagh et al. 2020; Parihar et al. 2021b; Munjal et al. 2022). According to extensive research, TME promotes cancer growth in several ways, particularly treatment resistance. It not only reduces drug penetration but also enables surviving cells the benefit of proliferation and antiapoptosis, aiding resistance and common morphological changes associated with illness (Sun 2016). Unavailability of oxygen or inadequate blood flow is a common TME condition in almost all malignancies due to the tumors' uncontrolled and rapid growth (Muz et al. 2015). Most cancers exhibit reduced oxygen availability (hypoxia), a TME characteristic caused by an imbalance between elevated oxygen consumption and insufficient oxygen delivery. Countless research analyses that a person with tumor hypoxia has a higher chance of mortality and metastasis. Independent of the clinical stage, it has been documented that hypoxia is an unfortunate prognostic indication at the point of diagnosis (Walsh et al. 2014). The transcription factor, HIF-1α triggered by hypoxia, mediates a significant portion of all these effects (You et al. 2021). Therefore, to achieve substantial advancements in cancer therapy and tumor prognosis, more effective techniques for screening anticancerous therapeutics and a deeper knowledge of TME employing sophisticated techniques, such as organs-on-chips technology, are required (Tsai et al. 2017). Cell culture and animal models currently involve in cancer research and antineoplastic drug screening to date and therefore serve as a

crucial link between the cell and clinical trials (Kitaeva et al. 2020). Although cancer animal models (animal experimental objects and related materials) can provide crucial in vivo evidence and testimony on tumor progression and drug molecule responses, the relevant statistics accomplished have immense variations among the animals utilized and are therefore exceedingly expensive (Mak et al. 2014; Parihar et al. 2022d). In laboratory-cultured cells as well as cancers from both humans and animals, hypoxia can be detected utilizing both direct (i.e., needle-type O_2 electrodes) and indirect methods (i.e., HIF-1/2α immunolabeling or downstream HIF-targets) (Godet et al. 2022). Furthermore, in vivo models employed for cancer screening and therapeutic development, such as mouse models, may not accurately depict the interaction since the human tumor cell lines' biological activity and tumor heterogeneity varies significantly from that of the underlying tumor tissue (Onaciu et al. 2020). Contrary to this, 2D and 3D cell culture models have extensively been employed for anticancer drug screening and studies of cell proliferation, signaling, migration, and treatment outcomes, including altered protein/gene expression (Parihar et al. 2022d; Edmondson et al. 2014; Kapałczyńska et al. 2018; Parihar and Dube 2022). These model systems entail the co-cultivation of various cell types in the matrix comprises hydrogel which include cells derived from patient. Although these cellular models are economical and simple to use and commonly exhibit excellent reproducibility, perhaps they couldn't be capable to replicate the TME in an organ or models, making them ineffective for studying the effects of complicated spatial organization and cell interaction (Barbosa et al. 2022).

A practical in vitro site for novel therapeutic designing should foresee drug efficacy in a shorter time frame, accurate, and significant outcomes with practical applicability (Parihar et al. 2010, 2013, 2021c; Parihar and Dube 2022). Microphysiological in vitro techniques are perhaps the utmost sophisticated forums for early clinical drug effect assay till known (Bai and Wang 2020). Nevertheless, existing platforms have limitations which include low throughput, exorbitant costs, and, most importantly, the incapability to procreate physiological drug exposures. As a result, there is an exigency to enhance and strengthen these networks, as well as develop better predictive systems capable of simulating drugs' in-vivo pharmacokinetic (PK) profiles (Norris et al. 2000). Microfluidics-based 3D culture models are coupled in tumoroid/organoids-on-chip technology (Duzagac et al. 2021). The fundamental goal of these techniques is to retain the cellular intricacy of cell models (mainly 3D cell culture with TME) and syndicate with the potential to simulate therapeutic testing in model organisms. The majority of available models investigate tumor invasion, progression, angiogenesis, and metastasis and thereby mimic the pathophysiology of human cancer. The more sophisticated they are, the lower will be their efficiency and the more complicated they become. A microfluidic chip comprising 2D or bare or enclosed 3D cultures continuously perfuses drugs at a consistent flow rate is possible using microfluidic technology (Kumar et al. 2022a; Li et al. 2012; Parihar et al. 2022b; Ranjan et al. 2022). These techniques are capable of simultaneously assessing a multitude of promising therapeutic leads as possible at varied concentration ranges (Cui and Wang 2019). The integration of gradient generators coupled with cell culture chambers is being used by some devices to

solve this challenge (Yahyazadeh Shourabi et al. 2021). Using cultural 2D or 3D models, however, varied chemical concentrations are administered to a limited proportion of replicate wells preventing the cell construct from being exposed to varied drug concentrations emblematic of the plasma PK profile. Moreover, significant advances incorporate PK-PD setups for 2D and 3D cultures with syringe pump-controlled flow rates that use custom-made PDMS chip models (Petreus et al. 2021).

14.2 3D Tumor Models on Chip for Measurement of Hypoxia

Drug discovery is still a difficult and expensive process due to low success rates in clinical trials. Approximately half of all medications couldn't make it to stage II and III clinical trials because of their futile in nature, although about one-third of failures are related to safety concerns such as a NTI (narrow therapeutic index) (Arrowsmith and Miller 2013). As casualty cases continue to escalate, innovative tools that allow for greater precision in drug discovery are considered essential (Dugger et al. 2018). The emergence of novel preclinical testing models that effectively mimic in vivo system and tumor microenvironment, along with targeted therapy, which has the potential to lead to future biomarkers and more precise pharmacological targets, are among the utmost promising fields that are anticipated to enhance drug development efficacies (Langhans 2018). Hypoxic cells have routinely been identified in 2D and 3D cell cultures by HIF-1 immunolabeling utilizing immunohistochemistry (IHC) or immunofluorescent (IF) techniques. Because HIF-1/2α proteins only have a modest half-life when exposed to oxygen, thereby immunolabeling can be difficult. Additionally, nuclear permeabilization is necessary for their detection since HIF-1 and HIF-2 reside in the nucleus in hypoxic settings. Although popular, in vivo three-dimensional models involving animal xenografts have ethical limitations too and cannot accurately replicate the biology and physiology of humans. Thereby, by modifying a multitude of three-dimensional tissue engineering techniques, 3D tumor models are produced that imitate in vivo TME in the body (Lara Rodriguez and Schneider 2013). As pioneered by Mina Bissell and her colleagues demonstrated in the 1980s when they conducted research on the significance of the ECM (extracellular matrix) in cell behavior, cultivating cells in 3D systems that imitate key tissue facets is comparatively more representative of the in vivo environment than simple 2D monolayers (Pampaloni et al. 2007; Ravi et al. 2015). 2D cultures are unable to replicate the intricate 3D tissue organization, biophysical and biochemical characteristics of ECM, and cell-cell interactions encountered in malignancies. High-throughput screening (HTS) cellular assays still frequently use traditional monolayer cultures, although 3D cell culture techniques for drug research are rapidly advancing (Ryan et al. 2016).

Building vascularized tissue in three-dimensional tissue engineering for non-animal substitutes has been a serious challenge for decades (Grover et al. 2018). Vascularization is required to provide sufficient oxygenation to thick tissue ($>$100–200 m) for persistent maintenance and functioning, for instance, assessments of toxins in sub-acute and chronic circumstances (Zhang et al. 2021). Additionally, it

is critical for improving therapies and simulating diseased tissue. Drug leads for neurological disorders, in particular, have a larger rate of failure during the bench-to-bedside transfer compared with former treatment (Gribkoff and Kaczmarek 2017). Only a small percentage of the candidates who made it through stage I of the clinical rigorous assessment got commercial approval, according to research (Sun et al. 2022). The causes for detrimental impacts on cerebral microvascular remain unknown, and it's unclear if they're caused by endogenous pathogenic systems or by the medications themselves. Many neurological illnesses, including brain malignancies, are linked to injury or malfunction of the brain's vascular system. Among the most prevalent and deadly forms of elderly brain tumor is glioblastoma multiform (GBM), which has an average survival span of only 12 months with the right treatment. Additionally, it is among the most vascularized cerebral tumor, and it's linked to a lot of ECM remodeling. Modeling the characteristics of GBM to comprehend their influence on cerebral vascularization, especially relevant to the modulation of angiogenic signaling pathways, has been a focus of research, as microvascular proliferation is a characteristic of GBM (Rodriguez et al. 2012; Hardee and Zagzag 2012). In methods for developing tumor-on-a-chip (ToC), three-dimensional tumor tissues are usually initially prepared according to standard culturing techniques before being introduced to the microfluidic chip for examination. Thus, research into tumorigenesis and the formulation of remedies for targeted therapy can both benefit from the application of a 3D cancer model. The common methods for developing 3D in vitro cancer models are being thoroughly covered.

14.2.1 Conventional Transwell Model

The BBB constituents' endothelial cells (ECs), astrocytes (ACs), and pericytes (PCs) are reconstructed in the co-cultivation transwell model (Fig. 14.1). To evaluate the motility of tumor cells in conjunction with a biological inclination, transwell inserts, often referred to as Boyden chambers, are frequently employed in traditional migration, invasion, and transendothelial migration studies. A polymeric porous membrane makes up a transwell insert, allowing cancer cells to traverse through the pores. The propensity of tumor cells to move through the pores is assessed using a migration assay. In glial cell co-cultures, positive glial input towards initiation of BBB characteristics is often more efficient (Abbott 2002; Garcia et al. 2004). Endothelial cells that were co-cultured with astrocytes in cell-cell interaction elevated the expression of close junction proteins like occludin (EC 1.6) and P-glycoprotein (P-gp). In co-culture settings, pericytes could be used instead of astrocytes or neurons (Zujovic and Taupin 2003; Nakagawa et al. 2009). As a result, a cell-cell interaction co-culture method is more structurally similar to in vivo condition (Malina et al. 2009). Accordingly, astrocytes and pericytes are assumed to partake a favorable influence on ECs on their own, but when combined as a tri-culture, they provide synergic stability of such a close junction configuration (Schiera et al. 2003).

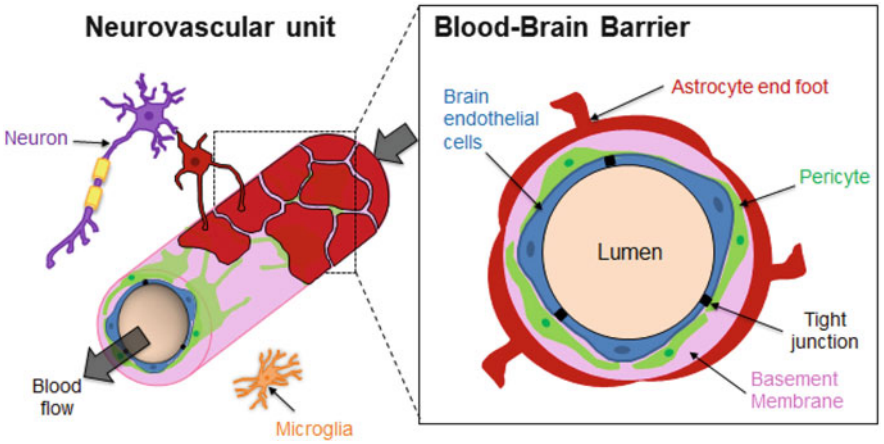

Fig. 14.1 The organizational structure of NVU and the BBB. NVU is a cellular and extracellular matrix-based structural and functional complex wherein ACs, microglia, BBB ECs, PCs, and neurons are all part of the NVU. The components of NVU cooperate synergistically to regulate the exchanges throughout the blood arteries and the brain

Tri-cultivation transwell model system encompasses the reconstruction of astrocytes, endothelial cells, and pericytes, the three BBB constituents. To generate a co-culture, ECs are full-fledged on the apical, and ACs are introduced to the basal surface of the membrane. A tri-culture can then be formed by adding pericytes to the in vitro system. Instead, the pericytes well developed on the basal membrane, while the astrocytes are produced on the culture plate (Nakagawa et al. 2007).

The highest TEER values were found in tri-culture models integrating pericytes. Tri-cultivation is the most accurate in vitro model that mimics in vivo scenario and should be investigated further.

Mono-cultivation Transwell Models

The development of cultured cell supplements with highly permeable membranes permitted in vitro permeability investigations with brain ECs (Grobstein 1953). Because of the favorable data from these models, transwell filters utilized as the benchmark modeling technique for in vitro BBB investigations ever since. To form a monolayer, ECs are cultured on a microporous membrane referred to as a monoculture. To improve cell adhesion, ECM such as fibronectin or collagen may be introduced to cover the membrane (Yue 2014). The simple monoculture transwell approach can simultaneously investigate a wide range of operations. For instance, drug permeation can be measured by an array of transwell filters over a wide range of concentrations. Additionally, BBB constituents like ACs and PCs have been demonstrated to affect the B-endothelial BB's cell characteristics. In comparison to ECs cultured alone, a syngeneic co-culture system comprising rat ECs and ACs has been shown to upsurge close junction confrontation amid ECs (Demeuse et al. 2002). Pericytes, that demonstrated to enhance ECs maturation and a thick barrier

development, are only used in a few transwell models. To improve the monoculture BBB model, astrocytes and pericytes must be explored.

14.2.2 Tumor Spheroids

Under non-adherent cell culture conditions, three-dimensional aggregations of cells can develop into tumor spheroid structures. The appearance, growth dynamics, nutrition transport, and connections amid cells and the matrix in the cancer spheroid mimics a minor tumor mass (Benien and Swami 2014). Due to its remarkable three-dimensional in vitro tumor model capabilities, the tumorospheres aids as an exceptional model. Disaggregated cells from primarily isolated tumorospheres and organotypic tissues, as well as sole- or numerous-cell suspensions from persistent cell lines, can all be used to create tumor spheroids. Patient- or murine-derived organotypic tumor spheroids (MDOTS/PDOTS) sustain the immunological milieu of the tumor microenvironment. To replicate ICB ex vivo, a 3D microfluidic system is used to develop MDOTS/PDOTS for a brief period. Multicellular organoids with corresponding immune cells were obtained after limited collagenase decomposition of fresh tumor tissues. P- or MDOTS been examined by flow cytometry (FC) or collagen injection into the device's central channel for anti-PD-1 or -CTLA-4 antibody exposure. They assessed the proportion of myeloid and lymphoid immune cells in a mass tumor with distinct spheroid cultures (S1 $>$ 100 m; S2 40–100 m; and S3 murine-derived organotypic tumor spheroids) after establishing that enzyme-mediated metabolism won't affect the expression of the surface antigen. In ex vivo 3D microfluidic culture, recapitulate sensitivity and resistance to PD-1 blockade. To assess the ex vivo response to PD-1 inhibition, anti-PD-1 antibody reacts with MDOTS for 3 or 6 days inside the device. Dual-labeling deconvolution fluorescence microscopy using AO (acridine orange), and PI (propidium iodide), was then used to see live and dead cells (Fig. 14.2a). The baseline survivability of MC38 MDOTS ($>$90 percent at Day 0) dose- and time-dependent death of MC38 MDOTS in response to anti-PD-1 using the MC38 syngeneic model (Woo et al. 2012) reacts to anti-PD-1 in vivo (Fig. 14.2b–c). Multiple independent replicates in different laboratories corroborated this result. Immune cell-derived MC38 spheroids without stromal cells had to be unresponsive to anti-PD-1 drugs for PD-1 (Fig. 14.2d). Despite comparable treatment, MDOTS produced from the PD-1-resistant B16F10 (Curran et al. 2010) (Fig. 14.2e–f) and Lewis lung carcinoma (LLC) models showed less cell death than MC38 MDOTS (Fig. 14.2f). The CT26 model (Duraiswamy et al. 2013), which is intermediately sensitive, showed little killing (Fig. 14.2g). Furthermore, co-treatment with an anti-CD8α Ab inhibited anti-PD-1-mediated death of CT26 MDOTS, suggesting a particular prerequisite for CD8+ T cells (Fig. 14.2h), which we also established with MC38 MDOTS. The MDOTS produced from explanted GL261 glioma tumors (Reardon et al. 2016) retained sensitivity to ex vivo PD-1 blockage in a CD8+ T cell-dependent manner. It is confirmed that CD45+ immune cells and CD8+ T cells survived ex vivo in the device after tumor cells died due to PD-1 inhibition. These findings show that using well-defined

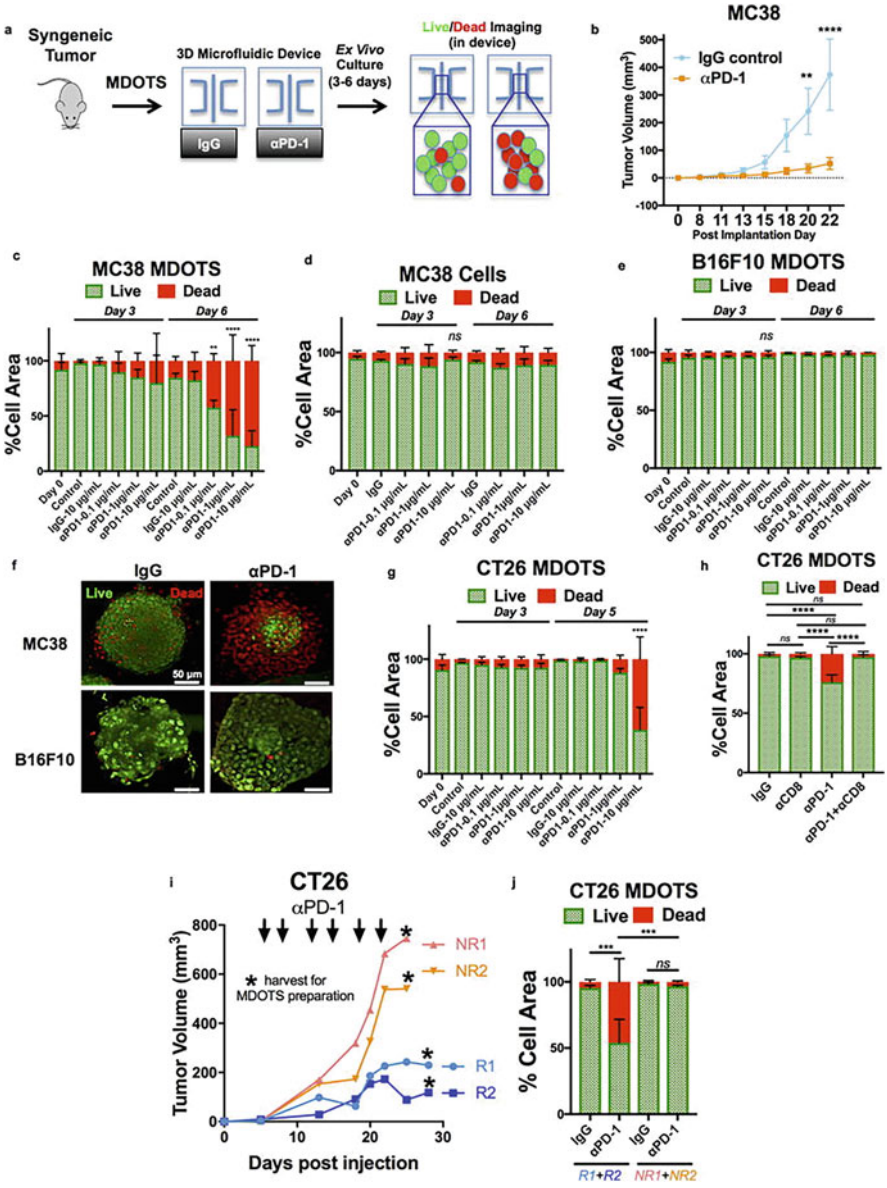

Fig. 14.2 Ex vivo PD-1 blockade MDOTS profiling. (**a**) Model for the MDOTS live/dead imaging process. (**b**) Rat anti-mouse anti-PD-1 antibody or isotype control IgG therapy on the MC38 tumor volume ($n = 10$). (**c**) Live (AO = green)/dead (PI = red) Day 3 and Day 6 quantification of MC38 MDOTS in response to IgG control or specified anti-PD-1 antibody dosages ($n = 4$, biological replicates, two-way ANOVA with Dunnett's multiple comparisons test). (**d**) Live/dead study of MC38 spheroids devoid immune cells ± anti-PD1. (**e**) B16F10 MDOTS ± anti-PD1 live/dead assay (**f**) MC38 and B16F10 MDOTS Day 6 ± anti-PD1 deconvolution fluorescent microscopy. (**g**) CT26 MDOTS ± anti-PD1 live/dead assay. (**h**) On Day 6, CT26 MDOTS conducted a live/dead assay in which isotype IgG controls (10 μg/mL) or anti-PD-1 (10 μg/mL) and anti-CD8 (10 μg/mL) were

mouse models, sensitivity and resistance to PD-1 blocking may be replicated ex vivo. Even when identical cell counts are implanted into syngeneic animals, stochastic tumor growth and response to ICB are common. Further, MDOTS from explanted CT26 tumors of various sizes was used to assess the influence of inter- and intra-tumor heterogeneity on PD-1 blockade sensitivity. When MDOTS from bigger CT26 tumors were compared to MDOTS from smaller tumors, there was less CD8 T cell infiltration and less ex vivo sensitivity to PD-1 inhibition. CT26 MDOTS were made from mice that responded (R) or did not respond (NR) to PD-1 inhibition in vivo and were then re-challenged with anti-PD-1 therapy in 3D microfluidic culture (Fig. 14.2i). CT26 MDOTS generated from PD-1-responsive tumors (R1 + R2) maintained PD-1 blockade sensitivity ex vivo, whereas MDOTS prepared from PD-1 non-responsive tumors (NR1 + NR2) maintained PD-1 blockade resistance (Fig. 14.2j). These findings show that tumor development and responsiveness to ICB heterogeneity found in vivo are conserved ex vivo in MDOTS.

MDOTS makes novel therapeutic combinations easier to test. The CT26 model was chosen due to its half sensitivity to anti-PD1, which could help to find new PD-1 blockade combinations that could overcome intrinsic resistance (Peng et al. 2016). The inhibition of CCL2 a chemokine alone did not improve PD-1-mediated CT26 MDOTS killing, indicating the need for alternate methods that decrease immune-suppressive signaling more extensively throughout the TME and reawaken T cells (Vanneman and Dranoff 2012; Benci et al. 2016). The impact of combining the MDOTS platform with Compound 1, a novel potent/selective TBK1/IKK inhibitor, showed the improved efficacy of PD-1 blocking. The kinases TBK1 and IKK promote autocrine/paracrine signaling (Zhu et al. 2014) as well as restrain T cell activation (Yu et al. 2015), suggesting that inhibiting TBK1/IKK could improve multiple strategies to overcome an immunosuppressive TME for tumor control in response to PD-1 inhibition. In contrast to the multi-targeted inhibitor momelotinib, they validated Cmpd1's efficacy and selectivity including the absence of JAK inhibitory action (CYT387). Cmpd1 boosted the production of IL-2 and IFN- from isolated CD4+ and CD8+ T cells from healthy adult patients as well as IL-2 from Jurkat human T-cell leukemia cells and decreased immune-suppressive cytokine synthesis by CT26 cell line spheroids with no cytotoxicity.

Ex vivo addition of Cmpd1 to PD-1 inhibition improved CT26 MDOTS death (Fig. 14.3a, b), which was related to lower CCL4, CCL3, and IL-1 levels and activation of cytokines implicated in activated innate immune responses (e.g., G-CSF) (Fig. 14.3c). Cmpd1 anti-PD-L1 was administered to Balb/c mice bearing CT26 tumors to see if MDOTS profiling predicted the in vivo response to combination TBK1/IKK inhibition (Fig. 14.3d–f). Cmpd1 + anti-PD-L1 had better tumor

Fig. 14.2 (continued) administered. (**i**) Treatment with anti-PD-1 to CT26 tumor volumes in Balb/c mice for the responder (R1 + R2) and non-responder (NR1 + NR2) indicating the moment of sample collection for MDOTS preparation. (**j**) Ex vivo isotype IgG control group or anti-PD-1 therapy and live/dead examination of CT26 MDOTS from R1 + R2 and NR1 + NR2 mice (Day 6)

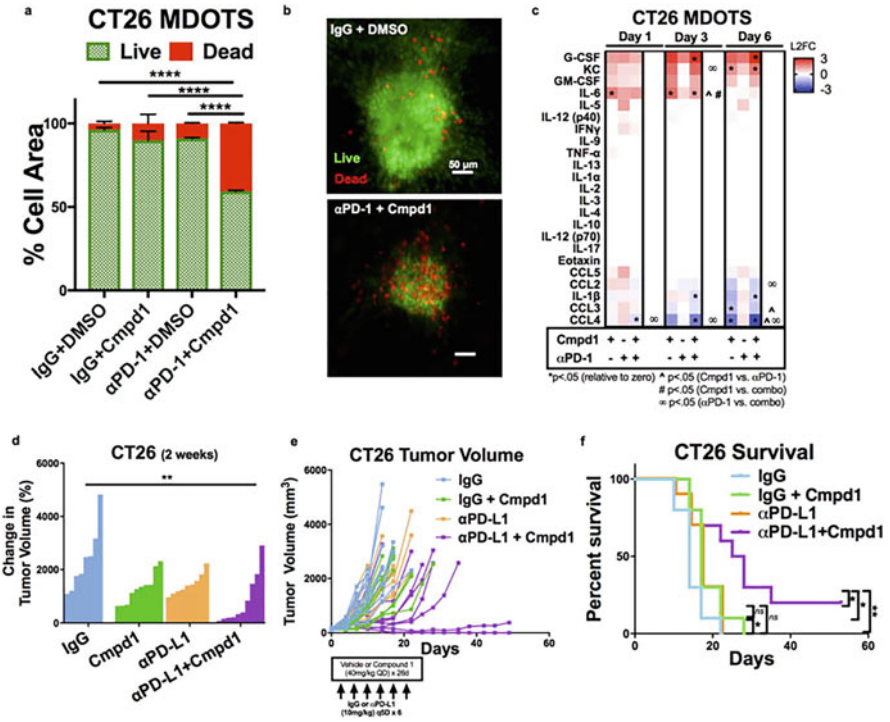

Fig. 14.3 Inhibition of TBK1/IKK improves PD-1 blockade responsiveness. (**a**–**b**) Following 6 days of treatment with IgG-DMSO, Cmpd1 (1 M), PD-1, and PD-1+ Cmpd1, the CT26 MDOTS was either alive (AO = green) or dead (PI = red). (**c**) Treatment of CT26 MDOTS with IgG + Cmpd1 (1 μM), αPD-1 (10 μg/mL), or αPD-1 + Cmpd1 (1 μM) cytokine heatmaps, with vehicle control, displayed as L2FC compared to isotype control IgG. (**d**–**e**) CT26 tumor volume implanted. (**f**) Percent survival following IgG + vehicle, IgG + Cmpd1, αPD-L1 + vehicle, and αPD-L1 + Cmpd1

control and longer survival than Cmpd1 or anti-PD-L1 alone (Fig. 14.3d–f), which was consistent with MDOTS profiling findings. Reimplantation of CT26 cells into animals with extraordinary responses to co-administered culminated in negligible proliferation, while EMT-6 implanted tumors grew properly, implying that Cmpd1 + anti-PD-L1 treatment induces immunologic memory of CT26 cells. As a result, MDOTS profiling accurately predicted the response to PD-1 inhibitor +/ TBK1/IKK blockade, demonstrating the use of ex vivo screening MDOTS for the development of combination immunotherapies.

14.2.3 Cancer Three-Dimensional Cell Culture in 3D Matrices

In contrast to 3D models that can imitate these conditions in vitro, 2D models cannot study cell-cell and cell-matrix interactions. Owing to the absence of preclinical

models that are appropriate for 2D cultures, 3D culture provides a practical pathophysiological TME and may contribute to drug discovery. Three-dimensional tumor models have been developed using tissue engineering techniques. A scaffold is an extracellular support structure that is biocompatible and chemically stable and functions as a cell adhesion model, growth, and tissue morphogenesis. Some biomaterials having ECM-like qualities can be used in inserts, and cells can also manufacture ECM proteins like collagen. Due to modifications in the physical and biological conditions of 2D and 3D cultures, 2D cells are more vulnerable to the effects of medications than 3D cultures because of their inability to retain normal morphology and variations in the arrangement of surface receptors on the cell. It's worth noting that there's mounting evidence showing cells cultivated in a 3D environment exhibit differences from cells cultured in a two-dimensional setting, retaining critical ECM signals. Consequently, suitable 3D culture provides a more appropriate physiological method of studying gene activity and cell phenotypic ex vivo. The preferred technique for succinct tissue construction of benign and malignant tumors in recent years is reconstructed 3D culture. As a result, the modifications, communications, and molecular and cellular signals that occur during tumor hypoxia-mediated malignant transformation can therefore be better understood using 3D culture.

Three-Dimensional Cell Culture Scales

Because of the unavailability of a single technique that can handle the requirements of all 3D cultured cells, many approaches have been created to meet the expanding need for cell culture. The influence of ECM molecules is ignored in 2D culture. Nonetheless, its density and packing play a crucial influence in the construction of a three-dimensional environment. The 3D model is inspired by the original microenvironment and is an in vitro recreation of the ECM. It maintains the ECM's geometric, mechanical, and biological qualities. Solid tumors grow by interacting with a variety of cell and non-cell components, using methods akin to those used in the initial phases of organogenesis. Several cells are combined in a unique setting and structured in such a way that they generate 3D tissues that resemble real tissue structures. The tumor microenvironment, which includes many cell types such as immune cells, stromal cells, and ECs, surrounds tumor cells in vivo. Other extracellular elements include the ECM, EVs, metabolites, growth factors, and cytokines (TME) (Riedl et al. 2017). The morphological and cellular structure formed by ECM interactions that are altered through oncogenesis can be studied using 3D culture models. As a result, in vitro 3D tumor models are a critical method for studying cancer growth and metastatic pathways. They are most advantageous because they permit tissue development derived from human cells and contain specific, physiologically significant components. Consequently, 3D culturing offers a more physiologically appropriate method of studying gene activity and cell phenotypic ex vivo. The characteristics of the selected cells show 3D artificial ECM imitation in which they grow; scaffold-based natural, synthetic, or hard biomaterials; signaling molecules; and cell culture bioreactors that support a biologically active environment. As a result, such variables should be assessed prior to selecting the best-suited

approach and methods. Scaffold-based culture (spheroids), scaffold-free (natural), or manufactured solid scaffolding (non-scaffold based) were exploited to study tumor hypoxia.

14.3 Tumor-Microvascular Model in Microfluidics

When it comes to tissue engineering and tumor biology, vasculature is indeed crucial. Additionally, the development of new vasculatures for nutrient delivery is essential for the proliferation and progression of the tumor. Chemical processes and evaluations carried out in microchannels and microstructures produced using semiconductor microfabrication approaches such as photo- and soft lithography (Betancourt and Brannon-Peppas 2006; Hacking et al. 2012). In the 1990s, an interdisciplinary branch of research named "Lab-on-a-Chip" or "Microfluidics" was formed by merging micro-/nano-device, chemical sensors, and analytical chemistry (Kimura et al. 2018). In general, in vitro-cultured cell lines are mostly inactive and lack physiological capabilities. This process also occurs in initially cultivated cells, and maintaining biological functions over long periods, even if they are regular right after harvest, is extremely challenging. Cells are cultivated in the semi-static condition in traditional methods, with the administration of experimental chemicals to cells relying solely on diffusion. However, in vivo, cells are exposed to chemical and physical stimulation from their ambiance, including stretching and shear stress, as well as oxygen and nutrients through blood flow. Variations in the environment and structure of cells in vivo and in vitro may lead to the loss or inactivation of cellular activities in culture. To bridge the gap between in vitro and in vivo environments, experts in the tissue engineering field, including those working in the mTAS and other domains, have been using microfluidic devices since the early 2000s (Gupta et al. 2016). Microfluidic approaches can manage spatial and temporal liquid conditions, cell adhesion, and mechanical stimulation of cells. To accurately recreate the TME in vitro, a functional microvasculature network must be developed in conjunction with a three-dimensional tumor model. It is possible to more effectively kinetically examine crucial cancer growth phases including intravasation, angiogenesis, and extravasation in a meticulous microenvironment by co-culturing vasculature and cancer cells on a microfluidic preferable platform (Pfisterer and Korff 2016). Organ-on-a-chip (OoC) which mimics organ functionalities using microfluidics has gotten a lot of press recently (Wu et al. 2020). To assist as models for cell-based assays during drug research, tissue and drug discovery disease models using OoC technology have been provided. This is especially true given the introduction of an iPS cell differentiation induction approach. Two early instances of organs-on-a-chip indicated enhancements in functional activity through the culture perfusion of 3D hepatocyte aggregations and evaluation of shear stress by EC vascular exposure and maintaining medium flow in a microchannel. This has evolved into a cutting-edge technology proficient in recreating normal cell behaviors in vitro thanks to recent developments in cell engineering, microfabrication, and imaging. Recent years have seen investments in OoC initiatives from the NIH

(National Institutes of Health), the FDA (Food and Drug Administration), the Defense Advanced Research Projects Agency (DARPA), the FP7 (Framework Program 7) in the European Union, and the AMED (Agency for Medical Research and Development) in Japan. This funding also demonstrates the size of the research aspirations for OoC technology.

Organ-on-a-Chip (OoC)

The mTAS researchers have proposed OoC devices that simulate a variety of organ and tissue processes. Although it is beyond the context of the study to give a comprehensive assessment of all the strategies, we do present an overview of in vitro models in this section.

(a) *Lung-tumor-on-a-chip*

Nearly all solid tumors have a characteristic microenvironment feature called hypoxia. The unchecked and rapid tumors growth which reduces the available oxygen due to insufficient blood flow. Intratumoral oxygen gradients facilitated by hypoxia contribute to the adaptability and heterogeneity of malignancies. Controlling the tumor parameters has been necessary throughout the entire 3D model-building process. By using light-sheet fluorescence microscopy, tumor spheroids, ECM buildup, or hypoxia may all be characterized phenotypically. Fluorescence imaging was used to track the 3D-3 model, which was made up of NSCLC cells, CAF, and monocytes, and is based on the alginate microencapsulation method. The improvements made in the creation of 3D models of NSCLC opened up several opportunities for investigations of hypoxia (Hsu et al. 2013). The "lung-on-a-chip," also identified as the "breathing lung," made by Harvard University's Ingber group is the most well-known OoC. A microporous membrane constructed of flexible polydimethylsiloxane, silicone, separates the two-layer channel structure vertically (PDMS). To mimic the form of the lung on a microfluidic device, they cultivated alveolar ECs on the top surface of the membrane and vascular ECs on the bottom side and employed flowing air and culture media through it. The internal pressure of the channel on each sides of the main channel was changed at a precise cycle to stretch and contract the membrane, simulating the expansion physiologically and contraction motions of the alveolus while breathing. Using this device, they mimicked inflammatory situations in which vascular ECs express a large amount of the integrin ligand (ICAM-1) following exposure to TNF-a and bacteria. Furthermore, following the development of ICAM-1, neutrophils flowing in the vascular side channel linked to vascular ECs; then traveled to the alveolar ECs surface side, through the vascular ECs and the membrane's pores; and engulfed the bacteria. Z. Xu et al. devised a framework for a successful drug sensitivity test using a microfluidic chip-based, three-dimensional co-culture (Xu et al. 2013). Fresh lung cancer tissues and a variety of stromal cell lines were produced in three dimensions (3D) under conditions that were similar to those found in vivo. To identify the most effective chemotherapy plans, various anticancer medications were administered to the cells using a gradient concentration generator within

the chips. To separate CTCs (circulating tumor cells) in NSCLC patients with high purity, J. Zhou et al. developed a unique multi-flow microfluidic (MFM) method (Zhou et al. 2019). Because of the specific oxygen regulator made possible by their minuscule size, investigations have conclusively shown that microfluidic devices offer a potential platform for the exploration of hypoxic TME.

(b) *Liver-tumor-on-a-chip*

Hepatocytes, the body's largest and primary organ for metabolism, a type of parenchyma cell make up 80% of all liver cells. Among the non-parenchyma cells are the ECs of sinusoid lumens, stellate cells, Kupffer macrophage cells, and pit cells (located in the same region as Kupffer to disrupt and kill CTC). High concentration of oxygen and particular shear stress are necessary for the normal survival of hepatocytes. To survive longer in vitro, hepatocarcinoma (HepG2) or its clonal derivation with significant contact-induced growth inhibitory (known as C3A) need more stringent environmental circumstances.

Because the liver is the primary organ involved in pharmaceutical synthesis, it's critical to estimate its metabolic capabilities and toxicity early on in the drug development process. Hepatocytes employed for in vitro testing, on the other hand, lose a lot of their natural capabilities and activities. The inversion of prodrugs like TF5 to metabolite drugs 5-FU, which causes hepatotoxicity, and doxorubicin to doxorubicinol and metabolism which led to hematological toxicity are two significant instances of the liver's contribution to drug metabolism. Accordingly, the liver must be co-cultured in microscale CCA (cell culture analogue) models to study the pharmacological properties of anticancer agents (Esch et al. 2010). Powers and colleagues suggested a microfluidic system that allows for the creation of 3D tissue architectures while in continuous perfusion (Fig. 14.4b) (Powers et al. 2002). In a cell culture chamber, 3D scaffolds were paired with structural support and a cell-retaining filter to allow culture media to flow across the top of the edge and through the 3D cell aggregates in every channel. A chamber for cell culture was created with flow rates that fulfill predicted cellular oxygen requirements while maintaining a healthy range of fluid shear stress. They revealed that this technology allows the development of hepatocellular aggregates resembling those found in hepatic acini and that they can uphold their shape and vitality for 2 weeks. Maintaining the polarized transportability of hepatocytes is also critical for studying drug reactions. Hepatocarcinoma is more sensitive to environmental factors than other cancer cells; hence it is essential to effectively recreate the tumor microenvironment. A significant challenge for 3D hepatocyte culture is inducing hypoxia and necrosis of surrounding inner cells in a functional realistic 3D structure. Hanging spheroid tissues were cultured in 3D spherical structures using hanging drop networks (HDNs) of media (without cell-surface adhesion) that have shown significant advancements toward biological and functional cancer microenvironments which include high gas exchange (O_2, CO_2) during incubation, the prevention of bubble formation in secretion, and cell injection (Frey et al. 2014). Additionally, oxygen-permeable chips induce central hepatocarcinoma cell death and

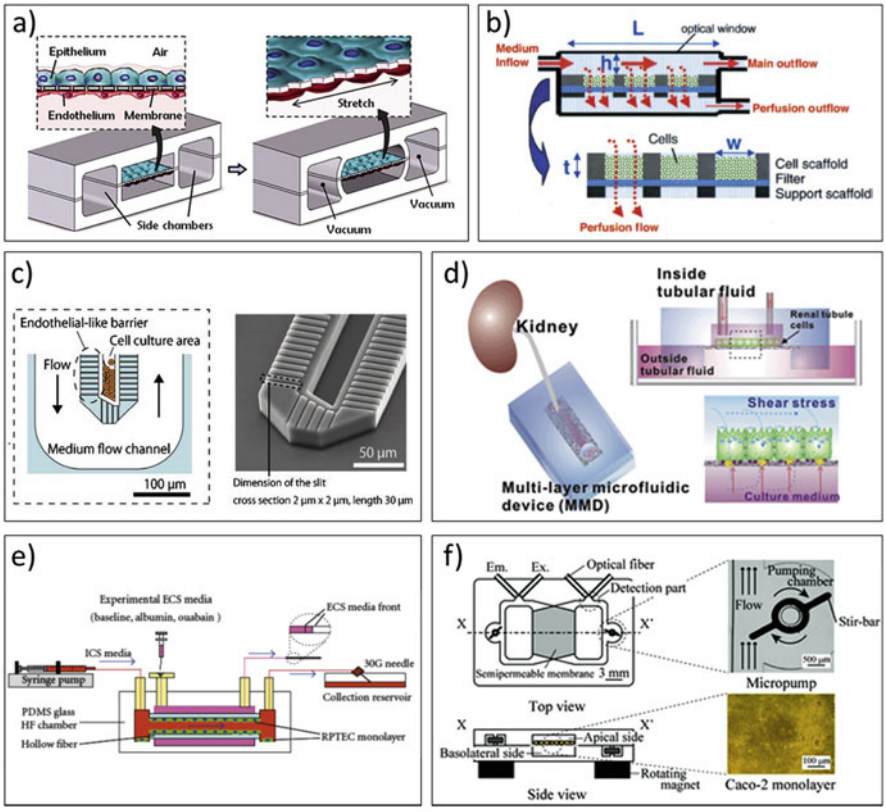

Fig. 14.4 Devices for OoCs: (**a**) by delivering a vacuum to the side chamber and mechanically stretching an elastic membrane creating the alveolar-capillary barrier, the lung-on-a-chip system mimics normal breathing motions. The American Association for the Advancement of Science (AAAS) owns the copyright to this image. (**b**) The perfused 3D liver culture microfluidic device. Wiley Periodicals, 2002. Copyright. (**c**) The liver-on-a-chip device has a hepatic cord-like shape. The American Institute of Physics (2011) owns the copyright to this image. (**d**) A porous membrane on the kidney-on-a-chip technology creates in vivo-like tubular habitats for collecting duct cells. The Royal Society of Chemistry (2009) owns the copyright to this image. (**e**) A tubular hollow fiber membrane is included in the kidney-on-a-chip device to mimic the tubular shape of the renal tubule. Hindawi Publishing Corporation (2013) owns the copyright to this work. (**f**) The gut-on-a-chip system comprises a two-compartment structure with stirrer-based micropumps and optical fiber inserts for each compartment, separated by a microporous membrane. The Royal Society of Chemistry (2008) owns the copyright to this image

hypoxia in 3D spheroid models (Anada et al. 2012). In the hepatic lobules, bile canaliculi are generated between consistently organized hepatocytes that spread radially and routinely from the central vein. Bile is discharged into the bile canaliculi and contains metabolic products biosynthesized in the cells. As a result, the bile canaliculi are mainly targeted for in vitro drug metabolism research. As an in vitro model for physiologic bile canaliculi development,

Nakao et al. (2011) designed a hepatic lobules device model that replicated the microstructure of the hepatic cord, a smallest unit hepatic lobule (Fig. 14.4c). The device's cell culture chamber was intended to arrange hepatocytes in two lines that resembled hepatic cords. Along with the hepatic cord-like structure, hepatocytes aligned progressively self-organize and create bile canaliculi. The drug response evaluation was then used to perform a drug metabolism test utilizing carboxydichlorofluorescein diacetate (CDFDA) as a model metabolite. The hepatocyte esterase hydrolyzes CDFDA to carboxydichlorofluorescein (CDF), a fluorescent substance that is discharged into the bile canaliculi. Herein, CDFDA-encompassing culture media was perfused into the blood vessel flow channel; CDF excretion into the bile canaliculi developed continually in the cells. The finding demonstrates how these processes keep tissues' form and polarity that may be replicated by simulating microstructures.

(c) *Kidney-tumor-on-a-chip*

Tubulointerstitial hypoxia has been identified as the ultimate pathway prominent to end-stage kidney disease (ESKD) as a result of studies conducted over the past two decades. CKD, or chronic kidney disease, is usually linked to a variety of varying levels of hypoxia damage in various tubular segments, based on the etiology and stage of the disease, which is a complex connection between oxidative stress, inflammation, and fibrosis. The kidney's resident cells are outfitted with defenses against hypoxia. Hypoxia-inducible factors (HIFs) play a key part in this process by transcriptionally activating genes. The kidney is an essential organ for both regulating metabolism and excretion. Because there are no adequate in vitro models, medication lead effectiveness and toxicity are investigated in the kidney entirely through animal testing throughout drug development. Drug dropouts in clinical trials, on the other hand, result from variations in metabolic pathways between humans and experimental animals, resulting in huge expenses in drug development. Preclinical testing that accurately identified nephrotoxic substances would result in considerable cost savings and the avoidance of nephrotoxic medications throughout development (Su et al. 2014). A study reported by Cho et al. stated that due to the nanoparticles' ability to agglutinate after being immunocaptured by released γ-glutamyl transpeptidase (GGT) and increasing the fluorescence measured in the outflow, 500 nm fluorescent polystyrene nanoparticles coupled with anti-GGT antibodies within the apical channel show the ability to track drug-induced nephrotoxicity (Cho et al. 2016). Additionally, it is anticipated that a highly accurate in vitro disease model would be employed as an effective drug screening method for potential therapies, providing fresh perspectives on the mechanisms underlying kidney disease. The most basic kidney-on-a-chip systems involved the attachment of human kidney-2 (HK-2) and Madin-Darby canine kidney (MDCK) epithelial cells to the lower surface of a microchannel and the application of biological shear stress (Frohlich et al. 2012). The shear stress caused by this device enhanced cell thickness, and expression of Na^+/K^+-ATPase, and promoted the production of cilia in the cells, according to the scientists. These findings imply that by employing a well-regulated shear stress

load, the physiological behavior of kidney-derived cells may be mimicked. To mimic the reabsorption function of renal tubules, two-layered kidney-on-a-chip devices having porous membranes have been developed (Fig. 14.4d) (Jang et al. 2013). The physiological reactions to variations in concentration of sodium and osmotic pressure of the apical channel were replicated in these investigations by injecting hormones like aldosterone and vasopressin into the device's basal channel. Further, they discovered that shear stress stimulates cilia, cell polarity, and P-glycoprotein expression and absorption of albumin/glucose in cells, in addition to changing cell orientation.

(d) *Gut-tumor-on-a-chip*

The gut is an organ that is primarily accountable for the absorption and digesting of nutrients; growing evidence indicates that it also plays a crucial part in the proper operation of other organs and the etiopathology of many disorders. Due to the existence of foldable microstructures called the intestinal villi and microvilli, the gut has a remarkably large surface area. Biological analysis-capable lab-on-a-chip devices have been made possible by recent developments in microfabrication methods and microfluidics. The intestines contain vital microbes that support digestion, immunological control, and defense against invading pathogens (Donaldson et al. 2015). The particular hypoxic environment in the intestines allows these mutualistic microbes to thrive. The most common material utilized for OoCs systems is polydimethylsiloxane (PDMS) which offers a non-toxic, gas-permeable, biologically inert, and surface with minimal adhesion to enable adequate O_2 and CO_2 exchange to their cells (Mata et al. 2005), but gas permeation is not ideal for creating the hypoxic environment seen in the intestines. The small intestine, in particular, acts as a barrier to orally delivered medications; thus it's crucial to know how it works during drug development. Kimura et al. (2008) developed a miniature intestine-on-a-chip device having an optical detecting mechanism to test this function (Fig. 14.4f). Small intestine model (Caco-2) cells were cultivated in two distinct compartments separated by a microporous membrane. Protracted monitoring and culture of the cells' polarization transport activities were used to assess the device's performance. The cells could be grown for more than 2 weeks, and online fluorescence studies of rhodamine 123 monolayer transport were successful; however, there was no physiological cell function. Kim and Ingber (2013) suggested a gut-on-a-chip system that allows Caco-2 cells to be subjected to stimuli like shear stress and cyclic mechanical strain to imitate peristalsis-like movements. Experts claim that under these physiological conditions, cells produced using the device are repurposed to naturally go through small intestinal cell differentiation and 3D villus morphogenesis. This apparatus was utilized to co-culture numerous commensal microorganisms in intestinal ECs and investigate the associated biological phenomena to duplicate more precise physiological circumstances. The outcomes concluded sensors in the system showed that hypoxic culture conditions can be sustained and the gut-on-chip device may be employed to examine intestinal pathophysiology and unravel ailment causes in vitro after demonstrating that this in vitro setting reproduced results from previous animal and human investigations.

(e) *Body-on-a-chip*

Humans are made up of tissues and organs that perform many biological functions and may be thought of as a complicated setting. As previously stated, it is challenging to anticipate interfaces amid tissues and organs via traditional in vitro cell culture methodologies; hence animal experiments are required to predict pharmacokinetics. Microfluidic systems comprising the activities of multi-tissues and multi-organs, dubbed "body- or human-on-a-chip," have been suggested in the OoC research field. Several tools may be employed to track ongoing or connected pharmacokinetic activities like ADME of multiple drug delivery pathways, and the information gathered could be utilized to build computational equations for anticipating the efficacy of medications. Since the 2000s, pioneers have led the globe in body-on-a-chip investigations. They created the micro cell culture analogue (μCCA), a device with numerous organ chambers, and co-cultured diverse organ-derived cells on it (Sung and Shuler 2009; Sung et al. 2014). These techniques were used to study organ interconnections utilizing cell-based tests employing tegafur, an anticancer medication. Their findings demonstrated that complicated biological reactions to a dosage scenario including oral or intravenous delivery, which had previously been explored using animal experiments, may be replicated using body-on-a-chip technology. Imura et al. (2010) created a device that includes absorption-related processes in the small intestine as well as metabolic functions in the liver (Fig. 14.5a). They demonstrated disparities in anticancer activity by employing medicines with varying therapeutic mechanisms and intestinal absorption rates using their technology. By incorporating a dialysis membrane into a system, they were also able to integrate renal excretion processes. Even though these systems were able to simulate various organ interactions in vitro, each biological parameter recorded that was not pathological (Kimura et al. 2015) built a device that replicates several physiological factors such as blood flow rate and organ volume ratios on the system (Fig. 14.5b) to achieve accurate pharmacokinetic predictions. This technology was utilized to investigate the efficacy of anticancer medications and proved that microfluidics devices may reliably restrict intestinal barrier absorption and provide pharmacological advantages owing to liver metabolism. We must keep in mind that these systems cannot replicate all biological reactions. According to Shuler's study, the importance of this device is not only to develop a smart minuscule human body but also to identify undiscovered reactions that can only be detected in instantaneous interfaces between organs. The information gathered by these sensors should help enhance the accuracy of mathematical models. To put it another way, pharmacokinetic models must be combined with this equipment to forecast unknown processes or events. By permitting the introduction of mixtures of cells and substances, the devices described here could be used in a variety of pharmacological toxicity tests, and a statistical centered on the testing data obtained with these devices could be in practical use as a pharmacokinetic prediction evaluation system. Certainly, Sung et al. (2010) presented a strategy for predicting cell behavior after anticancer drug exposure that combined data

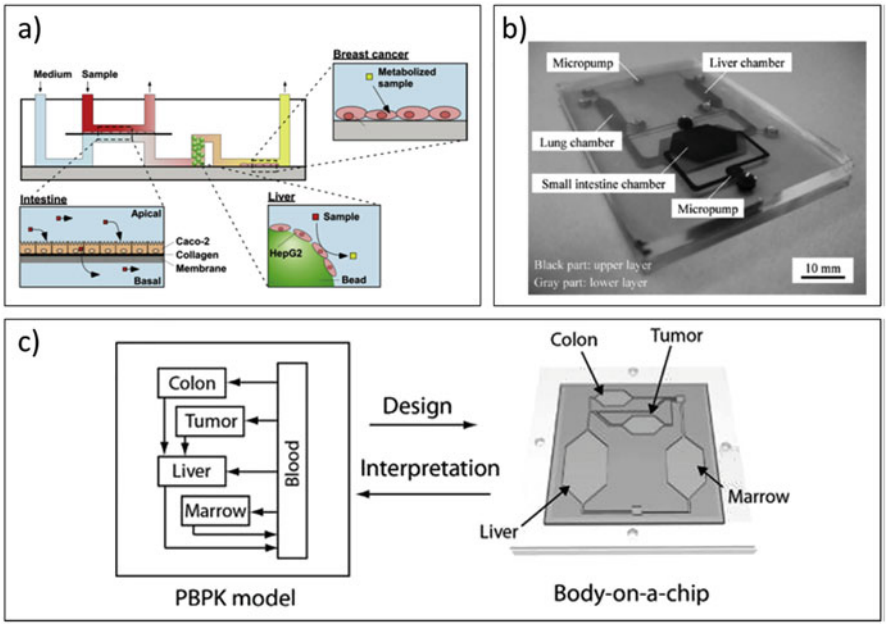

Fig. 14.5 Body-on-a-chip devices: (**a**) Small intestine and liver models are included in the body-on-a-chip system. The American Chemical Society (2010) owns the copyright to this image. (**b**) A device that measures a variety of physiological parameters. The Society for Laboratory Automation and Screening (2014) owns the copyright to this image (**c**) The PBPK modeling principle was used to design the body-on-a-chip technology. The Society for Experimental Biology and Medicine (2014) owns the copyright to this image

from mCCA with mathematical models to predict pharmacokinetics-pharmacodynamics behavior. As a consequence, study demonstrated that a body-on-a-chip technique may be combined with a statistics modeling method (Fig. 14.5c).

14.3.1 Mimicking TME Using Microfluidic Devices

Cancer is a multifaceted and diverse malignancy driven by the stroma's epigenetic, genetic, and cellular signaling. Three features of the TME are critical: (1) Cancer cells engage with stroma in the peri-necrotic niche to avoid detection by the immune system and to take on invasive and migrating characteristics that allow them to spread to distant organs. Due to a growth in tumor bulk, cancer cells' metabolic status is altered under hypoxia and ischemia (Brown and Wilson 2004; Byrne et al. 2014). Cancer cells are more likely to survive in harsh environments and develop metabolic resistance to many cancer treatments in a necrotic microenvironment that is significant in acidity, poor in oxygen and nutrients, and heterogeneous. (2) The tumor associated stroma in the perivascular forte cause new vasculature to develop in

response to nutrition (Charles and Holland 2010). As opposed to normal vasculature, tumor vasculatures are frequently immature and permeable. The perivascular and metastatic niches are both intertwined (Ren et al. 2015). Due to new vasculature, cancer cells detach toward circulating tumor cells and tumor-initiating initiating cells amid them might proliferate into secondary metastases when implanted in remote regions. (3) Cancerous cells engage in stromal interactions to avoid immune detection and take on invasive and migrating characteristics. A metastatic cascade is a complex sequential procedure that tumor cells go through. Recent research has suggested that tumor beginning cells, also known as cancer stem cells, are a small subset of CTCs. This suggests that the TMEs are essential for cancer stem cells to establish themselves in multiple organs and develop new metastases (Psaila and Lyden 2009).

14.3.2 Modeling Hypoxia and Necrosis

Hypoxia influences tumorigenesis and therapy tolerance (Abou Khouzam et al. 2021). A variation amid hyper-proliferative cancer cell proliferation, nourishment, and the vasculature gas supply induces ischemia in the surrounding tissue when the main tumor develops and its hyper-proliferating region grows (Lugano et al. 2020). The perivascular place, in part due to hypoxia, induces new vasculature to transport additional nutrients and gas. The new vasculature, on the other hand, is frequently aberrant and fails to compensate for the nutritional deficiency. The effects of chronic hypoxia in the tumor include the selection of survival cancer cell genotypes, metabolic shifts toward anaerobic glycolysis, an increase in pro-survival gene expressions, epithelial-mesenchymal transition (EMT), and treatment resilience (Martin et al. 2016). The chip material gas permeability in microfluidics allows for the creation of a hypoxic microenvironment to imitate the peri-necrotic area. For the fabrication of delicate lithographic microfluidic chips, polydimethylsiloxane (PDMS), a biocompatible silicone rubber having excellent gas permeability, has become a prominent material (Miranda et al. 2022). A low oxygen environment or an oxygen gradient can be induced by the flow of various gases, oxygen scavengers, or gas-equilibrated liquids in microfluidic networks. The gas environment can also be controlled by employing a poor gas-permeable thermoplastic for the microfluidic chip or by embedding a thin thermoplastic layer inside the chip (Chang et al. 2014). Zhang et al. (2015) employed SUM159 breast carcinoma cells on two-dimensional microfluidic devices to show enhanced mesenchymal migration and lactate generation amid hypoxia circumstances. Cell migration is aided by the acidic microenvironment created by metabolic reprogramming. Environmental acidification can slow cancer cell migration while also improving the efficacy of CSF-1R, HIF-1, and CCR4 therapies. Cell embedding hydrogel models may also be used to investigate the intervention of malignant cells to hypoxic conditions in 3D. Xu et al. (2015) found that hypoxia inhibited the growth and invasion of glioblastoma U87MG cells. Normoxia gas was pumped into one control channel close to the PANC-1, a pancreatic carcinoma cell, while hypoxia gas was pumped into other.

Microfluidic platforms were also employed to investigate the kinetic rate of a necrosis core formation in a 3D cell-embedded hydrogel tumor model. Over 6 days, Ayuso et al. (2016) used a 3D cell embedding hydrogel system to monitor the kinetic generation of necrosis cores in HCT-116 colon cancer and U-251MG glioma cells. Additionally, in situ on-chip studies of dynamic changes in glucose and oxygen concentrations, apoptosis, cell proliferation, formation of ROS, and therapeutic response are all possible. A significant strategic path is the development of more sophisticated microdevices to replicate various microenvironments for comprehensive kinetic studies of signaling cross talks among different microenvironments, such as elucidating the interrelatedness between necrosis and neo-angiogenesis in the cross talk of the peri-necrotic and perivascular area. Validating the in vitro necrotic tumor model to tumor lysis and including stroma to explore tumor-stromal cell interaction has indeed been difficult in the peri-necrotic niche. Tumor lysis is defined as the abrupt death of huge numbers of cells, resulting in metabolic abnormalities and the development of tumor lysis syndrome (TLS). To overcome these technological hurdles, new microdevice designs including (bio)sensors and active aerodynamics such as microvalves are required.

14.4 Application of Tumor-on-a-Chip

Tumor chips have been filled with patient-specific cells for targeted therapies, tailored for tumor metastases research such as tumor cell extravasation and micrometastasis formation, and arrayed for high-throughput screening applications (Bray et al. 2019). Organotypic tumor chips that can replicate complicated organ-level patterns of cancer development, spread, and therapy response seen in patients are rapidly becoming available. Multi-MPS were created to research drug pharmacokinetics (PK), pharmacodynamics (PD), and toxicity in more depth. Tumor chip models, in particular, enable innovative manipulation and closely managed real-time research of dynamic interactions between stromal, tumor cells, and TME, which is more difficult to achieve by traditional animal models and tissue culture.

14.4.1 Multiplexed Drug Screening

Among the most crucial steps for medication/lead candidates to undergo clinical trials that are based on pharmacological activity is drug screening. Preclinical testing is required after bio- and chemical synthesis to completely comprehend the efficacy, toxicity, and ADME (absorption, distribution, metabolism, and elimination) characteristics of the drug candidates. Organoids or organs-on-a-chip, which uses 3D cell culture methods to mimic human organ-specific microenvironments in vitro, have shown significant promise in overcoming the constraints of traditional animal models. Recent advancements in microfluidics technology have made high-throughput drug screening more economical and feasible. Microfluidic chips need a substantially smaller sample volume, in addition to having a cheaper cost and

higher processing speed. These chips may also be tailored to track the impact of anticancer medications on a variety of factors, such as cell migration. Organ-on-a-chip technologies and organoid models have arisen to better imitate the TME with human cells and enhance efficiency with higher throughputs for more effective translational cancer therapy research. Because tumor heterogeneity makes a "one-size-fits-all" approach to cancer treatment ineffective, substantial attempts have been undertaken to personalize cancer medicines for Individual or tiered healthcare organizations. Ex vivo screening on solid tumor samples and biopsies from patients has become a supplemental technique with the ability to accurately determine the most effective treatment plans. While next-generation sequencing (NGS) is employed to direct these strategies, ex vivo screening on solid patient tumor samples and biopsy has arisen as a complementing method, with the ability to establish ideal therapeutic regimens. To achieve this, several groups have been developing tumor-on-chip platforms that enable the cultivation of tumor samples obtained from patients as well as the assessment of their therapeutic response. Mazzocchi et al. (2018) exhibited abiding culturing of viable 3D spheroids in six chambers, followed by pharmacological testing. A peristaltic pump was employed to keep fluid or drug-infused media flowing continuously to the spheroids. The treatment reactions of patients were also replicated in the on-chip tumor spheroids, indicating that this method might be useful for precision medicine. Lim et al. (2018) cultivated size-controllable spheroids of breast tumor cells obtained from biopsies and treated them with the same chemotherapy medicines that the patients received. They also stated that their spheroids' medication responses were identical to the patients'.

14.4.2 Transport and Delivery of Nanoparticles

Because of tumor invasion, progression, and metastasis, the hypoxic TME is categorized by muddled vasculature and fast tumor growth. Hypoxic conditions reduce the effectiveness of cancer treatments such as chemotherapy, radiation, phototherapy, and immunotherapy, which has major consequences such as tumor recurrence and high mortality rate. Fabrication of efficient nanomaterials to alleviate hypoxic tumors has recently been the focus of research. The application of formulations with nanocarriers to administer precision medications and diagnostic agents to tumor locations has made tremendous progress in the burgeoning field of nanomedicines. By delivering enhanced coverage for the variables' bioactive components in the serum-rich setting, extending bloodstream circulation times, reducing side effects, increasing permeation and retainment effects, improving tumor-targeting effectiveness, increasing controlled release, and potentially integrating stimuli sensitivity for on-demand treatment, nanomedicines offer clear advantages over systemic therapy of free compounds (Blanco et al. 2015). Nanomaterials can be developed with characteristics that make administration smoother in a multitude range of settings such as enhancing oxygen-dependent tumor therapy that raises oxygen levels in tumors using oxygen-carrying nanomaterials (Khan et al. 2019), oxygen-generating nanomaterials (such as

nanozymes) (Liang and Yan 2019), and oxygen-economizing nanomaterials (Yu et al. 2019) and reduced oxygen dependence for tumor hypoxia treatment using gas-generating nanomaterials (Deng et al. 2018) and radical-generating nanomaterials (Lv et al. 2018). Due to the complexity of hypoxia tumors, a lot of research has also concentrated on hypoxic tumor treatment methods using nanomaterials that are less oxygen-dependent. Farokhzad and groups employed a simple microfluidic device with cancer cells monolayer on the bottom to explore the dynamic interactions of perfused particles with cancer cells (Farokhzad et al. 2005). PC3 and LNCaP a prostate cancer cell lines, grown in a microfluidic environment, revealed distinct uptake behaviors for polylactic acid (PLA) particles functionalized with and without aptamers recognizing PSA in a size- and flow-dependent way (PSMA). 3D configurations of interactions between NPs and malignant cells were established. Lee et al. (2009) employed inverse opal-structured hydrogel scaffolds to generate spheroids of HepG2 hepatocellular cancer cells. The vitality of the pristine spheroids was significant, and they had intact cell junctions. When these liver cancer organoids were treated with semiconductor CdTe NPs, their vitality was diminished, and the surface-located cell activities were compromised. Recent advancements in microfluidic on-chip tumor models have further integrated the benefits demonstrated in the first two examples. These advancements include the ability to replicate not only the systematic mobility of NPs with various parameters, such as size, shape, and surface characterizations but also the configuration of the pertinent frameworks.

14.4.3 Microfluidic Devices for the Analysis of Transcriptomic and Proteomic Factor

Droplets in a microfluidic device can be used to do transcription analysis at the single-cell level. Zhang et al. (2012) created a microfluidic system that used agarose droplets to do single-copy RT-PCR utilizing both sample and RT-PCR reagents. Significant variations in the expression of the EpCAM cancer diagnostic gene across different kinds of cancer cells were used to verify the platform. Hayes et al. (2016) employed microfluidic droplets in a separate investigation to assess ECM gene expression levels in colorectal cancer patient samples to see whether there was a link between metastatic potential and differential expression.

Quantitative characterization of cancer proteomics has the efficacy to revolutionize not just molecular diagnostics but also the discovery of new novel therapies. Using tissue samples and cultivated cell lines with as few as 1000 cells, Sun et al. (2010) designed a microfluidic cytometry imaging system that can do quantitative single-cell proteome analysis. Its therapeutic use was validated by examining four proteins involved in the mTOR signaling pathway in human brain tumor samples and contrasting the outcomes with those obtained via well-known clinical immunohistochemistry (IHC) procedures. On a broader scale, Xu et al. (2016) devised a biomimetic multi-organ microfluidic chip to examine changes in RANKL, CXCR4, and other markers' expression levels in the multiple "remote organs" after tumor cell invasion.

14.5 Challenges and Future Prospects

Tumor hypoxia emerges as a consequence of uncontrolled cell growth, altered metabolism, and aberrant tumor blood vessels, which diminishes the transfer of oxygen and nutrients. One of the key characteristics of solid tumors is hypoxia, which has been linked to cancer patients' poor prognoses. Three factors are significant inside the TME such as the following: (a) cancer cells' metabolic changes in the peri-necrotic niche are further driven by hypoxia in the necrotic core of initial tumor tissue, (b) the tumor and stroma in the perivascular area cause new vasculature to develop in response to nutrition, and (c) in the metastatic niche, cancer cells engage in interactions with stroma to circumvent the immune system and take on invasive and migrating behaviors to spread to distant regions. Apart from that, several crucial concerns should also be addressed to copiously exploit the efficacy of ToC techniques. For example, how to use such a system to model the mechanism of intra- and extravasation; how to enable tumor-associated tissues to sophisticated chip concerning self-organization; and if the construction of a tumor-on-a-chip system that enables a tumor to develop on a chip can be done with the bare minimum amount of components. To comprehend the augmented permeability and retention impact, along with the dormancy phenotype, alternative methodologies to statistically analyze tumor-matrix interactions along with matrix remodeling and growth factors must be established. Using a ToC approach, it's critical to depict the tumor's heterogeneity and progress. Depending upon biological and medical significance, ToC technologies possess varying unique designs and degrees of sophistication. Oversimplified or overcomplicated systems must be avoided, and proper complexity must be driven by demand. As a result, one or more of the following concerns may be included in a ToC system: (a) transport of culture media, cytotoxicity, elimination of waste, and cellular metabolism; (b) kinematic and physiological constraints, such as composite stiffness and inhomogeneity, adhesion molecules, and flow rates; (c) concentration gradients and physiological concentration levels of circulatory components; (d) structural features, such as 3D tumor constructs and microfluidics models; and (e) cell sources and types, such as culture, stromal, patient, stem, or progenitor cells. To precisely capture crucial characteristics of a tumor, it is essential to examine metastasis sites, recapitulate cancer-immune cell interactions, and conduct real-time, on-chip monitoring of key biochemical and biophysical variables. While premade tumor scaffolds offer certain benefits, self-organized tumor structures resulting from cell-cell interaction may be a superior paradigm for ToC platforms. For various tumors and/or to answer different questions, multiple tumor-on-a-chip models with varied characteristics and complexity are likely to be required.

The innovative ToC paradigm is effective in significantly altering oncology and cancer biology. Nevertheless, there are stumbling barriers in the creation of technology, such as design, optimization, analysis, and validation. Material selection determines the integrity of device qualities such as suitability, biocompatibility, and mechanical characteristics. The majority of the devices had been constructed on PDMS-based materials that have shown to be excellent for studying biological

phenomena but have serious drawbacks when employed with hydrophobic medicines. Alternative printable and moldable substitutes must be discovered to bypass this restriction, like off-stoichiometry epoxy resin, thiol-enes, and perfluorinated polymers. Integrating microdevices printing specialists with polymer and material scientists will be required for systematic modification and automation of chemical and physical parameters within the microfluidic device system. When it comes to device scalability, the creation of sound manufacturing practices, material selection, and user operability are the most important considerations. Furthermore, the shelf-life and sustainability of ToC devices must be determined. The ToC device has significant benefits of accurately influencing physio-chemical characteristics in the TME, co-culturing stromal cells with tumors, offering an optical screen for on-time microscopy assertion of molecular and subcellular mechanisms, and incorporating biosensors for detection and quantification.

14.6 Conclusion

The tumor-on-a-chip device might be more advantageous to animal xenograft models in terms of physio-chemical variances, biological diversity, affordability, and ease of statistical analysis. Altogether, to fully leverage the promise of ToC technology, material scientists, biophysicists, biomedical engineers, cell biologists, and oncologists must work together to build and optimize these systems for cancer research, drug discovery, and clinical application. Although proof-of-concept reports have demonstrated the efficiency of ToC systems for tumor research, major challenges remain in implementing the device in clinical settings, together with the authenticity of device implementations by correlating with traditional in vivo tumor models and data obtained via ToC technology and clinical tumor samples.

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Imaging the Hypoxic Tumor Microenvironment in Cancer Models

15

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Abstract

In hypoxic condition oxygen supply is disrupted, and it happens in myocardial infarction, stroke, or tumorous growth. If cells in normal tissue lose their oxygen supply abruptly, they commonly die, while in the tumor, cells become hypoxic over time due to the fast spread of cancer cells that led to deficiency in blood vessels which cause nutrient and O_2 deprivation. Tumor cells acclimatize to these changes by augmenting the production of multiple proteins that enable them to survive. These proteins suppress apoptosis, increase aggressiveness by promoting metastatic spread, switch metabolism from a mitochondria-dependent pathway to glycolysis, and promote the formation of new vasculature. Because of these pathophysiological consequences, patients with hypoxic tumors often have poor prognoses and treatment outcomes. To assess this hypoxic environment thereby improve the treatment efficacies, a number of molecular imaging systems have been progressed for hypoxia diagnostics in patients; these include invasive procedures like the use of oxygen polarographic electrode which measures tissue

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oxygen and phosphorescence quenching, noninvasive techniques such as magnetic resonance imaging (MRI) which detects oxygenation or lactate production, photoacoustic tomography (PAT) which detects sound waves produced by the absorption of light and provides oxygen saturation curve, radionuclide imaging PET (positron emission tomography), SPECT (single-photon emission computed tomography), and so on. This chapter summarizes and discusses currently accessible techniques that can be successfully used for imaging tumors along with the advantages and disadvantages. In addition, a brief insight into the mechanism of hypoxia in tumors has been presented.

Keywords

Tumor imaging · Hypoxia · Tumor microenvironment · HIF · VEGF

15.1 Introduction

Hypoxia is a physiological state marked by insufficient oxygen supply to tissue cells, where hypo refers to "less than the norm" (normal) and oxia refers to "oxygenation." In tumors, abnormal growth due to uncontrolled proliferation of cells and changes in metabolism lead to deprivation of oxygen demand from the preexisting blood vessels making them more susceptible to hypoxia, especially in the case of malignant solid tumors. In order to adjust to altered conditions, hypoxia-induced adaptive responses include suppression of apoptosis or autophagy and enhancement of angiogenesis. Vasculogenesis and increase in aggressiveness of the tumor, alteration in DNA repair pathways, and change in cellular metabolism also reduce antitumor responses (Ranjan et al. 2020; Parihar et al. 2021a, 2022a, b; Munjal et al. 2022). Besides these cellular adaptive responses influenced by hypoxia, reduced oxygenation in tumor tissue induces chemoresistance by altering drug transport and cellular absorption. The lack of oxygen essential led to the enhancement of cytotoxicity of several chemotherapeutics. Hypoxia also induces resistance to radiation therapy. Thus, knowing the extent and degree of hypoxia is imperative before the instigation of medication. A variety of approaches are being developed to evaluate hypoxia based on measuring oxygen levels in tissues directly or indirectly. Direct techniques include polarographic needle-based electrodes, near-infrared spectroscopy, phosphorescence imaging, blood oxygen-level-dependent imaging, electron paramagnetic resonance imaging, and magnetic resonance imaging. Indirect or noninvasive techniques include the measurement of immunolabeled exogenous and endogenous hypoxia markers, which can provide parameters related to oxygenation. Clinicians have been confronted to develop different treatment strategies. As a result, clinicians designed O_2 concentration measuring devices. Polarographic electrodes directly measure O_2 levels with needle-type probes, both optical and electrochemical. However, various problems obstruct their practical use, and better approaches for correctly detecting tumor hypoxia are entailed for hypoxia prognosis and therapeutic development. Indirect, noninvasive techniques, such as immunolabeling

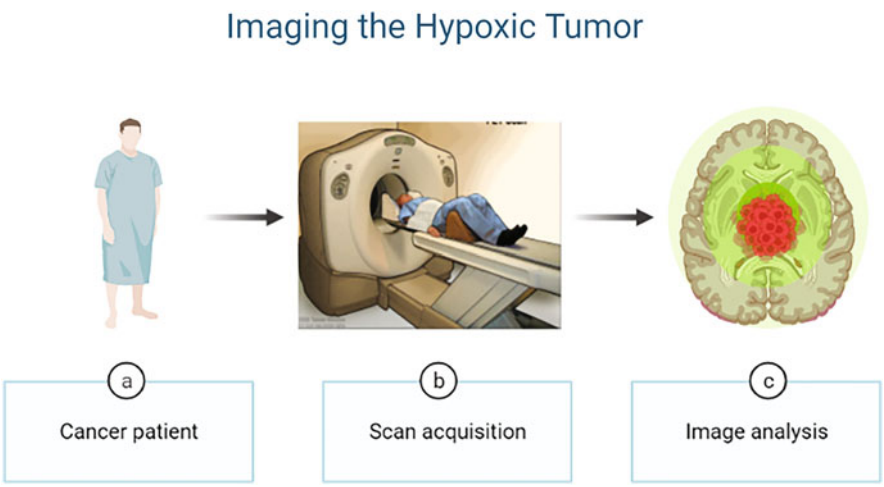

Fig. 15.1 General steps involved in imaging hypoxia tumor. Created with BioRender.com

endogenous or exogenous markers, have also been improved. Researchers have also used fluorescent, phosphorescent, and luminescent reporters to give real-time measurements of O_2 in living cells or tumors. Some modalities such as MRI (magnetic resonance imaging) and PET (positron emission tomography) are often used in medical imaging. Although several techniques for detecting tumor hypoxia use different pathways, very few modalities are licensed for use in clinical practice. The assessment of the extent of tumor hypoxia is a crucial step for the validation and development of treatment targeting hypoxia that will eventually be used in general clinical practice (Walsh et al. 2014; Godet et al. 2022). The general steps involved in imaging hypoxia tumors are shown in Fig. 15.1. The present chapter deals with the tools and techniques available for the measurement of tumor hypoxia along with their pros and cons.

15.2 Mechanism of Hypoxia in Tumors

Hypoxia is a pathological state that is characterized by a low-oxygen tension state. Under ordinary conditions, cells within developing tissue need to be supplied with oxygen. Although the oxygen supply is carefully controlled, there might not always be enough oxygen available. For instance, in a rapidly growing tumor, extreme hypoxia may develop. The core of the tumor has an insufficient supply of oxygen and nutrients which causes cells to become necrotic, while in the outer areas, cells live. Diffusion may provide oxygen to a small tumor with a diameter of lesser than 1 millimeter. The presence of oxygen corresponds to normal tissues. If tumor development persists, the percentage of oxygen in the central area of the tumor drops. When the tumor expands further, the O_2 can drop to below 0.02%, and the tumor cell experiences severe hypoxia. To avoid severe hypoxia and necrosis, tumor

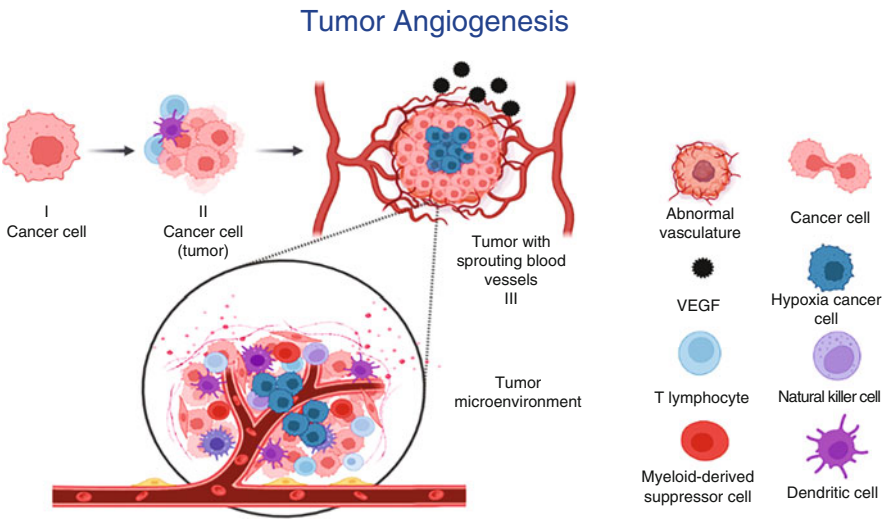

Fig. 15.2 The natural killer cell (NK cell) detects molecules related to stress on damaged cells. Dendritic cells (DC) turn on $CD8^+$ T cells, which then send tumor-corresponding antigens using their receptor. When NK cells and CD8+ T cells are activated, they let out granzymes and perforin. They poke holes in the membrane of the tumor cells, which makes them die through apoptosis, as shown in step II. However, when the tumor progresses, it may not display the chemicals that the immune cells recognize. Additionally, tumors can entice immune cells that inhibit the action of other immune cells, thereby promoting tumor development. These immunosuppressive cells include Tregs and a certain type of myeloid cells; therefore the tumor microenvironment consists of two opposing immune responses: one side of the immune system is attacking the tumor, while the other is helping it to grow, as shown in step III. The image is created with BioRender.com

may induce the growth of new blood vessels. This sprouting of new vessels from existing blood vessels is called angiogenesis (Weis and Cheresh 2011). The process starts when an small vessel of endothelial cell is activated by an angiogenic stimulus via vascular endothelial growth factor (VEGF) (Melincovici et al. 2018) which is responsible for capillaries sprouting as shown in Fig. 15.2 (Melincovici et al. 2018).

Alternatively, tumor cells may invade surrounding normoxic tissue areas. Angiogenesis, invasive tumor growth, and other adaptive reaction are regulated by the hypoxia-inducible factor, abbreviated HIF. It is a transcription factor consisting of alpha- and beta-subunits. The dimerization of one of the three distinct α-subunits with one of the two different β-subunits occurs. In analogy to the α-subunit, the β-subunit is not receptive to oxygen. Among the α-subunit, the function of HIF-1α and HIF-2α is most comprehended. The domain of the HIF-1α consists of an N-terminal basic helix loop helix (bHLH) motif which correlates with DNA. Two distinct transactivation domains can be found close to the C-terminal. These domains regulate how the HIF-1α gene is transcribed into mRNA. The helix domain, in addition to the helix loop, is required in dimerization with the HIFβ-subunit. The arginine residue (N^{803}) modulates the transcriptional activity, and the proline residues (p^{402} and p^{564}) are essential for protein stability. The HIF-2α contains

domains similar to those in HIF-1α. The bHLH domain, which is part of the dimerization motif, is found in the HIF-1β-subunit. The protein contains only one transactivation domain. As mentioned before HIFα-subunit contains prolyl residue which is critical to the stability of the protein. Human tissues primarily contain molecular oxygen levels between 10 and 30 micromolar. Under this normoxic condition, one or both the critical prolyl residue and HIFα proteins will be hydroxylated by a member of the prolyl hydroxylase domain family abbreviated PHD2. PHD2 is the major hydroxylase that hydroxylates HIFα. The PHDs are dioxygenases and contain both molecules of O2, in their product; also prolyl residues are the substrate of PHD dioxygenases. In the oxidative decarboxylation of α-ketoglutarate (an intermediate of the Krebs cycle), one O_2 atom is utilized. PHD dioxygenases required bivalent iron. The process produces hydroxy prolyl residue, carbon dioxide, and succinate, which is also a Krebs cycle intermediate. The von Hippel-Lindau protein, abbreviated pVHL, binds to HIFα when one or both of the essential prolyl residues are hydroxylated (Hayashi et al. 2019). The VHL gene is a tumor suppressor, which implies that it inhibits excessive or uncontrolled cell growth and division. The VHL protein is attached to the ubiquitin ligase complex, which also comprises cullin, elongin, and ring box protein 1 (ECR). An E2 ubiquitin-conjugating enzyme attaches ubiquitin to HIFα. The 26s proteasome breaks down polyubiquitinated HIFα. The C-terminal transactivation region of the HIFα protein comprises a crucial arginine and prolyl residue. The residue is hydroxylated by a dioxygenase called factor inhibiting HIF-1α or FIH-1. Similar to the prolyl hydroxylase domain, the FIH-1 contains both molecules of O_2, in its product. The oxidative decarboxylation of alpha-ketoglutarate utilizes a single oxygen atom. The product of the reaction is hydroxy arginine residue, carbon dioxide, and succinate. The hydroxylation of arginine residue inhibits the binding of p300 and CBP transcriptional co-activators. The PHD dioxygenases are unable to hydroxylate HIFα proteins in low-oxygen environments. HIFα-subunit along with a HIFβ-subunit binds to consensus sequences in the DNA. The p300 and CBP co-activator bind to HIF-alpha. The complex activates the transcription of the target gene (Burslem et al. 2017; Manuelli et al. 2021). Steps involved in both hypoxic and normoxic conditions are compared in Fig. 15.3.

15.3 Approaches for Imaging Tumor Hypoxia

Researchers are developing many approaches to measure hypoxia within tumors to overcome hypoxia-induced problems and to improve the therapeutic management of cancer. For assessing tumor hypoxia, reliable, noninvasive methods are desirable, so that patients can be selected for therapeutic management according to their needs. Various techniques have been anticipated to assess hypoxia in tumors based on direct or indirect measurements of oxygen concentrations. Tools and techniques which are currently available or under progress for the detection of tumor hypoxia are enlisted in Table 15.1. These are divided into three categories: methods that directly measure oxygen concentration, i.e., invasive methods, approaches that

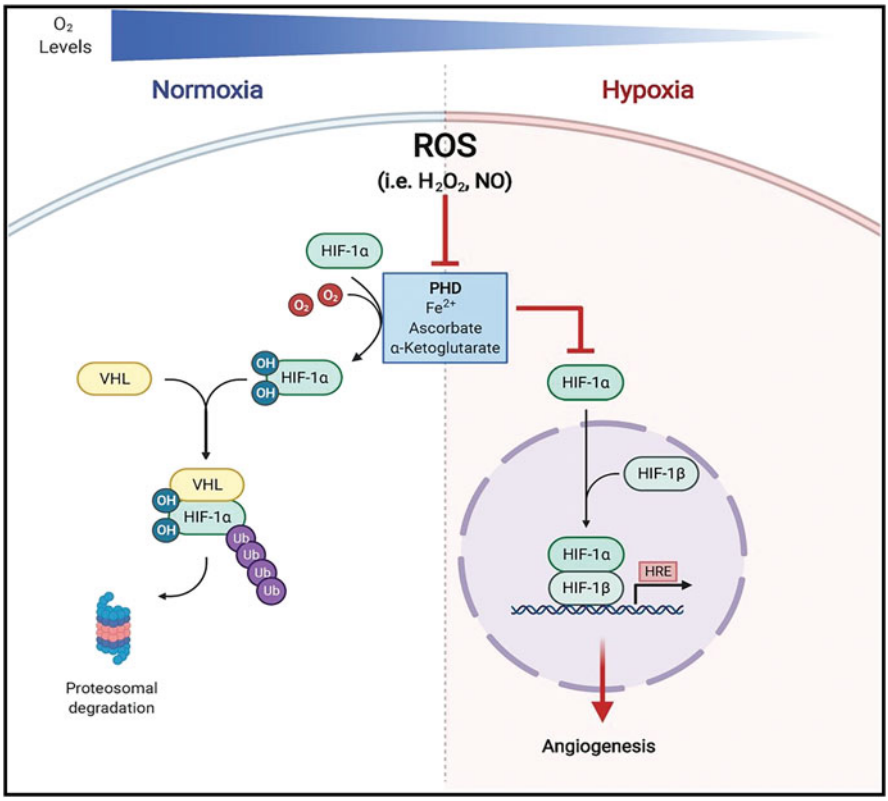

Fig. 15.3 The HIF-1 signaling pathway is represented schematically in normoxic and hypoxic environments. Under normal conditions (left side), PHD hydroxylates HIF-1α, causing it to interact with VHL. In hypoxia (right side), PHD activity is prohibited, preventing HIF-1α breakdown due to a deficiency of oxygen and high amounts of ROS (including H_2O_2 and NO). Consequently, HIF-1α gathers in the cytoplasm before translocating to the nucleus and making a transcriptionally active HIF-1 complex with HIF-1β. The HIF-1 complex detects and interacts with the hypoxia response element (HRE) sequence, increasing the transcription of HIF-1 targets, comprising those implicated in angiogenesis (reproduced with permission from (Manuelli et al. 2021))

report on physiologic processes involving oxygen molecules, and noninvasive methods that examine endogenous marker expression in response to hypoxia (Sun et al. 2011).

15.3.1 Invasive Approaches

A medical procedure in which the body is penetrated, usually by cutting or puncturing the skin or inserting equipment, is the invasive approach. Different types of invasive approaches for tumor imaging are discussed below.

Table 15.1 Methods currently available or under development for the detection of tumor hypoxia

Sn. no.	Methods	Advantages	Disadvantages	Approved clinical procedure	Ref.
A	Invasive approaches				
1	Oxygen polarographic electrodes	Gold standard technique	Limited sampling capabilities, highly invasive, measurements can't be repeated, appropriate only for tumors that are easily accessible, with the possibility of altering oxygen concentration	Approved	(Chaplin et al. 2011)
2	Phosphorescence quenching	Oxygen sensing is independent of tracer concentration, has an exceptional temporal resolution, and enables real tissue oxygenation	Decay and photobleaching	Europe only	(Kurokawa et al. 2015)
	Endogenous markers associated with hypoxia				
3	Hypoxia-inducible factor-1; carbonic anhydrase IX; glucose transporter 1; osteopontin	Provide primary indication about the cancerous condition	Less specific and the detection limit is moderate	Not approved	(Kudo et al. 2009; Carvalho et al. 2011)
	Noninvasive approaches				
4	MRI-BOLD (blood oxygen-level dependent)	There is no O_2 absorption; higher temporal resolution	Susceptible to disruption; unfavorable for tissue hypoxia	Preclinical	(O'Connor et al. 2019)
5	MRI-TOLD (tissue oxygen-level dependent)	Regardless of perfusion, short acquisition periods, hyperoxic gas challenge, and measurement of hypoxia throughout time	Throughput is relatively low and susceptible to motion artifacts	Preclinical	(Zhang et al. 2014)
6	MRI-fluorine	No oxygen is needed; real-time monitoring	Current clinical equipment is inappropriate	Not approved	(Chapelin et al. 2018)

(continued)

Table 15.1 (continued)

Sn. no.	Methods	Advantages	Disadvantages	Approved clinical procedure	Ref.
7	Near-infrared spectroscopy/ tomography	Authorized by doctors for use as pulse oximetry	Low-light attenuation, restricted tissue penetration, and confined to certain body sections	Approved	(Yu 2012)
8	Photoacoustic tomography (PAT)	Provide 3D tomographic images, high spatial resolution (~60 μm), and tissue penetration depth of ~30 cm	Restricted imaging window	Preclinical	(Zhang et al. 2020)
9	Hypoxia PET imaging	Noninvasive; very sensitive; quantitative; evaluation of the entire tumor volume; spatial mapping of hypoxia; repeated evaluation	Inadequate trace; susceptibility to false-positive findings; uptake in normal tissues; inadequate spatial resolution and tumor background ratio	Approved	(Fleming et al. 2015)
10	SPECT (single-photon emission computed tomography)	Noninvasive, tumor diagnosis on a macroscopic scale, high sensitivity, hypoxia detection throughout time	Resolution is restricted; fewer agents are accessible than with PET; it is difficult to measure hypoxia	Preclinical	(Daimiel 2019)

15.3.1.1 Oxygen Polarographic Electrodes

This method is based on the detection of oxygen molecules by electrochemical reduction using a polarographic electrode and is used extensively for measuring oxygen in both human tumors and animal studies. Although, it is a direct approach to determining tissue oxygen content, this approach is sometimes referred to as the "gold standard." The O_2 assessments entail injecting an electrode in a tumor or invasive lymph node and monitoring O_2 in submillimeter increments from different points per needle track as shown in Fig. 15.4 (Parker et al. 2004; Marland et al. 2020). The more regular the lesion form, the more representative the pO_2 values will be. In most cases, more than a hundred measurements are taken in suitable sites of the lesion, resulting in a composite illustration of the hypoxic condition of the lesion. Although, it is a lengthy procedure, the probes sample a tissue volume of 50–100 cells. Only surface lesions, such as metastatic lymph nodes, are accessible to the polarographic probes and have restricted sampling capacities (Parker et al. 2004).

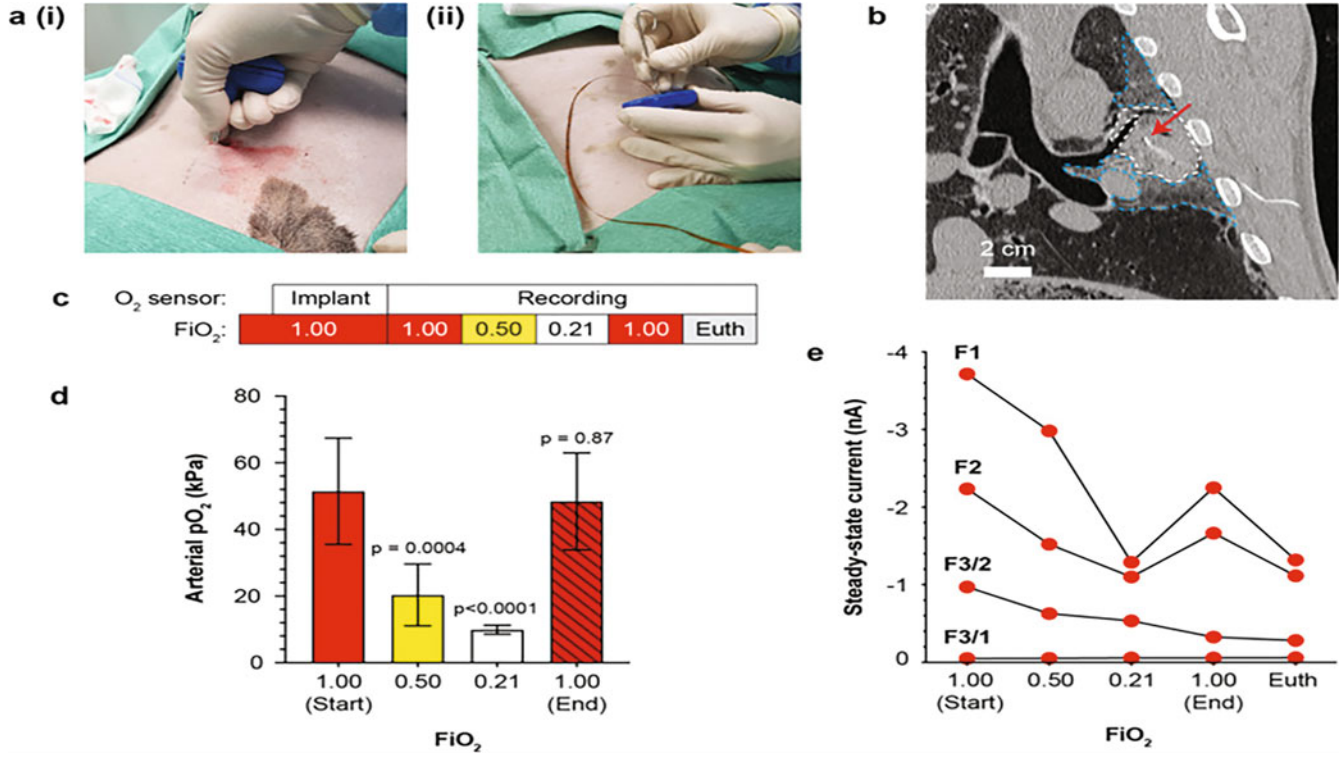

Fig. 15.4 Surgical insertion and sensor operation are conducted on lung tumors. (**a**) Images demonstrating the surgical implantation of a sensor: (1) putting a Jamshidi needle through the chest wall into a lung tumor; (2) insertion of the sensor and lead wire. (**b**) After implantation, a standard thoracic coronal CT scan reveals sensor placement (red arrow) inside tumor tissue. The tumor is encircled by areas that mimic secondary pneumonia or neoplastic foci (blue dashed outline). (**c**) Sensor installation and sequence diagram for FiO2. (**d**) Arterial blood partial oxygen concentration after every FiO_2 step ($n = 4$ repetitions of the experiment with three animals). The error bars depict the standard deviation between repetitions. (**e**) Typical sensor performance at every FiO_2 concentration over the final 5 mins of the FiO_2 stage. Each sensor's output is shown separately. (Reproduced with permission from (Marland et al. 2020))

For deep-seated tumors and to measure overall oxygen status, a polarographic electrode can be used under guidance CT. In addition, the proportion of pO2 tension varies minimally between primary tumors and lymph node metastases; thus node measures can be used as surrogates for the underlying tumor's hypoxic condition. Oxygen electrodes have a number of drawbacks that prevent them from being used in clinical practice on a regular basis. The approach is also quite intrusive, making repeat measurements extremely difficult. It is, however, challenging to create 3D oxygen maps using electrodes. Because the probe cannot distinguish between living and dead tissue, the probe exaggerates hypoxia in necrotic areas. When patients are given halogenated anesthetics (such as halothane), polarographic electrodes perform poorly, resulting in erroneous oxygen readings. In addition, the equipment handling needs a technically experienced operator, and inter-operator variance can be considerable.

15.3.1.2 Phosphorescence Quenching

In this technique, oxygen detection is dependent on the interaction between oxygen molecules and phosphorescence dyes. The dyes release their own light when exposed to a brief flash of light, and the intensity of emission decreases exponentially with the surrounding oxygen concentration (Kurokawa et al. 2015). Stern-Volmer constant tissue oxygenation may be assessed using factors such as the decay rate in the absence of oxygen. The advantage of this technique is oxygen measurements are independent of tracer (dye) concentrations as phosphorescence lifetime is measured instead of signal intensity. The decay rate is translated into tissue oxygenation using pre-calibrated factors such as the decay rate in the oxygen-deprived environment and the Stern-Volmer constant. Because phosphorescence duration rather than signal intensity is examined, the oxygen response is unaffected by tracer amount, unlike other oxygen-sensing systems. This high-temporal-resolution technology enables a real-time tissue oxygenation profile that seems to be challenging to get using conventional techniques. Both molecular reporters and physical needle probes have been used to assess oxygen concentration in vivo (Ziemer et al. 2005).

15.3.2 Endogenous Markers of Hypoxia

More recently, molecules involved in hypoxic response of tumor cells are considered as endogenous markers of hypoxia. Several proteins, including the glucose transporter 1 (GLUT1), carbonic anhydrase 9 (CA 9), and hypoxia-inducible factor-1 (HIF-1), can be detected immunohistochemically in archival pathologic substrate and are briefly explained below.

15.3.2.1 Hypoxia-Inducible Factor

HIF-1α is a transcriptional activator whose activity is controlled by oxygen levels (Ziello et al. 2007). It enhances several metabolic processes targeted at reducing hypoxia's negative impacts. Since it is continuously produced and degraded by

oxidation via oxygen, its deprivation is inhibited under low-oxygen pressure, resulting in elevated protein levels in numerous hypoxic malignancies. HIF-1α translates tumor hypoxia with several target expressions associated to hypoxia (Hu et al. 2003). Both hypoxia and normoxic signaling pathways are involved in activating HIF-1α expression in human cancer. When the phosphoinositide 3 kinase (PI3K), Akt, and mammalian target of rapamycin (mTOR) apoptotic pathways are switched on, the HIF-1 gene shoots up. Mutant forms of phosphatase and tensin homolog (PTEN), VHL, fumarate hydratase, or succinate dehydrogenase have also been shown to stimulate HIF-1 transcription (Schönenberger and Kovacs 2015). Growth factors like insulin growth factors and epidermal growth factor aid to maintain protein stability under normoxic conditions. The deposition of HIF-1α can also be stimulated by the production of mitochondrial ROS which oxidize iron in the active site of PHD, preventing it from hydroxylating HIF-1. HIF-1α expression is associated with reduced disease-specific survival (DSS) for people with colorectal cancer and gynecological cancer; even so, HIF-1α expression has been associated with lower disease-specific survival (DSS) in patients with colorectal cancer, and similar results have been found in patients with gynecological cancer (Imamura et al. 2009). Higher levels of HIF-1α observed in H&NC patients correlate with 5-year DFS in patients who had surgery (Walsh et al. 2014).

15.3.2.2 Carbonic Anhydrase IX

CA-IX (carbonic anhydrase IX) is an enzyme which simulates the reversible modification of bicarbonate anion to CO_2; also when oxygen pressure is less than 20 mmHg, its expression is increased. CA-IX is essential for the acidity of tumors; especially when the oxygen level is low, this can often make it harder for ionizable drugs to function, which is persistent with its function as a pH regulator of tissue. For example, in breast cancer patients receiving doxorubicin treatment, CA-IX expression was observed to be associated with poor progression-free survival (PFS) and overall survival (OS) (Pastorekova and Gillies 2019). Although CA-IX expression in H&NC patients did not have a substantial correlation with pO_2 levels or pimonidazole (PIMO) staining, this advocates that CA-IX expression is associated with factors other than the pO_2 level. Increased amounts of CA-IX protein are attributed to hypoxia in cervix cancers but not in colorectal adenocarcinomas (Pastorekova and Gillies 2019). In addition to its significance in H&NC, CA-IX has also been reported to be a poor factor for survival in non-small cell lung cancer (NSCLC), cervical cancer, and breast cancer. Patients with H&NC having radiotherapy with carbogen and nicotinamide HA are related to CA-IX expression. When a dichotomized cutoff value for low vs high CA-IX expression in sample biopsy was used, high CA-IX expression was associated with higher *LRC* and free from distant metastases. This is a surprising finding and emphasizes the complicated function of CA-IX expression in cancerous tumors.

15.3.2.3 Glucose Transporter-1

GLUT-1 is a membrane protein that aids in the transport of glucose across cell membranes (Navale and Paranjape 2016). This transporter is upregulated to fulfill

the increased glucose requirement of hypoxia cells, which arises from the increasing glycolysis that takes place under low-oxygen conditions. This protein is found in high concentrations in a variety of tumors, and its overexpression is linked to hypoxia in the head, neck, and cervix tumors. Clinical results deteriorate when this protein is expressed in H&NC and bladder cancer (Pezzuto et al. 2020).

15.3.2.4 Osteopontin

Osteopontin (OPN) belongs to the N-linked glycoprotein family of small integrin-binding ligands. It is secreted as a acidic glycoprotein, phosphorylated binds with numerous integrins via its arginine-glycine-aspartic acid (RGD) integrin-binding motif. OPN is displayed in several cells, such as macrophages, endothelial cells, and smooth muscle cells, and is engaged in vascular remodeling, cell adhesion, and immune functioning. Hypoxia increases OPN expression via the activation of Akt and activation of the ras-activated enhancer (RAE) in the OPN promoter. Furthermore, in patients with head and neck malignancies, plasma OPN levels have been observed to compare negatively with pO_2 levels. In stage IV head and neck cancer patients, tumor OPN expression was found to be linearly related to median pO_2 levels. OPN promotes integrin and MMP signaling pathways by binding to tumor cell surface receptors, increasing tumor cell invasion, migration, and adhesion.

15.3.2.5 Pimonidazole

PIMO is a lipophilic exogenous hypoxia marker with the hypoxia-targeting 2-nitroimidazole chemotype. PIMO was initially intended as a radiosensitizer and is found to be more effective than misonidazole (MISO); however, it unsuccessful to validate efficacy in later clinical testing. Currently, PIMO is used as an exogenous hypoxia marker. A few hours before tumor biopsy, PIMO is delivered directly into a vein or orally. Hypoxia in tissue samples can be found with the help of a PIMO metabolite staining kit that uses commercially available antibodies. A high linear correlation ($r2 = 0.81$) was found between the concentration of PIMO and the amount of oxygen in phantoms and animal tumor models. But there was no correlation between the presence of PIMO in needle biopsies and pO2 readings in women with uterine cervical cancer.

15.3.3 Noninvasive Approaches

Although the invasive methods and endogenous markers can offer a somewhat precise evaluation of tumor oxygenation, these techniques are biased and reveal partial evidence in the tumor area. Thus, there has been an increase in curiosity in using noninvasive methods. The following part illustrates several noninvasive techniques.

15.3.3.1 MRI-Based Measurements

MRI is a valuable tool for measuring hypoxia. In this approach, fluorocarbon reporter molecules are injected directly into a tumor; the absolute PO_2 may be

detected. The PO_2 measured by this method is consistent with electrodes (presumably interstitial PO_2). The general MRI/PAI imaging techniques have been shown in Fig. 15.5. The ability to assess regional PO_2 maps concurrently at 50–150 individual places is a significant advantage over the electrode method. Furthermore, once the reporter molecule is present, consecutive PO_2 maps may be created to highlight changes in oxygenation in response to therapies, such as hyperoxic gas breathing or vascular targeting drugs. Because of a shortage of human MRI equipment capable of 19F MRI, 19F oximetry measurements in patients have yet to be undertaken (Krohn et al. 2008). A kind of imaging technique called blood oxygen-level-dependent (BOLD) MRI distinguishes between oxy-Hb and paramagnetic deoxy-Hb. T2*-weighted imaging can identify changes in vascular oxygenation. Its vulnerability to changes in Hb concentration, which can be caused by variations in vascular volume and flow as well as the interconversion of oxy- and deoxyhemoglobin, is one major drawback. Instead of using quantitative measurements, this method provides a qualitative assessment of oxygenation changes (Murata 2007). This method is commonly employed in functional brain mapping, to study variations in blood flow as well as in tumor research. BOLD MRI is especially sensitive to oxygen manipulation therapy wherein hyperoxic gas breathing is used to alleviate hypoxia in conjunction with other therapies.

15.3.3.2 Near-Infrared Spectroscopy/Tomography

Near-infrared spectroscopy (700–900 nm) calculates the Hb/HbO_2 ratio using the absorption band of hemoglobin (Hb) and oxy-hemoglobin (HbO_2). This methodology does not detect oxygen content directly; rather, it translates the Hb/HbO_2 proportion into partial pressure of oxygen by using hemoglobin saturation curves. In clinical applications, one version of this method is routinely utilized for rapid oxygenation analysis using pulse oximetry. Additionally, methods based on the spectroscopic anomalies between Hb and HbO_2 are presented. Diffuse optical tomography, in particular, was utilized to rebuild the 3D oxygen concentration in breast cancer. The technique has minimal tissue invasion and is limited to parts of the body with lower-light diminution (Ali et al. 2004).

15.3.3.3 Photoacoustic Tomography (PAT)

It is an imaging method that provides both functional and anatomical data, while detecting tissue hypoxia noninvasively. PAT is an ultrasonic imaging method that recognizes sound waves produced by light absorption. Thermal elastic expansion occurs inside the tissue as a result of heat produced by the absorbed light. This expansion changes the pressure, and ultrasonic waves travel across the tissue. The ultrasonic source is detected and pinpointed by transducers, resulting in 3D tomographic pictures (Parihar and Dube 2022). PAT measures oxygen content by comparing the endogenous HbO_2 and Hb spectroscopic absorption changes. Derived from their varying response, oxygen saturation (SO_2) curves can give an approximation of how much oxygen is in the blood. PAT is predominantly an ultrasonic method, so it has a high spatial resolution (~60 μm) and can reach about 30 cm into the tissue. One major challenge is that the laser aperture confines the size of the

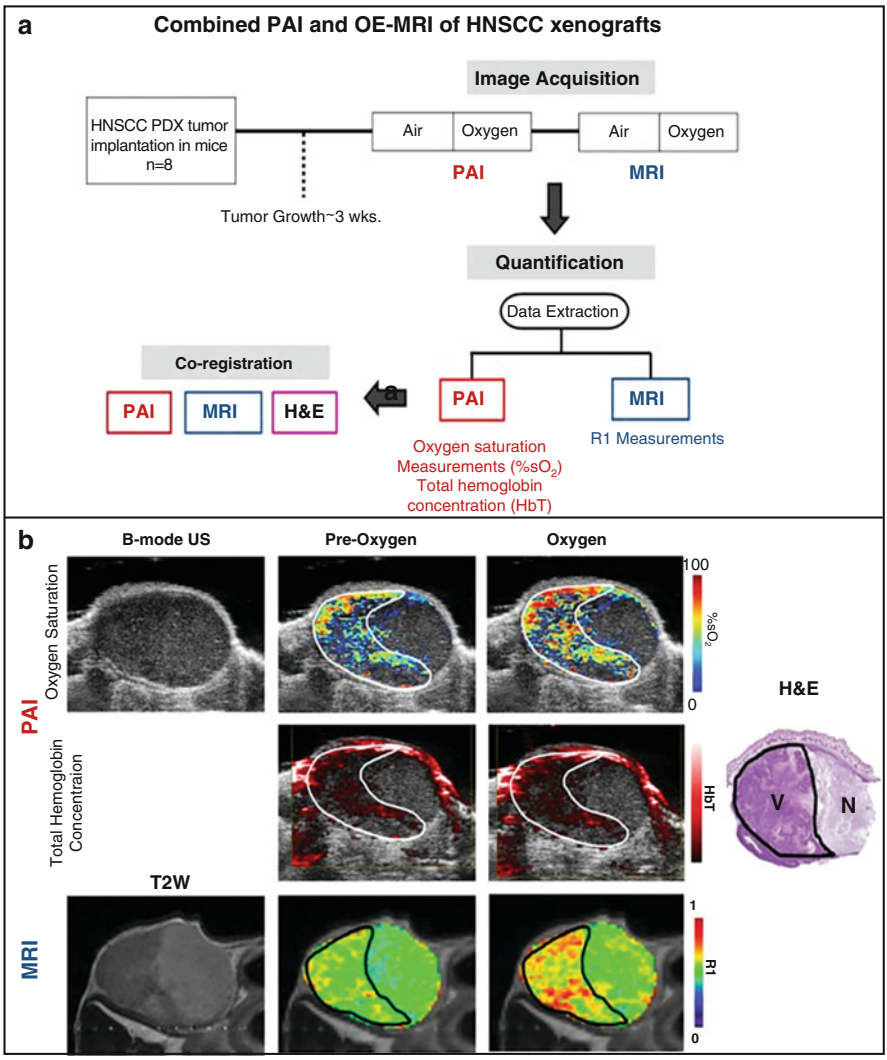

Fig. 15.5 (**a**) Protocol for MRI, histologic correlation of vascular hemodynamics, and oxygen-enhanced PAI (photoacoustic imaging) in HNSCC xenografts. Mice with tumors were given room air for 2 mins, then 100% oxygen for 6 mins (hyperoxia), before being given room air once more. (**b**) First row: B-mode PDX-HNSCC oxygen saturation maps in the US and co-registered PA after exposure to room air (pre-oxygen) and then after oxygen (100%; hyperoxia); hemoglobin maps showing the same tumor's hemoglobin levels before and after an oxygen challenge are shown in the middle row. Last row: the longitudinal relaxation rate (R1) color maps and the axial T2-weighted image of the tumor are shown using the same settings. ((Rich and Seshadri 2016) reprinted with permission)

imaging window. This imaging modality could be used to measure hypoxia because it has a good optical contrast, high structural resolution, and great depth penetration (Xia et al. 2014).

15.3.3.4 Hypoxia PET Imaging

Positron emission tomography (PET) imaging of hypoxia is a noninvasive approach which detects tumor hypoxia using radiolabeled reporters. These tracers are intravenously injected, and their tissue absorption is tracked using a PET camera (Fig. 15.6). However, in hypoxic situations, the lack of oxygen leads to the predominance of a chemically reduced species that localizes inside cells by dechelation or covalent binding to proteins rich in thiols (Xia et al. 2014). These tracers are detected in viable cells but not in dead cells because active enzymes such as cytochromes or nitroreductase are present in living cells to assist the accumulation of radiolabeled metabolites. The absorption of 2-nitroimidazoles and Cu-chelated complexes into hypoxic tissues is sufficient to generate the oxygen saturation necessary for detecting radioresistant hypoxic cells (1% oxygen volume or ~7 mmHg of partial oxygen pressure), making them exceptional hypoxia indicators. The selective demarcation of hypoxic cells is facilitated in vivo by differences in the absorption and washout of normoxic and hypoxic cells. Biomarkers based on PET provide extensive oxygenation info on tumors, and tests may be repeated. PET imaging using these

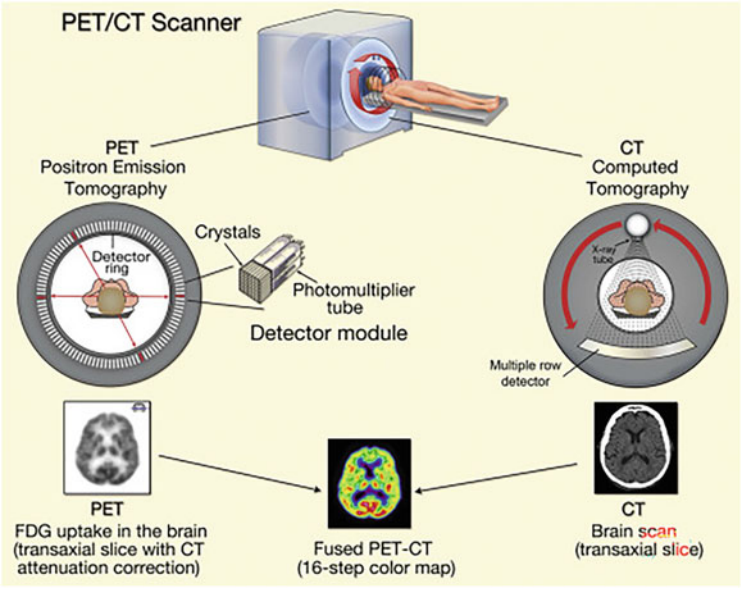

Fig. 15.6 A typical biomedical positron emission tomography (PET) imaging system integrates a cutting-edge PET scanner for molecular imaging. The software and hardware are designed to collect complementing data from a patient bed traveling through both scanners. For PET imaging, the bed slides in gradual increments dependent on the field of view of the PET detectors (usually 16.2 cm), with each acquisition requiring 3–6 mins. (Reproduced with permission from (Lameka et al. 2016))

tracers enables the visualization of the hypoxic condition of the whole tumor and concomitant lesions in metastatic or locally advanced cancer situations, producing a three-dimensional image of hypoxia that electrode- or biopsy-based methods cannot. Due to the relatively low temporal resolution (days between scans), it is not possible to monitor tissue oxygenation in real time. Moreover, since ^{18}F-fluorine has a relatively short half-life (t1/2 = 110 min), the tracer must be produced and examined in a few hours. For PET imaging, these are the tracers that are used:

(a) ^{18}F-fluoromisonidazole. The discovery of 2-nitroimidazoles containing radioactive ^{18}F-fluorine atoms for PET imaging of hypoxia was prompted by radiosensitizers of the same chemotype. The radiosensitizer MISO has been radiolabeled as ^{18}F-FMISO. The predominating PET tracer in this class is ^{18}F-FMISO, which has been widely studied for noninvasively detecting hypoxia in vivo via PET imaging. The in vivo bio-distribution pattern of ^{18}F-FMISO is determined by the fact that it is a modestly lipophilic molecule (partition coefficient = 0.40; log P = −0.40). The average amount of ^{18}F-FMISO excreted in urine by individuals is as little as 3% of the total amount administered. In humans, ^{18}F-FMISO is stable in plasma (92–96% intact at 90 mins post-injection) and is typically excreted in the urine as metabolites.

(b) ^{18}F-fluoroazomycinarabinofuranoside. ^{18}F-fluoroazo-mycinarabinofuranoside (^{18}F-FAZA) is another PET hypoxia imaging agent which is a ribose-containing, hydrophilic (partition coefficient = 1.1) agent with better clearance and hypoxia-targeting characteristics. In preclinical models, ^{18}F-FAZA diffuses into cells quicker than ^{18}F-FMISO and clears from body organs faster than ^{18}F-FMISO. Hepatic metabolism and biliary excretion, as well as urinary excretion, account for the vast majority of the tracer's elimination in humans. Consequently, tracer uptake in the liver, gallbladder, colon, and kidneys is typically moderate to high. While it is hard to determine the extent to which nimorazole mitigated the effects of hypoxia in tumors, the data demonstrate that ^{18}F-FAZA can be utilized to identify patients at risk of failure of treatment.

(c) ^{18}F-EF5 (pentafluorinated etanidazole). It is the third repetition of the fluorinated "EF" etanidazole by-product and an ^{18}F-radiolabeled analog of the exogenous hypoxia marker EF-5. It is extremely lipophilic (partition coefficient = 5.7) and contains many fluorine atoms (146). The brain is one of the physiological organs where ^{18}F-EF5 has a rapid and homogenous distribution, in contrast to other hypoxia tracers which are intended to have poor lipophilicity. Because of its enhanced lipophilicity, ^{18}F-EF5 has a blood half-life that ranges from 7.5 to 10 h, which exceeds the half-life of ^{18}F-EF3 in blood.

(d) ^{18}F-HX4 (^{18}F-flortanidazole). With better water solubility and faster background clearance than ^{18}F-FMISO, a newly discovered tracer from the 2-nitroimidazole family was produced, resulting in enhanced pharmacokinetic and tissue clearance characteristics. ^{18}F-HX4 is a water-soluble molecule whose molecular scaffolding contains a polar 1,2,3-triazole element (partition coefficient = 0.21; log P = -0.69). Consequently, the measurement of radiation of ^{18}F-HX4 is equal to ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and ^{18}F-FETNIM; its

bio-distribution is shown not only by lower brain and heart absorption than ^{18}F-FMISO but also by lower GI absorption, permitting imaging in the abdominal region. ^{18}F-HX4 is eliminated from normoxic tissues more promptly than ^{18}F-FMISO, allowing PET imaging to be performed at an earlier stage. Comparable to ^{18}F-FMISO in terms of metabolic stability, it maintains 82% of the tracer in human plasma for 135 mins after delivery.

(e) Copper (II) (diacetyl-bis(N_4-methylthiosemicarbazone)). Copper (II) (diacetyl-bis(N_4-methylthiosemicarbazone)) (Cu-ATSM) is a hypoxia tracer that uses chelated copper ion oxidation/reduction for selective deposition in hypoxic tissue. Copper isotopes that generate positrons have been used: ^{60}Cu ($t^{1/2}$ = 0.39 h), ^{61}Cu ($t^{1/2}$ = 3.33 h), ^{62}Cu ($t^{1/2}$ = 0.16 h), and ^{64}Cu ($t^{1/2}$ = 12.70 h). The tracer has undergone in vivo validation and is currently being evaluated in clinical studies. Shorter imaging times (as low as 30 min after injecting) and a high T/B ratio even after its higher lipophilic nature [log P = 2.2 (10)] are Cu-ATSM's primary advantages over 2-nitroimidazole derivatives. The tracer appears to concentrate in the liver of humans, with minimal accumulation recorded in the spleens and kidneys.

15.4 Pits and Falls of Hypoxia Imaging

So far very limited imaging modalities are available to physicians which can diagnose, stage, and treat human cancer: X-ray (computed tomography [CT] and plain film), positron emission tomography (PET), single-photon emission computed tomography (SPECT), ultrasound (US), magnetic resonance imaging (MRI), and optical imaging. Just four of these modalities (CT, SPECT, MRI, and PET) can image cancer in three dimensions in individuals. However, the inception and evolution of these imaging modalities were driven by historical breakthroughs in physics and/or chemistry rather than oncologists' requirements. Because of their inability to scan low numbers of cancer cells, all four 3D imaging methods fall short of tackling many of the complicated clinical concerns associated with cancer screening, staging, and treatment (Parihar et al. 2011, 2013, 2021b).

15.5 Challenges and Future Prospects

The following are some potential future directions for hypoxia imaging and targeted development: since hypoxia targeting is probably worthless in the later stages of cancer, it is crucial to provide solid information that hypoxic cells are therapy determinants in non-SCC and are not merely surrogate signs for severe cancer. Although difficult, such proof may be acquired in rodent research by following a strategy similar to the DAHANCA nimorazole experiments or advanced cell-tagging technologies that allow tracking the postirradiation destiny of tumor cells more directly. Conventional subcutaneous xenograft tumor models made from generations-old cell cultures are inadequate because they can have acquired or lost

certain hypoxia tolerance traits. Orthotopic tumors created using CRISPR/Cas9 in vivo gene editing technology and patient-derived xenografts grown directly from tumor biopsies might improve the research and produce more convincing evidence. When selecting illness models and therapy combinations, we must learn from the past to progress in the area of hypoxia targeting and imaging. Overall, the progress of hypoxia-targeting medicines has been dismal. Current preclinical and clinical experiments have been conducted in circumstances where the function of hypoxic cells is ambiguous, which may account for some of the disappointing results. It will be important to better define the nature of the interactions between tumor cells and the related microenvironment during the next 5–10 years. This will be useful in the development of novel cancer therapy techniques that generate a tumor-suppressive phenotype. Furthermore, the identification of novel biomarkers related to tumor stroma may allow for the further delineation of different gene signatures, which may be significant prognostically as well as predict responsiveness to targeted therapy. Successful modulation of angiogenesis in cancer will necessitate a shift from the bench to the bedside and back. Strategies must be examined not only for their impact on tumor development but also on endothelial tip cell sprouting, vascular maturation, endothelial progenitor cell recruitment, hypoxia, and other factors.

15.6 Conclusion

A common biological occurrence in malignant solid tumors, including neck and head, cervical, prostate, breast, and lung malignancies, is tumor hypoxia. Tumor hypoxia spreads irregularly and is not associated with tumor volume, grade, phase, or histopathology. Hypoxic tumors employ a range of survival tactics, which can cause a reduction in apoptotic capability, an elevation in proliferative potential, and the formation of new blood vessels, all of which contribute to an evolutionary selection toward a greater malignant phenotype. As a result, regardless of treatment mode, hypoxia impacts the curability of solid tumors. Given the convincing association between tumor hypoxia and poor treatment results, scientific research has shifted to investigating the efficacy of hypoxia-targeted treatments. Nevertheless, some trials needed a reliable technique for recognizing and selecting patient groups with hypoxic tumors. Thus according to various meta-analyses, the indiscriminate administration of hypoxia modification medication to a patient group with both normoxic and hypoxic cancers exhibited negligible benefits. Hypoxia-based patient lamination has not been employed in medical practice due to the intrusive sort of "gold standard" approach for assessing tumor oxygenation (pO_2 electrode readings). Sponsors are presently faced with the decision of including both tumor types in medical practice, which may dilute efficacy data and jeopardize the trial's success, or using an unapproved diagnostic technology (e.g., hypoxia PET imaging, MRI, etc.) to identify person with hypoxic tumors. This scenario makes the medical advances of hypoxia-based treatments difficult. Various studies, particularly secondary analyses from bigger trials, demonstrate that hypoxia evaluation predicts tumor grade, therapy response and can recognize high-risk person who requires therapy.

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Hypoxia-Targeting Drugs as New Cancer Chemotherapy Agents: Molecular Insights

16

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Abstract

Solid tumors have a condition of low oxygen level, termed hypoxia. Hypoxia is regarded as one important characteristic of the tumor microenvironment. Clinical investigations have shown that tumor hypoxia has many consequences, such as poor tumor prognosis, therapy failure, resistance to drugs, and overall reduced survival rate. It can lead to autophagy or inhibit DNA damage and mitochondrial activity and promote apoptosis by activation of several signaling pathways causing failure of chemotherapy, radiation therapy, and immunotherapy. Additionally, hypoxia has been shown to play a central role in producing more aggressive and advanced metastatic cancer by activating gene regulatory mechanisms and signal transduction pathways.

Furthermore, hypoxia-inducible factor-1α (HIF-1α) was involved in hypoxia-induced resistance to the therapy, and it is believed that knockdown of it could reverse the resistance to therapy. This has inspired cancer researchers to investigate the signal molecules for tumor hypoxia as potential molecular targets for cancer therapeutics. This chapter will overview various signaling pathways for hypoxia, its role in tumor proliferation, differentiation, progress in the development of hypoxia-targeting drugs, and its clinical perspective.

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Keywords

Tumor hypoxia · Chemotherapy · Cancer therapy · Tumor microenvironment · Hypoxia-inducible factor (HIF) · p53

16.1 Introduction

Hypoxia in tumors has been coined as the increase in oxygen demand due to growing vascularization in cancer cells which paves the way to elevated nutrients (Bowyer et al. 2017). Lower pH with an acidic environment, anaerobic respiration, high reactive oxygen species, altered metabolism, and uncontrolled angiogenesis with molecular interventions such as downregulation of DNA repair are a few major characteristics of hypoxia (Liou and Storz 2010; Chiche et al. 2010; Al Tameemi et al. 2019; Schönenberger and Kovacs 2015). Due to the diversification in physiological alterations, different hypoxia-suppression strategies, especially in chemotherapy, to enhance sensitization toward therapies have been introduced so far (Seo et al. 2014). This is also related to a reduction in sensitivity toward various treatment regimens, including chemotherapy, radiotherapy, and immunosuppression (Lee et al. 2010; Song et al. 2020).

Hypoxia is stated to exhibit tissue oxygen tension of less than 10 mm Hg, which is quite less than that in normal tissue ranging from 24 to 66 mm Hg (McKeown 2014). Contradictorily, tumors still expand in limited oxygen supply. The optimal reason for the drastic genomic changes is hypoxic conditions seeking cancer cells to adjust in deprived conditions for survival (McKeown 2014; Jain 2014). Activating various signaling pathways like hypoxia-inducible factors allows active targeting when dealing with tumor suppression (Schönenberger and Kovacs 2015). Tumor aggressiveness, overexpression of drug efflux protein, and autophagy with a decreased level of oxygen supply contribute to the different mechanisms through which resistance of chemotherapeutic drugs is encountered in the tumor microenvironment (Muz et al. 2015; Jing et al. 2019; Nagasawa et al. 2006). Therefore, the requirement to introduce a well-defined drug or prodrug moiety that can make cellular functional key differences between individual patients' tumor levels is yet to be established.

This chapter discusses the signaling pathways involved and altered during hypoxia including its role in tumor. Briefly the section enumerates various hypoxia-inducing factors in response to oxygen concentration. The description also includes the kinases regulating processes of angiogenesis and malignancies. The second section states the effect of hypoxia on tumor progression to metastasis and its role in drug resistance. It elucidates the role in attachment, extravasation of metastatic cells, and premetastatic niche formation. Resistance toward chemotherapy due to hypoxia classified as overexpression of drug efflux proteins, apoptosis inhibition, autophagy induction, and others has been deduced. Later, reactive oxygen species generation during radiation therapy and impairment in the effectiveness of radiation by the hypoxic environment have been explained.

Moreover, the effect of hypoxic conditions on immunotherapy has been correlated. The fourth section compiles hypoxia and chemotherapy. It covers the classification of different therapeutically active anticancer compounds based on their nature and chemical activity of drugs like quinine/nitro, enzymes, peptides, and metals. The subsections explicitly connect hypoxia treatment through enzymes as the novel trend of chemotherapeutic drugs and selectivity and specificity in designing peptides to regulate hypoxic conditions. Also, different peptide-targeting biomolecules are involved in the signaling pathway. Going more into detail, quinone-based compounds for hypoxia correlating to additional anticancerous activity through ROS and free radical formation have been deciphered. Prodrugs and their activation with changing molecular mechanisms during hypoxia are explored.

Further, the clinical relevance of the investigated drugs has been stated in Sect. 16.5. The effect of various factors like changes in lifestyle and therapeutics has been included. Blood flow modulators, increasing oxygen content, and bio-reductive therapies to overcome adverse conditions during hypoxia are pointed. The synergism for treating hypoxia through combinational treatments likewise includes antiangiogenic and vascular disruption agents with other therapies like radiotherapy. Lastly, a summary and outlook of the overall chapter have been stated.

16.2 Hypoxia-Induced Cellular Signaling Pathways

In aerobic metabolism, oxygen is essential as, during the metabolic pathway of oxidative phosphorylation, the oxygen serves as "electron acceptor," thus leading to the generation of ATP. Due to the metabolic activity and diverse blood vessel network, tissue oxygenation levels are maintained in the range of 2–9% of O_2 (average 40 mm Hg) depending on the tissue. Oxygen levels less than 2% are termed hypoxia, wherein there is decrease in O_2 levels at nonphysiological level (Muz et al. 2015). The hypoxic condition is commonly present in malignant tumors. In general, the cells utilize an oxidative phosphorylation pathway for energy generation. However, the tumor cells prefer the glycolytic pathway for energy generation which is independent of cellular oxygen levels (Zheng 2012).

Hypoxia causes a nonphysiological decrease in oxygen levels, a common characteristic of malignant tumors. Acute hypoxia, which is a brief and abrupt exposure to short-term hypoxia lasting for several minutes, can be reversed; the condition is known as cyclic hypoxia. This exposure to acute hypoxia is often reversible, and the cell can regain its normal oxygenation condition. However, this cyclic hypoxia can also increase intracellular reactive oxygen species (ROS) concentration and further mutations that lead to tumor formation and progression (Rofstad et al. 2010). The response to hypoxic conditions in a cancerous cell varies depending on exposure to hypoxia. Thus, hypoxia results in altered cell metabolism in cancer cells, leading to a cascade of pathways like HIF, PI3K, MAPK, and NF-κB pathways. These activated cellular pathways in the cancerous cell result in feedback mechanisms leading to suppressed or enhanced hypoxic effects. The signaling pathways in response to tumor hypoxia are briefly described in the chapter (Fig. 16.1).

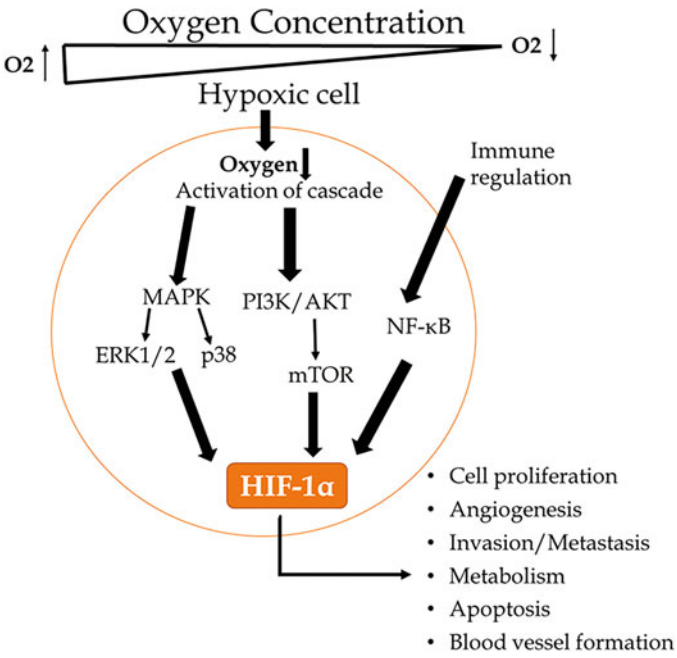

Fig. 16.1 Schematic diagram depicting the overview of mechanism behind hypoxia-induced pathways leading to various cellular responses

The progression of the cell cycle is an energy-driven process, wherein, every checkpoint in the cell cycle is highly regulated and the energy-restricted checkpoints are well controlled. The cell cycle is a well-regulated process. Microenvironmental oxygen is a crucial molecule in cellular metabolism and for driving energy from nutrients. Thus, a reduced level of O_2 in the cellular milieu sets the cells at stress, leading to the cell's hypoxic condition. The hypoxia-inducible factor (HIF) is a transcription factor that responds to the changing environmental oxygen and cell energy and coordinates a transcriptional activity, thereby regulating the cellular response to the dynamic cellular oxygen environment. Phosphoinositide-3-kinase (PI3K) is the family of lipid kinases that enhances several biological intermediates leading to alterations in the pathways. PI3K-AKT-mTOR is central in the signal transduction pathway, a key regulator controlling vital aspects of normal and cancer cell physiology. The PI3K-AKT-mTOR pathway at the cellular level regulates cell growth and proliferation, protein synthesis, metabolism, transcription, cell migration, and morphology. Thus, the PI3K/AKT/mTOR-targeting molecules target anticancer therapies (Rofstad et al. 2010).

Mitogen-activated protein kinases (MAPK) are the family of auto-phosphorylating protein kinase for activation or deactivation of their substrates (Rauth et al. 1993). The activation of MAPK is a cascade of consecutive phosphorylations of previous MAPKs. The MAPK pathway is followed by the

ERK1/2; p38 MAPK α, β, δ, and γ; and the c-JUN N-terminal kinase 1, 2, and 3 (JNK1/2/3) pathways. These pathways are triggered in response to various stimuli like growth factors, cellular and environmental stresses, proinflammatory stimuli, etc. (Owens and Keyse 2007). Nuclear factor-κB (NF-κB) and DNA-binding proteins family, which forms homo- or heterodimers are critical in regulating the adaptive and innate immune system. The NF-κB essentially regulates cell proliferation, migration, and invasion and promotes angiogenesis and metastasis. A constitutive stimulation of the NF-κB pathway is also well-documented in malignant and tumor cells. NF-κB can result in the progression of malignancy of the cancer cells by chronic stimulation of cancer cell proliferation, inhibition of cell death, chronic inflammation, and accumulation of cellular mutations. NF-κB and inflammation directly can stimulate the metastatic cellular propagation, increase cell extravasation in blood and lymphatic, and thus prevent cell death of circulating tumor cells.

The HIF-1α expression critically regulates the growth of a tumor and angiogenesis. The HIF protein synthesis is associated with the PI3K-AKT-mTOR pathway; inhibition of the mTOR pathway subsequently suppresses HIF-1α expression (Semenza 2013; Jiang and Liu 2008). The signals to the cell are transmitted via PI3K and MAPK pathways under normoxic and oncogenic conditions, thus leading to the regulation of HIF-1α. The resulting activation of PI3K in response to changing oxygen concentration results in increased HIF-1α protein (Semenza 2002; Bárdos and Ashcroft 2004; Laughner et al. 2001). Under hypoxic conditions, PI3K is a key regulator that transmits signals, upregulates HIF-1 protein synthesis, and transmits an extracellular signal in a HIF-1-independent mode (Zhong et al. 2000). In response to the PI3K pathway under normoxic or hypoxic conditions, VEGF activation is further subjected to complex regulation by HIF-1, PI3K, or MAPK pathways (Zhong et al. 2000).

16.3 Role of Hypoxia in Tumor Progression

Hypoxia activates the HIF-1 complex, a critical mediator of tumor aggressiveness and resistance to chemotherapy, which enhances the expression of target genes in several ways.

Angiogenesis Proangiogenic factors like vascular endothelial growth factors (VEGF), platelet-derived growth factor-b (PDGF-b), angiopoietin-2 (ANGPT2), stromal-derived factor-1a (SDF-1), and stem cell factor (SCF) are all upregulated by HIF-1a, which makes them more active. This makes it easier for plasma proteins and circulating angiogenic cells to reach the tumor microenvironment (Unwith et al. 2015).

Remodeling of surrounding stroma and bone marrow This is done by the activity of matrix metalloproteinases (MMPs), for example, MMP-2, MMP-9, and MMP-14. These MMPs are more active when HIF is present (Unwith et al. 2015).

DNA damage response Severe hypoxia (as little as 0.1% O_2) is thought to make genomes more unstable and thus more likely to become cancerous through a unique DNA damage response (DDR) that includes both ATR and ATM signaling (Begg and Tavassoli 2020).

mRNAs regulated by Hypoxia Hypoxia promotes tumor growth and metastasis by inhibiting Drosha and Dicer, two critical enzymes involved in the synthesis of miRNA, a small RNA that regulates gene expression in cancer (Shen et al. 2013).

Effects on metabolism Many genes, including glucose transporters, glycolytic enzymes, growth factors, and genes involved in erythropoiesis, iron transport, and nitric oxide synthesis, are regulated by HIF-1 in response to hypoxia in the body. HIF-1's dual role balances the proliferation and survival of cells (Koshiji et al. 2004).

Hypoxia and the Stem-like State The non-stem population can undergo a phenotypic change to resemble the stem-like fraction, promoting cell proliferation and self-renewal in the absence of oxygen. Non-stem cells cultured in hypoxia formed oncospheres at a rate double that of non-stem cells cultured in normoxia(Heddleston et al. 2009).

16.4 Role of Hypoxia in Tumor Metastasis

Escape from the Source
As a result of HIF-1-induced E-cadherin inhibition, cell-cell adhesion is lost, and c-MET expression is increased. c-MET promotes migration and invasion, and thus cancer stem cell (CSC) population increases (Loh et al. 2019).

Increase in Vascular Permeability
HIF-1 increases the density of angiopoietin-like 4 (ANGPTL4), VEGF, and SDF1 in the microvascular environment, which further promotes vascular dispersion of tumor cells (Hu et al. 2016).

Epithelial-Mesenchymal Transformation (EMT)
Expression of many transcriptional repressors for EMT includes Twist, Slug, ZFHX1B, Snail, Sip1, ZFHX1A, and zinc finger E-box-binding homeobox-1 (ZEB-1). These are controlled by HIF-1 (Lai et al. 2020).

Cancer Cell Survival in the Circulation
Anoikis is the induction of apoptosis in cells upon loss of attachment to the extracellular matrix (ECM) and neighboring cells. When HIF-1 inhibits a5 integrin, anoikis is inhibited, and tumor cells survive. HIF-1 also stimulates the expression of genes that promote tumor cell survival (Micalizzi et al. 2017).

Immunosuppression T cell proliferation and macrophage phagocytosis are essential for tumor development, and HIF-1 promotes pSTAT signaling, which results in increased production of immunosuppressive cytokines such as CSF1 and CCL2.

Arrest, Adhesion, and Invasion Processes at the Distant Organ Site.
Hypoxia promotes attachment and extravasation of metastatic cells to the endothelium via:

(a) "Ligand-induced cell adhesion molecule" (LICAM) expression.
(b) Formation of filopodia and lamellipodia.
(c) Cytoskeleton rearrangement by overexpression of vimentin and neuronal cadherin.
(d) PDGF band formation.
(e) Invadopodium formation: via tyrosine kinase (SRC) overexpression and colocalization with HIF-2a-induced MMP-2, MMP-9, and MMP-14 tyrosine kinase (FAK).
(f) Invasion of tumor cells is regulated by induction of certain enzymes, for example, matrix MMP-2 and lysyl oxidase, and upregulation of urokinase-plasminogen-activator receptor expression.

Premetastatic Niche
Before the tumor cells arrive, the target organ's microenvironment is changed to make it easier for the tumor cells to enter and grow. This is called a "premetastatic niche," and HIF-regulated VEGF and TNF-α help make this happen.

16.5 Role of Hypoxia in Resistance to Therapies

16.5.1 Resistance to Chemotherapy

Hypoxia reduces the effects of chemotherapy by various means, among which, increase in drug efflux, inhibition of apoptotic process, autophagy, and DNA damage inhibition are very prominent.

(a) Overexpression of drug efflux proteins (Singh et al. 2017)
Through MDR1/P-GP: seen in colon cancer cells in the case of 5-fluorouracil resistance.
Through ABCA2: seen in ovarian carcinoma cells in the case of estramustine resistance.
Through P-GP: seen in lung adenocarcinoma cells in the case of Adriamycin resistance.
(b) Apoptosis inhibition
Caspases 3, 8, 10, and Bak: seen in breast cancer cells in case of paclitaxel resistance.

Bid and Bax: seen in colon cancer cells in the case of Etoposide and oxaliplatin resistance.

p53 and NF-kB: seen in gastric cancer cells in the case of 5-fluorouracil and cisplatin resistance.

(c) Autophagy induction

Through Beclin-1: seen in HeLa cells. Retinamide (4-HPR) resistance and N-(4-hydroxyphenyl) resistance occur due to this.

Through HMGB2, ATG12, and miR-23b-3p: seen in gastric cancer cells for vincristine resistance.

Through p53: seen in colon cancer cells for cryptotanshinone and dihydrotanshinone resistance.

(d) DNA damage inhibition

Through *DNA–PKCs and Ku80:* seen in mouse embryonic fibroblasts for etoposide resistance.

Through TMEM45A: seen in breast Ca and liver Ca cells for etoposide and Taxol resistance.

(e) Mitochondrial activity

Through BAD: seen in the "human leukemic cell line" (HL-60) and "human lymphoma cell line" for Ara-c and doxorubicin resistance.

Through cytochrome, Akt, and ERK: seen in "oral squamous cell carcinoma cells" for cisplatin and 5-fluorouracil resistance.

16.5.2 Resistance to Radiotherapy

Radiation therapy mostly causes cell death and apoptosis by making reactive oxygen species (ROS) that damage the DNA of tumor cells in a way that cannot be reversed. Hypoxic cells need three times as much radiation as normal cells. If hypoxic tumor cells persist following radiation, the tumor may survive and develop into a more aggressive phenotype, which can be potentially fatal. Additionally, ROS can be produced by photodynamic therapy (PDT). Hypoxic tumor microenvironment impairs radiation effectiveness, but tumor oxygenation may enhance it. HIF-1 has a complicated and bidirectional interaction with radiation therapy. HIF-1 confers radioresistance, whereas HIF-2 acts as a tumor suppressor (Graham and Unger 2018).

16.5.3 Resistance to Immunotherapy

The most recent gene therapy approved by the FDA in the United States is chimeric antigen receptor (CAR)-T cell immunotherapy. Hypoxia is a big reason for failure of cancer immunotherapy. Anaerobic glycolysis by tumor cells makes much more adenosine, which further turns T cells less active. As a result of low oxygen levels in the tumor matrix, suppressor T cell concentration builds up in the

microenvironment. In addition, it is observed that HIF-1 also causes malfunctioning of adaptive immune cells (Graham and Unger 2018).

16.6 Hypoxia-Targeting Drugs

16.6.1 Hypoxia-Targeting Enzyme-Based Prodrug as a New Chemotherapeutic Drug

Hypoxia is when the tissue's oxygen level starts to lower down. The cancerous tumors that are vigorously spreading and growing utilize excessive oxygen for their growth, leading to hypoxic conditions. This negatively affects the treatment measures making the tumor resistant to the therapies available for cancer, such as radiotherapy, chemotherapy, and photodynamic therapy as well (Brahimi-Horn et al. 2007). As a hypoxic condition in cancer is concomitant with tumor malignancy, there is a growing need to develop therapies that include drugs that target hypoxia. Hence a considerable amount of work has been done on using enzymes for achieving this objective. Among the available enzymes, catalase (CAT) has been the major focus as it generates oxygen using hydrogen peroxide that is extensively generated inside the tumor mass. With the enhancement in nanotechnology, this catalase enzyme is combined with a nanomaterial that ultimately augments its efficiency. Encapsulation of CAT in cisplatin-loaded liposomes results in maintained and well-secured enzyme activity (Zhang et al. 2017). Free catalase enzyme being susceptible to enzymatic degradation also has a shorter half-life in blood and lesser cellular uptake, which can be overcome by linking the CAT molecule to the iron oxide NPs, therefore causing effective delivery and stability (Yen et al. 2019). CAT present in metal-inorganic nanocarrier and rapamycin results in its protection and collectively inhibits the hypoxia-inducible factor-1α, thus suppressing tumor growth (Liu et al. 2019). CAT was linked with hyaluronic acid polymer that selectively binds with CD44, which is abundantly expressed on cancerous cells to target the tumor appropriately. Due to this active targeting, a higher amount of CAT was seen to accumulate in tumors (Zeng Fiona Phua et al. 2019). Similar to the above concept, lactobionic acid (LA) was also combined with CAT causing increased cellular internalization. To further elevate the oxygen production, photosensitizer molecule Ce6 was provided that induce ROS generation directly affecting oxygen production (Cheng et al. 2020). Enzymes being highly efficient and specific have gained much more attention to reverse the hypoxic conditions to treat cancer. However, being a biomolecule also negatively impacts its ability which can be subdued with the help of nanotechnology.

16.6.2 Peptide-Based Drugs for Hypoxic Treatment in Cancer

Hypoxia is one of the crucial symptoms in many diseases such as malignant tumors, asthma, chronic obstructive pulmonary disease, anemia, congenital heart defects,

and many more. It is the condition where the tissue becomes deprived of oxygen. Hypoxic condition is the important characteristic of solid cancerous tumors that are developed. Malignant tumors have higher oxygen requirements for their growth, leading to hypoxic conditions in the surrounding environment (Barbara Muz et al. 2015). Hypoxic conditions cause increased expression of the HIFα gene that further promotes expression of genes such as vascular endothelial growth factor-A, leading to angiogenesis and, therefore, cell growth (Barbara Muz et al. 2015). The lower amount of oxygen surrounding the tumor makes it resistant to different therapies such as chemotherapy, radiotherapy, and photodynamic therapy. Hence, targeting and killing hypoxic cancer cells are the best ways to control the tumor.

Peptides, the short chains of amino acids linked through peptide bonds, are specifically used in such cases due to their safety, target specificity, and tumor permeability advantages. Peptides can overcome difficulties such as low drug/energy delivery efficiency, hypoxia-induced drug resistance, and tumor nonspecificity (Wang et al. 2021). Peptides attached to any anticancer drug molecule can act as a key that binds with the specific receptors on the cancerous cells, thus allowing easy and specific entry of that drug into the problematic cell. There is an increase in reductive stress in a hypoxic environment that acts on hypoxia-sensitive groups, causing cleavage of such groups. If drug moieties are attached to such groups, they can be selectively released at tumor sites. It is also possible to fabricate the peptide that can block the enzymes or the gene expression crucial for the growth of the tumor (Wang et al. 2021).

Many drugs are worked on using these peptides to target hypoxic conditions generated during cancer. Cyclo-CLLFVY1 is a kind of hexapeptide that inhibits the HIF-1 dimerization and transcription activity. Another such kind of peptide that prevents the expression of HIFα and therefore affects tumor angiogenesis is apolipoprotein A-I. GV1001 is derived from reverse transcriptase subunit of telomerase (hTERT), also shown to block the activity of HSP90, HSP70, and HIF-1α under hypoxic conditions (Kim et al. 2014). Carbonic anhydrase (CA) IX that is upregulated on the cell membrane cancerous cells is inhibited by 4-(2-aminoethyl) benzenesulfonamide (ABS) that is linked with short peptides that guide the way toward the tumor (Li et al. 2019). Studies have shown the invasiveness of tumors being increased if there is depletion in cGMP. The atrial natriuretic peptide can increase intracellular cGMP concentration, thus becoming a great option to treat triple-negative breast cancer and prostate cancer. Currently, one such breakthrough research has been done. The researchers have developed a nonpathogenic strain of *E. coli* expressing vessel dilator peptide that interacts with cancerous cells under hypoxic conditions, eventually causing enhanced survival rate of tumor-bearing mice and the beneficiary changes in cytokine profile (Mitra Samadi et al. 2021).

In conclusion, peptides are the most promising treatment and diagnosis options for cancer. Peptides being biomolecule, there is a biocompatibility issue that is quite less. With the help of nanoscience, conjugation of peptides with nanocarriers can be done to help them retain their activity and protection. Synthesis of peptides can be done according to the need, thus making them specific for specific tumors; with the help of the bioinformatics approach, its binding energy, pharmacokinetic/

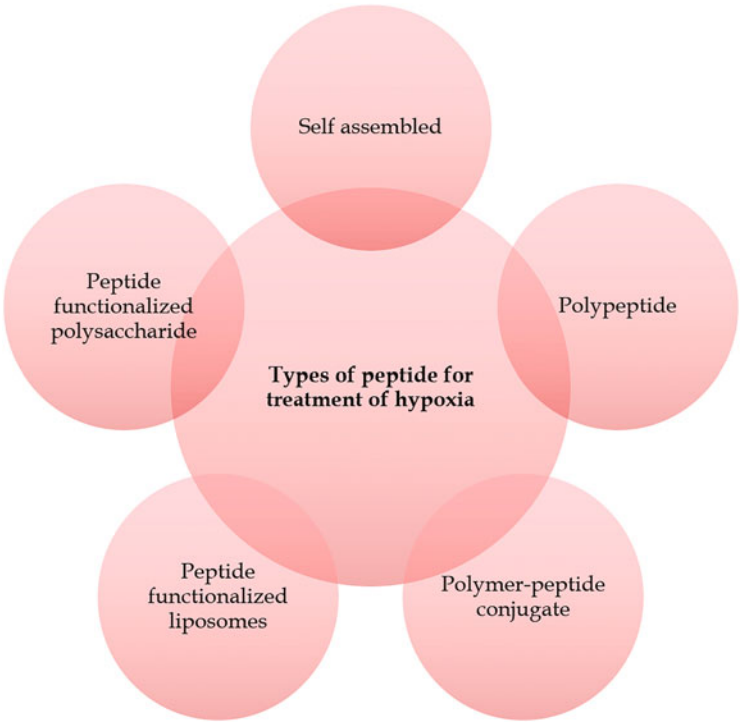

Fig. 16.2 Different types of peptides used for the treatment of hypoxia in chemotherapy

pharmacodynamics modeling properties, and many more things can be considered before the fabrication of peptides (Fig. 16.2). Hypoxia is the major hurdle in treating many diseases, especially cancer, ultimately stalling the recovery of the patients. The options mentioned earlier, including peptides, can subdue hypoxic tumors.

16.6.3 Hypoxia-Targeted Quinone-Based Drugs

Quinones are derived from aromatic compounds by rearranging the double bonds of even number of –CH = groups into –C– groups; hence these are the conjugated cyclic diones with two carbonyl groups, > C=O. Quinones are ubiquitously present in nature and humans as an endogenous moiety of significant importance. The quinones and their derivatives possess potential antitumor properties. Hence quinones such as anthracyclines have been clinically used for treating solid cancers. Quinones containing moieties of their derivatives indole, anthracene diones, anthraquinone, 1,4-naphthoquinone, and aza-naphthoquinone are shown to have anticancer activities. Quinones exert their antitumor effects by reduction and reoxidation in a redox cycling manner, which leads to the generation of reactive oxygen species

(ROS) and free radicals. Furthermore, quinones also lead to electrophilic arylation of critical cellular nucleophiles resulting in a cytotoxic or antitumor effect on the cell.

Taken ahead, as the quinones are ubiquitously present in nature, the naturally present mitomycin C is the widely used compound for hypoxia-activated prodrug. Similarly porfiromycin, a related compound to mitomycin C that shows enhanced selectivity against hypoxia, has been used (Rauth et al. 1993). The selectivity to hypoxia is achieved by one-electron reduction via NAD(P)H: cytochrome C (P450) reductase to semiquinone radical anion. This semiquinone is further reoxidized by molecular oxygen leading to hypoxia selectivity (Belcourt et al. 1998). Quinones are the substrate for DT diaphorase, which is a two-electron reductase. The potential of quinones as a hypoxia prodrug primarily relies on the relative metabolism by different reductases. Thus mitomycin C and porfiromycin are activated by reducing one and two electron, thereby achieving the cascade of action.

Further, these reduced species lead to guanine-guanine cross-links in the major groove of DNA (Tomasz and Palom 1997). In a clinical study with head and neck cancer, treatment with porfiromycin and radiation has shown promising results as an adjunction therapy for treating cancer. Quinone-based hypoxia-activated prodrug indoloquinone EO9, which is activated under aerobic conditions, mainly activated by DT diaphorase to hydroquinone which further goes through fragmentation to DNA cross-linking species (Tomasz and Palom 1997). Hence, in conclusion, the hypoxia-targeted quinone-based drugs possess a potential therapeutic agent against various tumors.

16.7 Clinical Perspective of Hypoxia in Cancer

Clinically significant hypoxia-targeting therapies include hypoxia-responsive prodrugs, hypoxia-selective gene therapy (transcription factor) that targets hypoxia-inducible factor-1 (HIF-1), and recombinant obligate anaerobic bacteria.

Prognostication of "Tumor Hypoxia"

Various hypoxia-related markers have been studied using different methods, demonstrating poor prognostic outcomes. Table 16.1 depicts results from various studies (Vaupel and Mayer 2007).

Modifiable Factors and Clinical Outcome

(a) Lifestyle

Smoking and Obesity

Preclinical studies indicated that high amount of carboxyhemoglobin seen in smokers could reduce the effectiveness of radiation therapy. Patients with non-small cell lung cancer who smoke during radiation had poorer results. TNF-alpha and IL-6 are two cytokines secreted by adipose tissue (Coppack 2001). Obesity and smoking are two modifiable habits affecting tumor oxygenation and radiation responsiveness.

Exercise

Table 16.1 Prognosis of various solid tumors with overt expression of hypoxia-induced factors

Factors	Clinical outcome
HIF-1a expression	Associated with worse survival in breast, head and neck, esophageal, stomach, lung cancers, and a few other tumor types
GLUT-1	GLUT-1 expression has been linked to a lower chance of survival in many types of cancer, including breast, head and neck, esophagus, bladder, stomach, colorectal, ovarian, and lung cancer (NSCLC)
CAIX	CA IX is found in many types of cancer, which helps cancer cells stay alive (apoptosis) and spread (invasiveness), for example, renal cell ca
HIF-1a target genes	The molecular substrates of many pathophysiological processes are affected by this
Hypoxia-induced proteins independent of HIF	Independent of HIF, nuclear factor B (NF-B), activator protein-1 (AP-1), and members of the unfolded protein response (e.g., GRP78) are hypoxia-induced factors. Their precise function in hypoxia-induced tumor progression is unknown. For example, "hypoxic" induction of NF-B is likely to result from reactive O_2 species produced by reoxygenation
Hypoxia detection using exogenous bio-reductive compounds	Certain molecules like 2-nitroimidazoles are called "exogenous hypoxia markers" (e.g., pimonidazole, EF5). While these are valuable tools, their prognostic utility is still not known clearly
Noninvasive hypoxia imaging	Nitroimidazole derivatives, fluoromisonidazole (18F-MISO), and copper-ATSM all had a detrimental effect on overall survival in various solid tumors

Source: (summarized from Cancer Metastasis Rev (2007) 26:225–239 DOI 10.1007/s10555-007-9055-1)

Systemic treatments, including aerobic exercise, may improve treatment effectiveness, reduce side effects, and improve the overall quality of life. Several studies on physical activity and cancer risk, tumor progression, and cardiovascular disease in cancer patients showed that increased perfusion pressure during exercise improves blood flow and decreases tumor hypoxia. According to new research, exercise may increase cyclophosphamide effectiveness by modulating tumor physiology. Beyond the potential for normalizing tumor vasculature and immunological benefits, exercise training as an adjunct to standard anticancer therapy has a low danger of severe side effects (Wiggins et al. 2018).

(b) Therapeutic Modifications

Radiation Dosing Schedule

Small radiation doses are given daily for weeks or months in conventional radiotherapy. Recently, stereotactic body radiation therapy (SBRT), which employs high doses of radiation in a single or a few treatment sessions, has become increasingly popular. The impact of SBRT on tumor hypoxia as a determinant of treatment outcome remains somewhat controversial. However,

preclinical studies and limited clinical findings suggest that tumor hypoxia is more important for SBRT than conventional fractionation.

Overcoming Anemia

In 1960s, studies showed that anemia was a negative prognostic factor in cancer patients undergoing radiation, and anemic patients treated with radiation reacted well to blood transfusions (Evans and Bergsjo 1965). However, erythropoietin therapy did not enhance responsiveness to radiation treatment and was perhaps related to a decrease in survival (Wright et al. 2007).

Blood Flow Modulators

In a rat model of liver cancer, noradrenaline effectively improved blood flow and drug delivery. Benzyl nicotinate enhanced the radiation response of a preclinical mouse tumor model, most likely by increasing the tumor's pO_2 (Hou et al. 2010). Other drugs studied in preclinical trials, such as pentoxifylline and nicotinamide, demonstrated a similar improvement in tumor blood flow and radiation response (Collingridge and Rockwell 2000).

(c) Increasing Oxygen Concentration

High Oxygen Content Breathing

Chambers of hyperbaric oxygen. Despite early encouraging results, hyperbaric chambers were discontinued in favor of other therapies due to patient discomfort, cases of oxygen poisoning, and chamber fires (Collingridge and Rockwell 2000).

Carbogen breathing. Numerous studies have demonstrated that carbogen enhances tumor oxygenation and radiation responsiveness. However, a trial of carbogen breathing in patients with advanced head and neck cancer suggested that carbogen breathing may be an option for patients who cannot receive concurrent chemotherapy and radiation (Collingridge and Rockwell 2000).

Hypoxic Cell Sensitizers

Radiation sensitizers act similarly to oxygen in hypoxic cells, enhancing radiation-induced DNA damage. Until now, the most notable effect of combining hypoxia cell sensitizers and radiation has been an increase in disease-free survival with no significant adverse effects (Collingridge and Rockwell 2000).

In contrast to hypoxia cell sensitizers, bio-reductive agents preferentially destroy hypoxic cells. These substances become cytotoxic under hypoxic conditions as a result of redox reactions (Collingridge and Rockwell 2000). Hypoxia selectivity is exhibited by various drugs, including quinones, nitroimidazoles, and benzotriazines. Tirapazamine, one of the most effective, induces DNA single- and double-strand breaks in low-oxygen environments and significantly increases the effectiveness of radiation in in vitro and preclinical animal models (Brown 1993).

(d) High Linear Energy Transfer (LET)

Another strategy for minimizing the loss of radiation sensitivity of cells residing in tumors in areas with low oxygen tensions is to use less oxygen-dependent radiation. It is believed that as the LET for radiation increases, the "oxygen enhancement ratio" decreases (Brown 1993).

(e) Targeting the Tumor Vasculature

Antiangiogenic therapy (AA). It has been demonstrated that combining AA treatment with radiation therapy improves tumor response. However, this restoration of tumor vasculature following AA treatment is not a consistent phenomenon, as it is dependent on the drug, the schedule, the growth phase, and the type of tumor.

Vascular disrupting agents (VDAs). While both AA and VDA treatments have the potential to affect tumor oxygenation and therapy response, a combination of the two classes may be required to exploit vascular targeting therapy's antitumor potential fully. Preclinical and early clinical data support the combination's therapeutic potential (Jameson and Clarke 2002).

Precision cancer medicine strategies. For tumor-specific targeting strategies, four approaches are being investigated, for example, hypoxia-activated prodrugs, hypoxia-selective gene therapy, targeting the hypoxia-inducible factor-1 (HIF-1) transcription factor, and the use of recombinant obligate anaerobic bacteria.

16.8 Summary and Conclusion

Hypoxia contributes to the poor prognosis associated with a variety of solid tumors. However, hypoxia does not behave uniformly in all patients, even when present. Hypoxia has the greatest impact on patient prognosis in patients whose malignancies exhibit a "hypoxic driver" feature, and thus the efficacy of hypoxia-targeted therapies varies. Hypoxia is a significant determinant of cancer behavior and outcome, occurring in some form in most solid malignant tumors in humans. It results in a complex and dynamic compensatory response, which includes genetic changes that favor the selection of hypoxia-adapted cells with an increased proclivity for local invasion, metastasis, and recurrence following surgery or radiation. The extremely low oxygen levels and necrosis found in solid tumors are unique characteristics that do not occur in normal tissues under normal physiological conditions and may thus be used to treat cancer. Since the discovery of hypoxic tumor behavior, most researchers have focused on radiosensitivity and the oxygen effect on hypoxic cells, until the discovery of HIF-1a by Wang et al. in 1995 (Wang et al. 1995). Following that, the hypoxia microenvironment has been extensively studied to understand the molecular mechanisms underlying tumor hypoxia better. This chapter discussed recent advances in hypoxia-targeting drugs, signaling pathways, and the mechanism and consequences of hypoxia in tumor growth, metastasis, and treatment resistance.

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Identification of Hypoxia-Targeting Drugs in the Tumor Microenvironment and Prodrug Strategies for Targeting Tumor Hypoxia

17

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Abstract

Hypoxia, which is a result of an imbalance between oxygen delivery and consumption, is frequently seen in solid tumors. ROS are produced as a result of hypoxia, which lowers genomic stability and downregulates DNA repair mechanisms. ROS overproduction is a characteristic of cancer cells and plays a variety of roles throughout the malignant tumor's natural history. From the first stages of cancer development to the evolution of the disease, ROS consistently contribute, either directly or indirectly. The hypoxic tumors are primarily reversed by anti-hypoxia medicines like MnO_2 or hemoglobin-based O_2 carriers. Cancer cells prefer aerobic glycolysis to oxidative phosphorylation, which leads to a buildup of reducing agents and enzymes. In comparison to other first-row metals on the periodic table, cobalt(III) complexes are kinetically inert and exhibit much slower rates of ligand substitution processes.

Keywords

Hypoxia · ROS · DNA repair mechanisms · Hemoglobin-based O_2 carriers · Ligand substitution

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17.1 Introduction

Wide range of solid tumors are often characterized with hypoxia which is a result of mismatch between oxygen delivery and consumption. Hypoxia has remarkable effect on tumor progression, for example, (1) selection of genotypes that favor hypoxia reoxygenation injury (TP53 mutations) and (2) change in gene expression that suppresses apoptosis and support autophagy (Wigerup et al. 2016). Hypoxia is also responsible for enhancing receptor tyrosine kinase-mediated signaling, angiogenesis, vasculogenesis, tumor invasion, and metastasis. Immune activity is also suppressed due to the hypoxia (Muz et al. 2015). In addition, hypoxia prompts generation of ROS that reduce the genomic stability and the downregulation of DNA repair pathways. Therefore, hypoxia often leads to the chemoresistance to the majority of the tumors (Begg and Tavassoli 2020). Therefore, an alternative strategy to overcome hypoxia is thus of paramount importance in the treatment of solid cancers. Strategic development of functional molecules is based on three parameters. (1) Anti-hypoxia agents like MnO_2 or hemoglobin-based O_2 carriers primarily reverse the hypoxia by generating O_2 (Li et al. 2021a), although the oxygenation strategy is limited by cytotoxicity, lower blood circulation, and poor penetration within a tumor, (2) hypoxia-active nanoparticles/agents are activated only in hypoxic tumor microenvironment (Wang et al. 2019), and (3) hypoxia-targeting agents target biomarkers of tumor hypoxia to improve the efficacy of the existing chemotherapeutic drugs. Considering the enhanced level of HIF-1 and mTOR pathways in hypoxia, hypoxia-targeting agents were developed for treating hypoxia (Burroughs et al. 2013a). The preferred aerobic glycolysis in cancer cells rather than the oxidative phosphorylation pathway results in the accumulation of reducing agents and oxidoreductases (NADH or NADPH, β-glucuronidase, alkaline phosphatase nitroreductase, DT-diaphorase, and cytochrome P_{450} reductase), making the cancer cell microenvironment as reducing (Jiang 2017). The reducing enzymes typically can reduce hypoxia-active or bio-reductive prodrugs through one- or two-electron reductions. In normoxic cancer cells, back oxidation of the reduced form of the prodrug to the oxidized form is kinetically favored and hence may result into the poor efficacy of the prodrug. However, in a hypoxic condition, the enhanced lifetime of the radical anion intermediate or the reduced form of the prodrug facilitates the forward reactions and makes the prodrugs to be converted into the cytotoxic agent efficiently. The deferential reversibility of the reduction-oxidation reaction ensures the prodrug activation in hypoxia condition, resulting in hypoxia-selective cell death. Among the several types of bio-reductive prodrugs, the unique redox and kinetic properties of cobalt complexes make them potential tools for the development of hypoxia-selective prodrugs. Cobalt(III) complexes are kinetically inert, and the rate of ligand substitution reactions is significantly lower than the other first-row metal on the periodic table. In contrast, cobalt in +2 oxidation state is labile making the ligand substitution reaction much faster and facile. These differences in lability of the cobalt complexes between these two oxidation states have enabled the development of Co(III)-based prodrugs that undergo reduction in biological systems to form labile Co(II) complexes resulting in subsequent release of cytotoxic drugs

and exhibit hypoxia-selective anticancer activity (Sharma et al. 2019; Renfrew 2014). There are several different classes of Co(III) complexes with the ultimate objective of selectively targeting hypoxic environments. The present book chapter illustrated recent advances on the development of Co(III)-based hypoxia-selective anticancer agents (Heffern et al. 2013).

17.1.1 Proteins Involved in Inducing Hypoxic Cancer Cells

There are several proteins which are responsible for these characteristics of hypoxic tumor cells, namely:

HIF-1α HIF-1α is an oxygen concentration-responsive protein. HIF-1α acts as oxygen concentration indicator in the human cells (Masoud and Li 2015). Generally it has short lifetime inside the cell. It gets synthesized via phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways which are independent of oxygen concentration inside the cell (Vaupel et al. 2001). However, the degradation process of this protein is controlled by oxygen-dependent degradation domain (Tianchi et al. 2017). In normoxia condition, when oxygen concentration is normal (between 10 and 20%), then P402 and P564 amino acid residues of HIF-1α undergo hydroxylation in the presence of O_2, 2-oxoglutarate, and active PHD (prolyl hydroxylation domain) (Lee et al. 2004a). HIF-1α gets identified by von Hippel-Lindau tumor suppressor gene and undergoes ubiquitination followed by proteasomal degradation by 26S proteasome (Ke et al. 2006) as both hydroxylation of P402/P564 needs sufficient amount of oxygen (Burroughs et al. 2013b). Hence in normoxia condition, HIF-1α gets metabolized, but deep-seated hypoxic tumor cells with poor concentration of oxygen lead to stabilization of HIF-1α (Lee et al. 2004b). Hypoxia-inducible factor-1α activates a number of cancer-causing gene, i.e., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoeitin-1 (ANGPT1), etc., and promotes cell survival, cancer metathesis, tumor invasion, and angiogenesis (Hong et al. 2004). In hypoxic condition when physiological oxygen concentration is low, all of the above explained processes get stalled, which results in the increase of concentration of HIF-1α in cell cytosol that enters to the cell nucleus, where it binds with previously present HIF-1β and forms heterodimer HIF-1α (Marxsen et al. 2004). HIF-1 activates transcription of more than 60 hypoxia-responsive genes. These genes are generally associated with cancer encoding angiogenic factors, survival factors, glucose transporters, and glycolytic enzyme (Ziello et al. 2007).

VEGF Vascular endothelial growth factor (VEGF) is induced in the hypoxic tumor cells by HIF-1α protein-dependent process. This protein plays an important role in increasing vascular permeability, elevation of interstitial fluid pressure, and endothelial proliferation. This further leads to angiogenesis. VEGF also inhibits dendritic cell maturation and induction of prostaglandins (Morfoisse et al. 2014).

GLUT-1 In hypoxic conditions HIF-1α protein is upregulated. The glucose transporter proteins (GLUTs) control the glucose metabolism into the cancer cells. Upon activation of the HIF-1α protein, the GLUT-1 protein gets overexpressed in the hypoxic tumor cells. This GLUT-1 protein generally shifts the glucose metabolism into the in the cancer cells toward glycolysis, and this process is associated with the formation of acidic byproducts of glycolysis pathway and lowering cancel cellular pH (Chung et al. 2009).

MMP Matrix metalloproteases are overexpressed by the HIF-1α protein. The MMP proteins get accumulated in the extracellular part of the tumor cells. The MMP proteins destroy the epithelial barrier, and this phenomenon facilitates the angiogenesis and tumor migration (Kessenbrock et al. 2010).

17.1.2 Challenges of Hypoxic Tumor Treatment

All these processes discussed above are involved in the hypoxic tumor microenvironment and induce the special characteristics of the hypoxic environment. The high concentration of reducing agents inside the cancer cells makes them resistive to many conventional antiproliferative drugs as the antiproliferative drugs generally are associated with the generation of reactive oxygen species (ROS) or DNA-binding properties (Calman et al. 1980; Yokoyama et al. 2017; Perillo et al. 2020). The lack of oxygen in these hypoxic tumor cells makes the generation of ROS difficult. Also the DNA-binding drugs are generally metal complexes, where metal centers get reduced in the presence of high concentrations of reducing agents (Li et al. 2017). And the lower pH of the hypoxic tumor cells affects the activity of slightly basic anticancer drugs like doxorubicin, where doxorubicin is converted into a charged form in the low pH of the hypoxic cancer cells, and it results in a lower accumulation of doxorubicin in the cells (Gerweck et al. 1999; Raghunand and Gillies 2000). The solid exterior of the hypoxic tumor cells also makes them resistant to ionizing radiations. Hence, we require an effective strategy to overcome the abovementioned properties of hypoxic cancer cells for their treatment.

17.2 Bio-Reductive Prodrugs as Hypoxia-Selective Anticancer Agents

17.2.1 Organic Molecules as Bio-Reductive Hypoxia-Selective Anticancer Prodrugs

Several hypoxia-activated prodrugs can be used for a hypoxic cell-selective activity like tirapazamine, AQ4N, PR-104, apaziquone, evofosfamide, etc. These drugs can be selectively activated in the hypoxic media using high intracellular reducing atmosphere and cytochrome P450 oxidoreductase, and they exhibited cytotoxicity by pH-dependent DNA-damaging ability and downregulation of hypoxic proteins

like HIF-1α, CA-IX, and VEGF. These bio-reductive compounds are currently in clinical trials for hypoxia-selective anticancer agents (Table 17.1).

17.2.2 Transition Metal Complexes as Bio-Reductive Hypoxia-Selective Anticancer Agents

Transition metal complexes also are emergent in hypoxia-selective therapy. Transition metal complexes provide a wide range of tunable properties like geometry, oxidation states, redox properties, etc. Hence generally these properties are now being exploited for hypoxia-selective treatment including hypoxia-selective drug delivery.

17.2.2.1 Platinum Complexes as Hypoxia-Selective Prodrugs

Among these transition metal complexes, Pt(IV) complexes are emergent for their bio-reductive properties. Pt(IV) complexes can get converted to Pt(II) by the high concentration of reducing agents present in the hypoxic tumor cells. This property of Pt(IV) complexes is used for hypoxia-selective delivery of anticancer drugs along with the DNA-binding ability of Pt(II) complexes which also attributes to the activity of the Pt(IV) complexes in hypoxia (Galsky et al. 2011; Wexselblatt and Gibson 2012; Vouillamoz-Lorenz et al. 2003). Brynzak et al. in 2016 explored the cytotoxic effect of satraplatin derivatives and satraplatin against the hypoxic cancer cell spheroids. The complexes exhibited cytotoxicity in the range of 6.4–0.20 μM but observed less drug localization into the tumor spheroids. The complexes get reduced in the hypoxic microenvironment into Pt(II) followed by release of cytotoxic agents (Brynzak et al. 2016). But the problem arises from the soft nature of the Pt(II) center, as they can get easily bound with intercellular sulfur-containing reducing agents like glutathione, and this factor drastically reduces the activity of the complexes, and in tandem, it may raise the heavy metal toxicity in the body.

17.2.2.2 Cobalt Complexes as Hypoxia-Selective Prodrugs

Here Co(III) complexes have emerged as an effective solution to overcome all these problems. Co is essential for our body as cobalamins. Co metabolism in the human body is also well explored. And Co(III) complexes are bio-reductive in nature, which makes them a suitable choice for hypoxia-selective chemotherapy. In the highly reducing environment of hypoxic tumor cells, the Co(III) center of the complex gets reduced to Co(II). The reduced Co(II) center readily releases a bidentate ligand into the hypoxic environment. This bidentate ligand is generally an anticancer drug. Hence Co(III) complexes themselves can act as hypoxia-selective prodrugs.

17.2.2.2.1 Ternary Co(III) Complexes with Bidentate Anticancer Agents

Tetradentate ternary N,N,N,N-based ligands like cyclams, cyclanes, and TPA provide a very good template for stable Co(III) complexes. The complexes can further be bound with bidentate cytotoxic agents for hypoxia-selective drug delivery under bio-deducible atmosphere. For a long time back, researchers have been utilizing

Table 17.1 Organic hypoxia-selective anticancer agents and their mode of action

Compound	Mode of action	Disadvantages	Reference
Tirapazamine	• It is the first reported hypoxia-responsive prodrug. • In hypoxic condition this molecule undergoes one-electron reduction, and by oxidative damage, it induces chromosome aberrations and pH-responsive DNA fragmentation. • The DNA fragmentation is caused by both purine and pyrimidine residue damage. • It also induces downregulation of HIF-1α, CA-IX, and cells. • It is currently under phase III clinical trials, and it is showing significant toxicity toward the hypoxic cancer cells.	Tirapazamine also shows activity in aerobic condition. • The phase I and II clinical trials help reveal some adverse effect like nausea, vomiting, granulocytopenia, and muscle cramping	(Saunders et al. 2000; Riley and Workman 1992)
AQ4N	• This compound is generally nontoxic toward the cells because of no DNA binding ability. But in hypoxic condition, it undergoes two-electron reduction process and converts to AQ4 which is a very good DNA binder. Thus the activity of the compound is reported to become 1000 times more potent in hypoxic condition. • It is currently under clinical trials and exhibited significant antitumor activity in phase I clinical trials associated with radiotherapy.		(Lalani et al. 2007)
PR-104	• In hypoxic condition this drug gets activated and acts as a DNA-inserted cross-linking agent. • Ovarian carcinoma, hepatocellular carcinoma, lung carcinoma. • This drug is under phase I and phase II clinical trials.		(Singleton et al. 2009)
EO9	• This drug exhibits its activity by one-electron		(Begleiter et al. 1997)

(continued)

Table 17.1 (continued)

Compound	Mode of action	Disadvantages	Reference
	reduction process in the hypoxic cancer cells. • pH-responsive activity of this drug has also been discovered, • In vitro studies suggest that this compound is effective against adenocarcinoma melanoma, central nervous system carcinoma, renal cancer carcinoma, oral carcinoma, and lung cancer carcinoma. • This bio-reductive drug is under phase I and phase II clinical trials for further application in the hypoxic cancer cells.	• In clinical trials this drug exhibited nephrotoxicity and proteinuria.	
TH-302	This compound is a prodrug of isophosphoramide mustard. In hypoxic condition the mustard part gets reduced and released from the molecule to show cytotoxic activities. It is found that cytochrome p450 plays an effective role in the reduction of the compound This compound is currently under clinical trials	Skin and mucosal toxicity along with thrombocytopenia, neutropenia, and myelosuppression is observed for this compound in the clinical trials	(Sun et al. 2012; Li et al. 2021b)

these types of Co(III) complexes as drug delivery agents. In 2006 Bonnitcha et al. used X-ray absorption near-edge spectroscopy (XANEX) in cellulo and reported the release of drugs by Co(III) complexes in cellular media via the conversion of Co(III) to Co(II) (Bonnitcha et al. 2006).

17.2.2.2.1.1 Cyclen- and Cyclam-Based sp^3 N,N,N,N Donor Ternary Co(III) Complexes Among the N,N,N,N donor ligands, cyclam and cyclens consist of sp^3 donor N atoms, and these complexes can exhibit bio-reductive properties under the hypoxic conditions. Ahn et al. in 2006 reported the synthesis of cyclen-based Co(III) complex with 8-hydroxyquinoline **(1)** and DNA minor groove alkylator ligand azaCBI **(2)**. These two bidentate ligands are known for their DNA-binding properties. The 8-hydroxyquinoline and azaCBI were released from the complex in hypoxic conditions. As evident from the HPLC in human plasma, the release of ligands was also observed in hypoxic HT29 cell lines, but in A549 cells, cytochrome P450 reductase is overexpressed. There the release of the ligands was not so prominent. The complexes were also found to be releasing the cytotoxic agents in

the presence of radiolytic radiations (Ahn et al. 2006). Lu et al. in 2011 reported N-alkylated cyclen Co(III) complexes of 1-(choromethyl)-3-(5,6,7-trimethoxyindol-2-ylcarbonyl)-2,3-dihydro-1H-pyrrolo(3,2-f quinoline-5-ol) **(3–8)** as radiolytically activable prodrug. The seco-6-azaCBI-TMI is an azaCBI derivative, and it also works as DNA minor groove alkylator. The complexes are lipophilic due to cationic charges, and they can enter the cells via passive diffusion. The compounds also can be reduced by outer cellular Mito reductases, and it exhibits some promising radiolytically activable prodrug properties. The IC_{50} value of the complex in the normoxic condition in SKOV3 and HT29 cell lines are 2.26 and 2.4 μM, respectively, whereas in the hypoxic condition, the IC_{50} values are 0.5 and 0.4 μM (Lu et al. 2011). In 2012, Hambley et al. synthesized several cyclen-based complex of cobalt with different bidentate oxygen co-ligands including hydroxamic acid, β-diketone, and catechol **(9–23)**. Hydroxamic acid complexes have a potential for targeting tumor under the acidic and hypoxic condition as the protonation of the acid changed the formal charge of metal which get reduced at a more positive reduction value under acidic conditions. These alter the characteristics of the complex cellular uptake. Under the alkaline condition, the hydroxamate forms dominate which increases the negative reduction potential allowing lower disassociation of ligand and lower cellular uptake (Bonnitcha et al. 2012). Chang et al. in 2013 reported several cyclen- and cyclam-based cross-bridged Co(III) complexes **(24–26)** with minor DNA groove alkylator bidentate ligand 8-hydroxyquinoline and azaCBI derivatives for their DNA alkylation properties to release them in the hypoxic cancer cell microenvironment and attain hypoxia-selective cytotoxicity. The complexes exhibited higher cytotoxicity in the hypoxic condition as compared to the free DNA alkylator ligand. For complex **25**, it was 81–212 times more cytotoxic in hypoxia than 20% oxygen concentration in a series of 10 human tumor cell lines. But the complex did not exhibit significant cytotoxicity in the xenograft model of HT29 cell lines. Hence this result limits the pharmacological activity of the complex in vivo (Chang et al. 2013). He also reported cross-bridged cyclam-Co(III) complexes with cytotoxic bidentate ligands and observed a 200-fold greater potency of the complexes in hypoxia than that of normoxia.

Other sp^3 4 N donor ligands like tren also are widely used for preparation of hypoxia-sensitive bio-reducible Co(III) complexes. Gopinathan et al. in 2014 reported two Co(III) complexes with trien and phen ligands **(27–28)**. The complexes bind with CT-DNA as a groove finder as evident from UV-Vis, Fluorescence spectroscopy, and cyclic voltammetry as well as viscosity measurements. The complexes were found to be cytotoxic against human liver cancer cells. The complexes also exhibited antibacterial and antimicrobial activities in vitro (Gopinathan et al. 2014). Kozsup et al. in 2021 also reported 16 Co(III) complexes **(29–37)** with N,N,N,N donor ligands like tris and bidentate ligand flavonoids. The flavonoids are polyphenolic natural products that have antibacterial, antioxidant, antiviral, cardioprotective, and antitumor activities. The tren complexes were found to be more stable. The tren complex **32** also exhibited slight hypoxia selectivity with moderate cytotoxicity (Kozsup et al. 2021). Also 8-quinolinol cobalt(III) complexes **(38–39)** were synthesized by Ware et al. using a tetradentate cyclen as an auxiliary

ligand. It showed that with the increase in charge density and hydrophilicity of the complex, their cellular uptake decreased and reduced the cytotoxicity. The complex shows quasi-reversible redox behavior at −440 and − 300 mV, but the cytotoxicity of the complexes has not been reported experimentally (Fig. 17.1).

17.2.2.2.1.2 Tpa- and Tren-Based sp^2 N,N,N and sp^3 N Donor Ternary Co(III) Complexes

Pyridine nitrogen are better sigma donors than that of other sp2 or sp3 nitrogen donor atoms. This is due to the fact that in pyridine nitrogen the lone pair over nitrogen atom resides in the orbital with higher percentage of p character. Hence there are several reports of pyridine containing sp^2 N,N,N and sp^3 N ligands like tpa and tren to form stable bio-reductive ternary Co(III) complexes.

Yamamoto et al. (2012) reported Co(III) ternary complexes **(40–43)** with coumarin fluorophore. The bidentate ancillary ligands are fluorescent mimics of hydroxamic acids. In hypoxic condition they are subjected to release the cytotoxic agents. Among the series of the compounds, complexes **42** and **12** exhibited the highest electron-withdrawing effects of hydroxamate moieties. The electronic-withdrawing groups of the nicotinic acid moieties decrease the stability of the Co (III) state. These complexes exhibited pH-sensitive delivery of fluorescent coumarin into the hypoxic and acidic tumor microenvironment (Yamamoto et al. 2012). That year, Bonnitcha et al. also reported the synthesis of several tpa- and tren-based complex of cobalt with different bidentate oxygen co-ligands including hydroxamic acid, β-diketone, and catechol **(44–53)** (Bonnitcha et al. 2012). The tren complexes have more negative reduction potential which is irreversible because of the interaction of the π-acceptor pyridyl rings with the metal center. The decrease in reduction potential increased solvolysis which further enhances the hindrance of waves for reoxidation. The tpa complex with the same ancillary ligand has a lesser negative reduction potential because of the σ interaction of amine with the metal centers and also observed greater reversibility due to π-back bonding interactions which decrease the ligand lability. The cytotoxicity of tpa complex with β-diketone is almost similar to the free ligand with IC_{50} values ranging from 74 μM to 66 μM in both hypoxic and normoxic conditions, while the tren complex shows a decrease in cytotoxicity indicating the masking of the ligand with tren ligand. Hydroxamic acid complexes have a potential for targeting tumor under the acidic and hypoxic condition as the protonation of the acid changed the formal charge of metal which get reduced at a more positive reduction value under acidic conditions. These alter the characteristics of the complex cellular uptake. Under the alkaline condition, the hydroxamate forms dominate which increases the negative reduction potential allowing lower disassociation of ligand and lower cellular uptake.

The cytotoxin curcumin is a natural anticancer drug isolated from *Curcuma longa* (turmeric) and has the potential to act as antiproliferative, antimetastatic, and antiangiogenic agent. Low solubility, rapid metabolism, and low bioavailability upon administration orally have posed a major problem in exploiting curcumin as anticancer drugs. In 2013, Renfrew et al. synthesized cobalt complexes with curcumin along with tris(2-methylpyridine)amine as ancillary ligands **(54)** which

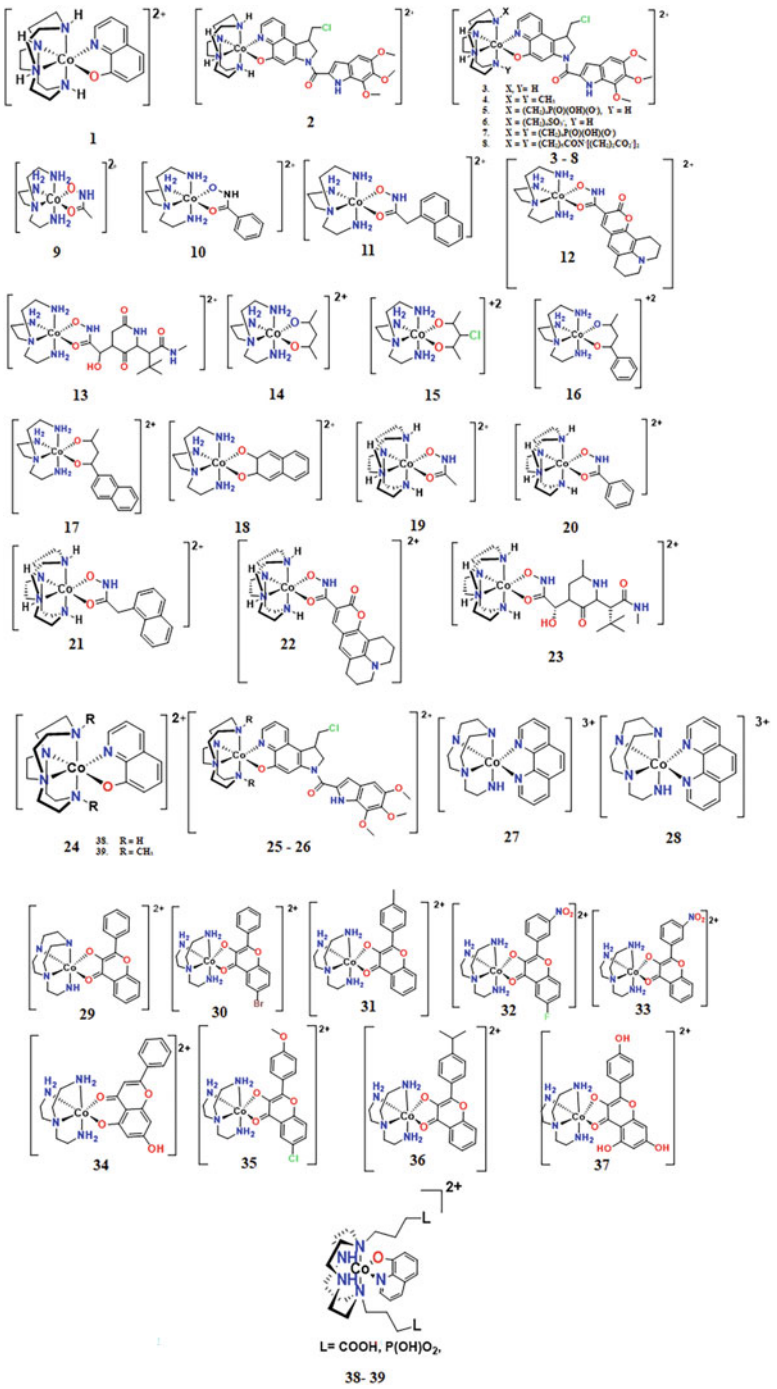

Fig. 17.1 Structure of cyclen- and cyclam-based sp^3 N,N,N,N donor ternary Co(III) complexes **(1–39)**

is found to increase the stability and solubility in aqueous medium. It also enhanced uptake and penetration in tumor hypoxic cells. The complexes showed one-electron reduction with IC_{50} value of around 30 μM against colorectal cancer cell line DLD-1. The study showed that the Co-TPA moiety didn't show any cytotoxicity alone but upon release of cytotoxin curcumin by reduction of the metal center (Renfrew et al. 2013). O'Neill et al. in 2017 reported the synthesis and anticancer activity of four TPA-based Co(III) complexes **(55–58)** and their structure-activity relationship. The complexes were characterized by ^{1}H, ^{13}C, ^{59}Co NMR, HRMS, IR, and UV-visible spectroscopy. The cyclic voltammetry of complex **55** exhibited a quasi-reversible redox character involving acetylacetone, but in a sustained reducing environment, the acetylacetone was lost from the complex, whereas **56** to **58** exhibited irreversible one-electron reduction character in the cyclic voltammetry, which concurs with the more facile release of acetylacetone ligand. The increase of carboxylic acid (-COOH) groups increases the stabilization of the complexes by inducing more electron density toward the pyridine moieties, thereby stabilizing Co (III) center. The relaxivity (r_2/r_1) ratio was also found to be in the range of 4.0–8.7. The complexes were found to be almost nontoxic against DLD-1 colorectal cancer cells as greater than 60% of cell viability at the highest concentrations. In **56** to **58,** there is a decreased cellular uptake for the decrease in surface area to volume ratio. The complex TPA3 also exhibited an increase in MRI signal intensity in the inner cellular region but not in the outer region indicating the formation of Co(II) inside the cells (O'Neill et al. 2017). Buglyó et al. in 2017 reported 16 Co(III) complexes **(59–75)** with 4 N donor tripodal ligands with hydroxamate ligand derivatives. The cyclic voltammetry suggests that Co(III)abap complex possesses Co(III) to Co (II) reduction potential far below than that of the biological reduction range. From the structure-activity studies, they have also found that the +3 oxidation state is highly stabilized by the presence of a doubly protonated form of benzo hydroxamate. The in vitro studies also signify the release of hydroxamates in biological media (Buglyó et al. 2017). Kozsup et al. in 2021 also reported 16 Co(III) complexes **(76–83)** with N,N,N,N donor ligands like tpa, tren, and bidentate ligand flavonoids. The flavonoids are polyphenolic natural products that have antibacterial, antioxidant, antiviral, cardioprotective, and antitumor activities. The tren complexes were found to be more stable. Tren complex **83** also exhibited slight hypoxia selectivity with moderate cytotoxicity (Kozsup et al. 2021). Palmeira-Mello et al. in 2020 reported three Co(III)-based complexes: $[Co(esc)(py_2en)]ClO_4 \cdot (CH_3OH)_2$, $[Co^{III}(esc)(TPA)]ClO_4 \cdot 3H_2O$, and $[Co^{III}(bipy)_2(esc)]ClO_4 \cdot 2.5H_2O$ **(84–86)**. In air argon and dioxygen conditions, Co(III) center gets reduced to Co(II) by biologically relevant reducing agents like ascorbic acid, cysteine, and glutathione. The reduction of the metal center is observed to be more facile in hypoxic conditions, and the rate of reduction is **86** > **85** > **84**. Complex **86** exhibited cytotoxicity against HCT-116, but not against HT-29 or HEK293. Complex **85** was also reported to exhibit electrostatic DNA-binding abilities (Palmeira-Mello et al. 2020a). Cobalt complexes **(87–91)** with esculetin and different ancillary ligand py2en, TPA, and bipy have been synthesized. Esculetin (6,7-dihydroxycoumarin) is a derivative of coumarin which is present in plants and has antibacterial and anticancer activity. It was also found to

inhibit angiogenesis-induced vascular endothelial growth factor (VEGF) and caspase-mediated apoptosis. All the complexes showed an irreversible reduction with a large ΔE value ranging from 0.30 V to 0.41 V. Since the complex reduction potential depended on the electron-donating potential of the ancillary ligand, there was an observed anodic shift with the highest in py2en complex followed by TPA and bipy complex. The reduction potential of py2en complex and bipy complex was −0.27 V and − 0.22 V which falls under the biological reduction window, while the complex 3D had −0.08 V which is outside the reduction window. Although py2en complex reduction potential is under a biological window, it could not be reduced by biological reductase. TPA and bipy complex showed a reduction by decreasing the absorbance at around 390 nm of all the reducing agents with more effective conversion or dissociation of esculetin in lower oxygen level shown by complex bipy complex in spite of its reduction potential outside the biological window. Cytotoxicity assay against HCT-116 and HT-29 cell lines showed that py2en and TPA complexes have $IC_{50} > 100$ μM in both normoxic and hypoxic conditions and bipy complex shows higher toxicity with IC_{50} value 31 μM in HCT-116 hypoxic cells only. All complexes showed electrostatic DNA interactions (Palmeira-mello et al. 2020b). Batista et al. synthesized another cobalt complex **(92–94)** with triazole ligand and ancillary ligands TPA, Py2en, and $Py2enMe_2$. A class of triazole, i.e., carboxyamidotriazole, is a promising cancer therapy drug and has many pharmaceutical applications. The oxime moiety of the triazole ligand is bidentately linked to the metal providing the stability from immature reduction. The effect of the ancillary ligand has been shown effective in the reduction potential of the metal center. The replacement of pyridine and tertiary amine in TPA ligand by two secondary amines in py2en observed a cathodic shift, and again when it was replaced by methylated py2en, there was an observed anodic shift in the complex. The CH_3 groups act as steric hindrance groups that keep the nitrogen atom away from the cobalt instead of increasing the basicity of amines through inductive effects. These increased the bond length, and hence the ability of N-atom for σ-donation readily decreased causing an anodic shift. The hypoxia selectivity of the complex has been demonstrated using ascorbic acid depleting in the presence of oxygen (Batista et al. 2018).

Melphalan is a derivative of amino acid phenylalanine anticancer drugs of the nitrogen mustard class. The toxicity of the drugs was shown by alkylation of DNA bases, rendering replication and transcription of DNA. The aziridinium form as an intermediate attacks the nucleic acid bases of DNA, and the availability of lone pair in the nitrogen mustard atom defined its reactivity. So in order to tune the reactivity of the nitrogen mustard family, a large alkyl or aryl group has been attached. In 2020, De Souza et al. synthesized a Co(III) L-phenylalanine complex with TPA, Py2en, Py2enMe, and bipy as ancillary ligands **(95–97)**. The electron donor capability of the ligand has been observed by the cathodic shift in the potential with the py2en ligand the most negative attributed to its σ-donor ability of two pyridyl and two 2^0 amine group followed by TPA due to the presence of three pyridyl and one 3^0 amine group. For complex with Py2enMe, a positive cathodic shift is observed due to the presence of the CH_3 group which causes the steric hindrance, and the bipy complex which has

the weakest σ-donor and π-acceptor shows a positive reduction potential. ΔE value of all complexes ranges from 0.14 V to 0.20 V showing irreversibility reduction. Ascorbic acid reduction shows O_2-dependent dissociation of L-phe at pH 7.4 by TPA, Py2en, and Py2enMe complex, while bipy complex showed independent disassociation unaffected by the presence of oxygen or change in pH (De Souza et al. 2020) (Fig. 17.2).

17.2.2.2.1.3 Bimetallic N,N,N,N Donor Ternary Co(III) Complexes

Quinizarin is an anthracycline class of drugs that shows anticancer activity against breast, lung, and ovarian cancers as well as neuroblastomas and leukemias. Quinizarin is present in a variety of anthracycline which is a class of antineoplastic drugs as a redox-active moiety and known to inhibit the growth of bacteria and have antiproliferative activity. Hence there are few reports of quinizarin-based bimetallic Co(III) ternary complexes with both cyclen and tpa type of ligands. Kozsup et al. in 2020 synthesized cobalt(III) complexes **(98–101)** with quinizarin. Tris (2-aminoethyl)amine (tren) and tris(2-pyridylmethyl)amine (TPA) are taken as ancillary ligands. The two ligands show reversible reduction potential at −450 to −800 mV, and the sulfonated ligand shows less negative reduction potential by 150 mV assigned to the electron-withdrawing potential of the sulfur atom. Both the complexes showed affinity toward HSA (human serum albumin) in which $quinSH_3$ ligand shows two binding sites on albumin. The complexes do not seem to have any affinity for HSA, but their reduction potential falls under the biological reduction window and has the potential to reduce under hypoxia conditions and initiate ROS generation (Kozsup et al. 2020a). The cytotoxic activity of both the complex was identified by Crlikova et al. in 2020 in HeLa cells, HCT-116 cells, MCF-7 cells, A2780 cells, and A2780 cisR. TPA is more potent than tren complex with IC_{50} ranging from 26.4 to 15 μM in all cell types. The TPA compound which is more lipophilic as compared to tren compound showed higher cellular accumulation in HCT-116 cells and showed greater DNA association by intercalative DNA-binding mode and increasing the rigidity of DNA, while TPA complex is proposed to bind with DNA by groove-binding mode. For antiproliferative efficiency, TPA complex was higher because of its redox potentials and its ability to form radicals that can cleave DNA by generating singlet oxygen via hydroxyl radicals (Crlikova et al. 2020). Kozsup et al. in 2020 reported four Co(III) complexes **(102–105)** with ternary N,N,N,N donor ligands like tris, tpa, and bidentate ligands quinH2 and quinSH3 (Fig. 17.3). The complexes exhibited binding affinity toward human serum albumin protein. The sulfonate complexes make the reduction potential of Co(III) center slightly more negative, and it also increases the binding affinity of the complexes toward HAS (Kozsup et al. 2020b).

17.2.2.2.2 Bio-Reductive Co(III) Complexes with Acetylacetonate Ligands

Acetylacetonate type of ligands also provides stability to the cobalt(III) complexes, and utilizing this type of ligands, we can attach cytotoxic agents like nitrogen mustard for bio-reductive drug release. These types of complexes showed promising results against the hypoxic tumor cells which lower pH levels.

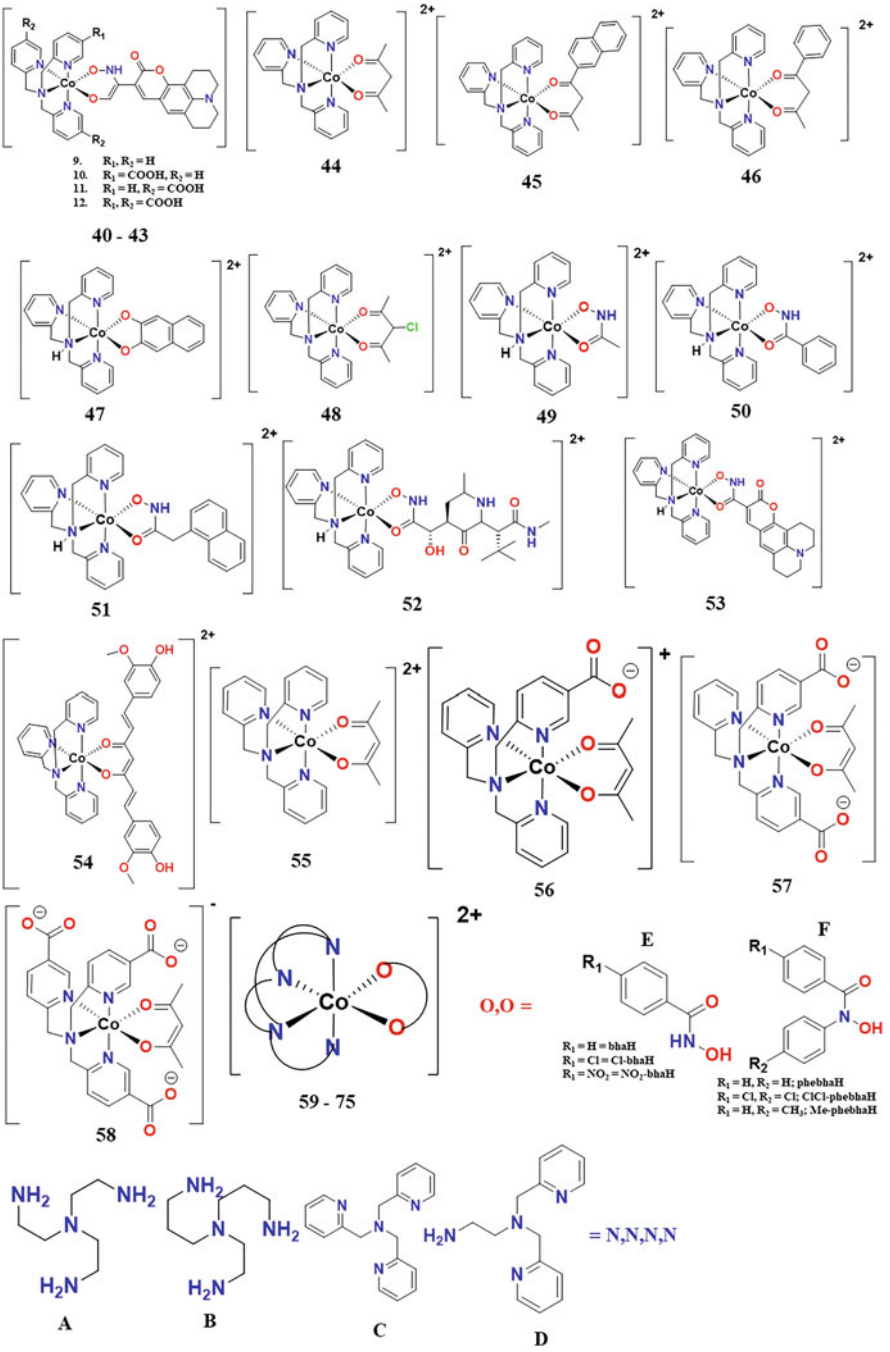

Fig. 17.2 Structure of tpa- and tren-based sp2 N,N,N and sp^3 N donor ternary Co(III) complexes (**40–97**)

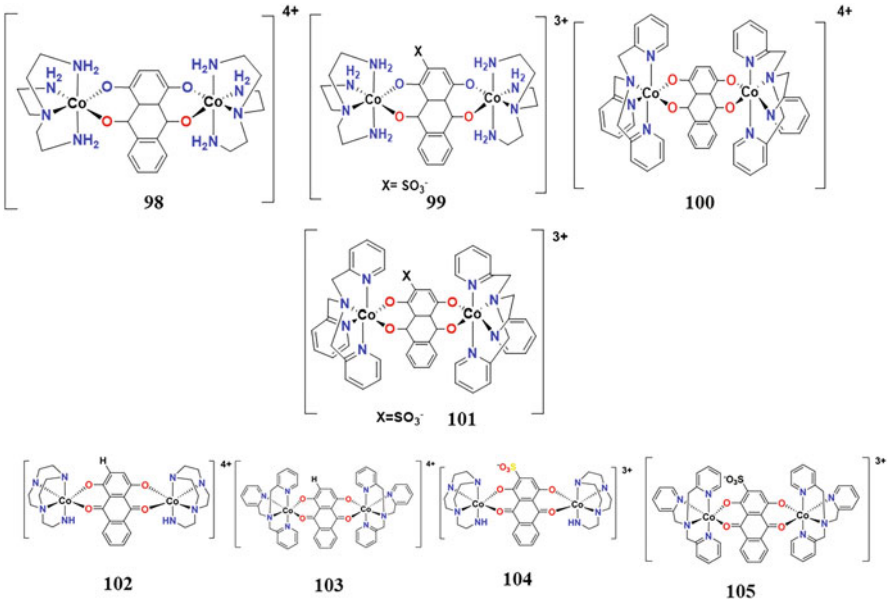

Fig. 17.3 Structure of bimetallic N,N,N,N donor ternary Co(III) complexes **(98–105)**

17.2.2.2.2.1 Binary Co(III) Complexes with Acetylacetonate and Nitrogen Mustard Ligands

Since 1942 nitrogen mustard compounds are known as very good alkylating agent. The labile Cl groups present in the nitrogen mustard compounds attribute to the cross-linking properties of the compound to DNA, and thereby they can prevent DNA replication and gradually lead to tumor cell death. Hence the release of nitrogen mustard compounds as cytotoxins in hypoxic tumor microenvironment is an effective strategy for hypoxic cancer treatment.

Ware et al. in (1991) showed that cobalt complexes with nitrogen mustard **(106–109)** could be hypoxia-selective cytotoxins. The cytotoxicity and selectivity of the compounds were evaluated in cell lines AA8 and UV4. The complexes showed similar cytotoxicity with the free ligand which showed that the toxicity was due to the release of the ligand after reduction. Acac derivatives as ancillary ligand with the chlorine substituent having higher reduction potential (−0.13 V) were shown to have large aerobic toxicity, while the complex with meacac ligand showed higher cytotoxicity in hypoxia conditions (Ware and Wilson 1991). Ware et al. in 1993 reported two types of Co(III) complexes with acac and nitrogen mustard ligands **(110–113)**. For improvement of the cytotoxic efficacy of the complex, they also prepared a similar Co(III) complex with anticancer nitrogen mustard drug melphalan. The melphalan-based complex exhibited higher cytotoxicity, where IC_{50} values were ranging from 34 ± 0.8 to 1.36 ± 0.06 μM in AA8 cell line and subline UV4. These melphalan-based complexes exhibited the release of melphalan in a hypoxic environment, thus resulting to the higher cytotoxicity of the

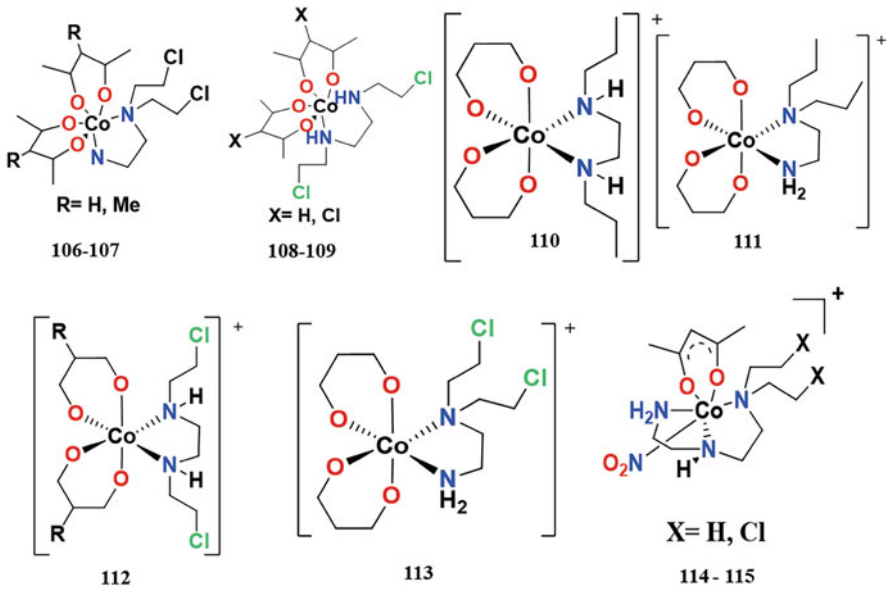

Fig. 17.4 Structure of binary Co(III) complexes with acetylacetonate and nitrogen mustard ligands **(106–115)**

melphalan-based complexes (Ware et al. 1993). In 2000, Ware et al. also synthesized a tridentate nitrogen mustard complex with cobalt(III) **(114–115)** but found it to be less toxic than the bidentate nitrogen mustard which might be due to deactivation or masking of the mustard group in coordination with metal center (Ware et al. 2000) (Fig. 17.4).

17.2.2.2.2.2 Binary Co(III) Complexes with Acetylacetonate and Other Bidentate Ligands

Thamilarasan et al. in 2016 reported three Co(III) complexes with acac ligand and bidentate ligands, bpy, en, 2-pic, and N_3 **(116–118)**. The complexes were found to be binding with CT-DNA and BSA. The complexes also exhibited pBR322 DNA cleavage in the presence of MPA, promoted by singlet oxygen generation. The complexes were also found to be cytotoxic against MCF-7 cell line (IC_{50} is in order **116** > **118** > **117**) (Thamilarasan et al. 2016). Mathuber et al. in 2020 reported Co(III) acetylacetonate complex with tyrosine kinase inhibitor **(119–122)**. Tyrosine kinase is a protein that triggers the loss of apoptosis in cancer cells. The methyl substitution of the complex increases the lipophilicity of the complex. The complexes exhibited significant stability in the blood plasma. In hypoxic conditions and higher concentrations of glutathione (GSH), the tyrosine kinase inhibitor gets detached from the complex and inhibits tyrosine kinase activity as evident from Western blot experiments. The complexes exhibited higher cytotoxicity in A431 cells at 0.1% oxygen concentration hypoxic environment (IC_{50} up to 7.2 μM) than

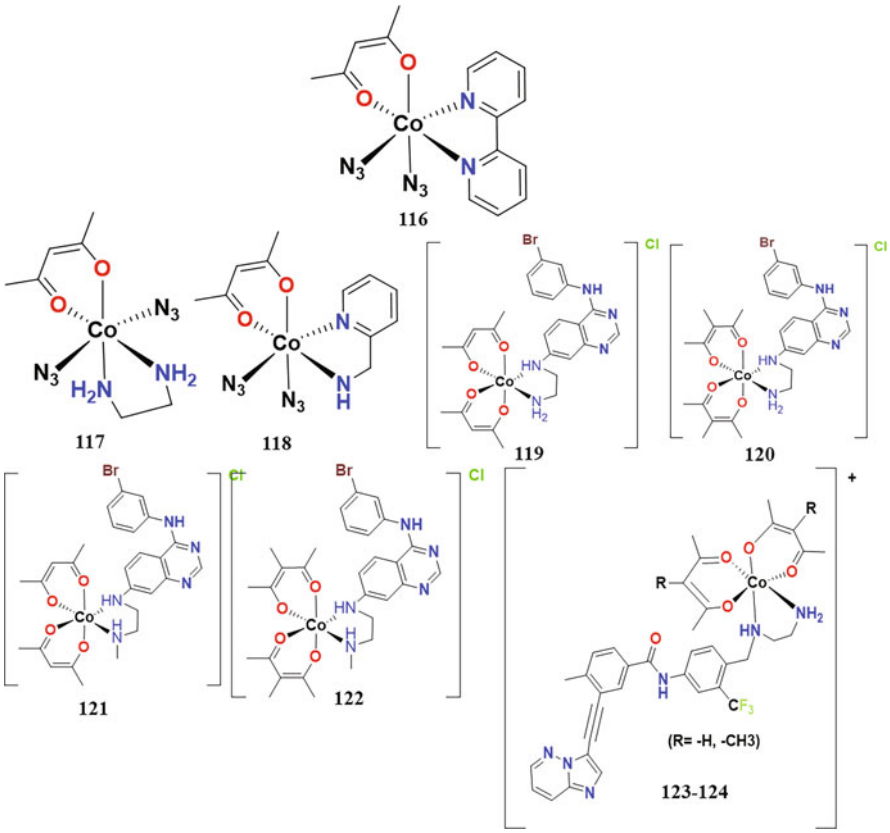

Fig. 17.5 Structure of binary Co(III) complexes with acetylacetonate and other bidentate ligands **(117–124)**

normoxia (IC_{50} 22.9). The cytotoxicity of the complex at 0.1% oxygen concentration in hypoxic environment is higher than the free tyrosine kinase inhibitor itself (Mathuber et al. 2020).

Ponatinib is known to inhibit tyrosine kinase which played an important role in cell growth, proliferation, or differentiation by catalyzing in the transfer of ATP. It targeted the Abelson kinase (ABL), FGF receptor (fibroblast growth factor), and PDGF receptor (platelet-derived growth factor). Ponatinib is a clinically approved and highly active anticancer drug. In 2021, Mathuber et al. synthesized a cobalt complex **(123–124)** with ponatinib using methyl acetone (meacac) and acetylacetone (acac) as ancillary ligands. The fluorescence emission wavelength of ponatinib at 470 nm was highly quenched in the complexes as the ligand-based fluorescence was difficult to observe due to the shorter lifetime of the triplet excited states (Fig. 17.5). The two complexes showed a single reduction irreversible cathodic peak which can be assigned to the reduction of Co(III) to Co(II). The cobalt meacac ponatinib complex was shown to have lower reduction potential implying more stability and

slow release of ligand than their corresponding acac complex with ponatinib and erlotinib derivatives which the group synthesized previously for EGRF inhibitors. Due to the -CH_2- spacer present in ponatinib, the ethylenediamine moiety is not in direct contact with quinazoline ring system, and hence the cathodic peak potential decreases which increases the stability of the complex in blood serum. The complexes show inhibition of FGFR- and ABL-dependent human cancer cells while only the acac complex exhibit toxicity in the leukemic K-562 model in vivo (Mathuber et al. 2021).

17.2.2.2.3 Co(III) Complexes with Schiff Base Ligands

N,N donor-type Schiff base ligands can also form stable Co(III) complexes, but their main activity is generated from type-I photo-processes by hydroxyl radical generation. These types of compounds can be utilized against normoxic cancer cells. King et al. in 2017 reported nine Co(III) bis(thiosemicarbazone) complexes **(125–133)**. The stability of the complexes in the phosphate buffer medium was found to be dependable on the axial ligand of the complexes. The stability trend was found to be NH_3 > imidazole > benzylamine. The equatorial bis(thiosemicarbazone) ligand dictates the cellular uptake and the cytotoxicity of the complexes. The diacetyl bis (thiosemicarbazone) complexes exhibited higher cellular uptake than that of pyruvaldehyde bis(thiosemicarbazone) and glyoxal bis(thiosemicarbazone) complexes. In hypoxic conditions, the cytotoxicity trends were also found to be similar. But the difference in cellular uptake or cytotoxicity was not drastic (King et al. 2017). Garcia et al. in 2016 reported four Co(III) complexes with Schiff base ligands and bidentate cytotoxic ligands **(134–136)**. The reduction potential of the complexes was in the biological redox window. Complex **135** exhibited a reduction by sodium dithionite followed by the release of the ligands. The complexes were also found to be binding with human serum albumin protein that suggests the facile transport of the complexes through blood (Garcia et al. 2017). In 2021 Gowdhami et al. reported two Schiff base Co(III) complexes **(137–138)** and reported their activity to inhibit the growth of A549 and MCF-7 cells. The complexes were reported to exhibit antiproliferative properties by the generation of ROS. The complexes also lead to express m-RNA and apoptotic genes, thereby triggering the apoptotic death of the cancer cells (Gowdhami et al. 2021). Cobalt(III) complexes derived from salen ligand **(139)** by areal oxidation with tert-butylated and trans-DACH ancillary ligands showed cytotoxic effects toward Burkitt lymphoma and leukemia cell lines (Fig. 17.6). The ligand didn't have any toxicity, and the Co(III) salen complexes showed maximum apoptosis at 50 μM concentration, and also it showed a reduction of cell proliferation of the Burkitt lymphoma by 80%. The inhibition was proved to be caused by apoptosis activating the caspase-3 responsible for induced cell death and also decreasing the mitochondrial membrane potential. The compound showed a cytotoxic effect against Nalm6 cell (NDau) which is a daunorubicin resistance cell with IC_{50} value of less than 70 μM (Hopff et al. 2020).

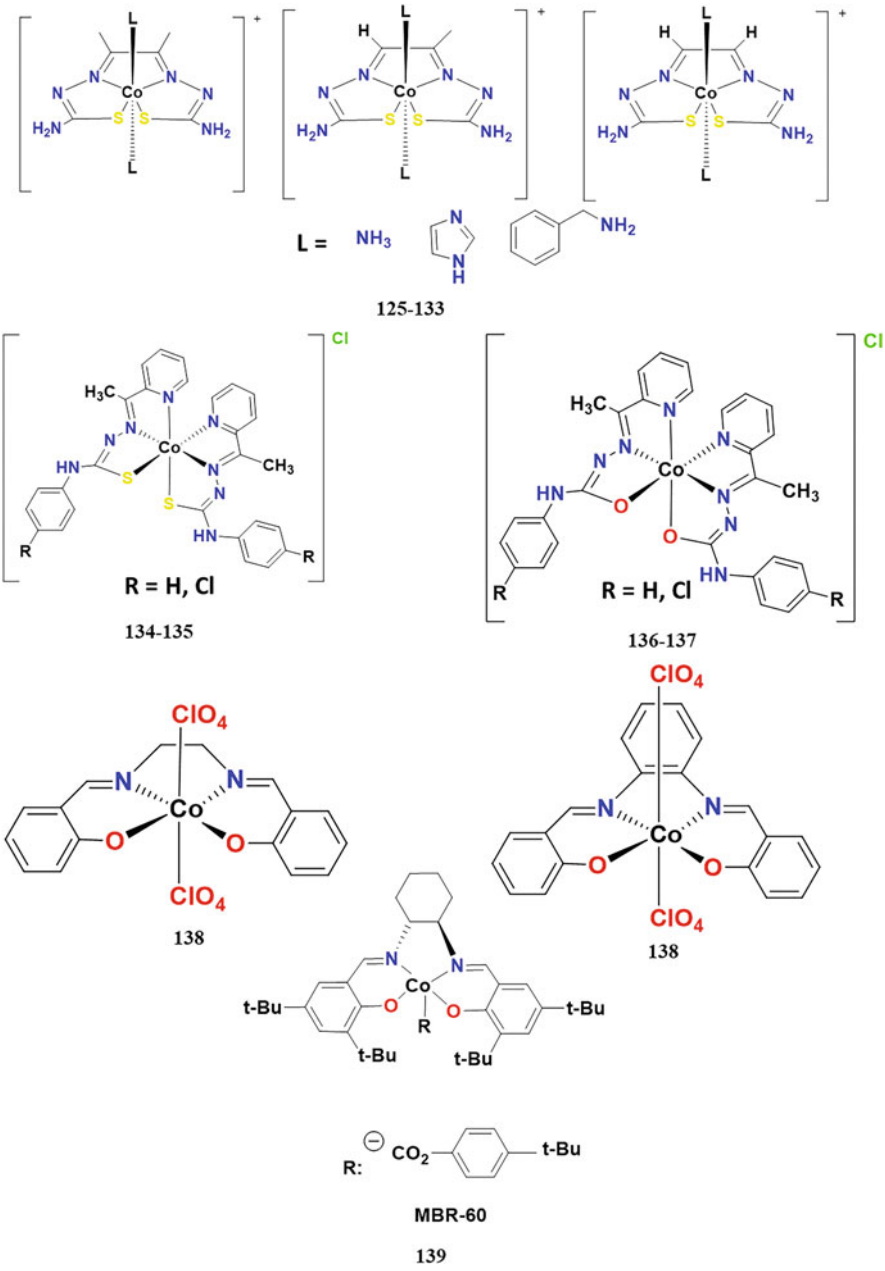

Fig. 17.6 Structure of Co(III) complexes with Schiff base ligands **(125–139)**

17.2.2.2.4 Co(III) Complex with Phenanthroline-Based Ligands

Sarkar et al. in 2021 reported a series of Co(III) complexes **(140–145)** with general molecular formulae $[Co(B)_2(L)]ClO_4$; here B is N,N donor phenanthroline-based ligand, and L is a catechol-based O,O donor ligand. The complexes exhibited d-d transition band around 700 nm with π- π* band at 403 nm. The complexes exhibited light-activated cytotoxicity against HeLa and MCF-7 cell lines. The complex **145** was also reported to exhibit light-activated DNA cleavage and generation of hydroxyl radical via type-I photo-redox process (Sarkar et al. 2021). Suntharalingam et al. synthesized a cobalt(III) cyclam complex **(146–148)** having nonsteroidal anti-inflammatory drug (NSAID)-bonded monodentate to it. NSAID is a type of drug which inhibits the production of prostaglandin which is an effector for inflammation (Fig. 17.7). The production of prostaglandins is mediated by cyclooxygenase COX-1 and COX-2. COX-2 is overly expressed in some cancer stem cells and plays a major role in the proliferation of the cell. Therefore inhibition of COX-2 has been exploited for cancer drug targeting. The cyclam complexes were less potent due to facile reduction of the metal center and immature release of NSAID ligand. For increased improvisation, they synthesized cobalt complexes with diflunisal bidentate NSAID ligand having salicylate moiety which stabilized the Co(III) center. The cytotoxicity of the complexes was observed in HMLER and HMLER-shEcad cell lines. IC_{50} value of all complexes ranges from 13.9 to 0.1 μM and 8.2 to 0.3 μM in both the cell lines. In breast cancer cell line MDA-MB-231, the complex shows the highest toxicity, and the complex with 1,10-phenanthroline releases diflunisal in the reducing environment and kills the cancer stem cells by damaging DNA and downregulation of COX-2 (Abe et al. 2018) (Table 17.2).

17.2.2.2.5 Other Types of Co(III) Complexes with Anticancer Activities

Ware et al. in 1991 reported the synthesis and hypoxia-selective cytotoxicity of Co (III) complex with molecular formulae [Co(Az)4(NO2)2]Br.2H2O.LiBr **(149)**. The crystal structure and the NMR spectroscopy suggested the formation of symmetric complexes. Cyclic voltammetry proves the complexes were also converted to Co (II) in an irreversible manner (Ware et al. 1991). Hypoxic conditions in the complexes were also reported to release aziridine in a facile manner. Denny et al. in 1997 reported a series of Co(III) complexes with general formulae [Co(trop)2(L)] +, where trop is tropolonate anion and L is a hypoxia-selective cytotoxic agent **(150–153)**. The complex possesses significantly higher reduction potentials than Co (III) (acac) complexes. The Co(III) (trop) complex with nitrogen mustard ligand possesses IC_{50} values similar to free nitrogen mustard ligand. But the complex 3-methyl acac exhibited significant hypoxia selectivity in AA8 and UV4 cell lines. The IC_{50} value ranged from 3100 μM to 4.6 μM, and the q ratio was found to be in the range of 0.7 to 47 (Ware et al. 1997). Gust et al. in 2004 reported three Co-alkyne complexes **(154–155)** and found that the complexes exhibit growth-inhibiting properties against LAMA-84, K-562, SD-1 leukemia, and U-937 lymphoma cells. The IC50 values were found to be in the range of 7.7 to 18.6 μM. The complexes were sensitive against LAMA-84 cell line but found to be insensitive against K-562 cells (Ott et al. 2003). Trofimov et al. in 2019 reported Co(II) complex of

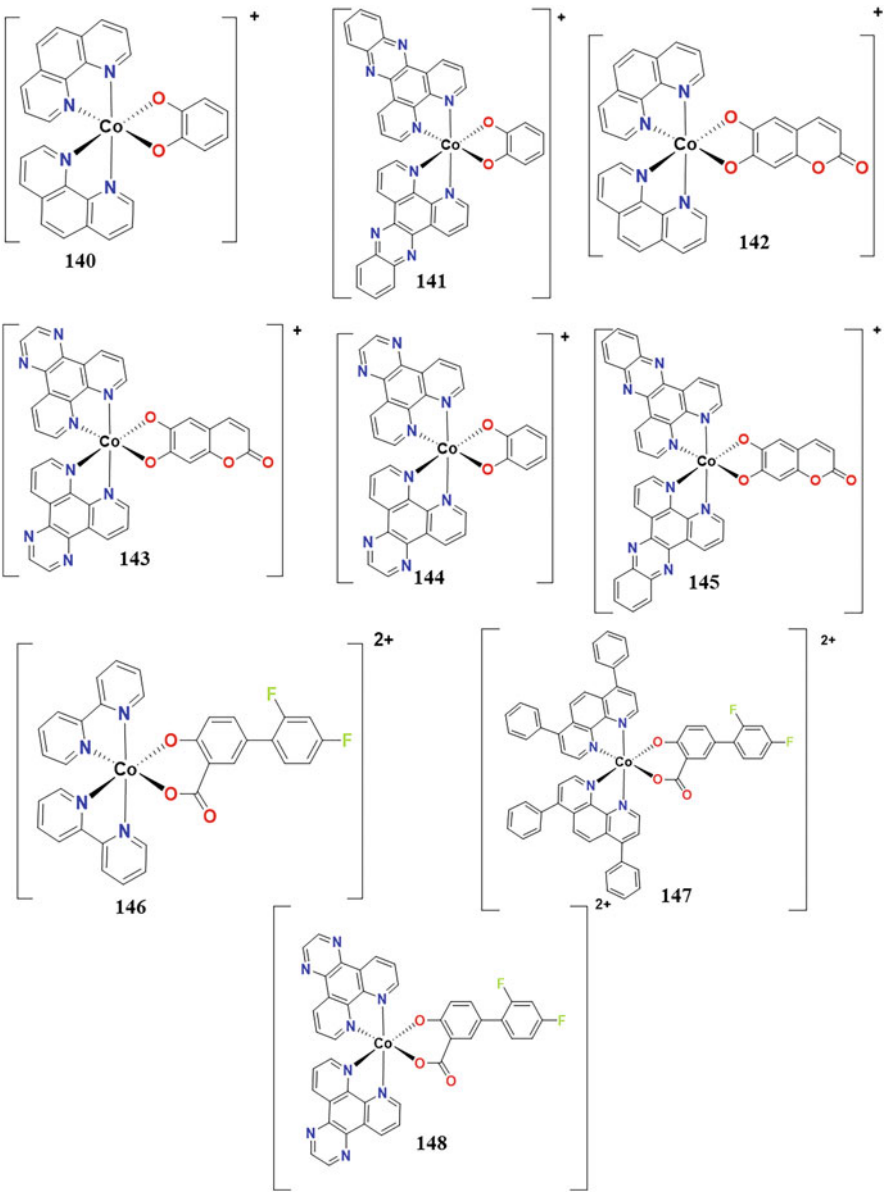

Fig. 17.7 Structure of Co(III) complex with phenanthroline-based ligands **(140–148)**

N-allylimidazole **(155),** and it was reported to exhibit anti-hypoxic effects against all types of acute hypoxia, and thereby it increases the lifetime of white nonlinear mice dosage. The complex had more anti-hypoxic properties than mexidol and hypoxen. Hence the complex is a suitable candidate for hypoxia-related clinical trials

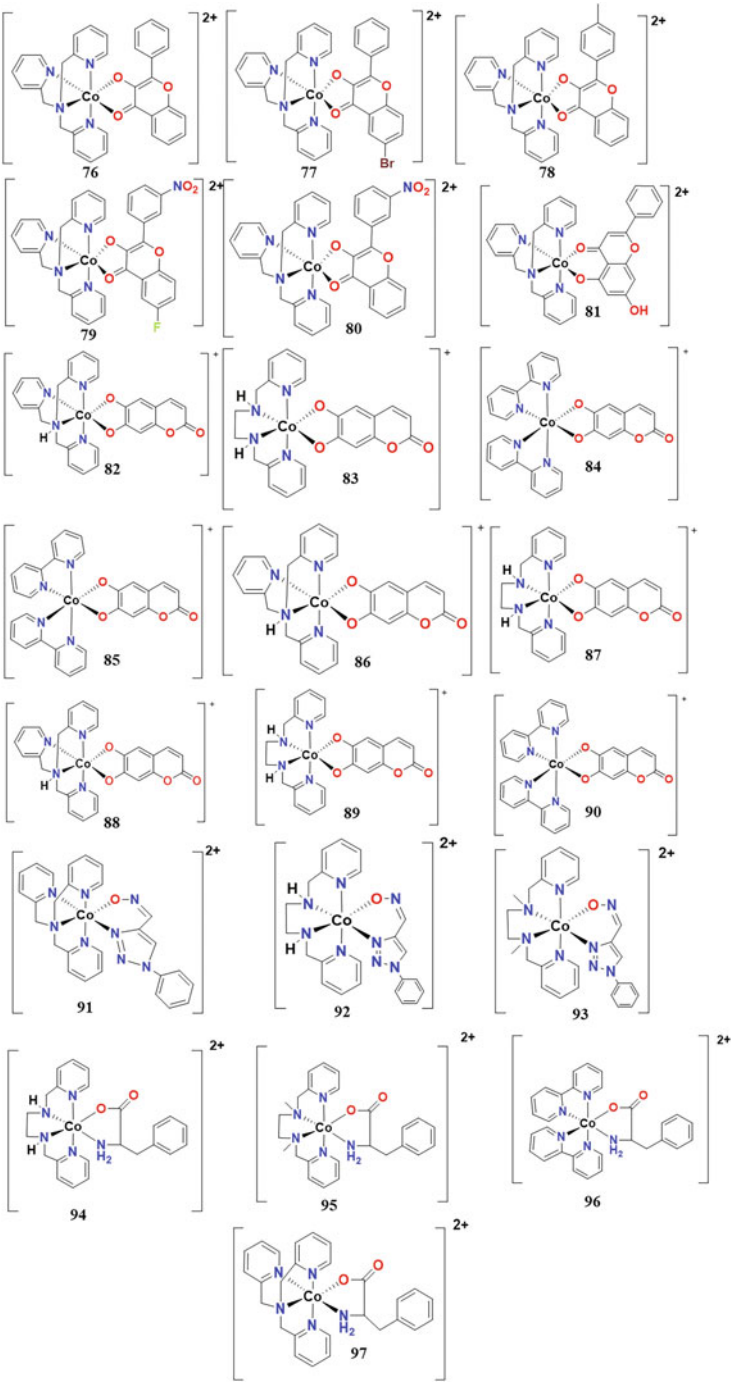

Fig. 17.2 (continued)

Table 17.2 Cytotoxicity of Co(III)-based prodrugs against different cancer cell lines

Compound	Cell line	IC_{50}		Mode of action	References
		Normoxia	Hypoxia		
2	HT-29	4.94 nM	0.43 nM	Release of cytotoxic azaCBI derivative in the hypoxic condition	Ahn et al. (2006)
3	SKOV3	38 nM	3.7 nM	Release of cytotoxic azaCBI derivative in the hypoxic condition	(Lu et al. 2011)
3	HT29	69 nM	5.6 nM		
4	SKOV3	7.9	0.4		
4	HT29	14	0.67		
5	SKOV3	4.5	0.91		
5	HT29	1.43	0.28		
6	SKOV3	2.96	0.53		
6	HT29	1.08	0.12		
7	SKOV3	0.98	0.25		
7	HT29	1.6	0.56		
8	SKOV3	0.74	0.22		
8	HT29	1.2	0.41		
9	DLD-1	–	>200	Release of cytotoxic naacH derivatives in the hypoxic condition	(Bonnitcha et al. 2012)
10		–	>200		
11		–	>200		
12		–	>200		
13		–	>200		
14		–	>200		
15		–	>200		
16		–	>200		
17		–	>200		
18		–	>200		
19		–	>200		
20		–	>200		
21		–	>200		
22		–	140		
23		–	184		
24	SKOV3	44	0.67	Release of 8-hydoxyquinoline in the hypoxic condition	(Chang et al. 2013)
24	HT29	66	1.2		
25	SKOV3	110	0.52	Release of seco-CPyI-TMI in the hypoxic condition	
25	HT29	63	0.71		
26	SKOV3	<3.7	0.40	Release of seco-CPyI-TMI in the hypoxic condition	
26	HT29	–	–		
27	HepG2		59.89	Release of phenanthroline	(Gopinathan et al. 2014)
28			464.6		
33	A431	44	88		(Kozsup et al. 2021)
34		17	26		

(continued)

Table 17.2 (continued)

Compound	Cell line	IC_{50}		Mode of action	References
		Normoxia	Hypoxia		
35		55	91	Release of flavonoid derivatives in the hypoxic conditions	
36		13	17		
37		>100	>100		
33	A549	>100	>100	Release of flavonoid derivatives in the hypoxic conditions	
34		14	17		
35		89	73		
36		6.8	8.9		
37		>100	>100		
40	DLD-1	–	40	Release of fluorescent coumarin in hypoxic condition	(Gopinathan et al. 2014)
41		–	43		
42		–	46		
43		–	58		
44	DLD-1	–	>200	Release of bidentate ligands like hydroxamic acid, β-diketone, and catechol	(Kozsup et al. 2021)
45		–	74		
46		–	>200		
47		–	>200		
48		–	>200		
49		–	>200		
50		–	>200		
51		–	>200		
52		–	188		
53		–	31		
54	DLD-1	–	30	Release of cytotoxic curcumin in hypoxic condition	(Yamamoto et al. 2012)
55	DLD-1	–	> 4 mM	–	(Renfrew et al. 2013)
56		–	> 4 mM	–	
57		–	> 4 mM	–	
58		–	> 4 mM	–	
79	A431	68	64	Release of flavonoid derivatives in the hypoxic conditions	(Buglyó et al. 2017)
80		>100	>100		
81		15	22		
82		14	23		
79	A549	34	33		
80		72	48		
81		5.2	6.9		
82		6.3	7.3		
83	–	–	>100		
84	HCT-116	–	>100	Release of coumarin moiety	(Palmeira-Mello et al. 2020a)
	HCT-29	–	>100		
85	HCT-116	–	31		
	HCT-29	–	>100		

(continued)

Table 17.2 (continued)

Compound	Cell line	IC_{50}		Mode of action	References
		Normoxia	Hypoxia		
87	HCT-116	>100	>100	Release of coumarin moiety	(Palmeira-mello et al. 2020b)
	HT-29	>100	>100		
	HEK-293	>100	>100		
88	HCT-116	60	31		
	HT-29	>100	>100		
	HEK-293	>100	>100		
89	HCT-116	–	>100	Release of coumarin moiety	
	HCT-29	–	>100		
98	HeLa		30	Release of anthracycline ligand	(Kozsup et al. 2020a)
	HCT-116		56.3		
	MCF-7		26		
	A2780		34		
100	HeLa	–	26.4		
	HCT-116	–	28		
	MCF-7	–	15.1		
	A2780	–	16		
	A2780-cisR	–	19.6		
106	AA8/UV4	IC_{50} (AA8/UV4) = 12		–	(Ware and Wilson 1991)
107	AA8/UV4	IC_{50} (AA8/UV4) = 48		–	
108	AA8/UV4	IC_{50} (AA8/UV4) > 10		Release of nitrogen mustard ligands	
109	AA8/UV4	IC_{50} (AA8/UV4) > 10			
112	AA8	–	34	–	(Ware et al. 1993)
113	AA8	–	1.36	–	
114	AA8/UV4	IC_{50} (AA8/UV4) > 10		Release of nitrogen mustard ligand	(Ware et al. 2000)
115	AA8/UV4	IC_{50} (AA8/UV4) > 10			
116	MCF-7		49		(Thamilarasan et al. 2016)
117	MCF-7		98		
118	MCF-7		92		
119	A431	22.9	7.2	Release of EGFR inhibitor	(Mathuber et al. 2020)
120	A431	51.9	23.5		
121	A431	15.1	11.9		
122	A431	58.6	19.9		
123	Leukemic K-562	–	6.8 nM	Release of ponatinib	(Mathuber et al. 2021)
	UM-UC-14	–	8 nM		
124	Leukemic K-562	–	5.5 nM		
	UM-UC-14	–	10 nM		

(continued)

Table 17.2 (continued)

Compound	Cell line	IC_{50}		Mode of action	References
		Normoxia	Hypoxia		
125	HeLa	>500	>500		(King et al. 2017)
	A549	>500	>500		
	MRC-5	–	–		
126	HeLa	>500	>500		
	A549	>500	>500		
	MRC-5	–	–		
127	HeLa	>500	>500		
	A549	>500	>500		
	MRC-5	–	–		
128	HeLa	>500	>500		
	A549	>500	>500		
	MRC-5	–	–		
129	HeLa	22	15		
	A549	16.9	21.8		
	MRC-5	–	–		
130	HeLa	28	17		
	A549	4.2	8.6		
	MRC-5	–	–		
131	HeLa	21	15		
	A549	12	9.2		
	MRC-5	>250	–		
132	HeLa	7.4	3.7		
	A549	2.6	2.9		
	MRC-5	>250	–		
133	HeLa	7.3	6.3		
	A549	5.8	8.2		
	MRC-5	>250			
138	MCF-7		3.55		(Gowdhami et al. 2021)
	A549		10.78		
	3 T3		85.15		
139	BJAB-FADDdn		<50 μM	Release of MBR 60	(Hopff et al. 2020)
	Nalm6		<70 μM		
140	HeLa		>50		(Sarkar et al. 2021)
141	HeLa		28.9		
142	HeLa		5.0		
143	HeLa		20.1		
144	HeLa		10.0		
145	HeLa		1.6		
146	HMLER		13.9		(Abe et al. 2018)
	HMLER-shEcad		8.2		

(continued)

Table 17.2 (continued)

Compound	Cell line	IC_{50}		Mode of action	References
		Normoxia	Hypoxia		
	CSC spheroid cell lines		>133		
147	HMLER		0.1		
	HMLER-shEcad		0.3		
	CSC spheroid cell lines		0.8		
148	HMLER		3.9		
	HMLER-shEcad		2.1		
	CSC spheroid cell lines		22.8		
150	AA8		3100		(Ware et al. 1997)
151	AA8		150		
152	AA8		120		
153	AA8		0.69		
157	DLD-1	–	20.4	Release of anthraquinone-2-carboxillic acid	(Kim et al. 2011)
158	DLD-1	–	1.9	Release of coumarin	

(Trofimov et al. 2019). Bryce et al. developed a fluorescence probe model for hypoxia cell target delivery by two cobalt(III) cyclam complexes with the fluorescent ligand anthraquinone-2-carboxylic acid and coumarin **(157–158)** (Fig. 17.8). The cytotoxicity of the complex was observed in DLD-1 colon cancer cells and found that the coumarin cyclam complex was more toxic with IC_{50} value 1.3 μM in normoxic and 1.9 μM in hypoxic conditions. Due to the presence of negatively charged oxygen donor in both the fluorophore, there is greater stabilization of the metal center with more negative reversible reduction potentials. These compounds are also able to release drugs at the pH of the cancer cells. The release of coumarin is not complete in hypoxia and the delayed release of the ligands enhanced the penetration of the compounds inside the cells in the reduced cellular environment (Kim et al. 2011).

17.3 Summary and Conclusion

Among all types of cancers, hypoxic tumor cells are specifically very difficult to treat because of their solid outer surface which prevents the permission of the radiation used for treatment. As these type of tumors are distantly localized from the blood

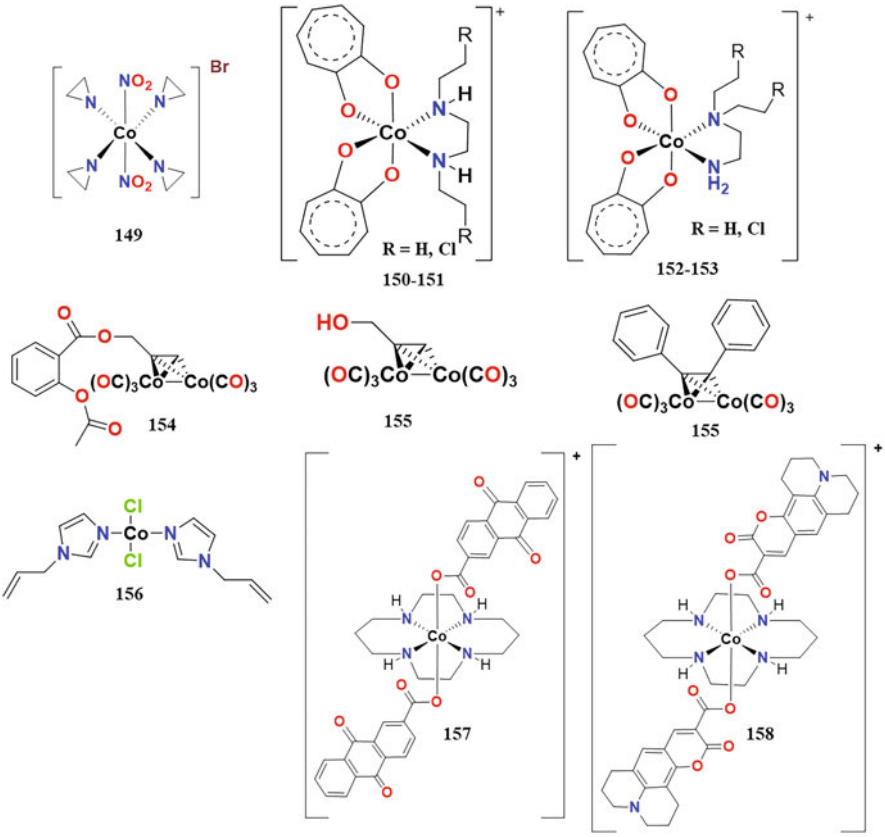

Fig. 17.8 Structure of other types of Co(III) complexes with anticancer activities **(149–158)**

vessels. Hence it is very difficult to supply chemotherapeutic drugs to this kind of cancer cell. Also, the lack of oxygen supply in these cells makes the activity of the chemotherapeutic drug more difficult as the chemotherapeutic drugs generally generate reactive oxygen species and activate the apoptotic mechanism in the cancer cells by inducing oxidative stress. And some chemotherapeutic drugs act as DNA binders, but the lack of oxygen adds the presence of reducing agents like glutathione and NADPH making this type of drug practically useless. Hence to treat this type of cancer, we require bio-activable single agents that can be activated in a lower pH environment. Several organic molecules are implemented for this kind of cancer cell, but the use of transition metal complexes gives us tunability and dual activity for our active agent against this type of cancer. Platinum complexes are also used for this type of cancer cell treatment. But the drawback is that platinum complexes get easily bound with sulfur-containing reducing agents like glutathione and lose their activity, and these cases generate a possibility of heavy metal toxicity in the patient's body. To overcome this drawback, researchers have implemented Co(III) complexes as

bio-reducible prodrugs; for two decades researchers have been developing Co(III) complexes for hypoxia-selective drug delivery. This type of complex provides an excellent template for attachment of bidentate donor ligands as drugs with N,O or O, O donor sites, and in the hypoxic condition, these bidentate ligands get released in the cancer cells and show their activity. Cobalt(III) complexes with ternary nitrogen-based donor ligands like cyclam, tpa, tpb, tren, etc. are frequently used for this type of metal complex. Along with them bidentate ligands like acetylacetone complexes also can release nitrogen mustard-like drugs. Other Co(III) complexes with Schiff base and phenanthroline-based ligands are also reported to exhibit hypoxia-selective activity. There are also reports of Co(III) phenanthroline-based complexes that absorb at 700 nm and can generate hydroxyl radical by inducing a photoinduced type-I process. This type of complex can further be modified for organ-selective drug delivery into the hypoxic cells or specific hypoxic cell targeting rather than normal cells. This way the Co(III) complexes can open a wide range of scope, which can be used to treat these types of hypoxic cancer cells. Hypoxia is also associated with several proteins like HIF-1α, MMP, VEGF, etc.; hence by exploiting the bio-reductive nature of Co(III) complexes, we can release the inhibitors for those proteins and other potent anticancer agents for hypoxia-selective treatment.

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Hypoxia-Induced Apoptosis in Cancer Development

18

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Abstract

According to World Health Organization reports, cancer is the leading cause of mortality, accounting for approximately ten million deaths or 1 in 6 deaths globally. The cancer care burden is increasing, putting enormous physical, emotional, and economic pressure on individuals, families, communities, and healthcare systems worldwide (Prager et al. ESMO Open 3:e000285, 2018). Many patients are losing their lives each year due to the inaccessibility to high-standard healthcare facilities and early and proper diagnosis and treatment, especially in health systems belonging to the countries which fall under low- and mid-income categories (Alkire et al. Health Affairs (Project Hope) 37:988–996, 2018). Cancer can be cured and prevented if a proper lifestyle is maintained and a correct diagnosis and effective treatment regimen are followed. Early detection, world-class treatment, and survivorship care improve cancer patients' survival rates and life expectancy in countries with advanced systems.

A variety of factors are involved in cancer progression in the human body. One of the significant factors is a low level of oxygen or hypoxia in the tumor; the pancreatic tumor with 0.3% O_2 is the most severe, and the brain tumor with 1.7% O_2 is the most minor (Koong et al. Int J Radiat Oncol Biol Phys 48:919–922, 2000; Rampling et al. Int J Radiat Oncol Biol Phys 29:427–431, 1994). The poor prognosis of cancer patients has been linked to hypoxia which has been proved to be the most common characteristic of solid tumors. Cancer cell metabolism

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promotes therapeutic resistance by inducing cell quiescence under hypoxic conditions (Semenza Oncogene 29:625–634, 2010). In tumor cells, the interaction of a complex cell-signaling network including HIF, PI3K, MAPK, and NF-kB pathways is activated by hypoxia, creating a positive and negative feedback mechanism leading to the amplification or depletion of hypoxic effects (Luo et al. BioMed Res Int 2014:409272, 2014).

Hypoxia can induce or resist apoptosis or programmed cell death, one of the essential factors cancer cells must evade for their survival, growth, and continued proliferation. Whether the cell will become apoptotic or antiapoptotic depends on the severity of hypoxia (Greijer and van der Wall. J Clin Pathol 57:1009–1014, 2004). This chapter discusses in detail how hypoxia is extensively connected to apoptosis and how, under severe conditions, it can lead to the development and progression of cancer with implications for treatment for the disease.

Keywords

Hypoxia · Prognosis · Cancer · HIFs · Apoptosis · Cancer development

18.1 Apoptosis

Apoptosis is an essential cellular process that allows cell death induced by a tightly regulated suicide program at the molecular level, activating an intrinsic enzyme that causes the degradation of genomic DNA, nuclear proteins, and cytoplasmic proteins (Wong 2011). The cell is fragmented into apoptotic bodies bound by a plasma membrane containing cellular organelles, which contain cytoplasm and may or may not have nuclear pieces. During the process of apoptosis, the cell's plasma membrane is not damaged, but some structural changes occur in this stage (Wong 2011). For example, in healthy cells, phosphatidyl serine is present in the inner leaflet of the plasma membrane. Still, when the cell is damaged, phosphatidyl serine is flipped to the outer side of the plasma membrane (Lee et al. 2013). Macrophages easily identify these types of apoptotic fragments, and these macrophages phagocytose these apoptotic fragments without causing any inflammation or blocking any tissue damage (Lee et al. 2013). An apoptotic cell exhibits structural and morphological features, including cellular shrinkage, blebbing in the plasma membrane, mitochondrial swelling and release of cytochrome c, chromatin condensation, and DNA fragmentation (Fink and Cookson 2005).

Apoptosis plays a pivotal role in removing faulty cells. If DNA damage is beyond repair, then apoptosis is induced. DNA damage occurs from radiations or cytotoxic drugs directly or by producing free radicals. During this stage, P53-dependent pathway comes into action. They initially try to repair the DNA, but if this repair mechanism fails, P53 triggers apoptosis via the mitochondrial or intrinsic pathway (Hirsch et al. 1997). Cytotoxic T lymphocytes are essential in inducing apoptosis of the virally infected cells and killing tumor cells.

In the human body, the development of cancer is influenced by a variety of factors (Koong et al. 2000; Rampling et al. 1994). The relationship between tumorigenesis and apoptosis has a long history because it is known that some oncogenic mutations impair apoptosis, which leads to the beginning and spread of tumors. Other oncogenic alterations promote apoptosis and selectively emphasize this process over others during multistage carcinogenesis. Also, it is well-known that most cytotoxic anticancer agents induce apoptosis, opening up the possibility that cancer treatment failure can be related to defects in apoptotic programs as the same mutations that suppress apoptosis during tumor development can also reduce treatment sensitivity. Apoptosis provides a bridge linking cancer genetics with cancer therapy (Lowe and Lin 2000).

Some well-known oncogenes contribute to apoptosis by promoting or suppressing it, such as the BCl-2 family member proteins identified in mammalian cells. The study of BCl-2 oncogene established the importance of apoptosis in tumor development (Gross et al. 1999). With BCl-2, BCl-Xl is also a potent death suppressor and is upregulated in some tumor types (Green and Reed 1998). On the contrary, BAX, a death promoter, is inactivated in some colon cancer types and hematopoietic malignancies. Similarly, p53 was the first tumor suppressor gene linked to apoptosis. p53 mutations occur in most human tumors and are a critical factor of study regarding cancer (Schmitt et al. 1999). In apoptosis, the cell is broken down from within with the help of caspases. Caspase needs to be activated to trigger apoptosis and can be induced by tumor suppressor genes such as p53. Caspase activation can happen via two distinct pathways called the intrinsic and extrinsic pathways (Schmitt et al. 1999).

18.1.1 Intrinsic Pathway

The first pathway of apoptosis is the intrinsic pathway, also called the mitochondrial pathway for apoptosis. The apoptotic factor BCl-2-associated X protein (BAX), which leads to cell death, regulates mitochondria to release apoptotic substances like cytochrome c. This pathway can be activated by cells with damaged DNA or oncogenes. Deprivation of growth factors, excess Ca2+, DNA-damaging chemicals, oxidants, and microtubule-targeting medications are additional triggers for this pathway. The BCl-2 protein family controls the pathway as a whole. The overexpression of BH3-only proteins is caused by various apoptotic stimuli, activating BAX and BAK (Lomonosova and Chinnadurai 2008). Once triggered, BAX and BAK oligomerize, which causes permeabilization of the mitochondrial outer membrane (MOMP) (Lopez and Tait 2015). MOMP is the point of no return and the defining event of intrinsic apoptosis. Further, intermembrane proteins such as cytochrome c, a second mitochondria-derived activator of caspase (SMAC) (Hassan et al. 2014), can be released due to permeabilization (Fig. 18.1).

Evidence shows that inhibitors of apoptosis (IAPs) check cancer cells' natural inclination to undergo apoptosis (Berthelet and Dubrez 2013). For this reason, abnormal cells with high basal levels of caspase-3 and caspase-8 activity and active

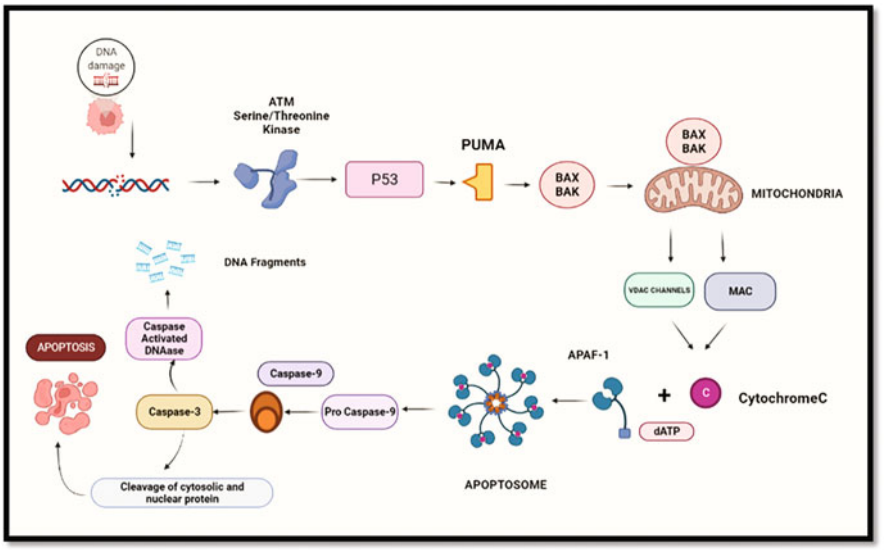

Fig. 18.1 This figure describes the steps involved in the intrinsic pathway of apoptosis. DNA damage acts as an initiation of apoptotic signals. When DNA lesion is in the form of double strand breaks, the first molecule that is being recruited is ATM serine/threonine kinase which in turn activates p53 protein. This protein regulates one more protein that is PUMA (p53-upregulated modulator of apoptosis) which is a proapoptotic protein. PUMA then activates the BAX protein (BCl-2-associated X protein) in the cytosol. This activated BAX becomes mitochondrial membrane-bound. Then this BAX opens VDAC channels (voltage-dependent anion channels) of mitochondria. When a cell is damaged or if it stops receiving survival signals, BCl-2 and BCl-Xl are blocked in turn. BAX and BAK (BCl-2 homologous killer) then punch a series of channels in mitochondria, undergoing a series of reactions in the presence of reactive oxygen species and calcium ion cardiolipin leading to the leakage of cytochrome c into the cytoplasm. The cytochrome c then combines with APAF-1 in the presence of dATP. This biochemical reaction forms a complex called the apoptosome, further releasing dADP after consumption of dATP. Apoptosome then recruits procaspase-9 and converts into its activated form, caspase-9. Caspase-9 then activates caspase-3, which acts as an endonuclease and activates caspase-dependent DNAs within the nucleus. After this DNA fragmentation takes place. Caspase-3 cleaves cystolic and nuclear proteins that mark the death of the cells

caspase-3 fragments in the absence of apoptosis were found in various tumor cell lines and cancer tissues. High levels of IAP expression were also found in tumor cells, indicating that elevated IAP expression specifically reduced the high-baseline caspase activity in tumor cells. IAP-targeting techniques are therefore considered to be a viable strategy for improving the efficacy of cytotoxic drugs against cancer (Baig et al. 2016; Finlay et al. 2017; Saelens et al. 2004).

Cancer cells usually have mutations in the genes that control the mitochondrial pathway. Such alterations are typically connected with treatment resistance because the bulk of anticancer drugs causes apoptosis in cancer cells by activating the intrinsic pathway (L. Chen 2018). For instance, chromosomal translocation of the BCl-2 oncogene into the immunoglobulin heavy chain gene locus results in

overexpression of BCl-2 in around 85% of human follicular lymphomas. Since members of the BCl-2 family with several BH domains that promote apoptosis are overexpressed to promote oncogenesis, these family members function as tumor suppressors (Adachi et al. 1989; Tsujimoto et al. 1984, 1985; Tsujimoto and Croce 1986). In addition to genetic modifications, the aberrant expression of BCl-2 family proteins is primarily controlled at the transcriptional or posttranscriptional level.

Along with BCl-2 family proteins, Apaf-1 activity is diminished or missing in ovarian cancer, melanoma, and leukemia. The intrinsic route is also affected by mutations in the tumor suppressor gene p53, the most prevalent genetic flaw in human tumors (Delbridge et al. 2016).

18.1.2 Extrinsic Pathways

The second pathway is referred to as the extrinsic pathway because initial signals come from outside the cell. The extrinsic pathway requires an external stimulus, usually from an immune cell, commonly from the subsets of T lymphocytes. The signaling molecule and receptors can mediate this apoptosis pathway in two ways. The first is the FAS pathway or apoptotic signal pathway, and the second is the TNF pathway or tumor necrosis factor pathway.

18.1.2.1 FAS Pathway of Apoptosis

In the extrinsic pathway, the FAS receptors are expressed in its membrane constitutively at any given time. The lymphocytes have a surface molecule known as FAS ligand or FAS-L. The extrinsic pathway is initiated when FAS-L binds with FAS receptors on the surface of the targeted cells. The FAS receptors are transmembrane proteins, and on its cytosolic side, there are also other proteins, namely, FADD (FAS-associated death domain). This naturally sets off a chain of intracellular events, resulting in apoptosis. The FADD proteins will bind to an inactive zymogen called procaspase-8. Zymogens are inactive enzymes incapable of inducing cell death; only when these proenzymes or procaspases become fully functional they can execute the program for apoptosis and cause cell death (Fig. 18.2).

Cancer cells have developed a variety of defense mechanisms to avoid extrinsic cell death induction. A rise in antiapoptotic molecules or a fall in proapoptotic protein levels or activity can be the main inhibitors of the signaling to cell death in response to death receptor ligation. For instance, the surface expression of death receptors may differ depending on the cell type and may be downregulated or missing in cancer resistant to treatment. Drug-resistant leukemia or neuroblastoma cells display significant downregulation of CD95 expression (Friesen et al. 1997). Human malignancies frequently include CD95 mutations indicating tumor-suppressing properties of CD95 (Friesen et al. 1997). Decoy receptor3 (DcR3) is also amplified or overexpressed in lung, colon, and glioblastoma carcinomas. DcR3 functions as a decoy receptor by competitively binding CD95L (Pitti et al. 1998), and it can obstruct CD95-triggered apoptosis.

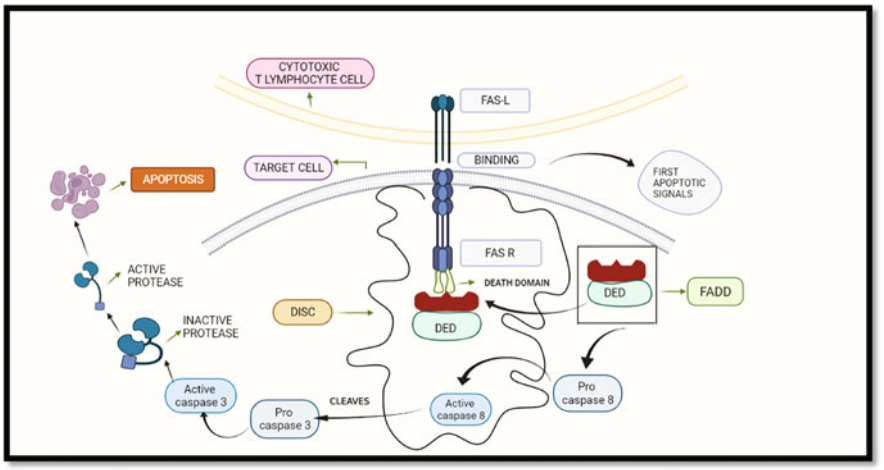

Fig. 18.2 In the figure below, it can be seen that on the one hand, there is a cytotoxic T lymphocyte cell, and to that cell, there is a bound FAS ligand (FAS-L), a signaling protein in apoptosis. On the other hand, there is a target cell that needs to be eliminated by apoptosis, and to that cell, a FAS receptor (FAS R) is attached on its membrane. This FAS R acts as a death receptor. The binding sends the first apoptotic signal when the FAS-L and FAS R bind together. FAS R contains death domain in its intracellular path. After binding and interaction between ligand and receptor molecule, FADD adaptor molecule is recruited. This FADD molecule comes in and binds with the death domain of FAS R. Death effector domain (DED) of FADD molecule further recruits a molecule called procaspase-8, which gets activated to its fully functional form, which is caspase-8. Finally, a bunch of molecules exists together, including FAS R, FADD, DED, and caspase-8 enzyme, forming a single complex molecule called DISC (death-inducing signaling complex), starting the cascade part of the process at this point. This process is carried out as caspase-8 cleaves procaspase-3 into its active form, that is, caspase-3. The active caspase-3 acts on inactive apoptotic substrate (caspase-dependent DNAs, proteolytic enzymes, or proteases in their inactive form) and converts them into their active apoptotic effectors (caspase-dependent DNAs, proteolytic enzyme, or proteases in its active form) which in turn degrades DNA and nuclear lamina, respectively, eventually inducing cell death

Human cells have been found to contain two splice variants of FLIP, a long form (FLIPL) and a short form (FLIPS), both of which lack the catalytic site of caspase-8 and caspase-10. Consequently, caspase activation is inhibited by recruiting FLIP to the DISC instead of procaspase-8 or procaspase-10, thus evading apoptosis. Numerous tumor cells have high FLIP expression, which has been linked to resistance to TRAIL or CD95 as well as chemotherapy-induced apoptosis.

18.1.2.2 TNF Pathway of Apoptosis

Tumor necrosis factor can be considered one of the most potent inducers of programmed cell death or apoptosis. TNF stimulates the mechanism of cell death and cell survival simultaneously. NF-kB-dependent genes undergo activation, which in turn regulates the cell survival or proliferative effects of TNF, whereas activation of caspases regulates cell death. Apoptosis is induced by TNF primarily through

activation of type 1 receptors, whose death domain simulates the expression of more than a dozen signaling proteins that are part of an apoptotic cascade. In the TNF pathway, the extracellular apoptotic signaling molecule is TNF-α, a cell-signaling protein cytokine produced by activated macrophages, natural killer cells, and CD4-positive cells (Rath and Aggarwal 1999).

TNF is regulated by two receptors, TNFR1 (TNF receptor-1) and TNFR2 (TNF receptor-2). TNFR1 is expressed primarily by all human tissues and is the primary signaling receptor for TNF-α. TNFR2 binds with TNF-α, and TNF-β is expressed mainly in immune cells and controls limited biological responses.

In the TNF path, the extracellular apoptotic signaling molecule is TNF-α, a cell-signaling protein cytokine produced by activated macrophages, natural killer cells, and CD4-positive cells in response to inflammation, infection, or any other environmental stresses. On the cell's surface, TNF-α acts by binding to its two receptors TNFR1 (p55) and TNFR2 (p75). TNFR1 is considered the most potent mediator of cytotoxicity of TNF-α. After binding TNF-α to its two receptors, it recruits signal transducers that activate at least three distinct effectors. These effectors activate caspases and two transcription factors, activation protein-1 and NF-kB (nuclear factor-kappaB), with the help of complex signaling cascades and networks (J. et al., 2008; Varfolomeev et al. 2008). TRADD protein or TNFR-associated death domain binds to TNFR1 at first. Then subsequently, TRADD recruits FADD (FAS-associated death domain), RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain), MADD (MAPK-activating death domain), and RIP (receptor-interacting protein). Recruitment, oligomerization, and activation of caspase-8 result from TRADD and FADD binding to TNFR1. Activated caspase-8 initiates a proteolytic cascade that comprises other caspases, for instance, caspase-3, caspase-6, and caspase-7, inducing apoptosis (Ndebele et al. 2008; Rath and Aggarwal 1999) (Fig. 18.3).

The idea that persistent inflammation encourages tumor formation and progression is supported by an increasing amount of epidemiological and clinical data. TNF is a potent pro-inflammatory cytokine that can link inflammation and carcinogenesis by acting as an endogenous tumor promoter (Nabors et al. 2003). According to recent research, TNF is implicated in every stage of carcinogenesis, including cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis. Several preneoplastic and malignant tissues also express TNF at greater levels. Additionally, the advancement of malignant disorders like chronic lymphocytic leukemia, Barrett's adenocarcinoma, prostate cancer, breast cancer, and cervical carcinoma is linked to the elevated TNF expression level in precancerous and tumor cells (Wang and Lin 2008).

TNF knockout mice expressing cyclooxygenase-2 and microsomal prostaglandin E synthase-1 demonstrated a considerable reduction of hyperplastic tumors with lower cell proliferation compared to TNF wild-type K19-C2mE mice, demonstrating the crucial role of TNF in tumor promotion. TNF mediates tumor progression by activating the NF-kB or a PKC and AP-1-dependent pathway. In mouse epidermal JB6 cells (Yang et al. 2006), TNF treatment dose-dependently promoted NF-kB activation and tumor development. Since TNF was unable to activate NF-kB in the

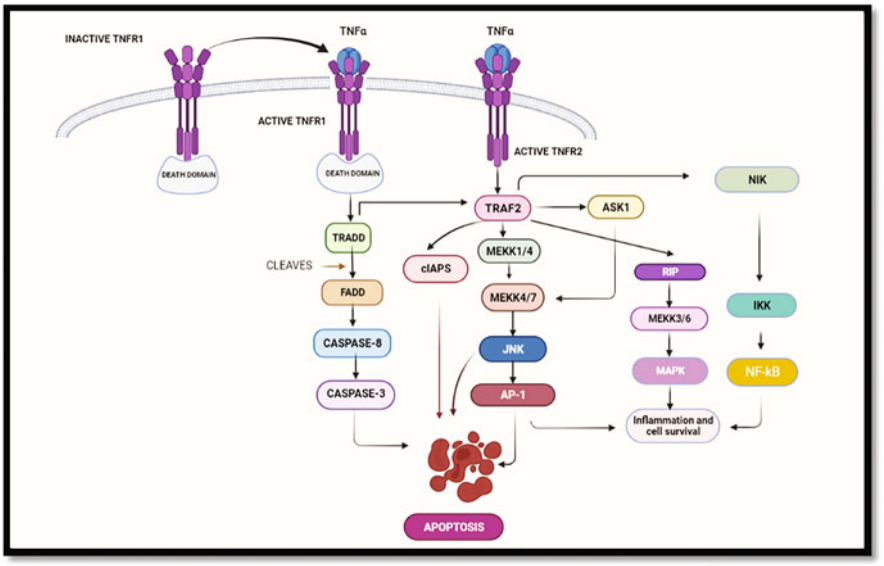

Fig. 18.3 The figure shows the signaling pathway of TNF-α. Different pathways are activated by TNF-α inducing apoptosis, inflammation, and cell survival. TNF induces apoptosis by caspase-8 binding to FADD, promoting inflammation and survival mediated through TRAF2 (tumor necrosis factor receptor-associated factor 2) via JNK (Jun N-terminal kinase)-dependent kinase cascade, MEKK kinase cascade, and NF-kB activation by RIP (receptor-interacting protein)

transformation-resistant JB6 mouse epidermal cells (P-cells), NF-kB activation is crucial for TNF-induced tumor development. Additionally, TNF may encourage the growth of tumors by activating platelet-type 12-lipoxygenase and secretory leukocyte protease inhibitors (Suzukawa et al. 2002).

TNF can directly affect leukocyte activation, function, and survival during cancer growth, inhibiting antitumor immune responses. However, it can also change the phenotype of cancer cells to make them less noticeable to T cells and express immune-suppressive chemicals.

18.1.3 Evasion of Apoptosis in Tumor Cells under Hypoxic Condition

Mutations in tumor cells make them apoptosis-resistant. Cancerous cells can control apoptotic pathways through transcription, translation, and posttranslational mechanisms, such as phosphorylation. The expression of pro- and antiapoptotic genes can be altered by cancer cells to escape apoptosis. Stabilizing or destabilizing antiapoptotic or proapoptotic proteins cause inhibition of apoptosis (Sharma et al. 2019). Cancer cells are constantly under pressure to develop mechanisms to evade apoptosis. This occurs mainly by acquired mutations and changes in gene expression. These, in turn, disable the critical components of the intrinsic pathway of

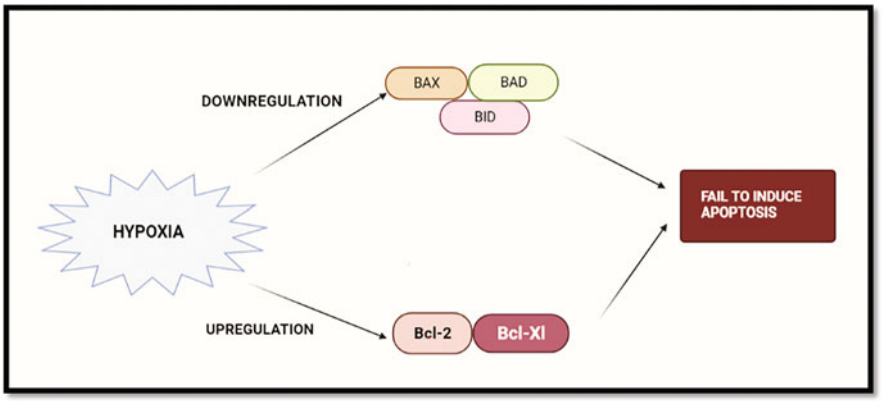

Fig. 18.4 Hypoxia-induced mechanism of resistance to apoptosis. Hypoxia regulates the expression levels of antiapoptotic and proapoptotic proteins, resulting in apoptosis resistance

apoptosis. The two principal mechanisms by which apoptosis evasion occurs are loss of TP53 function and overexpression of antiapoptotic molecules of the BCl-2 family (Strasser et al. 1990) (Fig. 18.4). Two methods mainly cause loss of TP53 function: firstly, the mutation of the TP53 gene, and secondly, the amplification of MDM2. Mutation in the TP53 gene is commonly reported in tumor cell diagnosis, and it is more frequently found in tumors that relapse after therapy (Benard et al. 2014; Rivlin et al. 2011).

The amplification of MDM2 impairs the function of P53 indirectly. MDM2 encodes the inhibitor of P53, so when amplified, it disrupts P53 function (Oliner et al. 2016). The loss of P53 function prevents the upregulation of PUMA (a proapoptotic BH3-only molecule) in response to various stresses or any damage preventing the cell from facing cell death. Overexpression of the antiapoptotic molecule BCl-2, for example in follicular lymphoma, which involves translocation at the 14th and 18th chromosomes, prevents cell death (Sharma et al. 2019). This translocation fuses the BCl-2 gene to transcriptionally active immunoglobin heavy chain gene leading to increased expression of BCl-2 (Correia et al. 2015). This causes the cell to escape from cell death. Follicular lymphomas are slow-growing as it arises because of reduced cell death rather than explosive proliferation.

The induction of apoptosis via the mitochondrial pathway depends on the balance between the antiapoptotic and proapoptotic BCl-2 family (Browne et al. 2012). Hypoxia modulates most of these proteins' abundance, subcellular location, or posttranslational modifications. Proapoptotic molecules like BAX, BAD, and BID shows decreased expression under hypoxic condition (Harrison et al. 2011). The increase in the expression of antiapoptotic molecules like BCl-2 and BCl-Xl in hypoxia leads to the dramatic reduction of caspase activity. Consequently, Hypoxia also reduced caspase activation, DNA fragmentation, and poly(ADP-ribose) polymerase, resulting in the evasion of programmed cell death.

18.2 Apoptosis Reprogramming under Hypoxia

Reprogramming of somatic cells incorporates vigorous chromatin remodeling and pluripotency network induction for resetting the epigenome to an embryonic stem cell-like structure. Metabolism occurring during this process generates reactive oxygen species or ROS. An increase in ROS level can cause DNA damage, cell senescence, and apoptosis. ROS restricts reprogramming cell survival during the increase of generation of iPSC under hypoxia. Additionally, reprogramming factors leading to ROS generation are unsuitable for iPSCs formation, which represses the mitochondrial content and oxidative stress in iPSCs and hESCs (human embryonic stem cells) (Hanna et al. 2009).

A shift from oxidative to glycolytic metabolism is vital for reprogramming cells to pluripotency. This shift requires HIFs in a very strategic manner. To start this metabolic switch and acquire pluripotency, two HIFs, HIF-1α and HIF-2α, are needed. Experimental studies found that HIF-2α is necessary during the initial stage of iPSC reprogramming which aids in the promotion of metabolic switch. However, prolonged HIF-2α stabilization suppresses iPSC formation through TRAIL-induced restriction of caspase-3 signaling. Earlier it was stated that iPSC generation is prevented by activating apoptotic caspases during reprogramming. But later, it was found that HIF-2α restricts caspase-3 activity with the help of reprogramming factors and iPSC formation with the use of TRAIL. Apoptotic and antiapoptotic signaling is activated by binding TRAIL to its cognate receptors DR4 and DR5. However, the antiapoptotic pathway is activated if TRAIL binds with its decoy receptor2 or DcR2 (Degli-Esposti et al. 1997; Russo et al. 2006; Sanlioglu et al. 2005).

Reprogramming cells hold characteristics similar to those progressing toward becoming aggressive tumor cells. So, it can be stated that cancer progression is a slow reprogramming process. Firstly, cancer cells and reprogramming cells initially change their metabolism from oxidative to highly glycolytic. In addition, HIFs control the switch regulation in both cell types. Although cancer cells and reprogramming cells are TRAIL sensitive, cancer stem cells and pluripotent stem cells are TRAIL-resistant. All these similarities depict somatic cell reprogramming as an essential mechanism in cancer progression in the future (Ui et al. 2007).

Tumor metabolic reprogramming, mediated by hypoxia in vitro and in vivo, continues to yield unexpected results, revealing its impact on cell survival, tumorigenesis, and tumor progression via immune escape, angiogenesis, and metastasis and resistance to radiotherapy and chemotherapy.

Due to its ability to downregulate the antitumor immune activity in multiple ways, hypoxia-driven immuno-metabolic alterations have recently been studied in detail. Aldolase A (ALDA), phosphoglycerate kinase 1, and pyruvate kinase M (PKM) are three crucial glycolytic enzymes that are upregulated by HIF-1, stimulating the glycolytic metabolism in immune cell types. First of all, it can encourage the development of highly glycolytic cells. However, the extracellular glucose that immune cells need for ATP generation and the synthesis of lipids, amino acids, and nucleotides is less available due to the increased flux of glucose

absorption by tumor cells. Therefore, immune cell malfunction may result from glucose deficiency, leading to cancer development (Domblides et al. 2019; O'Neill et al. 2016; Semenza et al. 1994; Vito et al. 2020).

It has been demonstrated that the hypoxia and HIF-1 pathways control angiogenesis by regulating the transcription of genes involved in angiogenesis and antiangiogenic factors. Through an HIF-1-independent mechanism, tumor metabolic reprogramming caused by hypoxia also contributes to angiogenesis. The ability to infiltrate and spread from the primary tumor to distant areas is given to malignant cells by the molecular regulation of the epithelial to mesenchymal transition (EMT), which increases glycolytic metabolism and the development of new blood vessels. The metabolic flipping of tumor cells under hypoxic conditions, typified by the acidic microenvironment, also supports tumor cells' migration and metastasis (Qiu et al. 2017).

18.2.1 Hypoxia-Inducible Factor (HIF): The Master Regulator of Hypoxic World and Apoptosis

18.2.1.1 Hypoxia-Inducible Factors

Cells within growing tissues need adequate oxygen supply; under pathophysiological conditions, the oxygen supply in tissues becomes insufficient, causing severe hypoxia in rapidly growing tumors. In the case of tumor cells, oxygen may be supplied by diffusion, and with tumor growth, the percentage of oxygen in the central area of tumor can drop below 0.02% (Bertout et al. 2008). Tumor cells become hypoxic and eventually become necrotic. In order to avoid this, cancer cells may induce angiogenesis. Alternatively, tumor cells may invade surrounding normoxic tissue areas (Lugano et al. 2020). The hypoxia-inducible factors or HIFs regulate angiogenesis and other adaptive reactions. This heterodimeric transcription factor maintains oxygen homeostasis and adapts to low oxygen levels mediated by cellular and systemic responsiveness. HIFs are master regulators of oxygen homeostasis, which play a significant role in the development, physiology, postnatal period, and pathogenesis of various diseases.

HIF consists of an α and β subunit. The α subunits are sensitive to oxygen, whereas the β subunits are not. HIF-α subunits consist of three isoforms; they are HIF-1α, HIF-2α, and HIF-3α. HIF-β subunit consists of three paralogues which are Arnt-1, Arnt-2, and Arnt-3. HIF-1α subunit contains an N-terminal basic helix-loop-helix DNA-binding domain. This domain guides the transcription of the HIF-1α gene into mRNA (Park et al. 2010; Weidemann and Johnson 2008) (Fig. 18.5a).

The HIF-2α subunit contains a domain similar to those in HIF-1α. Both HIF-1α and HIF-2α undergo the same proteolytic regulation. But tissue expression is much more limited in the case of HIF-2α. Both isoforms are critical for the hypoxic response. They possess the ability to form a complex with HIF-1β (Fig. 18.5b). HIF-1β is encoded by ARNT or aryl hydrocarbon receptor nuclear translocator gene in partnership with AhR to form a heterodimeric complex necessary for AhR

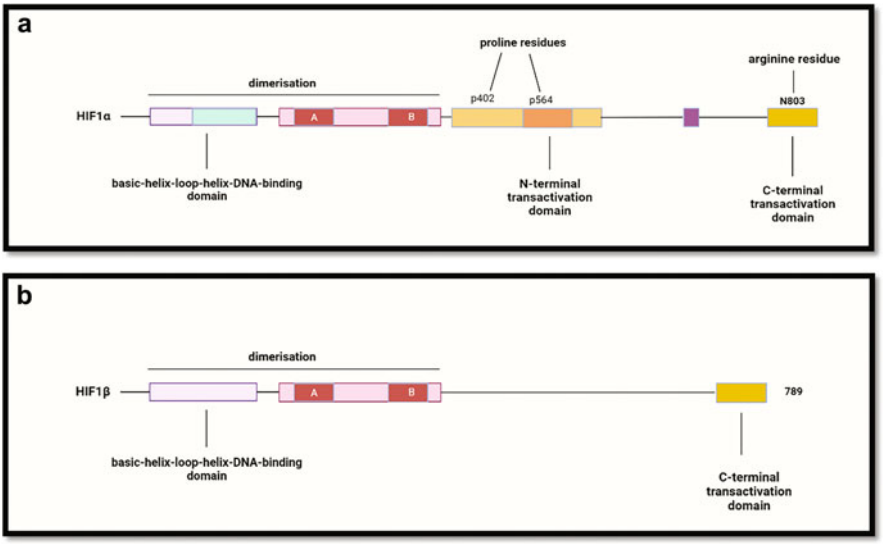

Fig. 18.5 (**a**) In this figure different domains of HIF-1α subunit are shown. The subunit contains an N-terminal basic helix-loop-helix motif which associates with the DNA. Near the C-terminal, two transactivation domains can be distinguished. This domain guides the transcription of HIF-1α gene into mRNA. In addition to the helix-loop-helix domain, an adjacent domain is essential for dimerization with a HIF-β subunit. The proline residues are crucial for protein stability, and the arginine residue regulates transcriptional activity. (**b**) In this figure different domains of HIF-1β are shown. HIF-1β subunit contains the basic helix-loop-helix DNA-binding domain which is a part of the dimerization motif. The protein contains only one transactivation domain at C-terminal

activity. Ligand binding subunits require this protein to translocate from the cytoplasm to the nucleus (Weidemann and Johnson 2008).

HIF-1α plays a crucial role in cancer cell adaptor glycolysis and angiogenesis. Among the HIF proteins, HIF-1α is highly overexpressed in most solid tumors, and their metastases due to which has been more characterized in the signaling pathways involved in tumor progression by regulating the expression of various growth factors, for example, insulin-like growth factor (IGF2), transforming growth factors (TGF), and platelet-derived growth factor (PDGF) which activates tumor development under hypoxic condition (Harris 2002). Transcription of more than 1000 genes encoding proteins involved in genetic instability, immortalization, cell proliferation, and metabolic reprogramming mediated by HIF-1α activation influences every crucial aspect of cancer metabolism, development, progression, and metastasis (Dewhirst et al. 2008).

18.2.1.2 HIF Regulation

Human beings require oxygen to survive. In order to transport this oxygen, human body needs red blood cells. The production of red blood cells in the bone marrow is stimulated by a hormone called erythropoietin (EPO). The mechanism which enables the kidney to produce EPO in response to decreasing oxygen levels is still

unknown. It was reported that HIF is an essential factor for the transcription of several genes, including EPO.

18.2.1.2.1 HIF Regulation Under Normoxic Condition

HIF-α subunit contains two proline residues critical to the proteins' stability. Under the normoxic condition, one or both of the essential residues of proline and HIF-α proteins will be hydroxylated by a member of prolyl hydroxylase domain family abbreviated PHD. PHD2 is the major hydroxylase that hydroxylates HIF-1α. These PHDs are substrates of PHD dioxygenases. The PHD dioxygenases with the help of bivalent iron produce hydroxy prolyl residue, carbon dioxide, and succinate; this takes place when one or both of the critical proline residue have been hydroxylated by pVHL protein which binds to HIF-α.

The VHL gene is a tumor suppressor gene, and inactivating germline mutations predispose individuals to tumors such as clear cell renal carcinomas. VHL inactivation is found in approximately half of all sporadic renal cell carcinomas. The pVHL protein associates with ubiquitin ligase complex containing elongin, cullin, and RING-box protein called ECR complex. E2 ubiquitin-conjugating enzyme attaches ubiquitin to HIF-α. Polyubiquitylated HIF-α is degraded by the 26 s proteasome, thus targeting HIF-1α for degradation (Fig. 18.6). In addition to the proline residues, HIF-α protein contains a critical arginine residue in the C-terminal transactivation domain. The residue is hydroxylated by a dioxygenase called factor-inhibiting HIF-1α or FIH-1. The hydroxylation of the arginine residue inhibits the binding of the p300 and CBP transcriptional coactivators (Weidemann and Johnson 2008).

18.2.1.2.2 HIF Regulation under Hypoxic Condition

Under hypoxic conditions, PHD activity decreases due to insufficient availability of cofactors like Fe (II) or 2-oxoglutarate. After the stabilization, HIF, together with HIF-1, generates a heterodimeric transcription factor inside the nucleus which

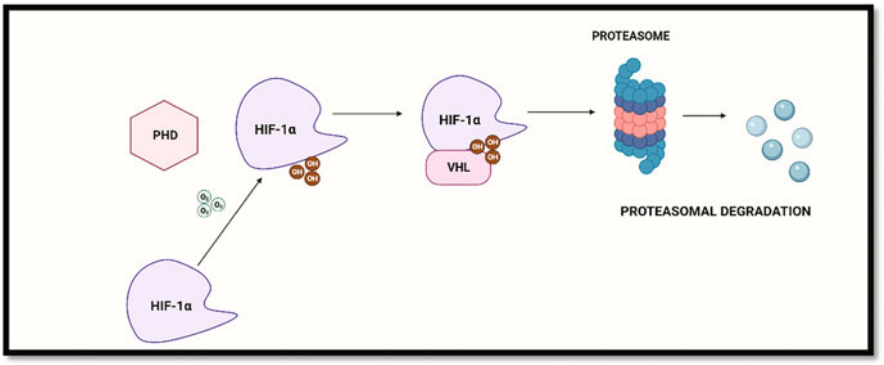

Fig. 18.6 The figure shows that under normoxic conditions, the enzyme HIF prolyl hydroxylase hydroxylates HIF-1α. This allows binding with VHL protein which then targets the complex for degradation in 26 s proteasome

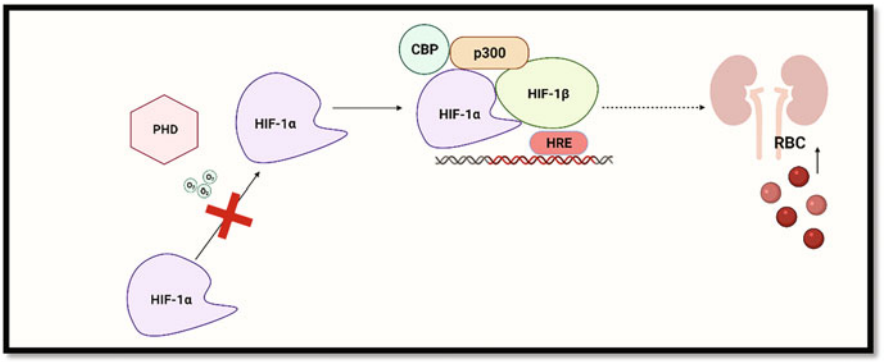

Fig. 18.7 In the figure given below, it is shown that under hypoxic conditions, no oxygen is available to allow HIF-prolyl hydroxylase to hydroxylate HIF-1α. The protein remains functional, allowing it to bind the hypoxia element together with other transcriptional factors inducing gene transcription, for example, EPO

promotes hundreds of targeted gene expression by binding to hypoxia response elements in their regulatory region. FIH disruption occurs when hypoxia occurs. On a cellular level, HIF activates the expression of several genes involved in angiogenesis, cell growth, and the regulation of apoptosis (Masoud and Li 2015).

Under hypoxic conditions, HIF-α protein is no longer hydroxylated. A HIF-α subunit is associated with a HIF-β subunit, binds to consensus sequences in the DNA, and affects glucose metabolism (Fig. 18.7). SCLC2A1, a glucose-encoding gene, reacts to both HIF-1α and HIF-2α. HIF-1α induces the formation of enzymes involved in the glycolysis mechanism. HIF-1α stimulates the synthesis of hexokinase 1 and 2. Eventually, HIF-1α causes the simulation of phosphoglycerate kinase synthesis, which catalyzes chemical reactions leading to the generation of ATP from ADP without oxygen, establishing the importance of HIF-1α for glycolysis regulation. Under aerobic conditions, decarboxylation and pyruvate oxidation occur, leading to the acetyl-coenzyme A generation. HIF-1α stimulates the synthesis of pyruvate dehydrogenase kinase. In this way, the oxidative metabolism of glucose is reduced under anaerobic conditions and stimulates the synthesis of lactate dehydrogenase and, consequently, the synthesis of lactate. To summarize, HIF-1α favors the anaerobic metabolism of glucose under hypoxic conditions (Masoud and Li 2015).

18.2.1.3 HIF and Apoptosis

Under hypoxic conditions, apoptosis is induced by hyperpermeability of the inner mitochondrial membrane, leading to the release of cytochrome c. The inhibition of the electron transport chain at the inner membrane of mitochondria induces hypoxia-mediated apoptosis. There is a reduction of membrane potential due to restriction of proton transport by insufficient oxygen. The reduced mitochondrial-derived ATP activates BAX or BAK, which releases cytochrome c in the cytoplasm (Fig. 18.8). Due to low ATP, reactive oxygen species or ROS generation participates in the apoptosis process induced by hypoxia (Brusselmans et al. 2001).

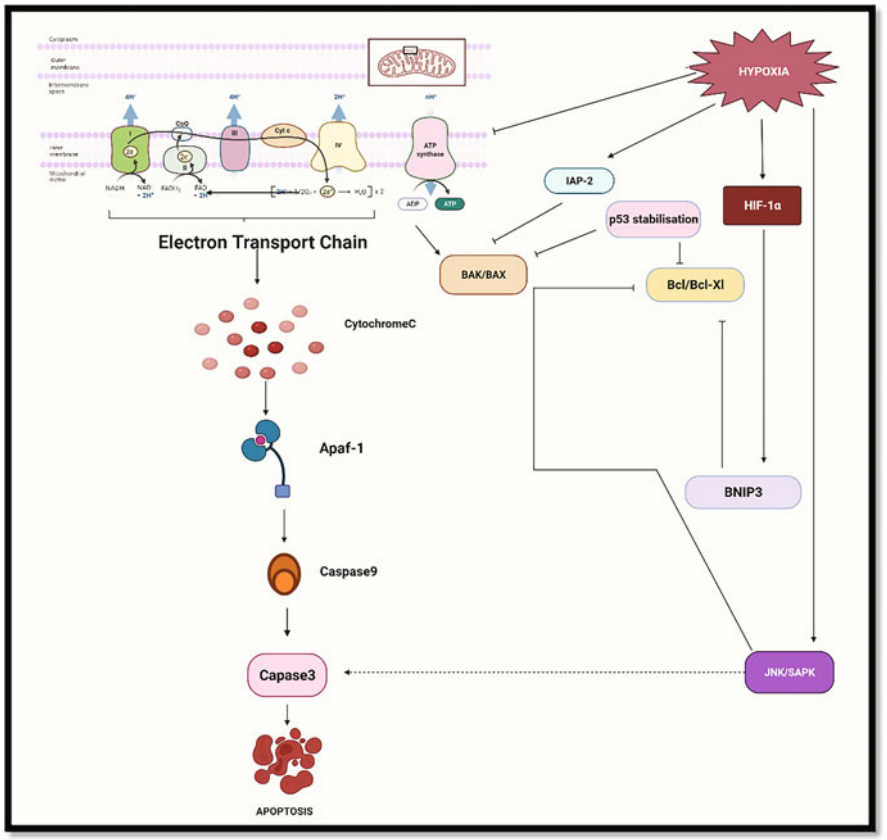

Fig. 18.8 In the diagram below, hypoxia-induced signaling pathways are depicted schematically. In these pathways, HIF-1α is shown to be involved. A direct interaction is indicated by a solid line, while an indirect interaction is indicated by a dashed line

In contrast to the proapoptotic effects of hypoxia, cells become apoptosis-resistant under hypoxic conditions. In addition, BAX accumulation in the inner mitochondrial membrane releases cyto-C into the cytosol, drastically dropping in the hypoxic environment, blocking the cascade, and leading to apoptosis. Severe hypoxia, along with ATP, is necessary to promote apoptosis. Cells are blocked from undergoing apoptosis if oxygen levels are above 0.5% (Aragonés et al. 2001; Santore et al. 2002). A phosphoinositide 3-kinase (PI3K) and its downstream target Akt mediate cell survival under a mild hypoxic situation (Alvarez-Tejado et al. 2001). Both PI3K and Akt signaling pathways are crucial for the survival of the cell and cell growth and proliferation because they restrict inhibition of the apoptotic activity of BCl-Xl by BAD (Alvarez-Tejado et al. 2001).

HIF-1α mediates hypoxia-induced apoptosis. In wild-type embryonic stem (ES) cells, hypoxia combined with hypoglycemia lowers proliferation and induces apoptosis. The lower rate of hypoxia-induced death in HIF-1α mutant ES cells may

explain why tumors develop quicker than wild-type cells. HIF-1α can cause apoptosis in two ways (McClintock et al. 2002). Firstly, it can improve the stability of the p53 tumor suppressor gene's product. When cells are exposed to environmental stress or DNA damage, p53 causes programmed cell death by controlling proteins like BAX, or it can cause cell growth arrest via p21 (D. Chen et al. 2003). Secondly, the proapoptotic proteins BNIP3 (BCl-2/adenovirus E1B 19 kDa-interacting protein 3) and NIX, a BNIP3-like protein, are overexpressed in hypoxic peri-necrotic areas of tumors (D. Chen et al. 2003; Hansson et al. 2002).

18.2.2 p53 and HIF: Communications among Master Regulators

18.2.2.1 HIF and p53 Interaction in Cancer Progression

p53 is one of the most frequently altered genes in solid tumors. p53 transcriptional activity is disrupted by conformational changes caused by missense mutations in the DNA-binding domain, resulting in p53's inability to regulate the cellular response to hypoxia effectively. Low oxygen pressure also selects cells with the p53 mutation, leading to increased metastatic potential and decreased apoptosis.

Hypoxia is associated with more aggressive tumor phenotypes and poor treatment responses, while p53 mutations promote cancer progression (Hockel et al. 1996). This mainly entails HIF-1 stabilization and overexpression of its target genes. In several studies, the overexpression of the hypoxia marker CA IX was linked to lower patient survival, less differentiated cancers of higher grade, and poorer therapeutic response. Increased expression of HIF hydroxylases, which inhibit HIF-1 and are themselves regulated by hypoxia, has been proposed as a poor prognostic factor in non-small cell lung tumors, whereas inhibiting them lowers the survival of glioblastoma cells (Andersen et al. 2011).

Hypoxia develops resistance to frequently used anticancer medications, either through downregulation of drug target genes or because oxygen deprivation renders the drugs ineffective. The first-line chemotherapeutics (doxorubicin, etoposide, and cisplatin) cause DNA damage, triggering p53 to initiate apoptosis (Li et al. 2015). HIF-1 reduces the sensitivity of cells to therapy by altering the expression of its target genes, albeit this effect varies by cell type. Hypoxic cells divide more slowly and are found farther away from working blood arteries. As a result, medications cannot reach locations with low oxygen levels and perform less effectively than in actively dividing cells (Brown 2000).

18.2.3 HIF-Independent Response

A broad range of human cancer cells thrive in hypoxic extracellular environments that are induced by the HIF (hypoxia-inducible factor) transcription factors. This change in the metabolism of cancerous cells is due to the overexpression of HIF, primarily HIF-1 (Greijer and van der Wall 2004). Clinical studies are under assessment to create drugs that can work as a suppressor or HIF inhibitors, but the concern

for this is the evidence providing instances of the hypoxic cancer cells and their ability to adapt to the HIF inhibitors and cause drug resistance. So, understanding the pathways independent of HIF-1 factors is crucial to preventing treatment failures and challenges.

HIF-1 is an essential factor in several pathways.

18.2.3.1 Allosteric regulation of glycolytic enzymes

According to a series of experimental studies and results on Hepa1 c4 cells, it was found that these cells are deficient in HIF-1β subunit that is a necessary component of both HIF-1 and HIF-2 active complex. So they cannot form any dimeric complexes, hence ruling out the chances of HIF-1-induced gene transcription. This makes Hepa1 c4 cells a model cell line to analyze the drug resistance of cancer cells toward HIF-1 inhibitor-based drugs (Kierans and Taylor 2021).

- Even with the deficiency of HIF-1/2, the Hepa1 c4 cells were able to match the rate of upregulation of glycolysis as their wild type cultured under hypoxic conditions (wild type has HIF) (Semenza 2012).
- When studied in vivo, the growth of Hepa1 c4 tumors were slower than wild-type tumors, with significantly lower expression of several glycolytic enzymes and lower levels of ATP; still assessing the amount of glucose uptake using FDG-PET could not show any difference between Hepa c4 and Hepa WT tumors, and this suggests that glycolysis in c4 tumors is not affected by the absence of HIF-1 pathway (Semenza 2010).
- Another two HIF-1-independent pathways that might upregulate glycolysis signaling are PI3K-Akt and c-Myc. They show a little lower or the same expression in Hepa c4 compared to Hepa wild-type tumor cells. There was an increase in AMP/ATP ratio in these two types, c4 tumors being a higher ratio as that of wild type (Semenza 2010, 2019).
- With reference to these experimental results, it is suggestive that allosteric PFK-1 activation by small molecule metabolites can be a mechanism by which solid cancer cells adapt to HIF-1/2 deficiency under hypoxic conditions (Semenza 2010, 2019).

18.2.3.2 Alternate Glucose Uptake & Creatine Metabolism During Hypoxic Conditions

- Cancer cells deficient in HIF-1α subunit have their HIF-2-dependent pathways still present providing a potential development of drug resistance mechanisms dependent on HIF-2 (Fallah and Rini 2019).
- Studies have shown that HCT116 colorectal cancer cells under hypoxic conditions could adapt to deficiency of HIF-1 aided by HIF-2 pathways (Fallah and Rini 2019; Kim et al. 2006).
- Aldolase has been shown to be an essential step for HIF-independent pathways during hypoxia glycolysis. This was shown by Valli et al. They administered a conversion of fructose-1,6-bisphosphate to DHAP at aldolase level rather than the

conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by PFK as occurs in c4 cell tumors (Kim et al. 2006).
- The uptake of glucose also implies that the glucose from glycolysis can be used to support other metabolic pathways like the pentose phosphate pathway, lipid pathways, and glycogen pathways.
- This study also highlights the importance of creatine metabolism for providing energy buffers during high ATP demands. These ATP buffers help the metastatic cancer cells to meet their energy requirements despite the absence of oxygen and general ATP production pathways during hypoxic conditions (Papandreou et al. 2006).

18.2.3.3 Myc and HIF-1 Deficiency

- Myc are transcription factors that can activate the proliferation of genes by binding to enhancers and bringing in HATs (histone acetyltransferases). So, this factor can promote tumor growth in many ways and result in rapid progression. Activation of Myc results in upregulation of glutamine metabolism, and that results in the formation of glutamine to glutamate. And there have been studies supporting those malignant cells using glutaminolysis (process of converting glutamine into TCA cycle metabolites by an enzymatic pathway) during hypoxia (Leek et al. 2005).
- Increased glutaminase, the enzyme mainly responsible for glutamine to glutamate conversion, was found in increased concentration during clinical studies (Das et al. 2019; Gordan et al. 2007).
- It has been shown that an increase in HIF-1α decreases the expression and an opposite behavior with HIF-2α promotes the expression and functioning of Myc. This combination of Myc and HIF-2α also works together to downregulate the expression of p53 (Das et al. 2019; Munksgaard Thorén et al. 2017).

18.3 HIF and Apoptosis in Physiological and Pathophysiological Conditions

HIF is a heterodimer that includes one of three α subunits and a β subunit. HIF-β expression is found in a normally regulated manner, while HIF-1α is triggered under hypoxic conditions. In most mammalian cells, HIF-1α acts as a transcriptional activator and helps regulate gene expression in response to hypoxia. HIF-1α causes positive regulation of glycolytic enzymes and glucose transporters, shifting it toward a glycolysis-mediated pathway (Fukuda et al. 2007). It can give an idea that HIF-1α can cause modulation in aerobic metabolism. HIF-1α promotes regulation of the majority of the HIF target genes, whereas there is a scarcity of exclusive HIF-2-α-dependent genes. The physiological and pathological adaptation for protecting cells from apoptosis is triggered by this HIF "switch" between HIF-1α and HIF-2α.

18.3.1 Role of HIF in Physiological and Pathophysiological Conditions

Many tissues physiologically operate at levels as low as 5% oxygen or even as low as 1% oxygen. Hence hypoxia may not be considered equivalent to ambient oxygen concentration (21% oxygen) (Keeley and Mann 2019). Hypoxia refers to a low oxygen level (usually less than 2%) in a particular organ, tissue, or cell type compared to the normal state. Hypoxia is a condition in which the body is deprived of oxygen for an extended period (acute hypoxia, e.g., ischemia) or a short amount of time (chronic hypoxia, e.g., kidney diseases, cancer).

Recent research indicates that HIF-1α may contribute to the pathophysiology of cancer. The partial pressure of oxygen reaches to low as 10 mmHg in the hypoxic core of quickly growing insufficiently vascularized cancer cells (Helmlinger et al. 1997), HIF-1α can be maintained. In vitro hypoxia lasting only 3 hours can stabilize HIF-1α in cancer cells (Zhao et al. 2015). In this situation, HIF stabilization alters glycolysis, nutrition intake, waste disposal, angiogenesis, apoptosis, and cell migration, which aid in the survival and dissemination of the tumor (Luo et al. 2014; Nagaraju et al. 2015; Parks et al. 2016; Xie et al. 2016). Tumors in the kidney, retina, and adrenal tissues result from mutations in the von Hippel-Lindau gene, which also causes constitutive overexpression of HIF-1α and HIF-2α (Krieg et al. 2000). In the VHL syndrome, high levels of HIF-1α cause the overexpression of growth factors such as transforming growth factor (TGF), platelet-derived growth factor, and vascular endothelial growth factor (VEGF), which activate downstream receptor tyrosine kinases leading to the development of cancer.

18.3.1.1 Role of HIF in Cancer

Hypoxia in the tumor microenvironment is characterized by reduced oxygen delivery and consumption. The increase in hypoxia is caused by the restriction of oxygen diffusion in avascular primary tumors, as well as greater oxygen demand due to cancer cell hyperproliferation. Many facets of tumor growth, including cancer cell survival, proliferation, epithelial-to-mesenchymal transition (EMT), invasion, angiogenesis, treatment resistance, and metastasis, are dependent on molecular and cellular responses to hypoxia. Hypoxia stabilizes HIFs at the molecular level, allowing cells to respond to hypoxia by transactivation of downstream genes. HIF-1 and HIF-2 have been linked to advanced stages of cancer and poor patient survival. Furthermore, HIF signaling is required for cancer cells to promote their potential to spread (Seagroves et al. 2001; Semenza 2001).

During tumor growth, physiological oxygen diffusion in the microenvironment is a limiting factor, which encourages the production of new blood vessels (angiogenesis) to provide needed oxygen and nutrients for tumor propagation. Multiple pro- and antiangiogenic factors play a role in tumor angiogenesis. Stabilized HIF-1α/β attaches to HRE (hypoxia response element) of target genes and transactivates their expression as a transcription factor (Greer et al. 2012). Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGF-B), fibroblast growth factor (FGF), plasminogen activator inhibitor-1

(PAI-1), matrix metalloproteinases (MMP-2 and MMP-9), interleukin 8 (IL-8), and angiopoietins (ANG-1 and ANG 2) are among these genes which a play vital role during tumor angiogenesis (Lin et al. 2017; Lv et al. 2017). Furthermore, under the supervision of HIF-2, VEGF and PAI-1 have been discovered to increase tumor angiogenesis (Raval et al. 2005; Skuli et al. 2009).

18.3.1.2 Overexpression of HIF-1α in Human Cancers

In common malignancies, immunohistochemical examination of human tumor tissues revealed substantial upregulation of HIF-1, which is a result of both intratumoral hypoxia and genetic changes. While hypoxia appears to be a universal stimulation for HIF-1 production, the impact of genetic changes (other than VHL loss of function) appears to be dependent on the signal transduction pathways active in a given tumor cell. Because HIF-1 has been shown to regulate transcription of the genes encoding VEGF, glucose transporters, and glycolytic enzymes, these findings provide a molecular mechanism linking oncogene gain of function and tumor suppressor gene loss of function with tumor vascularization (the angiogenic switch) and increased glucose uptake and lactate production (the Warburg effect) (Iyer et al. 1998; Seagroves et al. 2001; Semenza 2001).

HIF-1α upregulation is linked to vascular density in tumor samples from brain tumors, ovarian carcinoma, and ductal carcinoma in situ, showing that HIF-1α activity plays a role in the angiogenic switch. The combined overexpression of HIF-1α and the antiapoptotic protein BCl-2 is related with treatment failure in early-stage esophageal cancer. HIF-1α upregulation was linked to tumor apoptosis and patient survival in one research of non-small cell lung cancer (Koukourakis et al. 2001). HIF-1α overexpression is associated with apoptosis in ovarian cancer expressing wild-type p53, but it is not associated with apoptosis in tumors expressing mutant p53. The combination of mutant p53 and HIF-1α overexpression is linked with increased risk of patient mortality (Koukourakis et al. 2001).

Primary tumors with the highest degree of HIF-1 overexpression have a considerably increased risk of radiation therapy resistance. This has been found in squamous cell oropharyngeal carcinoma. As a result, immunohistochemical examination of HIF-1 expression in tumor tissues may be utilized to identify deadly tumors, and some extensive treatments are required to increase patient's life expectancy (Aebersold et al. 2001).

There are two key conclusions that can be drawn. First, the activation of HIF-1 expression may contribute to hypoxia tumor cells' resistance to radiation or chemotherapy, as well as the enhanced aggressiveness of severely hypoxic tumors. Second, research studies indicate that for each cancer, it will be important to see if HIF-1 overexpression, alone or in combination with other markers like p53 or BCl-2, can identify a subset of patients with more aggressive disease for whom standard therapy will not be enough to ensure a high chance of survival.

18.4 Implications for Cancer Therapy

The molecular events responsible for the antitumor activity of most cancer medicines were incompletely defined because they were discovered via empirical screens. Our knowledge of cellular damage responses and physiological cell death pathways has advanced over the last decade, leading to new insights into drug-induced cell death (Lowe and Lin 2000). Apoptosis is induced by a variety of medicines with varying structures and specificities, and it is now thought that apoptotic pathways have a role in the cytotoxic activity of most chemotherapeutic agents (Lowe and Lin 2000). These findings suggest that cells can understand a drug-induced insult in the same manner that they interpret a physiological insult like hypoxia or growth factor deficiency. Emerging techniques for identifying specific tumors have the potential to revolutionize the creation of hypoxia-targeted drugs, much as they have in other areas of cancer medication. Given the diversity in cancer hypoxia at the level of the greater human population, even within a single disease subtype, the effective development of hypoxia-targeted medications is undoubtedly a futile goal unless hypoxic tumors can be detected proactively. One of the cancer therapies targeting apoptosis and hypoxia is to repair hypoxia-mediated apoptosis; one crucial technique for that is to pharmacologically activate p53. Small molecules like APR-246 also known as PRIMA-1 is developed for reactivation of p53 which in turn helps in restoring mutant p53's transcriptional activity and RITA leading to the blockage of MDM2-mediated p53 degradation. Although cell death in aerobic and hypoxic cells was comparable, RITA also caused a DNA damage response that seemed to help activate p53-dependent apoptosis (de Lange et al. 2012).

18.5 Implication of Cancer Therapy Targeting HIF

Several novel chemotherapeutic drugs currently in clinical trials may have antitumor effects in part due to their reduction of HIF-1α expression. Rapamycin, for example, works by blocking the serine/threonine kinase FRAP, which increases HIF-1α mRNA translation into protein (Laughner et al. 2001). Rapamycin also inhibits hypoxia-induced HIF-1α expression at higher dosages, showing that FRAP is important for HIF-1α synthesis in these cells. HIF-1α is constitutively expressed in multiple prostate cancer cell lines, and rapamycin can suppress HIF-1α and VEGF expression. HER2neu receptor tyrosine kinase (RTK) activity is important for PI3K activation in breast cancer cells, whereas the EGF receptor appears to be important in gliomas and prostate cancer cells, and autocrine activation of the IGF receptor increases PI3K signaling in colon cancer cells. PI3K signaling is also stimulated by gain-of-function mutations in the oncogenes HRAS and CSRC (Blancher et al. 2001; Zhong et al. 2000).

Inhibitors of the RTK-PI3K-FRAP pathway have a variety of additional effects that aren't related to HIF-1; therefore their potential to suppress HIF-1α production in many tumors may be limited. A particular low-molecular-weight inhibitor of HIF-1 activity is required for proof-of-principle preclinical research as well as

development of clinically relevant medicines. Using a cell-based reporter assay, the National Cancer Institute's Developmental Therapeutics Program is currently conducting high-throughput screening of its drug library for inhibitors of HIF-1-dependent gene transcription. HIF-1 inhibitors appear to be most suited for cancer types when large levels of HIF-1α overexpression are linked to treatment failure or mortality (Yu et al. 1999).

HIF-1α appears to mediate resistance to chemotherapy and radiation, according to recent research. Inhibition of HIF-1α activity could thus be a key component of antiangiogenesis therapy in combination (Höckel and Vaupel 2001; Semenza 2003).

Many innovative therapeutic medicines that target signal transduction pathways have antiangiogenic properties, in addition to those that have been developed expressly as antiangiogenesis agents. This effect appears to be attributable to the fact that inhibiting signal transduction pathways causes HIF-1α levels to drop. Topoisomerase I inhibitors block HIF-1α expression via an unclear mechanism (Rapisarda et al. 2002), according to a preliminary screen of the NCI Diversity Set of small molecule chemotherapeutic drugs utilizing a cell-based test. HIF-1 levels and xenograft growth were both reduced by the small drug YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole) (Yeo et al. 2003). Although YC-1 has been shown to enhance soluble guanylate-cyclase activity, this impact is not needed for HIF-1α suppression. The mechanism through which YC-1 lowers HIF-1α levels has yet to be discovered. HIF-1α interacts with the chaperone HSP90, and the HSP90 inhibitor 17-allyl-aminogeldanamycin (17-AAG) causes HIF-1α degradation without requiring VHL, implying that HSP90 is essential for HIF-1α stability (Isaacs et al. 2002; Mabjeesh et al. 2003; Zagzag et al. 2003). Thioredoxin-1, a redox regulator, also has a beneficial effect on HIF-1α expression. HIF-1α expression and xenograft growth are inhibited by thioredoxin inhibitors. Finally, 2-methoxyestradiol (2ME2) has been found to reduce HIF-1 levels as well as VEGF mRNA expression in cultured cells via disrupting microtubule polymerization. 2ME2 inhibits tumor xenograft development and vascularization in vivo. YC-1, topoisomerase I inhibitors, 17-AAG, thioredoxin inhibitors, and 2ME2 all have the ability to lower HIF-1 levels, block VEGF and other HIF-1 target genes, and affect xenograft growth and vascularization. As a result, it's possible that these drugs' anticancer effects are due in part to their inhibition of HIF-1 (Mabjeesh et al. 2003).

An antiangiogenic agent combined with an HIF-1 inhibitor could be particularly effective, as the angiogenesis inhibitor would cut off the tumor's blood supply while the HIF-1 inhibitor would prevent the tumor from adapting to the resulting Hypoxia. There is most certainly a therapeutic window for inhibiting HIF-1 activity under these extreme intra-tumoral hypoxia situations. The remarkable effects of overall HIF-1 impairment on vascular development in mice further suggest that inhibiting HIF-1 could enhance the action of angiogenesis inhibitors on endothelial cells and lower the risk of drug resistance (Blagosklonny 2001; Yeager et al. 2001).

18.6 Conclusion

The cancer care burden is growing, putting enormous physical, emotional, and financial strain on individuals, families, communities, and healthcare systems all over the world (Prager et al. 2018). Many patients die each year as a result of a lack of access to high-quality healthcare facilities and timely and appropriate diagnosis and treatment (Alkire et al. 2018). Hypoxia plays a pivotal role in cancer development and progression. It is responsible for influencing tumor neovascularization, metabolism, cell survival, and apoptosis (Luo et al. 2014). In addition to that coming up with a cure due to contribution of hypoxia to cancer stem cell characteristics resisting treatment is the most challenging part for the medical field. Transcriptional mechanism activated under hypoxic condition which incorporates HIF, PI3K, and MAPK pathways is capable of regulating cancer adaptive processes (Dengler et al. 2014). Under pathophysiological situation, hypoxia is associated with increasing tumor progression leading to the depletion in patients' survival rate. Clinical observation may aid in predicting patients who actually require HIF-targeted treatments. CXCR, VEGF, PDGF, and TGF are among of the HIF target genes that have been linked to cancer. HIF-regulated apoptosis along with oncogene activation holds a huge impact on tumorigenesis and cancer therapy.

Existing chemotherapy can cause wearing side effects which can cause major problems in patients. Despite chemotherapy targeting only tumor cells, toxic effects of these agents can harm the vulnerable intestinal epithelial cells and hair matrix keratinocytes. The toxicity of these chemotherapeutic drugs is mainly caused due to cell apoptosis mediated by p53 (Johnstone et al. 2002). Theoretically it may be possible to inhibit p53 activity in order to block the side effects of cancer therapy, but it will eventually lead to survival of normal cells without affecting p53 mutant tumor cells, but it will be of no help to the patients, whereas if p53 blocker and anticancer drug are combined together and administered to a patient, it can help in the removal of toxicity and maintain antitumor effect. There is inadequate amount of research work on which the clarity of how hypoxia-related apoptosis creates an impact on prognosis can be confirmed. Understanding how hypoxia regulates apoptosis in solid tumors could help researchers better understand how tumors behave and how hypoxia affects antitumor therapies to develop, test, and improve techniques for turning research findings into safe and effective cancer treatments.

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Hypoxia in Drug Resistance and Radioresistance

19

Kwang-Yu Chang, I-Li Lin, and Chun Hei Antonio Cheung

Abstract

Tumors mostly experience hypoxia or hypoxic stress as they progress, especially for tumor cells that are located far from the functional blood vessels. Pathologically, tumor hypoxia is known to play an important role in tumor progression, and it also contributes to the development of chemoresistance and radioresistance. Among different intracellular signaling pathways, the hypoxia-inducible factor-1 (HIF-1) signaling pathway is the most studied and known for its importance in regulating the survival of cells, including cancer cells, under hypoxic conditions. In the following sections, the molecular biology of hypoxia and the HIF-1 signaling pathway will be briefly reintroduced. Importantly, the role of hypoxia in tumor chemoresistance and radioresistance will be discussed in detail.

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Keywords

Cancer · Chemoresistance · Hypoxia · HIF-1α · Radioresistance

19.1 Introduction

Hypoxia is mostly defined as a reduction in the normal level of oxygen (referred as normoxia) available to cells or tissues. Among different intracellular signaling pathways, the hypoxia-inducible factor-1 (HIF-1) signaling pathway is the most studied and known for its importance in regulating the survival of cells, including cancer cells, under hypoxic conditions. HIF-1 is a heterodimeric transcription factor composed of an alpha (HIF-1α) and a beta [HIF-1β (aryl hydrocarbon receptor nuclear translocator, ARNT)] subunit. At the molecular level, HIF-1α is an oxygen sensor in which its expression is induced under hypoxic conditions, whereas HIF-1β is mostly constitutively expressed in cells. Under normoxic conditions, the amino acid residues Pro-564 and Pro-402 of HIF-1α are post-translationally hydroxylated by oxygen-sensitive prolyl hydroxylases (PHDs). Then, the E3 ubiquitin ligase von Hippel-Lindau protein (VHL) binds to the hydroxylated HIF-1α and promotes the degradation of HIF-1α by proteasome (Yu et al. 2001). The protein ubiquitination effects of VHL on HIF-1α can be counteracted by TAR (HIV-1) RNA binding protein 2 (TARBP2). Activation or upregulation of TARBP2 has been shown to reduce the expression of VHL and tumor necrosis factor receptor-associated factor 6 (TRAF6, another E3 ubiquitin ligase), resulting in the reduced ubiquitination level of HIF-1α and the enhanced protein stability of HIF-1α (Li et al. 2021). The amino acid residue Asn-803 of HIF-1α can be hydroxylated by factor inhibiting HIF-1 (FIH-1) through an oxygen-dependent mechanism under normoxic conditions (McNeill et al. 2002). Hydroxylation of the amino acid residue Asn-803 is known to inhibit the interactions between HIF-1α and the transcriptional coactivators E1A binding protein p300 (p300)/CREB-binding protein (CBP) (Lando et al. 2002). Hypoxia triggers the interactions between p300/CBP and HIF-1α (Arany et al. 1996). Together with HIF-1β, the p300/CBP-HIF-1 transcriptional complex binds to the hypoxia response elements (HREs) of different genes to promote the synthesis of proteins that are important for cell survival. Interestingly, it has been shown that the tumor suppressor P53 binds to p300 and interferes with the interactions between p300 and HIF-1α, leading to the inhibition of HIF-1α and the induction of death in cells under severe hypoxic conditions. However, HIF-1α dissociates p300 from P53 in cells under mild hypoxic conditions (Ye et al. 2019).

Pathologically, upregulation of HIF-1α in cancer and cancer-associated fibroblast (CAF) cells is known to promote aggressive behavior development, tumor angiogenesis, and metastasis (Yoo et al. 2011). Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein known to trigger angiogenesis upon binding to the receptors VEGFR1 and VEGFR2 on cells. Upregulation of HIF-1α promotes the production and secretion of VEGF from cells. At the molecular level, hypoxia induces HIF-1α and G-protein estrogen receptor (GPER) expression, leading to

VEGF production in CAFs (De Francesco et al. 2013). In addition, hypoxia represses the expression of neuropilin-2 (NRP2), subsequently inhibits the activity of SEMA3F, and increases the production of VEGF in cancer cells and the secretion of VEGF from cells in a HIF-1α-dependent manner (Coma et al. 2011). HIF-1α also negatively regulates the expression of E-cadherin (CDH1), in which downregulation of this molecule promotes the epithelial-mesenchymal transition (EMT) of cancer cells and tumor metastasis (Esteban et al. 2006). Interestingly, a recent study showed that hypoxia induces HIF-1α-dependent disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) expression and subsequently mediates the ectodomain shedding of heparin-binding epidermal growth factor (EGF)-like growth factor, which is a known ligand of EGFR, in breast cancer cells (Wang et al. 2021a). The binding of heparin-binding EGF-like growth factor on EGFR activates the EGFR signaling and induces breast cancer cell invasion (Wang et al. 2021a). He et al. showed that hypoxia also promotes the expression of microRNA-224 (miR-224) in a HIF-1α-dependent manner and upregulation of miR-224 promotes the growth, migration, and invasion of gastric cancer cells (He et al. 2017).

19.2 Hypoxia, Apoptosis, and Drug Resistance

Hypoxia modulates the expression of various anti−/pro-apoptotic molecules known to play an important role in the development of drug resistance. Oligomerization and mitochondrial membrane localization of BCL2-associated X (BAX) mediates mitochondrial membrane permeabilization and the subsequent release of cytochrome c, which are the early initiation steps of intrinsic apoptosis. Similar to BAX, BH3 interacting-domain death agonist (BID) is a pro-apoptotic member of the BCL2 superfamily. It has been demonstrated that hypoxia decreases the expression of BAX and BID and induces resistance to etoposide and oxaliplatin in human colon cancer cells (Erler et al. 2004). A past study showed that hypoxia induces temozolomide (TMZ) resistance in glioma cells. Hypoxia upregulates the expression of the miRNA, miR-26a, in an HIF-1α-dependent mechanism. Overexpression of miR-26a decreases BAX and Bcl-2-associated death promoter (BAD, a pro-apoptotic member of the BCL2 superfamily) expressions and prevents the release of cytochrome c from mitochondria to the cytoplasm (Howells et al. 2011; Ge et al. 2018). Of note, the release of cytochrome c from mitochondria is crucial for the activation of CASP9 (caspase-9) and the formation of apoptosome during the early stages of intrinsic apoptosis.

Nowadays, it is known that hypoxia not only modulates the expression of various BCL2 family members but also regulates the expression of different inhibitor of apoptosis protein (IAP) family members in cancer cells. Members of the IAP family are characterized by the presence of the conserved baculoviral IAP repeat (BIR) domain, and functionally, they negatively regulate apoptosis by inhibiting the activity of different caspases through both direct interactions and indirect modulations in cells. Baculoviral IAP repeat containing 5 (BIRC5, also known as survivin) is an IAP family member. Unlike other IAPs, BIRC5 is known as a

multifunction protein that exhibits both anti-apoptotic and promitotic functions (Cheung et al. 2020). Emerging evidence also suggest that BIRC5 exhibits anti-autophagic functions in cancer cells (Cheung et al. 2020; Lin et al. 2020; Cheng et al. 2021a). Given the molecular functions of BIRC5, it is unsurprising to see that overexpression of BIRC5 induces multidrug resistance in cancer cells. At the molecular level, HIF-1α positively regulates BIRC5 expression (Chen et al. 2012). Hypoxia upregulates BIRC5 expression in cervical and gastric cancer cells, and overexpression of BIRC5 induces cellular resistance to cisplatin (Bai et al. 2013; Sun et al. 2014). It is worth noting that besides being upregulated in cancer cells experiencing hypoxic stress, hypoxia also promotes the expression of BIRC5 in human umbilical vein endothelial cells (HUVECs) through both HIF-1α/VEGF-dependent and HIF-1α/VEGF-independent mechanisms (Conway et al. 2003). Moreover, upregulation of BIRC5 induces angiogenesis in ischemic brain (Conway et al. 2003).

19.3 Hypoxia, Cell Cycle Arrest, Senescence, and Drug Resistance

Cancer cells can acquire drug resistance through induction of cell cycle arrest and activation of cellular senescence. A study showed that chronic intermittent hypoxia triggers a senescence-like phenotype in human white preadipocytes in part through a cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as P16)-dependent mechanism (Polonis et al. 2020; Rayess et al. 2012). Although P53 is traditionally considered as a pro-apoptotic molecule, recent evidence suggest that it also exhibits pro-survival effects in cancer cells under certain circumstances. For instance, prolonged hypoxia causes HIF-1α and P53 upregulation and promotes glycolysis in lung cancer cells (Guo et al. 2018). Upregulation of P53 subsequently activates its downstream target cyclin-dependent kinase inhibitor 1A (CDKN1A, also known as P21) and induces cell cycle arrest at the G0/G1 phase (Guo et al. 2018). As cancer cells are most sensitive to the DNA-damaging agent, cisplatin, in the S phase of the cell cycle, cell cycle arrest at the G0/G1 phase exerts a protective effect against cisplatin treatment in cancer cells (Guo et al. 2018). It is worth noting that opposite role of hypoxia on senescence has also been reported. Sullivan et al. demonstrated that hypoxia induces HIF-1α-related doxorubicin resistance in breast cancer cells through inhibition of the drug-induced senescence (Sullivan et al. 2008). Another study showed that hypoxia promotes lung cancer cells escaping from cisplatin-induced senescence via reduction of P53 and CDKN1A (Olszewska et al. 2022). Geroconversion is a process that describes the conversion from proliferative arrest to irreversible senescence of cells. Accordingly, hypoxia suppresses CDKN1A-induced senescence through an mTOR pathway-dependent, but HIF-1-α-independent, mechanism and maintains the reversible quiescence potential of HT-p21–9 cells (Leontieva et al. 2012).

Dysregulation of cyclin-dependent kinase regulatory subunit 1 (CKS1B) is associated with the pathogenesis of a variety of human cancers and closely related

to drug resistance (Shi et al. 2020; Zhan et al. 2007). Interestingly, hypoxia has been shown to induce transient copy gain for the CKS1B gene in breast cancer cells (Black et al. 2015). Downregulation of CKS1B inhibits the proliferation, invasion, and angiogenesis of retinoblastoma cells (Zeng et al. 2019). Noticeably, CKS1B upregulation promotes CDKN1A degradation and inhibits senescence in multiple myeloma cells, again, suggesting that hypoxia plays differential roles on senescence induction in different cell lines/types or in cells under different circumstances (Gu et al. 2013; Welford and Giaccia 2011).

19.4 Hypoxia, Metabolic Programming, and Drug Resistance

Besides altering the expression of different pro-apoptotic and anti-apoptotic molecules, hypoxia also modulates various metabolic processes, which leads to the development of drug resistance in cancer cells. Thymidylate synthase is an enzyme that plays an important role in the biosynthesis of thymidylate, a precursor for DNA biosynthesis and the main molecular target of the widely used anticancer drug 5-fluouracil (5-FU). In cells, the primary active metabolite of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), binds to and forms a stable complex with thymidylate synthase, inhibiting the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), thereby suppressing DNA replication. FdUMP can also be converted to fluorodeoxyuridine triphosphate (FdUTP) and subsequently be misincorporated into DNA, causing DNA damage. It has been shown that hypoxia decreases the protein expression level of thymidylate synthase (decreases the rate of DNA replication) and various checkpoint signaling molecules (prevents cell cycle arrest and inhibits the induction of apoptosis) like ataxia telangiectasia mutated (ATM) kinase and checkpoint kinase 1 (CHEK1/ChK1), attenuating the pro-apoptotic effects of 5-FU in hepatocellular carcinoma cells (Li et al. 2017).

Mitochondria have been noted for their central roles in intracellular metabolic reprogramming of cancer cells that are related to the induction of resistance to different chemotherapeutic agents (Oliva et al. 2011; Guerra et al. 2017). Under normoxia and physiological conditions, cells mostly favor the use of the oxygen-dependent mitochondrial oxidative phosphorylation (OXPHOS) to produce ATP. In contrast, hypoxic cells (including tumor cells) generally exhibit enhanced glucose metabolism (i.e., anaerobic glycolysis) and reduced OXPHOS partly through an HIF-1-dependent mechanism (Guillaumond et al. 2013; Lum et al. 2007; Rodríguez-Enríquez et al. 2010). In fact, even at normoxic conditions, cancer cells tend to use aerobic glycolysis instead of oxidative phosphorylation to produce energy (i.e., Warburg effect). Under hypoxia, HIF-1α-upregulated Hes-related family BHLH transcription factor with YRPW motif 1 (HEY1) represses PTEN-induced kinase 1 (PINK1) expression and reduces mitochondrial cristae and mitochondrial mass (Kung-Chun Chiu et al. 2019). Hypoxia can also indirectly alter the activity of

mitochondria. Hypoxia induces oxidative stress and inflammation through modulating the activity of endoplasmic reticulum, NADPH and xanthine oxidase, nitric oxide synthase, and lipoxygenase. Hypoxia-induced oxidative stress causes lipid peroxidation, in which it subsequently generates 4-hydroxynonenal (4-HNE) as a byproduct. 4-HNE impairs mitochondrial function, lowers ATP production, and decreases mitochondrial oxygen consumption in cells (Galam et al. 2015). Additionally, HIF-1 promotes the expression of various glucose transporters like glucose transporter 1 (GLUT1) and glucose transporter 3 (GLUT 3) (Hayashi et al. 2004; Lauer et al. 2020). Moreover, hypoxia induces the translocation of GLUT1 to the plasma membrane in vascular endothelial cells (Mamun et al. 2020).

As aforementioned, the role of the hypoxia-promoted glycolysis on drug resistance has been widely studied. For example, Wang et al. demonstrated that the use of wogonin (an O-methylated flavone) restores the sensitivity of the hypoxic colon cancer cells to various chemotherapeutics through downregulation of HIF-1α, glycolysis-related proteins like hexokinase-II (HK2), pyruvate dehydrogenase kinase 1 (PDHK1), and lactate dehydrogenase A (LDHA) and reduction of glucose uptake (Wang et al. 2014). Besides PDHK1, hypoxia and HIF-1α are known to positively modulate the expression of pyruvate dehydrogenase kinase-3 (PDK3). Functionally, PDK3 inhibits the conversion of pyruvate to acetyl-CoA; thus, upregulation of PDK3 inhibits mitochondrial respiration and promotes glycolysis in normal and cancer cells (Lu et al. 2008). It has been demonstrated that HIF-1-α-induced PDK3 expression decreases the sensitivity to paclitaxel in hypoxic cancer cells (Lu et al. 2008). Even though hypoxia has widely been demonstrated to promote a switch from oxidative phosphorylation to glycolysis for ATP production, a study reported an interesting finding that hypoxia upregulates peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) expression and increases mitochondrial biogenesis, oxidative phosphorylation, mitochondrial energy metabolism, and antioxidant enzyme expression via PGC-1α, promoting the induction of 5-FU resistance in colorectal cancer cells (Yun et al. 2019).

Interestingly, hypoxia not just directly modulates the metabolism of individual cancer cells but also promotes intercellular communications between cancer cells, transferring the "metabolic regulatory signal" from one to another, resulting in increased drug-resistant populations within the tumor mass. Pyruvate kinase M2 (PKM2) is a rate-limiting enzyme that catalyzes the last step of glycolysis. It has been shown that hypoxia further increases the expression of PKM2 and HIF-1α and the level of drug resistance in the cisplatin-resistant non-small cell lung A549 cancer cells. In addition, hypoxia promotes the exosomal secretion of PKM2 from the cisplatin-resistant non-small cell lung A549 cancer cells (Wang et al. 2021b). The secreted PKM2 then increases PKM2 expression and subsequently upregulates reduced nicotinamide adenine dinucleotide hydrogen (NADH) and glutathione (GSH) levels in the cisplatin sensitive cells, leading to the induction of drug resistance (Wang et al. 2021b).

19.5 Hypoxia, Angiogenesis, and Drug Resistance

Hypoxia promotes the development of disordered blood vessel network. Even though the new blood vessels can deliver extra nutrient and oxygen to tumor cells in response to hypoxic stress, they are often fragile and without coordinated formation. Thus, these fragile and uncoordinated new blood vessels mostly cannot deliver anticancer drugs efficiently, which limits the effects of drugs in the hypoxia tumor area. From the experience of bevacizumab (Avastin®) usage in patients with colorectal cancer, even though this anti-VEGF antibody corrects the tumor neovasculature for better transportation of chemotherapeutic agents to the tumor, vascular normalization by bevacizumab results in tumor hypoxia, which promotes the induction of drug resistance and increases the aggressiveness of cells (Fan et al. 2011; Bensaad and Harris 2014). For instance, Ueda et al. showed that bevacizumab induces acute hypoxia- and cytokine secretion-related cancer progression in patients with refractory breast cancer (Ueda et al. 2017). Although antiangiogenic therapy has increased the progression-free survival of many cancer patients, the effect on overall survival is little (McIntyre and Harris 2015). It is also known that hypoxia affects the effectiveness of antiangiogenic therapy (McIntyre and Harris 2015).

19.6 Hypoxia, Intratumoral Immunity, and Immunotherapy

Hypoxia-related secretome from tumor cells modulates the immune environment. Cytokines secreted by hypoxic tumor cells promote the infiltration of pro-tumoral myeloid-derived suppressive cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs) into the hypoxic tumor area. These cells help in reshaping tumor immunity and microenvironment for adaptation (Murdoch and Lewis 2005; Wenes et al. 2016; Casazza et al. 2013). It has been reported that placental growth factor (PGF) and C-X-C motif chemokine ligand 16 (CXCL16) secretion by hypoxic breast cancer cells facilitate the recruitment of mesenchymal stem cells (MSCs) to breast tumors (Chaturvedi et al. 2014). C-C motif chemokine ligand 5 (CCL5) secretion by infiltrating MSCs subsequently stimulates colony stimulating factor 1 (CSF1) secretion by breast cancer cells, leading to the recruitment of TAMs and myeloid-derived suppressor cells (Chaturvedi et al. 2014). Another study showed that interleukin-4 (IL-4) and interleukin-10 (IL-10) drive the polarization of TAMs into type 2 macrophages (M2), possessing immunosuppressive activities and promoting tumor progression (Mantovani et al. 2002). Under hypoxia and inflammation, excessive induction of HIF-1α can alter T cell differentiation, leading to more inflammation that was related to T_H17 accumulation and induced Treg suppression (Dang et al. 2011). It has been shown that hypoxia downregulates the proliferation of $CD4^+$ T cells and inhibits the differentiation of T_H1 cells in a colitis-associated colon cancer model (Westendorf et al. 2017). Hypoxia also upregulates the expression of PD-1 ligand 1 (PD-L1) on MDSCs, macrophages, dendritic cells, and tumor cells via an HIF-1α-dependent mechanism, and blockade of PD-L1 under hypoxia increases MDSC-mediated T cell activation

(Noman et al. 2014). Pharmaceutical abrogation of HIF-1α decreases PD-L1 expression on malignant cells, leading to the reactivation of tumor-infiltrating lymphocytes (TILs) and tumor eradication (Bailey et al. 2022). Recent success in combining VEGFR inhibitors with anti-PD-1/PD-L1 inhibitors to treat clear cell renal cell carcinoma (ccRCC) pursues the synergistic strategy from effects of both drugs (McIntyre and Harris 2015).

19.7 Hypoxia and Radiation Therapy Resistance

Low levels of oxygen in tumors often reduce the effectiveness of radiation therapy (RT) in patients, and free molecular oxygen is known as a potent radiosensitizer (Fig. 19.1). In clinical situation, the oxygen enhancement ratio (OER) is given by the dose of radiation used in hypoxia divided by the dose of radiation used in physiological normoxia (or in air) to achieve the same effect. Generally, low linear energy transfer (LET) radiations have a high OER, which means their therapeutic effects rely more on the presence of oxygen. Mechanistically, ionizing radiation generates reactive oxygen species (ROS, including O_2^-, OH^-, and H_2O_2) by the radiolysis of water, leading to the induction of DNA/subcellular organelle damage and the activation of apoptosis in cancer cells (Yamamori et al. 2012). Ionizing radiation also induces mitochondrial ROS production in cells (Yamamori et al. 2012).

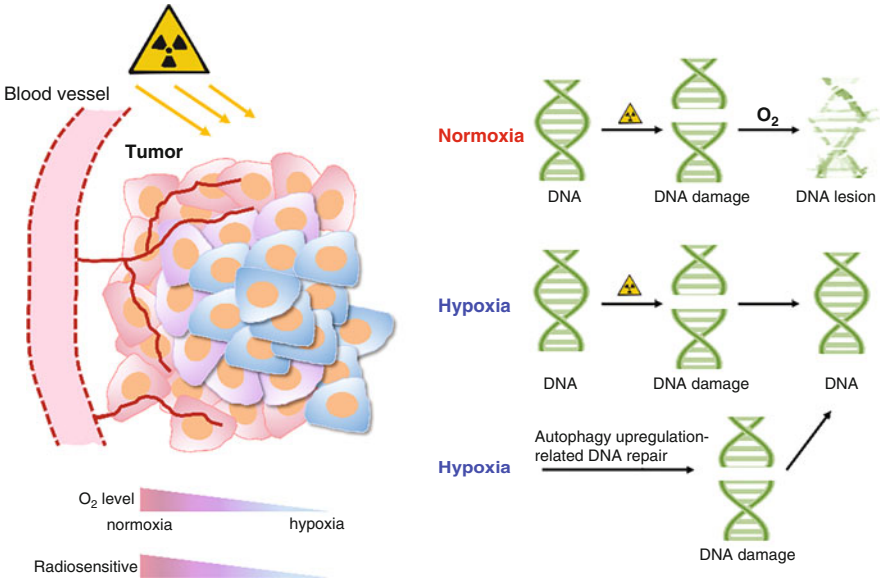

Fig. 19.1 The effects of hypoxia (or oxygen) and autophagy on the repair of radiation-induced DNA damage in cancer cells

Although increased radiation sensitivity of cancer cells in the presence of oxygen than in hypoxic conditions (i.e., the oxygen effect) has widely been observed, the biological/chemical role of oxygen in ionizing radiation-induced cell death still remains incompletely understood. It is believed that the presence of molecular oxygen is crucial for "fixing" DNA lesions induced by ionizing radiation as it reacts with radiation-induced DNA radicals to stabilize them (i.e., oxygen fixation hypothesis) (Robert Grimes and Partridge 2015; Rakotomalala et al. 2021). Thus, the presence of molecular oxygen makes DNA repair became more difficult and promotes DNA damage accumulation in the radiation-treated cells (Fig. 19.1). The degrees of effect of irradiation also seem to be related to the levels of mitochondrial oxygen consumption and ATP generation (Richardson and Harper 2016). Irradiation increases mitochondrial respiration and mitochondrial ATP production. As aforementioned, hypoxia induces a metabolic switch from oxidative phosphorylation to glycolysis to generate ATP. Therefore, hypoxia decreases the irradiation-induced mitochondrial ROS production and the mitochondrial ROS-induced DNA double-strand breaks (Richardson and Harper 2016).

Hypoxia decreases the radiosensitivity through both oxygen directly involved and indirectly involved mechanisms (Wiechec et al. 2022). As described in previous sections, hypoxia (i.e., reduced oxygen level) induces BIRC5 (survivin) expression, which leads to the induction of multidrug resistance. It has been demonstrated that upregulation of BIRC5 also induces radioresistance in cancer cells (Rödel et al. 2005; Asanuma et al. 2000; Chakravarti et al. 2004). Of note, BIRC5 depletion/inhibition decreases the expression of various nonhomologous end joining (NHEJ) and homologous recombination (HR)-involved proteins like XRCC6 (also known as Ku70), RAD51 recombinase (RAD51), and MRE11, suggesting that BIRC5 plays a role in the repair of DNA double-strand breaks (Cheng et al. 2021a; Véquaud et al. 2016; Jiang et al. 2009). Thus, hypoxia-induced BIRC5 upregulation may reduce radiosensitivity through increased DNA repair efficiency and decreased caspases activity in cancer cells. Besides BIRC5, hypoxia induces the expression of human epididymis protein 4 (HE4, a secreted glycoprotein) via HIF-1α, and it has been shown that overexpression of HE4 decreases the sensitivity to radiation in the hypoxic gastric cancer cells (Peng et al. 2019). However, the mechanism underlying the radiosensitivity role of HE4 is still unclear.

Autophagy has widely been demonstrated as a positive regulator of the DNA damage repair response (DDR), and it plays an important role in the maintenance of genomic stability (Hewitt and Korolchuk 2017). It regulates mitochondrial and reactive oxygen species homeostasis in cells (Tal et al. 2009; Yamauchi et al. 2019). Thus, it is unsurprising to see that upregulation of autophagy could affect the DNA-damaging effects of radiation therapy. A study by Zou et al. showed that hypoxia-induced autophagy contributes to radioresistance in cancer cells (Zou et al. 2014). In fact, irradiation, by itself, also triggers the induction of cyto-protective autophagy in cancer cells, and inhibition of autophagy potentiates the effects of radiotherapy (Apel et al. 2008).

19.8 Conclusion

Given the roles of hypoxia in the induction of anticancer drug resistance and radiotherapy resistance, various efforts have been devoted into the development of intratumoral reoxygenation therapy (Gogna et al. 2012). Hyperbaric oxygen therapy (HBOT) is a well-established treatment for decompression sickness, and the potential of HBOT as part of the anticancer treatment is undergoing investigations (Chen et al. 2021; Wu et al. 2018). The use of oxygen-generating nanoparticles as a chemosensitivity-restoring agent for hypoxic tumors is also undergoing intensive investigations (Cheng et al. 2021b; Ruan et al. 2021). Various efforts have also been devoted in the development of HIF-1α-targeting small molecular inhibitors (Yu et al. 2017; Salman et al. 2022; Fallah and Rini 2019; Ban et al. 2017). However, the biology of hypoxia and the pathological role of hypoxia in tumor progression and drug resistance are highly complicated (e.g., differential mechanisms in different cells, intratumoral environments, and oxygen levels), and further studies are still required for better understanding the molecular pathways that are affected by hypoxia and to explore the possibility of targeting molecules, other than HIF-1α, in interfering with the hypoxia-signaling pathway to restore the sensitivity to different chemotherapeutics and radiotherapy. Development of biodegradable, highly hypoxic tumor-specific, oxygen fine-releasing, agents may also be clinically useful for the future anticancer treatment.

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