Hardeep Singh Tuli *Editor*

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Dedicated to My Beloved Parents

Preface

Cancer is a major cause of death worldwide and is known to be the biggest killer in the twenty-first century. It has been ranked second in mortality rate following cardiovascular diseases in most of the countries. Every year, the number of people being diagnosed with cancer is increasing very fast. Due to the lack of significant improvement in diagnosis, treatment, and prevention, cancer has or will soon become the number one killer in most parts of the world. Induced side effects and acquired resistance against anticancer drugs create the hassle for the treatment of cancer and the enthusiasm for the development of new approaches.

Further, application of safe compounds with strong anticancer properties may open new avenues in the fight against this devastating disorder. Therefore, different antitumor mechanisms of drugs, that is, cell cycle arrest, apoptosis induction, antioxidant, anti-inflammatory, antiproliferative, antiangiogenic, anti-invasive, antimetastatic, and proapoptotic properties, are summarized in this book. In addition, this book will introduce readers to the various aspects of drug interactions in recognized cellular processes and will explore the various anticancer targets in different phases of drug development in clinical trials along with new drug targets for personalized cancer. The dataset presented in this book could be a valuable basis for the understanding of cancer biology and the initiation of the human clinical trials with patients suffering from different cancerous diseases, either alone or in combination with traditional therapies. It will help to understand the cancer biology as well as drug mechanisms of action. Various undergraduate and post-graduate students will also be benefited to learn various cancer regulatory processes.

Ambala, Haryana, India — Hardeep Singh Tuli

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Contents

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History of Oncotherapies in Cancer Biology

1

Vaishali Aggarwal, Katrin Sak, Mehak Arora, Ashif Iqubal, Ajay Kumar, Saumya Srivastava, Anjana Pandey, Satwinderjeet Kaur, and Hardeep Singh Tuli

Abstract

Cancer is the second leading cause of death worldwide, just behind cardiovascular diseases. In fact, there was an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 around the world. Due to the continuously increasing global prevalence of malignancies, novel efficient therapeutics and treatment strategies are highly needed. The most common types of cancer treatment modalities include surgery, chemotherapy, and radiation therapy. At that, over 50% of all cancer patients receive chemotherapy in some stages of their disease. Although modern drugs are very efficient to kill tumor cells, they also affect normal healthy cells often causing intolerable side effects. In addition, drug resistance to chemotherapeutic agents is problematic. Several signaling pathways

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are found to be associated with cancer progression and survival. This book chapter presents an overview of various oncotherapies in cancer.

Keywords

Radiotherapy · Chemotherapy · Biomarkers · Personalized medicine · Clinical trials

1.1 Introduction

Cancer is a major burden of morbidity and mortality all around the world. Each year, tens of millions of people get the diagnosis of cancer and more than half of them lose their life due to this dreadful disease [1]. Currently, malignant tumors rank the second most common cause of death worldwide just following cardiovascular disorders [2]. However, cancer cannot be considered as only the disease of the modern era as it has been existed with the people already from the ancient Egyptian and Greek times [3].

The most common therapeutic strategies nowadays applied in the fight against malignant tumors include surgery, chemotherapy, and radiotherapy. From the Egyptian times until the beginning of the twentieth century, the main approach to cancer treatment comprised of surgical eradication of superficial tumoral lesions and alleviation of pain using various herbal extracts [3]. Discovery of the X-rays and radium in the end of the nineteenth century gradually led to the introduction of modern radiotherapy in 1920 [3]. The use of chemotherapy in cancer treatment began in the years of the Second World War when it was found that nitrogen mustard can retard the development of lymphomas [4]. This discovery was further followed by the synthesis of several alkylating agents and antimetabolites, including chlorambucil, cyclophosphamide, methotrexate, and 5-fluorouracil [4]. The progress in research and introduction of modern technological solutions in the second half of the twentieth century brought along the development of numerous novel cancer drugs. Still, despite all these advancements we have not yet won the battle with cancer. Moreover, all the conventional therapeutic modalities are associated with different adverse effects on normal healthy tissues, causing additional distress and aggravation of quality of life of patients. Such often intolerable side effects include hematological toxicity, bone marrow toxicity, neurotoxicity, cardiotoxicity, hepatotoxicity, and nephrotoxicity among several others [5]. In addition, intrinsic and/or acquired resistance of malignant cells toward the therapeutics is also a great problem hindering the successful treatment of tumors [6]. Therefore, we are still faced with the urgent need to find novel, more efficient, and safe treatment modalities in combating malignant disorders. In this book chapter, we present a thorough review about the modern methods of oncotherapies that will probably find wider application in the clinical settings in the near future and lead us closer to the final aim of efficient cancer cure.

1.2 Radiotherapy in Cancer

The meaning of radiotherapy is to use the radiations to treat cancer. Radiotherapy works by damaging the DNA within cancer cells and destroying their ability to reproduce [7]. When damaged cancer cells are destroyed by radiation, the body naturally eliminates them [8]. Radiotherapy is either governed externally or internally. In external radiotherapy, radiations are delivered by using a linear accelerator [9]. It is used to treat many tumors including cancer of head, neck area, lungs, colon, and breast. In contrast, using internal radiotherapy, radioactive sources are given inside the patient. It is used to treat cancers of the eye, esophagus, uterus, bladder, and cervix [10]. Radiotherapy can be used to: cure the cancer completely and make other treatments more effective; for example, it can be combined with chemotherapy or used before surgery [9]. There are a variety of benefits of radiotherapy such as in organ preservation, destroying cancer cells, treating noninvasive tumor, and improving treatment cost [11–13]. However few disadvantages of radiotherapy associated like painful, causes skin irritation, nausea, fatigueness, diarrhea, hair loss, immunosuppression, and damage to surrounding tissue [14, 15].

1.3 Anticancer Drugs to Chemotherapy

The treatment for cancer involves chemotherapy besides surgery and radiotherapy [16]. The use of anticancer drugs in chemotherapy involves DNA interactive agents (cisplatin), antimetabolites (5-fluorouracil), topoisomerase inhibitors (topotecan), antitubulin agents (paclitaxel), hormone (tamoxifen), monoclonal antibodies (cetuximab), etc. [17–19]. The cytotoxic chemotherapy agents exhibit their effects by disruption of the cell cycle, resulting in apoptosis. This may involve the interaction, with DNA and/or protein involved in cell division [20]. The fast-dividing normal cells as that of bone marrow, hair follicles, gastrointestinal tract, etc. are affected, resulting in undesirable effects along with the development of drug resistance that may be due to multidrug resistance (MDR), cell death inhibition, alteration in drug metabolism, enhancement of DNA repair, and gene amplification [21]. In the recent years, there is an emphasis on the development of novel targeted therapies that block biological transduction pathways and/or specific cancer proteins that are involved in tumor growth and progression [22]. The natural products and their variants act as microtubules inhibitors which are quit effective in the treatment of various malignancies including solid tumors [23–27]. The various types of the natural compounds from the plants have been reported in the literature, to selectively inhibit the microtubule activity, mitosis, and the cellular signaling events and showed less toxicity in the chemotherapy [22, 28, 29]. Finally, stimulated immune system along with the use of a smart drug delivery system (SDDS) aimed at the death of cancer cells, minimizing the death of normal cells and undesirable side effects.

1.4 Diagnostic Biomarkers to Molecular Basis

Of all the life-threatening diseases, the structure of cancer is very unique, as the disease-laden cells invade healthy and normal cells around the area and tissues [30, 31]. From there, they start metastasizing them to different other sites of a human body [32, 33]. As the process of cells becoming cancerous continues inside the body, a number of genetic and epigenetic mistakes occur and some of them define the contribution of protein in cell survival, invasion, and getting metastasized [34–36]. Targeted therapy is a very promising cure that kills the cancerous cells without causing any harm or any major side effect to the normal cells surrounding them [37, 38]. However, after multiple researches, it was finally traced that the success or the effectiveness of targeted therapies entirely depends on the nature of the target [39, 40]. One more very influential factor was the development of the agents that could impact only the targeted areas. This was a complex situation, as some targets found in patients with chronic myelogenous leukemia (CML) were very unique to cancer cells [41, 42]. Some were expressed at higher levels in the patients suffering from some very unique types of cancer, and here, it is important to know that some of them were even expressed toward normal cells, thereby presenting the risks of toxicity [41, 42]. Because of this, it was established that even if the targets were unique to cancer cells, there are some nonspecific effects that would occur for sure if the targeting agents affect other proteins [23, 24, 27, 32, 33, 38]. But since every patient with cancer is unique, the real challenge is to ensure the delivery of right treatments and right targeting agents that would not affect the surrounding cells or damage/infect them [43]. This can be done very easily by some very complex tests known as biomarker tests for molecularly targeted therapies. They are known to have a potential to reveal before the medical teams about the most effective and safest targeting agents or treatments [44, 45]. Medical care experts have assumed these biomarker tests as a key to offer the most precise and the least hazardous treatments.

Precision Medicine

But to advance further in the process of using precision medicines, several tests are needed that have to be accurately done and they should be reliable and not to mention that they have to be properly validated as well [46, 47]. In the pursuit of precision, the tests to be conducted need too much accuracy and at the same time, they have to be appropriately implemented in clinical practices as well [48–51]. Then, one more very important thing was required, i.e., collecting and sharing of the information about the results of the patients to whom treatments were given post these biomarker tests [52–54].

In easier words, to find precision medicine and targeting agents, it is important that these biomarker tests are done right, as this will help oncologists in optimizing the treatments of each individual patient and improve the chances of getting cured [52–54]. The precision of these tests is required for one more reason that their results would help oncologists in understanding the role of genetics in the disease in a better

manner [55, 56]. In short, this can be said that the exactness, accuracy, or precision of these biomarker tests for molecular targeted therapies are crucial because any inaccuracy is as bad as a wrong medicine.

Biomarker Tests

Ever since these biomarker tests came into existence, lots of researches have been conducted and in fact, they have evolved entirely. The first major breakthrough came way back in 2001 from the draft sequence of the human genome [57]. Since then, these biomarker tests have been assumed as a rapidly evolving and improving field in terms of precision medicine and targeting agents [45, 46]. To understand these biomarker tests in a better way, it is important to understand definitions and terminologies, as with this information only, you would be able to cope up with the rapidly evolving field of these tests [58, 59].

Biomarker tests can be termed as a characteristic that has to be observed as well as evaluated as a sign of a normal biological process [58, 59]. In other words, you can call them as a test to measure out numerous things such as macromolecules (DNA, RNA, proteins, lipids), cells, or processes. Note that all these things are an indicator of normal or irregular biological state in an organism. Where these biomarker tests for molecularly targeted therapies can be used? If you see the studies conducted in the past, a whole new light of knowledge would emerge from them because these biomarker tests have many different uses in clinical practices [45–47]. This could include

- Disease screening tests for prostate-specific antigen
- Diagnostic tests (pathologic or histologic assessment of a tissue biopsy)
- Treatment and posttreatment monitoring tests (detection of treatment complications or subsequent disease advancement)
- Prognostic tests for estimating risk or time to clinical outcomes (e.g., aggressive cancers have a poorer prognosis than more indolent cancers)

Types of Biomarker Tests

Studies further reveal that other than these, biomarker tests can also be used to predict patient's response to specific treatments and targeted agents [60–62]. Few of these biomarkers are as follows:

- BRAF = B-RAF proto-oncogene
- Serine/threonine kinase
- ER = Estrogen receptor
- HER2 = Human epidermal growth factor receptor 2
- PGR = Progesterone receptor
- PIK3CA = Phosphatidylinositol-4

- 5-Bisphosphate 3-kinase
- Catalytic subunit alpha
- PTEN = Phosphatase and tensin homolog

Clinical Uses of Biomarkers

Healthcare providers across the globe have been using these biomarker tests to provide tailored treatments to individual patients based on their patient history and an analysis of how their body would respond to a particular treatment [63]. There is a dedicated section of these tests, which examines each individual patient's ability to metabolize a particular drug or targeting agent [64, 65]. This is followed by another decision that has been designated as the task of studying biomarker tests for specific aberrations in biological mechanisms of action. As far as biomarker tests for molecularly targeted therapies are concerned, a number of tests can be carried out for clinical use. This could range from single analyte tests to guide the use of a single class of therapy to a suite of multiple, but separate, tests for single analytes [59]. As mentioned above, these tests would guide the use of different therapies in a specific clinical context. Lastly, using these biomarker tests, the entire genome can also be analyzed with the help of next-generation sequencing and the good thing in this context is that rapid technological advancements have made these tests more accurate, faster, and more affordable for a common man [60–62].

1.5 Personalized Medicine: Bigger Picture Ahead of Time

Personalized medicine (PM) is an integration of personal profiles of genes or proteins for strengthening of healthcare at personalized level by aiding the emergent technologies "-omics," including genomics, transcriptomics, pharmacogenomics, and proteomics [66]. Currently, for optimizing and selecting the cancer patient's therapeutic care, PM has exploited the systematic usage of genetic information in contrast to conventional cancer therapies that involve family history of patients and lifestyle [67]. National Institutes of Health (NIH) has defined personalized medicine as emerging medicine branch that uses genetic profile of individuals, for making decisions on disease diagnosis and treatment [68]. It targets the factors having positive effects on that disease to provide the timely, appropriate, and correct treatment to the right person [69].

Cancer therapeutic drugs are not equally effective for all patients. Due to advance high-throughput genomics and proteomics tools available for cancer molecular mechanism understandings, it became easier to disclose the genes that are responsible for drug responses. PM is a revolution for healthcare regimen due to its ability to integrate genetic information, to increase the drug efficacy for treatment, and to introduce new healthcare business [69]. There is a huge variability across diseases, that is, 38–75% patients do not respond to a drug or treatment. In the case of cancer, average response rate of drug is minimum at 25%. In addition, adverse drug reaction

is also a problem. In USA, 16% of the approved drugs have shown the disadvantageous drug reactions [70]. Due to the personalized medicine healthcare pattern, doctors or clinicians can make ideal selections to maximize the effectiveness of treatment, simultaneously adverse drug reactions risks can be avoided, and researchers can improve drug and medical device research process for enabling early detection of disease [69].

Based on predictive biomarkers, molecular diagnostic tools provide valuable facts and figures of patients associated with genetically defined subgroups who would take advantage of specific therapy. For example, a 16-gene signature was used by a diagnostic device OncotypeDX® (Genomic Health, USA), to assess the recurrence risk in estrogen receptor positive breast cancer patients [71–73]. Likewise, MammaPrint® (Agendia, the Netherlands) practices on a 70-gene expression profile for assessment of distant metastasis risk in breast cancer patients of early stage [74]. In the case of lung cancer, based on recent modern genetic studies, epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), Cbl proto-oncogene (CBL), MET proto-oncogene, and receptor protein kinase (MET) are being used as targets for therapeutic purpose [75]. Crizotinib has shown significant results in non-small cell lung cancer treatment by inhibiting ALK [76].

Personalized medicine is getting huge attention of researchers and clinicians for its remarkable potential and countless applications. The notable introduction of recent high-throughput tools combined with improved cancer molecular profile knowledge provides a stable platform for novel molecular targets identification.

1.6 Cancer Therapies Successful in Clinical Trials

For the past 5 years the success of many clinical trials has been witnessed, and a number of newer as well as existing drugs (Poly (ADP) ribose polymerase (PARP) inhibitors, monoclonal antibodies, and cyclin-dependent kinase (CDK) 4/6 inhibitors) were approved by the US Food and Drug Administration (FDA). In February 2018, based on the outcome of MONARCH 3 trial (NCT02246621), FDA has approved a combination of Abemaciclib (CDK) inhibitor and Anastrozole (aromatase inhibitor) for the treatment of HR+ epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer [77]. Another recently approved drug is the combination of Nivolumab [62, 76] and Ipilimumab (monoclonal antibody) for advanced melanoma (CheckMate067 study) [78]. The outcome of the CheckMate067 study (NCT01844505) has shown a survival rate of 52% when administered with a combination of Nivolumab and Ipilimumab, whereas 44% and 26% patients survived with Nivolumab and Ipilimumab, respectively, for a period of 5 years [78]. Based on the findings of this study, in 2018, Nivolumab/Ipilimumab was approved as a first-line therapy in advanced melanoma [78]. CheckMate 067 was further expended in triple combination (NCT02130466) for advanced melanoma [79]. In this study, Dabrafenib (BRAF inhibitor), Trametinib (MEK inhibitor), and Pembrolizumab (PD-1-blocking antibody) were compared with Dabrafenib, Trametinib, and placebo. The triple combination improved the survival

duration for 16 months, whereas, in Dabrafenib, Trametinib, and placebo-treated group, survival duration was only 10.3 months [79]. Further, it is well known that survival rate for metastatic non-small cell lung cancer (NSCLC) for 5 years is only 5%, but the outcome of KEYNOTE-001 trial (NCT02220894) using Pembrolizumab (monoclonal IgG4 antibody) has shown increased survival rate (up to 25%), and this drug was approved as a first-line therapy for metastatic NSCLC on April 11, 2019, by the US FDA [80, 81]. For the treatment of chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL), a controlled randomized trial (NCT02242942) was conducted for Venetoclax (selective BCL-2 inhibitor) and based on the outcome of progression-free survival (PFS) and response rate (85%), this drug was approved for CLL/SLL on April 11, 2016 [82]. Similarly, based on the outcome of MURANO and CLL14 trial (NCT02005471 and NCT02242942), a combination of Venetoclax, Rituximab (monoclonal antibody) and Venetoclax, Obinutuzumab (anti-CD20 monoclonal antibody) were approved on June 8, 2018, and on May 15, 2019, respectively, as a chemotherapy-free first-line treatment of CLL [83–85]. Further, 2020 has witnessed the successful outcome of PROfound trial (NCT02987543) using Olaparib (PARP inhibitor) and Enzalutamide (androgen antagonist)/abiraterone (antiandrogen) for metastatic castration-resistant prostate cancer and PRIMA trial (NCT02655016) using Niraparib for newly diagnosed advanced ovarian cancer. The outcome of these two trials has achieved a statically significant end point of PFS [86, 87]. Mutation of BRAF V600E in metastatic colorectal cancer is associated with a very poor survival rate, once initial therapy fails. Thus, the combination of Encorafenib (BRAF inhibitor), Binimetinib (MEK inhibitor), and Cetuximab (EGFR) inhibitor) was accessed in BEACON trial (NCT02928224), and the outcome showed longer duration of survival with increased response rate (26%) leading to its approval of this combination on April 8, 2020 [88, 89]. The US FDA very recently approved the combination of Ibrutinib (Bruton's tyrosine kinase inhibitor) and Rituximab (approved on April 21, 2020) as a drug for CLL and SLL. Approval was based on the successful outcome in E1912 trial (NCT02048813), where statically significant PFS was achieved after the follow-up of 33.6 months [90].

1.7 Conclusion and Future Directions in Oncotherapies

The advancements in the field of cancer therapies have transitioned their way from surgical therapies and radiotherapies to chemotherapy. Further, advancements in chemotherapy have made it possible to realize the potential of immunotherapies. These novel targeted therapies are increasingly being looked up to for cancer-specific treatment, and a number of immunotherapies have also been approved by FDA in the past few years for treatment of renal cell carcinoma and melanoma of the lung, to name a few. Also, with the ongoing research and translation into clinical trials, new oncology therapeutic drugs are being constantly envisaged to deliver best in care therapies. With the public–private partnerships, comprehensive cancer care centers are being established to extend best in care therapies and treatments to cancer

patients. These promising approaches present a way ahead in oncotherapies for treatment of carcinomas for which we still do not have a potential cure.

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Electrochemical Sensors and Biosensors for the Detection of Cancer Biomarkers and Drugs

2

Tuğba Ören Varol

Abstract

Cancer has been posing a global health concern due to an increasing number of people who have been struggling day by day. The fight against this global health threat can be accomplished with efficient early diagnosis and theranostic strategies. Cancer biomarker detection and anticancer drug monitoring utilizing the unique features of analytical techniques constitute a vital part of developing powerful cancer diagnosis and treatment methodologies. Hence, electrochemical sensors and biosensors offer practical, sensitive, selective and accurate detection of cancer biomarkers and anticancer drugs with low-cost and portable devices for on-site and in vivo analysis by holding a potential to be an alternative to conventional techniques. A general consideration about the electrochemical sensors and biosensors for the cancer diagnosis and treatment has been given in this context by presenting basic principles of electrochemical sensor and biosensor fabrication and their applications in recent years. Besides, it has been attempted to trigger readers to gain knowledge about the requirement and potency of electrochemical sensing and biosensing strategies in terms of cancer diagnosis, treatment and drug development studies by discussing pros and cons of electrochemical sensors and biosensors and predicting future perspectives.

Keywords

Electrochemical sensor · Biosensor · Cancer biomarker · Anticancer drug · Cancer diagnosis · Nanomaterial

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2.1 Introduction

Cancer has been evaluated as a global epidemic that emerges as a second leading cause of death worldwide, and it is thought that there are more than 200 types of cancer along with a high heterogeneity within a tumour tissue [1–6]. The early prognosis is a prominent factor to inhibit and treat carcinogenesis to save the life of a cancer patient by increasing the survival rate through a successful treatment [6–8]. However, uncontrollable cell proliferation engenders the invasion and metastasis of cancer cells to the more distant locations of the body. Moreover, the recognition of cancer cells by immune system is tricky due to the classification of these self-derived cells as safe by the fact that cancer cells can easily coordinate the activity of inflammation cells through the regulation of inflammatory factors, transcriptional factors and growth factors [5, 9–11]. In spite of the misdiagnosis possibility arising from the contradiction in the benign and malignant lesions differentiation and lower sensitivity, imaging technologies based on X-ray, ultrasound, magnetic resonance and cytological or histopathological techniques have been clinically used for the cancer diagnosis [6, 12, 13]. Polymerase chain reaction (PCR), DNA sequencing, southern blotting, enzyme-linked immunosorbent assay (ELISA) and flow cytometry are other widely employed sensitive and precise methods with the disadvantage of laborious, expensive and complicated analytical procedures [12, 14]. Thus, efficient methodologies for the accurate, low-cost and practical diagnosis of cancer at early stage have been strictly required.

Cancer treatment plays a crucial role as much as the early diagnosis of cancer for the survival of the patient. Nowadays, chemotherapy has been commonly applied compared to other treatment methodologies such as radiotherapy, surgery, immunotherapy and targeted therapies. In chemotherapy, a wide variety of anticancer agents can be administered as a single dose or combined doses of different agents [15, 16]. However, it is well-known that as a result of adaptation, serious side effects of anticancer agents restrict the survival rate of patients and limit the treatment efficiency, even though an initial response to chemotherapy has been observed [15, 17]. The aforementioned issue, namely drug resistance, has a particular importance in the period of chemotherapy, since it may cause dosage limitations and toxicity also paving a path for the development of new anticancer drugs [17, 18]. During the process of new drug development, as well as the monitoring in vivo and in vitro cancer cell responses, analytical characteristics of active pharmaceutical ingredients should be examined and corresponding concentrations that form the response should be quantified for the determination of metabolic fate, pharmacological activity and even the most suitable pharmaceutical formulation by keeping in mind that lower amounts of the analyte of interest are involved in the medium [19–21]. In order to meet the demand within this objective, fast, sensitive and selective analysis techniques have been gaining considerable attention over the past decades. Furthermore, a large number of studies based on electrochemical, spectroscopic, chromatographic and radiometric methods have been devoted so as to develop novel strategies in the field of pharmaceutical and biomedical analysis for the early

detection of cancer-related biomarkers and anticancer drugs in recent years [6, 12, 22–30].

Among the foregoing methods in the interest of sensitive, accurate and practical detection of cancer biomarkers and anticancer drugs, electrochemical methods have currently come into prominence. Although the majority of studies in this field covers spectroscopic and chromatographic methods owing to the advantages of higher sensitivity and simultaneous analysis of different analytes with improved resolution, longer analysis time, bulky and expensive equipment and requirement of larger amounts of biological samples and well-trained staff are challenging issues to be taken into account. Alternatively, electrochemical methods have suggested direct and practical detection of analytes without the need of sample pretreatment and derivatization procedures prior to measurement even in coloured and turbid matrices in most cases with low cost, improved sensitivity and accuracy [5, 6, 20–22, 26, 31–33]. Moreover, the fabrication of miniaturized electrochemical point-of-care (POC), lab-on-a-chip and organ-on-a-chip devices has provided on-site and real-time monitoring, offering a promising tool for the development of ameliorated strategies in terms of cancer diagnosis and treatment [14, 34–39].

2.2 A General Outlook to Electrochemical Sensors and Biosensors

As defined by the International Union of Pure and Applied Chemistry (IUPAC), chemical sensor is "a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal." In the specified definition, the chemical information implies a chemical reaction originated from the analyte or a physical property of the examined system [40–42]. Chemical sensors can be utilized in qualitative and quantitative analysis since a selective, continuous and reversible response to the amount or the activity of the interested species occurs. A chemical sensor is composed of a transduction element, called transducer, and a recognition layer. The recognition layer interacts with the analyte of concern through a physical or chemical interaction, and the chemical change generated from the physical or chemical interaction is converted into electrochemical, optical, mechanical or thermal signal via transducer in a measureable format consequently amplified by a signal processor for data management. In case of a chemical interaction proceeds via a biochemical mechanism owing to the presence of a biological component (e.g. enzyme, DNA, antibody etc.) in the recognition layer, then the analytical device is termed as biosensor [34, 40, 41, 43–46] (Fig. 2.1).

Biosensors can be categorized according to the signal transduction type and biorecognition elements. As previously mentioned that signal transduction is based on electrochemical, optical, mass detecting and enthalpic principle also valid for sensors, whereas the classification of biorecognition elements depends on the usage of biological components with catalytic feature (e.g. enzyme, cell, tissue or microorganism) and affinitive feature (e.g. antibody, nucleic acid or aptamer) [12, 47–

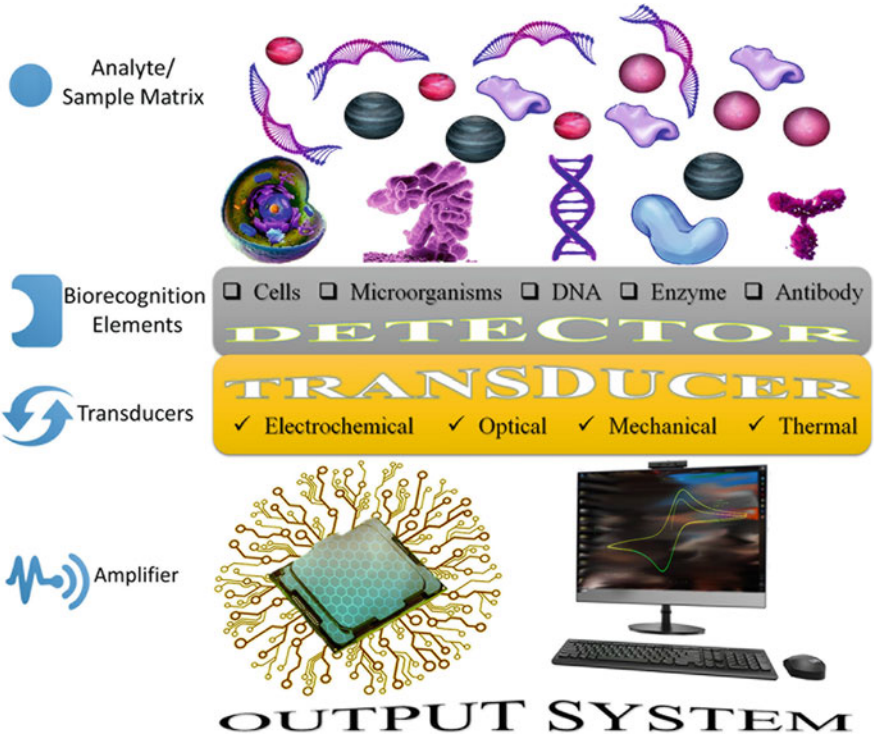

Fig. 2.1 Schematic illustration of biosensor components

49]. However, regardless of the origin of biorecognition element and signal transduction principle, analytical characteristics of higher sensitivity, selectivity or specificity, a broad range of linearity, rapid response time, improved repeatability/reproducibility and stability should be met for the fabrication of an ideal sensor or biosensor [14, 43].

In electrochemical sensors and biosensors, analytical signal is obtained through a change in potential, current, conductivity or resistance because of the chemical reaction between analyte and recognition or biorecognition elements [14, 43, 50]. Therefore, the design of recognition and biorecognition layers in the construction of electrochemical sensors and biosensors constitutes the vital stage, in which the success of sensing and biosensing strategy is closely related with the idea of providing specific interaction of analyte with (bio)recognition element by supressing other non-specific interactions at the same time [4, 12, 51]. Aforementioned sensing and biosensing strategies depend on the nature and the characteristic of monitoring species, so that further considerations will be specially discussed over recent publications in terms of cancer biomarker and anticancer drug detection in the upcoming sections.

As mentioned previously, transducer, the other remaining component of an electrochemical sensor or a biosensor, is capable of converting the energy produced

during the analyte–(bio)recognition element interaction mainly into the electrical signal. In electrochemical techniques, chemical reactions involving electron transfer take place at electrode surface or in solution interface. Therefore, during an electrochemical sensing and biosensing process, electron transfer results in electricity generation based on a chemical reaction called redox that denotes oxidation and reduction of species by applying an external voltage or via chemical energy release leading to a change in the quantity of electrical magnitudes such as current, potential and resistance [19, 46, 52–54].

General classification of the electrochemical methods is established on the differentiation between measuring of transport properties in ionic species and electrochemical equilibria, charge transfer reactions and interfaces [55]. According to measured electrical magnitude as a function of potential, impedance or time under static or dynamic conditions, these methods are subclassified as potentiometry, voltammetry, amperometry and electrochemical impedance spectroscopy (EIS), which have been conventionally employed in electrochemical sensing and biosensing applications [12, 14, 34, 46, 54, 55]. Among these electrochemical methods, voltammetry and electrochemical impedance spectroscopy have a particular importance. The application of advanced voltammetric techniques, cyclic voltammetry, pulse voltammetry (differential pulse, square wave) and stripping voltammetry, has not only contributed in electrochemical signal boosting but also enabled to explain electrochemical reaction mechanisms [12, 46]. Electrochemical sensor and biosensor fabrication procedure along with analyte–(bio)recognition element interactions can be examined stepwise by evaluating capacitance and charge transfer resistance changes by using EIS. However, since lower excitation potential has been applied in EIS, the damage of biological microenvironment due to electrode heating has been eliminated in real-time analysis, providing an efficient tool for cancer cell, protein and nucleic acid biomarker detection [6, 14, 34, 56].

In order to perform an electrochemical measurement, main requirement is an electrochemical cell, in which redox reactions take place, aside from an electrochemical analyser with suitable circuitry and software [46]. Three-electrode configuration, consisting of working, reference and counter electrodes, is extensively used in electrochemical cells for this purpose (Fig. 2.2). These electrodes are immersed into a solution at a certain concentration and ionic strength (generally buffer solution) containing the analyte. Working electrode is the core of this configuration due to the presence of recognition or biorecognition layer. Current, formed as a result of redox reaction from analyte–(bio)recognition element interaction, passes through working and counter electrodes; while the potential of working electrode is controlled in relation with reference electrode that has a constant and reproducible potential [46, 57]. As a consequent of transduction and data management process, the analytical data is shown as a voltammogram, amperogram or Nyquist plot according to electrochemical technique applied in the measurement.

Working electrode properties such as size, geometry and fabricated material profoundly affect the performance of sensor and biosensor. Redox behaviour of the analyte and background current generated by sample matrix components are prominent factors to be taken into consideration in sensor and biosensor fabrication

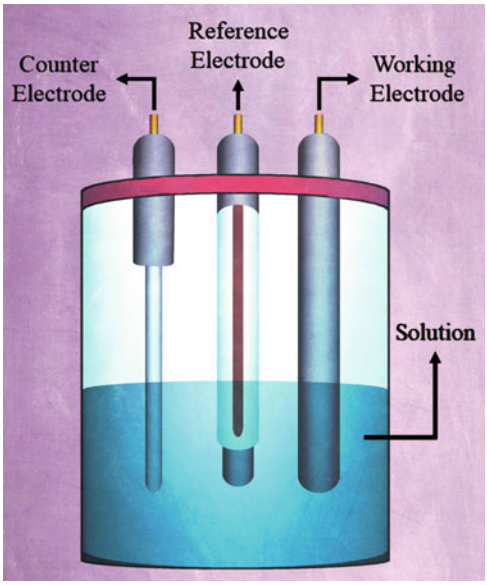

Fig. 2.2 A typical three-electrode configuration composed of working, reference and counter electrodes

as well as toxicity, mechanical strength and potential window. Carbon-based (e.g. glassy carbon, carbon paste) and metal (e.g. gold, platinum, silver) electrodes have been extensively utilized in sensor and biosensor design, and many attempts have been made to explore novel and functional materials so as to fabricate modified electrodes with enhanced properties for the detection of cancer biomarkers and anticancer drugs [34, 41, 46, 58–67]. Furthermore, the design of three-electrode configuration in a single electrochemical platform, as in commercial glucometer test strips, opened a new era offering disposable and miniaturized sensing and biosensing strategies in the field of clinical and biomedical analysis [19, 36, 50]. Outstanding examples of the fabricated electrochemical sensors and biosensors comprising the modification of working electrodes with superior featured materials in terms of cancer diagnosis and treatment will be presented in the following sections.

2.3 Electrochemical Sensing and Biosensing Strategies in Cancer Biomarker Detection

According to the definition of the National Institutes of Health Biomarkers Working Group, biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological/pathogenic process or pharmacological responses to a therapeutic intervention" [12, 68]. The term, biomarker, states a biological molecule such as a cell, enzyme, protein, hormone or DNA fragment, which exists in blood, tissues and body fluids, indicating a normal or abnormal condition or a disease [14, 69]. Owing to their diversity and great number, cancer biomarkers are classified into enzymatic tumour markers, embryonic and carbohydrate antigens,

protein, hormone and oncogene markers [12, 24, 70]. Even though cancer biomarkers can be simply determined using overexpressed proteins released in bloodstream and cancer cell surface receptor proteins, the levels of biomarkers in body are extremely low at the initial stage of cancer that early diagnosis of the disease is not feasible in most cases. Apart from the early diagnosis, sensitive and accurate detection of cancer biomarkers can be informative in terms of the determination of prognosis and examination of cancer course in a patient treated by chemotherapy, surgery or radiotherapy [5, 12, 71]. However, monitoring a single cancer biomarker is not useful at all, since most cancer types are diagnosed based on the presence of multiple biomarkers addressing the need for efficient diagnostic methodologies in the same platform with improved specificity [72–74].

Computed and positron emission tomography, magnetic resonance and ultrasound imaging, biopsy and endoscopy have been currently employed in cancer diagnosis by offering several advantages and also limitations such as overpriced instruments, limited sensitivity and physical or chemical damages. Among the genomic and proteomic techniques such as PCR, DNA quenching and fluorescence in situ hybridization, ELISA has gained popularity with the widespread use in laboratories and hospitals. However, laborious analysis procedures, complicated instrumentation and insufficient sensitivity leading to false negative results have restricted its availability as a cancer diagnostic tool [12, 25, 75]. These obstacles in cancer biomarker detection have changed the scope of the researches, so that minimally and non-invasive methods to overcome the pointed limitations have shown a rising trend, nowadays. As also recommended by World Health Organization, an ideal diagnostic test should meet the following criteria of affordability, sensitivity, specificity, being user-friendly, rapidity and robustness, being equipment-free and deliverable to end users [12, 36, 76]. When considered cumulatively, electrochemical sensors and biosensors are uniquely suited for the efficient detection of cancer biomarkers. Researchers have mainly focused on the design of bioreceptors and redox tags for the multiplexed bioassay based on the development of signal amplification techniques by using various materials emphasizing the importance of nanomaterials within this objective [14, 28, 77].

Electrochemical Immunosensors

Immunosensor is a kind of biosensor in which analyte–biorecognition element interaction is provided through the formation of an immunocomplex between a specific antigen and its corresponding antibody [46, 78, 79]. Even though the basic principle resembles with the principle of immunoassay techniques, immunocomplex formation and signal transduction occur in the same platform in electrochemical immunosensors; whereas in immunoassay techniques, biorecognition process of antigen is carried out in a different medium [80–82]. Antibodies are immunoglobulins produced by B lymphocyte cells as an immune system response to foreign species, namely antigen. In immunosensor fabrication, immunoglobulin G is the most preferred glycoprotein, which has two identical light

chains of about 25,000 Da with two heavy chains of about 50,000 Da constructing a Y-shaped molecule that is held together by non-covalent interactions and disulphide bonds. Y-shaped immunoglobulins possess physiological regions of action containing a site called "paratope" specific to a site called "epitope" on an antigen, providing a lock and key mechanism for antibody–antigen binding [80, 83, 84].

While fabricating immunosensors, monoclonal and polyclonal antibodies can be utilized; however, polyclonal antibodies can bind to antigens at different locations with variable affinity, and as the identical products of single parent cell, monoclonal antibodies are capable of forming more specific interactions with antigens. Hence, the binding site stereospecificity of an antigen is the indication of its antibody selectivity, which is characterized by larger binding constants [41, 46, 85].

Electrochemical immunosensor fabrication requires the immobilization step in which the biorecognition element, generally an antibody, is attached on the electrode surface. Additionally, there are several studies in the literature based on the immobilization of antigens on the electrode surface to detect target antibodies [80, 86–88]. Biorecognition elements can be immobilized on electrode surface by using physical adsorption, covalent binding, embedding, crosslinking, self-assembly and Langmuir–Blodgett techniques [24, 80]. Regardless of the applied immobilization technique, an efficient immobilization demands the retention of biological activity and proper distribution of biorecognition elements with well-organized orientation on the electrode surface [80, 89].

Detection strategies in electrochemical immunosensors are based on label-free and labelled approaches. In label-free approach, the analytical signal, produced by antigen–antibody interaction, is directly measured without the need of any labelling species. Despite the rapid and real-time analysis feasibility, background signals arising from the non-specific adsorption of co-existing proteins in the sample diminish the sensitivity. In order to overcome the limitation arisen from co-existing protein interferences, electrode surface can be treated by suitable agents such as bovine serum albumin, catalase or surfactants to eliminate non-specific interactions [90, 91]. In label-free immunosensors, EIS is a widely used technique by offering facile monitoring of increasing electron transfer resistance due to the antigen–antibody immunocomplex formation on the electrode surface. Pulsed voltammetric techniques and amperometry have been also utilized within this purpose [92–96]. In labelled approach, antibody or antigen is generally labelled with enzymes such as horseradish peroxidase, glucose oxidase and alkaline phosphatase. The interaction of labelling enzyme on antigen or antibody with its substrate forms an electroactive product, leading to obtain an indirect response of the immunoreaction. Apart from enzymes, nanomaterials such as noble metal nanoparticles (e.g. Au, Pt), carbon nanotubes (CNT), graphene oxide, polymer-metal nanoparticle composites, quantum dots and mediators (e.g. ferrocene, Prussian blue) have been also used in signal amplification [24, 34, 46, 73, 97, 98].

Labelled immunosensors can be operated through a competitive and a non-competitive strategy. In the competitive strategy, analyte antigen competes with the labelled antigen to bind the immobilized antibody on the electrode surface. The obtained signal for labelled antigen is inversely proportional to the analyte

antigen amount. In the non-competitive strategy, conventionally known as sandwich-type immunosensing, large antigens with an ability of binding to two antibodies can be detected. In this strategy, antigen in the sample is sandwiched between the immobilized antibody (capture antibody) and the tracer antibody (labelled antibody) after successive washing steps for each incubation stage, and electrochemical signal generated from the label is monitored to determine the antigen amount [46, 80, 99–103].

Compared to label-free immunosensors, non-specific adsorption of co-existing molecules have been restrained in labelled immunosensors; however, the binding efficiency of antigen-antibody is closely related with effective labelling of tracer antibody or antigen, which requires the proper selection of the labelling agent among a wide variety of material [89, 104]. In Table 2.1, recent studies based on labelled and label-free electrochemical immunosensors for cancer biomarker detection have been presented.

Electrochemical Nucleic Acid Biosensors

Even though the development of electrochemical immunosensors for protein biomarker detection constitutes the majority of studies in the field of electrochemistry for cancer diagnosis, electrochemical nucleic acid biosensors have received considerable attention during the past decades. In nucleic acid biosensors, single-stranded DNA (ssDNA) is generally used as a biorecognition element due to the ability of hybridization with its complementary strand generating a specific response, so that the detection of the complementary DNA or RNA has become possible owing to the probe–target pairing approach [41, 125, 126]. DNA-based biosensors, also termed as genosensors, can be utilized for the monitoring of genomic and genetic details of a patient through a facile route offering an alternative to direct sequencing methods in practical applications. Additionally, it should be also mentioned that nucleic acid sequences of several pathogens have been also detected for the diagnosis of diseases in relation with influenza, hepatitis B and human papilloma viruses by using electrochemical DNA sensors [125, 127–129].

DNA hybridization that proceeds on the electrode surface can be examined on the basis of Watson–Crick base-pair recognition phenomena. In a similar manner with electrochemical immunosensor fabrication, DNA fragment to be used as a probe is immobilized on the electrode surface. After the immobilization procedure, probe–target hybridization is generally accomplished by immersing the probe-immobilized electrode into target DNA-containing solution. Electrical signal generated as a result of hybridization process can be detected via an electroactive indicator such as enzyme and redox labels by measuring current changes, or direct monitoring of hybridization-related changes like capacitance or conductivity can be carried out [126, 130]. According to the study reported by Wang et al., a dual-probe electrochemical DNA biosensor was fabricated to detect double-stranded DNA (dsDNA) of acute promyelocytic leukaemia-related gene. In this study, genosensor was designed based on "Y" junction structure with restriction enzyme, endonuclease, assisted

Table 2.1 Recently developed electrochemical immunosensors for cancer biomarker detection

Cancer biomarker	Immunosensor	Method	Linear range	Limit of detection	Reference
Alpha-fetoprotein (AFP)	Isoorientin/anti-AFP modified GCE	DPV	0.001–10 ng/mL	0.0002 ng/mL	[105]
Alpha-fetoprotein (AFP)	Ab_2 label/AFP/BSA/Ab_1/D-Au NPs/GCE	Amperometry	20 fg/mL–100 ng/mL	6.7 fg/mL	[106]
Carbohydrate antigen 15-3 (CA15-3)	CoS_2-GR-AuNPs/Ab/SPE	DPV	0.1–150 u/mL	0.03 μ/mL	[107]
Carcinoembryonic antigen (CEA)	Anti-CEA/PEDOT/Ag@BSA/rGO/CNTs-COOH/Au	LSV	0.002–50 ng/mL	1.0×10^{-4} ng/mL	[108]
Carcinoembryonic antigen (CEA)	Anti-CEA/MWCNTs/GNPs/HNF/CPE	EIS	0.4–125 ng/mL	0.09 ng/mL	[109]
Carcinoembryonic antigen (CEA)	SPCE/GNP-MnO_2/Fe_3O_4@Au-anti-CEA	LSV, EIS	0.001–100 ng/mL	0.10 pg/mL, 0.30 pg/mL	[110]
Carcinoma antigen 125 (CA-125)	Ab/CysA-AuNPs/Ag-DPA-GQDs/GCE	DPV	0.001–400 U/mL	0.001 U/mL	[111]
CD59	Anti-CD59/GrONPs/PG	CV	1 fg/mL–10 ng/mL	1 fg/mL	[112]
Cytokeratin 19 fragment antigen 21-1 (CYFRA21-1)	BSA/Ab_1/GA/3D-G @Au/GCE	DPV	0.25–800 ng/mL	100 pg/mL	[113]
Epithelial cell adhesion molecule (EpCAM)	Anti-EpCAM/rGO@TiO_2/ITO	DPV	0.01–60 ng/mL	0.0065 ng/mL	[114]
Interleukin-1β (IL-1β)	AP-strept-biotin-dAb-IL-1β-cAb-IgG-MWCNTs/SPCE	DPV	10–200 pg/mL, 200–1200 pg/mL	5.2 pg/mL	[115]
Interleukin-6 (IL-6)	PPCE/IL-6 receptor modified ITO	EIS	0.02–16 pg/mL	0.006 pg/mL	[116]
Interleukin-8 (IL-8)	Anti-IL8/β-Ag_2MoO_4/ITO	DPV	0.001–40 $\times 10^4$ pg/mL	90 pg/mL	[117]
Lymphocyte activation gene-3 (LAG-3) protein	SiO_2-Ab_2/LAG-3/BSA/bio-Ab_1/streptavidin/rGO-SnO_2/HNMs/AuPt/GCE	Amperometry	0.01 ng/mL–1 μg/mL	1.1 pg/mL	[118]
Neuron-specific enolase (NSE)	GCE/Au@MOFs/Ab_1/BSA/NSE/MnO_2 UNs/Au@Pd^Pt NCs-Ab_2	DPV	10 fg/mL–100 ng/mL	4.7 fg/mL	[119]
Neuron-specific enolase (NSE)	PPD-GR-AuNPs/Ab/SPE	DPV	1–1000 ng/mL	0.3 ng/mL	[120]

Prostate-specific antigen (PSA)	GC/MOF-CHIT/Ab_1/PSA/Ab_2-QDs	DPV	0.001–100 ng/mL	0.45 pg/mL	[121]
Prostate-specific antigen (PSA)	Anti-PSA/GO/SPCE	DPV	0.75–100 ng/mL	0.27 ng/mL	[122]
Receptor activator of nuclear factor-κ⍰ ligand (RANKL)	HRP-DAb-AuNPs/MWCNTs/RANKL/ bCAb-Strep/p-ABA-SPCE	Amperometry	10.4–1000 pg/mL	3.1 pg/mL	[123]
Squamous cell carcinoma antigen (SCCA)	Co_3O_4@CeO_2-Au@Pt-Ab_2/SCCA /BSA/ Ab_1/D-Au NPs/GCE	Amperometry	100 fg/mL–80 ng/ mL	33 fg/mL	[124]

CV cyclic voltammetry, *DPV* differential pulse voltammetry, *LSV* linear sweep voltammetry

cyclic enzymatic amplification strategy. Signal amplification is based on the repeated cycles of hybridization, cleavage and separation steps. In the first step, hybridization occurs between the capture DNA probe and the target DNA by forming DNA duplexes with restriction sites. In the second step, DNA duplexes are cleaved by endonuclease enzyme and target DNA is released to be identified by another capture DNA probe to start a new cycle of hybridization, cleavage and separation in the final step. The requirement of special sequences of target DNA limits the applicability of the strategy. For this purpose, two separate detection probes containing a capture and an assisted probes complementary with partial sequences of two strands of the target dsDNA were used to improve the hybridization efficiency. Limit of detection (LOD) was found as 47 fM, indicating a promising tool to develop integrated devices with PCR systems and electrochemical DNA biosensors [131].

DNA methylation is evaluated as an indication of cancer in head and neck squamous cell carcinoma and has a silencing effect on tumour suppressor in cancer development [132, 133]. DNA methylation of O^6-methylguanine DNA methyltransferase (MGMT) gene in head and neck cancer cell lines was detected by using a recently developed genosensor. MGMT promoter methylation probe sequence was immobilized on gold electrode with the help of mercaptoacetic acid and 11-mercaptoundecanoic acid self-assembled monolayers, and electrochemical detection was carried out by EIS technique. The obtained results clearly showed an apparent discrimination between the methylated and non-methylated DNA with a detection limit of 0.24 pM [133].

MicroRNAs (miRNAs) are small non-coding RNA molecules consisting of 18–24 nucleotides with the ability of controlling gene expression via binding target messenger RNA in order to induce messenger RNA degradation or repression in protein translation. Due to the gene expression regulatory feature, miRNAs play a critical role in cell proliferation, cell cycle progression and apoptosis apart from functioning as tumour suppressors and oncogenes [12, 75, 134]. Circulating miRNAs existing in body fluids such as plasma, serum, saliva and urine can be evaluated as ideal non-invasive cancer biomarkers owing to their tissue-specific and dysregulated expression profiles in cancer and higher stability in body fluids [75, 135–137]. Thus, many attempts have been made to detect miRNA as a potent non-invasive cancer biomarker by academia and also industry that miRNA-based diagnostic kits are available on market. However, there are also limitations such as difficulty in miRNA amplification and isolation owing to the short length structure of miRNA. Besides, multiplexed and in vivo analysis of miRNAs with single nucleotide specificity is still demanded [138]. In order to correspond this demand, electrochemical genosensors have been fabricated based on labelled and label-free strategies by offering low-cost and portable devices for commercialization [12, 138]. Salimi et al. developed an amine-functionalized graphene-based genosensor for monitoring miRNA hybridization. miRNA-155 was selected as a model, since it is overexpressed in many types of cancer (e.g. breast, colon and cervical cancer) [139]. Genosensor was fabricated onto glassy carbon electrode by modifying amine-functionalized graphene to provide an efficient platform for miRNA-155 probe immobilization via crosslinking with glutaraldehyde and highly

conductive layer, preventing the electrode surface passivation. Target and probe miRNA-155 hybridization was detected by using DPV responses in the presence of 5 mM $Fe(CN)_6^{4-/3-}$ redox probe. The authors claimed that the proposed genosensor is capable of detecting miRNA-155 at femtomolar level [140]. There are also papers in the literature reporting the development of electrochemical biosensors for the sensitive detection of miRNA-21, miRNA-34a, miRNA-122b, miRNA-141, miRNA-197, let-7a and let-7b as cancer biomarkers [141–148]. Multiplexed detection of miRNAs has been an emerging issue in cancer diagnosis as mentioned previously. Construction of electrochemical biosensors based on ssDNA or hairpin DNA to detect multiple miRNA targets has been reported in the literature [149, 150]. The use of these one-dimensional capture probes in multiple miRNA detection is lack of sensitivity due to the decreasing accessibility of molecules to capture probes considering the high surface disturbance and uncontrolled density. Alternatively, three-dimensional nanostructured DNA capture probes suggest an improved capture efficiency with minimal non-specific adsorption [151, 152]. Hence, in another study by Xu et al., a novel DNA circle capture probe containing multiple target recognition sites was designed for the simultaneous detection of miRNA-21 and miRNA-155. For this purpose, DNA circle capture probe was attached on the top of the tetrahedron DNA nanostructure immobilized on gold nanoparticle deposited on glassy carbon electrode. The single strand chain in DNA tetrahedron nanostructure was hybridized with capture probe consisting of two recognition sites, and then the hybridization of target miRNA-21 and miRNA-155 was employed via helper strands by triggering mimetic proximity ligation assay to capture ferrocene and methylene blue labels. The proposed technique showed wider linear ranges between 0.1 fM and 10 nM with LOD values of 18.9 and 39.6 aM for miRNA-21 and miRNA-155 from cancer cell lysates, thus offering novel and efficient strategy for multiple miRNA detection [153].

Aptamers are synthetic short oligonucleotides with 30–40 nucleobases of RNA or ssDNA, which have been widely used in the fabrication of electrochemical biosensors termed as aptasensors. Aptamers enable specific binding of target and oligonucleotide analyte in a similar manner with conventional nucleic acid biosensors. However, aptamers are capable of binding various types of target analytes including proteins, biologically important small molecules and even organisms by folding into a three-dimensional structure to interact through their complementary shapes rather than their sequences. The advantages of thermal stability, facile modification with demanded functional groups and in vitro synthesis have made aptamers favourable biological elements to design novel biosensors for cancer biomarker detection. Nevertheless, complex ingredients of sample matrixes due to the presence of macromolecules and ions may cause non-specific interactions, leading to a significant limitation for the utilization of aptasensors in cancer diagnosis as commercialized devices [12, 14, 41, 125]. In spite of the limitations to be overcome, electrochemical aptasensors still offer an efficient strategy for the detection of a wide variety of cancer biomarkers. Current studies have demonstrated the efficacy of electrochemical aptasensors in singular and multiplexed detection of

cancer biomarkers as well as cancer cell and cancer cell-released exosome quantification [154–166].

In addition to electrochemical immunosensors and nucleic acid-based biosensors, electrochemical cytosensing strategies deserve a special mention owing to the capability of detecting circulating tumour cells released from primary and metastatic tumours inducing metastasis and even death of the patient. On basis of the fact that cancer cells overexpress a significant amount of proteins, enzymes or receptors on the cell surface or within the cell, novel sensing and biosensing strategies have been developed so as to fabricate efficient platforms for cancer cell detection as a precise diagnostic tool to determine the appropriate treatment method [5, 167]. In this point of view, the interaction of cell surface biomarkers, in other words overexpressed proteins, enzymes or receptors by cancer cell, with the recognition layer of the sensing/biosensing platform establishes the principle of the biosensor called cytosensor. Since each cells of different types of cancer have significant and unique surface characteristics, selective detection of the interested cancer cell by discriminating normal cells and other cancerous cells is possible by designing cytosensor biorecognition layer with specific and affinitive agents [35, 167, 168]. In electrochemical cytosensing strategies, aptamer-based direct and sandwich assays utilizing enzyme and nanomaterial signal probes and displacing DNA/nanostructured probes as well as advanced functional material-modified electrochemical platforms have become a popular area of research in the past decade [167, 169–181]. Furthermore, electrochemical cytosensing approaches devoted to cell type identification and cell counting could be considered as alternative and complementary techniques to flow cytometry [167, 182].

2.4 Electrochemical Sensing and Biosensing Strategies in Anticancer Drug Detection

In recent years, advances in drug discovery and development studies based on in silico, in vitro and in vivo methodologies have made a considerable impact on diagnosis, treatment and prevention of the diseases. There is no doubt that pharmaceutical analysis has a vital role in this progress by providing an analytical knowledge to researchers from drug formulation to marketing stages [21, 183, 184]. Owing to the fact that cancer imposes a global health concern based on the estimation of more than 25 million new cases by 2050, the development of efficient treatment and theranostic strategies are urgently demanded [15]. For the time being, chemotherapy is the most commonly applied strategy in the cancer treatment, which requires anticancer drugs with higher specificity of action and undesired side effects, thereby also demanding accurate and precise analytical techniques to obtain useful information about the quantity, purity, stability, toxicity and therapeutic index of anticancer drugs in pharmacokinetic and pharmacodynamic trials [15, 17, 184–186]. Therefore, sensitive and selective detection of anticancer drugs in biological fluids and pharmaceutical formulations could give a consideration about the efficiency of anticancer drugs to be utilized in cancer treatment.

Electrochemical techniques offer sensitive, selective and practical detection of anticancer drugs in pharmaceutical formulations and biological materials with low-cost and potential miniaturized devices as mentioned in previous sections. Furthermore, electrochemical sensing and biosensing strategies access in vivo pharmacological activity prediction of a drug through the investigation of its electrochemical redox characteristics. The examination of the related redox reactions gives insights into understanding the interaction mechanism of anticancer drug with living cells, and also its bioavailability and metabolic fate by gaining analytical knowledge about the electrochemical behaviour as well as possible interaction of anticancer drug with sample matrix components [21, 33, 183].

Electrochemical anticancer drug monitoring strategies are based on the fabrication of electrochemical sensors and biosensors with superior materials to amplify the signal bearing on the specific interaction of anticancer agents with the recognition elements in a similar way with cancer biomarker detection. Apart from the biological materials to construct biosensors for this purpose, literature survey reveals the utilization of nanomaterials as signal boosting materials in majority due to their electrocatalytic activity, larger surface area-to-volume ratio, improved conductivity and biocompatible nature [187–195]. Thus, the rising trend in the fabrication of new state-of-the-art electrochemical sensors and biosensors for anticancer drug detection is based on the synthesis of hybrid and composite nanomaterials with synergetic effect [61, 192, 196–201]. In addition, it should be mentioned that metal-organic frameworks and molecularly imprinted polymers have recently comprised a hotspot in this field [63, 202–206].

Since most of the pharmaceutically active compounds tend to be easily oxidized or reduced compared to remaining excipients in the pharmaceutical formulations, direct detection of anticancer drugs is available by measuring their oxidation and reduction signals, leading to fabrication of a wide variety of electrochemical sensors [33, 183]. In Table 2.2, successful examples of electrochemical sensors fabricated in the past five years for the sensitive detection of anticancer drugs are presented.

It is likely that most of the electrochemical anticancer drug detection studies are based on the development of electrochemical sensors. Besides, there are also many attempts to fabricate electrochemical biosensors, in which aptamers, peptides, DNA and cancer cells have been used as the biorecognition element, to quantify the anticancer drug amount and examine drug–cancer cell or drug–DNA interactions. Hence, electrochemical biosensors assure highly efficient tools for monitoring cell viability and drug resistance as well as in situ pharmacokinetic assays as also demonstrated in the reported studies [220–226].

2.5 Conclusions and Future Perspectives

Electrochemical sensing and biosensing strategies have been receiving tremendous attention in order to fabricate efficient platforms for non-invasive cancer biomarker detection, therapeutic drug monitoring and investigation of drug–target interactions over the last decade. As mentioned in sections earlier, electrochemical sensors and

Table 2.2 Electrochemical sensors fabricated in the past 5 years for the sensitive detection of anticancer drugs

Anticancer drug	Sensor	Method	Linear range	Limit of detection	Reference
Capacitabine	AuNPs/SGNF/GCE	DPV	0.05–80 μM	0.017 μM	[207]
Cisplatin	GQDs-thio/npGCE	DPASV	0.2–110 μM	90 nM	[61]
Dacarbazine	MIP-NSPs@PGE	DPASV	0.09–50.88 ng/mL	0.02 ng/mL	[208]
Docetaxel	Au-MWCNTs/GCE	DPASV	0.3–3.3 μM	90 nM	[199]
Doxorubicin and Methotrexate	CuNPs-CB-Nafion/GCE	SWV	0.45–5.1 μM 2.2–25 μM	0.024 μM 0.090 μM	[209]
Epirubicin	Fe_3O_4-SWCNTs/MOCTICl/CPE	SWV	0.02–700 μM	7 nM	[189]
Epirubicin and Methotrexate	Ce-ZnO/GCE	DPV	0.01–600 μM 0.01–500 μM	2.3 nM 6.3 nM	[210]
Epirubicin and Topotecan	CPE/1-BPr/CuO-NPs	SWV	0.03–800 μM 0.7–800 μM	0.008 μM 0.3 μM	[211]
Etoposide	GNWs/silicon wafer	CV	0.05–1 μM	4.36 nM	[212]
Flutamide	HF/HBP-GO/PGE	SWV	0.1–110 μM	0.029 μM	[213]
Flutamide	FC/MWCNTs/CPE	SWV	0.1–110 μM	0.001 μM	[190]
Imatinib	HF-PGE	DPV	0.01–200 μM	7.39 nM	[214]
Imiquimod	MIP/Au/GO/GCE	SWV	0.02–20 μM	0.006 μM	[202]
Methotrexate	ITO/PVS/BM	SWV	1–62.5 μM	0.595 μM	[215]
Methotrexate	g-C_3N_4@V_2O_5/SPCE	DPV	0.025–273.15 μM	13.26 nM	[216]
Methotrexate	*f*-CNTPE	SWV	0.01–1.5 μM	2.9 nM	[217]
Nilutamide	CeV/CNF/GCE	DPV	0.01–540 μM	2 nM	[217]
Nilutamide	β-CD-AuNP/GO/SPCE	DPV	0.01–193 μM	0.4 nM	[218]
Nilutamide	*f*-MWCNT/GCE	DPV	0.01–21 μM 28–535 μM	0.2 nM	[188]
Paclitaxel	GCE-graphene-EAu	DPV	0.01–2 mM	0.005 mM	[196]

Pemetrexed	CPE/Pd/CNF/$[M3OA]^{+}[NTF2]^{-}$/ Nafion	SWV	1–35 nM	0.33 nM	[197]
Raloxifene	Nd_2O_5 NPs@GO/GCE	Amperometry	0.03–472.5 μM	18.43 nM	[198]
6-thioguanine and 6-mercaptopurine and Azathioprine	ERGO-IL-Chit/CSE	Amperometry	0.2–10, 10–250 μM 0.4–10, 10–400 μM 0.3–10, 10–400 μM	0.05 μM 0.11 μM 0.09 μM	[219]

DPASV differential pulse anodic stripping voltammetry, *SWV* square wave voltammetry

biosensors have a great impact on sensitive, selective, practical and low-cost detection of cancer biomarkers and anticancer drugs in biological samples and pharmaceutical dosage forms, owing to well-engineered design of electrode surfaces with appropriate biological elements and materials with superior properties. It should be also emphasized that electrocatalytic activity, biocompatibility, larger surface area and enhanced electrical conductivity make nanomaterials (e.g. CNT, graphene, metal nanoparticles, quantum dots, hybrid and composite nanostructures) indispensable components of electrochemical sensors and biosensors, and multifunctional nanomaterials are being fabricated to enable more sensitive, selective, precise and accurate analysis. Besides, the development of point-of-care, lab-on-a-chip and organ-on-a-chip electrochemical devices has paved the way for their widespread utilization in laboratories and hospitals. Even though many successful attempts have been made as a proof of concept, there are several challenges to be overcome for commercialization and replacing the current technology in this field. Multidisciplinary approaches including materials science, engineering, medicine, biology, chemistry and bioinformatics will enable to overcome the limitations from sensor or biosensor design to commercialized device production.

Multiple cancer biomarker detection still requires much more attention since electrochemical techniques suffer from complexity of sample matrixes containing various interfering species, which may be eliminated by designing multi-array platforms. However, long-term stability and reusability should also be taken into account in electrochemical sensor and biosensor design for cancer biomarker and anticancer drug detection. Long-term stability and reusability are also related with the retention of immobilized biomaterial activity, especially for enzymes, that biomimetic materials could be an alternating choice in electrochemical sensor and biosensor fabrication within this scope. Although electrochemical sensor and biosensor fabrication face with limitations of signal amplification, storage and device integration, they are still promising candidates to be potentially used as novel and complementary technologies in cancer biomarker and anticancer drug detection in future.

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Cell Cycle Arrest: An Impending Therapeutic Strategy to Curb Cancer

3

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Abstract

Eukaryotic cell division is divided into several phases and each of these phases has their own control mechanisms. Failure of any of these control mechanisms may lead to development of errors which may be propagated to up-coming generations leading to development of carcinogenic phenotype. Therefore, cell cycle has become an attractive target in anticancer research which is mainly focused on dealing with the regulators and checkpoints involved in the progression of cell cycle. The major components involved in controlling the cell cycle are cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs). Apart from these, an efficient DNA repair system and the proper assembly of spindle fibers also contribute to smooth progression of cell cycle. Therefore, in addition to the great dependency of anticancer research on cyclins, CDKs, and CDKIs, DNA repair system and assembly of spindle fiber

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also contribute to the foundation of anticancer research. In this chapter, we describe cell cycle and its importance in anticancer research, the clinical studies based on cell cycle to curb neoplastic development, and approaches used in anti-tumor research to counter cancer progression.

Keywords

Cell cycle · Cyclins · Checkpoints · Cancer · Anticancer therapy

3.1 Introduction

The cell cycle is a coordinated sequence of events that deals with duplication of genomic material and subsequent distribution of duplicated genetic material leading to the division of cells [1]. In the case of eukaryotes, the cell cycle has been categorized into several phases including Gap 1 (G1) phase, DNA synthesis (S) phase, Gap 2 (G2) phase, and Mitosis (M) phase. In first three phases, a cell prepares itself for division, and in M phase, segregation of chromosomes occurs followed by division of cells [2]. The M phase is progressed by initiation of prophase where nuclear envelop is disappeared and chromosomes become visible as chromatids. Prophase is followed by the alignment of chromosomes in metaphase, segregation of sister chromatids in anaphase, and subsequent movement of chromosomes at opposite poles in telophase followed by the division of genetic material leading to next interphase which is characterized by G1, S, and G2 phases as shown in Fig. 3.1 [3, 4]. The interphase is although a resting phase, but prepares a cell for the actual M phase, since a cell performs a normal metabolic role in interphase to duplicate its genetic material in S phase followed by DNA proof-reading, and preparation of M phase by the end of G2 phase. Additionally, G0 phase is a part of cell cycle in which cells are quiescent but have the potential of division under proper stimulus. Strict regulation of all the events in cell cycle is important for duplication of genetic material with high fidelity and its transfer in next generation with great accuracy since, even subtle errors in the cell cycle may lead to the fatal outcomes that may manifest in the development of complex diseases such as cancer. This chapter aims to provide a glimpse of the cell cycle and its crucial component with emphasis on the regulation of cell cycle in development as well as prevention of cancer.

3.2 Regulation of Cell Cycle by Interacting Partners

Several regulatory components are involved in the hassle-free progression of the cell cycle. These components work in a fashionable manner. Cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs) are the key components involved in

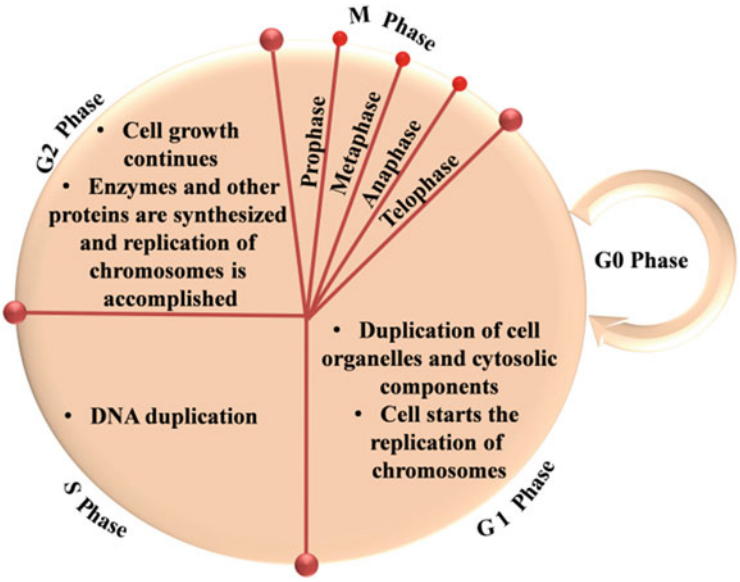

Fig. 3.1 Different phases of the cell cycle. Cell cycle comprises G1 phase, S phase, G2 phase, and M phase. Duplication of genetic material and cell organelles to assist in remaining cell cycle phases starts in G1 phase. S phase is represented by actual duplication of genetic material while in G2 phase, a cell continues to grow by completing its genetic content. M phase is demonstrated by actual segregation of chromosomes followed by division of cells. In G0 phase, cells undergo quiescence and may participate in division under the effect of proper signal

Table 3.1 The functional role of CDKs and cyclins in different phases of cell cycle (adapted and modified from Bai et al. [5])

CDKs	Cyclins	Cell cycle phase
CDK1	Cyclin A	G2/M transition
CDK1	Cyclin B	M
CDK2	Cyclin A	S
CDK2	Cyclin E	G1/S transition
CDK4	Cyclin D1, D2, and D3	G1
CDK6	Cyclin D1, D2, and D3	G1

regulating the cell cycle which perform in a coordinated manner to ensure proper progression of the cell cycle. The following few sections are briefly focused on the description of each of these regulatory components. Additionally, different interacting partners involved in progression of cell cycle are given in Table 3.1 below.

Cyclins

Cyclins are proteins known to regulate the progression of the cell cycle by their ability to complex with appropriate CDK partners. The expression of a particular

cyclin occurs in a particular phase of cell cycle, therefore, there is a sequential change in the expression pattern of cyclins which is dependent on specific cell progression phase.

Of the two types of cyclins, including cell-cycle related cyclins, viz. Cyclin A, B, D, and E, and non-cell cycle-related cyclins, viz. Cyclin C and H, cell-cycle related cyclins such as cyclin D and E play a pivotal role in G1 to S phase transition of the cell cycle [6]. Similarly, cyclin A forms the complex with CDK1 and CDK2 and plays a key role in S and M phase transition. The accumulation of cyclin A starts during the S phase and is down-regulated before commencement of M phase [7]. Similarly, cyclin B regulates the M phase and is required for a cell to enter and proceed through M phase. Therefore, cyclic change in the levels of cyclins is necessary in cell cycle progression.

Cyclin-Dependent Kinases (CDKs)

CDKs are about 300 amino acid proteins that contain binding motifs favoring the binding of appropriate cyclins. On binding to cyclins as their preferred binding partners, CDKs become catalytically active [8, 9]. Unlike cyclins, the expression of CDKs remains constant throughout the cell cycle, and several members of CDK family switch their association with cyclins, and their functional activities vary in accordance with a particular cell cycle phase. Notably, four different CDKs, namely, CDK 1, 2, 3, and 4 are responsible for governing the progression of the cell cycle [10]. In this way, at the G1/S phase transition, CDK4/6 and CDK 2 are required to make the cells to enter in S phase. CDK2 remains active throughout the S phase, and its activity declines after the cell exits S phase [9]. Similarly, CDK 1 is active during the G2 phase with persistent activity during mitosis [6]. CDK 1 associates with cyclin A and B, and acts on the interface of the G2/M phase. The accumulation of cyclin A and B and their degradation at the initiation of anaphase leads the cells to enter and exit mitosis, respectively. Therefore, periodic changes in the activities of CDKs are required for transition in phases of the cell cycle.

CDK Inhibitors (CDKIs)

CDKIs are up-regulated in response to a variety of anti-proliferative signals. CDKIs are known to regulate the activity and functions of CDK family members [11]. CDKIs are majorly categorized in two families, namely, CIP/KIP family of universal cyclin/CDK inhibitors, and INK4 family. The members of CIP/KIP family include p21 $^{Waf1/Cip1}$, p27 ^{Kip21}, and p57 ^{Kip2} proteins, and are known to bind and inhibit both cyclins, through their conserved LFG residues present in their cyclin box motif, and CDKs concurrently [12]. On the other hand, the members of INK4 family including p16 ^{INK4a}, p15 ^{INK4b}, p18 ^{INK4c}, and p19 ^{INK4d}, specifically bind and inhibit cyclin D, CDK4, and CDK6 (Fig. 3.2) [13].

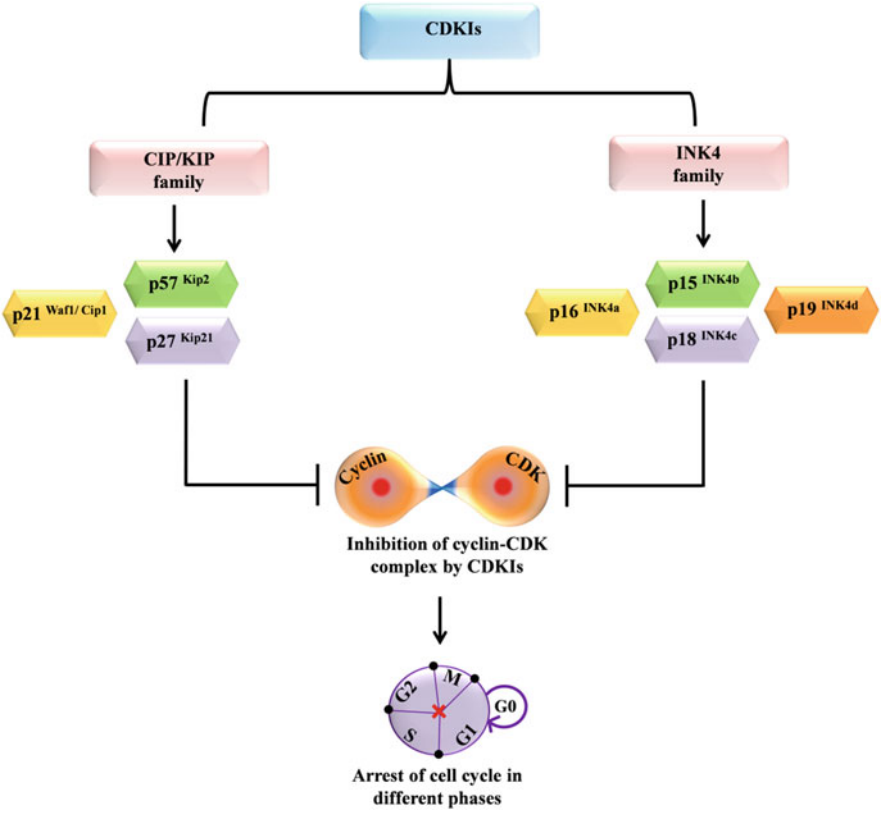

Fig. 3.2 Different families of CDKIs controlling the cell cycle. CDKIs of CIP/KIP family include p21 $^{Waf1/Cip1}$, p27 ^{Kip21}, and p57 ^{Kip2}, while CDKIs of INK4 family include p16 ^{INK4a}, p15 ^{INK4b}, p18 ^{INK4c}, and p19 ^{INK4d}. The members of both CDKI families work in a coordinated manner so as to inhibit the progression of cell cycle under certain circumstances

It is noteworthy that the relative concentration and distribution of the members of these two families determine the progression of the cell cycle. For instance, p21 plays a significant role in the inhibition of CDK kinase activity and inhibits the replication of DNA. Additionally, it is also known to arrest the cell cycle in G 1 phase so as to allow a cell to repair its DNA damage; which is seen when p53 is up-regulated (Fig. 3.3) [14]. Therefore, CDKIs act as a surveillance system to regulate the faithful progression of the cell cycle.

3.3 Cell Cycle Checkpoints

The status of the cell cycle progression from one phase to next is ensured by chronological activation as well as inactivation of a plethora of *regulatory gates* which are known as cell cycle checkpoints. These checkpoints monitor the status of

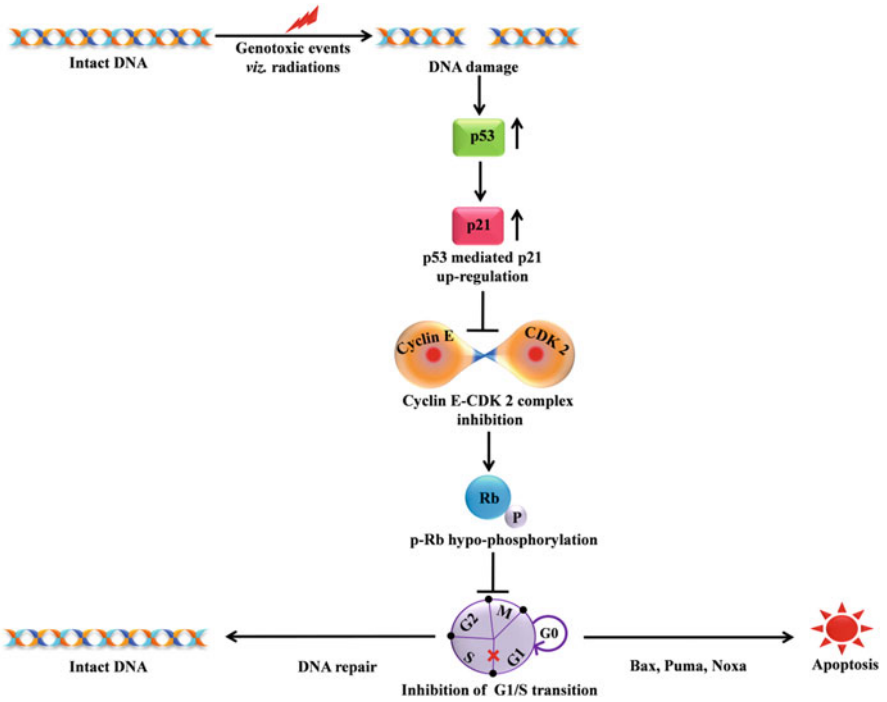

Fig. 3.3 Regulation of cell cycle under genotoxic stress. When DNA is damaged, p53 dependent up-regulation of p21 leads to inhibition of cyclin E-CDK2 complex resulting in hypophosphorylation of Rb protein which is accomplished by inhibition of cell cycle, DNA repair, and apoptosis

dividing and non-dividing cells [15]. Functionally, checkpoints are subsets of gene products that function in a sequential and controlled manner to ensure the fidelity in the cell cycle progression. If any of these checkpoints are mutated or altered, they confer independence in the cell cycle progression; which was otherwise dependent on successful completion of on-going cellular progression. Cells can arrest the progression of the cell cycle transiently so as to overcome the stress, viz. DNA damage. Otherwise, if the stress is irreversible, then checkpoints can direct a cell to programmed cell death. Alteration in the reliability of checkpoints can manifest with an expansion of DNA damage and permanent genetic lesions over several generations. It is noteworthy that cell cycle checkpoints are often hampered in cancerous cells resulting in the propagation of tumorigenic growth [16]. Hence, a cell has to pass through a huge number of internal checkpoints to ensure proper forwarding of genetic information to daughter generation [3, 17, 18]. The following few sections are focused on the type of cell cycle checkpoints and their importance in cancer.

G1/S Checkpoint

The inhibition of G1 phase cyclin and CDK complexes plays a significant role in maintaining the G1/S checkpoint [19]. As discussed earlier, CDKs can be negatively regulated by CDKIs. Among CDKIs, the members of the INK4 family are known to inhibit CDK4 and CDK6 during the G1 phase, while the members of CIP/KIP family can inhibit the activity of CDKs in all phases of the cell cycle (Fig. 3.2), thereby firmly maintaining the G1/S checkpoint. Furthermore, when a normal cell faces the genotoxic insult, transcription of p21, an important member of the CDKI family is up-regulated by p53 protein. Subsequently, p21 binds and inactivates cyclin E-CDK2 complex leading to hypophosphorylation of pRB followed by arresting the cell cycle from G1/S transition, allowing a cell to repair DNA damage, accumulate apoptotic factors such as Puma, Bax, Noxa, and up-regulate oxidative stress response as shown in Fig. 3.3. Additionally, p16 arrests the cell cycle in the G1 phase in p53 independent manner in response to DNA damage by abrogating cyclin D/CDK4 and cyclin D/CDK6 dependent pRB phosphorylation [20, 21]. Therefore, G1/S checkpoint acts by targeting two important tumor suppressor pathways which are often deregulated in a variety of human cancers.

S Phase Checkpoint

The S phase checkpoint, also known as intra-S phase checkpoint, operates to avoid the duplication of damaged DNA to transfer in mitosis further. This checkpoint is regulated by two different signaling pathways which include ATM/ATR-Chk1-Cdc25A and ATM-Nbs1-SMC1 [22]. DNA damage induced by ionizing radiations of UV radiations may provoke either of these pathways to arrest the cell cycle in the S phase. ATM or ATR results in phosphorylation of Chk1 that in turn phosphorylates Cdc25 A on serine residues maintaining the required concentration of Cdc25 A. The augmented functional activity of Chk1 and Chk2 leads to Cdc25 A down-regulation resulting in subsequent inhibition and inactivation of Cdk2-cyclin E complex in response to genotoxic insult [23]. ATM-mediated phosphorylation of Nbs1 on Ser 343 residue and some other residues results in activation of Nbs1-Mre11-Rad50 complex which is involved in S phase arrest [24, 25]. Similarly, cohesin protein SMC1 is also phosphorylated by ATM on Ser 957 and Ser 966 depending on the phosphorylation status of Nbs1, which is essential in S phase arrest of the cell cycle. Several other components including BRCA1, FANCD2, MDC1, and p53 BP1 are also involved in intra-S checkpoint [22, 26].

G2 Phase Checkpoint

If a cell feels genotoxic stress, then the cell can trigger a checkpoint mechanism arresting the cell cycle in G2 phase. For instance, ATM (ataxia-telangiectasia mutated)- and ATR (ATM and Rad3-related)-dependent signaling can arrest the

cell cycle in G2 phase by inhibiting CDK1 as a consequence of DNA damage. If a cell is exposed to ionizing radiations, ATM-dependent checkpoint kinase 2 (Chk2) activation can be seen. Whereas if a cell is exposed to ultraviolet radiation insult, ATR dependent Chk1 activation is prevalent [27]. Chk1 and Chk2 are known to phosphorylate Cdc25 C, thus generate a docking site for 14-3-3 proteins which leads to nuclear export and cytoplasmic sequestration of phosphatases followed by inhibition of CDK1 resulting in G2 phase arrest of the cell cycle [27].

Previously, studies have revealed that sustained G2 arrest can be mediated by p53 as a consequence of DNA damage in cancerous cells [28, 29]. p53 leads to transcriptional up-regulation of 14-3-3σ and p21 thereby inhibits G2 progression as a consequence of cytoplasmic sequestration and thus inactivating CDK1-cyclin B complex, respectively [29–32]. Additionally, once accumulated, p21 may cause the arrest of the cell cycle in G2 phase (Fig. 3.3) by disturbing the interaction of proliferating cell nuclear antigen and Cdc25 C [33].

Mitotic Spindle Checkpoint

The attachment of microtubules and chromosomes is under the strict control of mitotic spindle fiber checkpoint. This checkpoint monitors the accurate segregation of chromosomes during anaphase. Kinetochore associated proteins including MAD2, BUBR1, BUB1, BUB3 proteins are key components of mitotic spindle checkpoints [34]. Out of these, MAD2 and BUB are known to directly interact and inhibit APC machinery preventing the entry of cells in anaphase in case of mitotic spindle fiber dysfunction. Similarly, BUB1 and BUB3 also contribute to mitotic arrest in case of spindle dysfunction [34].

3.4 Dysregulation in Checkpoint Leading to Cancer

Cancer is the second leading cause of death in developed countries including United States [35, 36]. Abnormal cell proliferation due to the loss of cell cycle checkpoints is a key hallmark of cancer and also crucial for cancer progression [37–39]. Indeed, modulation in the machinery of cell cycle progression occurs in a variety of cancers. A healthy cell considers such modulations as a genetic insult which results in dysregulation of tumor suppressor genes which are considered as a suitable target for the implication of anticancer regimens [40]. For instance, regulation of cell cycle progression by tumor suppressor Rb protein plays a central role in curbing tumor development since oncogenic modulation in cyclins, CDKs, and other regulators of pRB is prevalent in a plethora of human cancers, viz. retinoblastoma, osteosarcoma, and many other cancers [41]. In cancers where pRB protein encoding is normal, even a subtle alteration in the alteration in signaling pathways regulating pRb can be frequently observed with augmented levels of cyclin D and cyclin E, deletion of p 16, and enhanced amplification of genes encoding CDK4 and CDK6 [41]. It is noteworthy that nearly half of the metastatic breast cancers are manifested with

increased expression of cyclin D as compared to normal breast epithelium in the vicinity [42]. In support of this, previously it has been speculated that transgenic mice overexpressing either human cyclin D1 or cyclin E in breast cells are more prone to develop breast adenocarcinomas [43, 44]. Likewise, sarcomas, melanomas, gliomas, and breast cancer have also shown amplification in CDK4/6 encoding genes [45]. Therefore, cell cycle dysregulation as a consequence of an alteration in cell cycle machinery is a major phenomenon detected in various cancer types.

Alteration In Cellular Checkpoint Proteins

The molecular events of checkpoint proteins play a crucial role in cell cycle regulation and these checkpoints altered during cancer progression [46]. Gene encoding cell cycle checkpoint proteins may undergo several genetic alterations leading to the development of cancer. For instance, mutations in p53 are one of the most often reported genetic alterations in human cancers [21]. Germline mutations in p53 are responsible for Li–Fraumeni syndrome which is manifested with provoked incidences for the development of breast cancer, brain tumors, and sarcomas [47]. The normal function of p53 may be altered by several cellular proteins such as Mdm2. This protein binds with p53 and leads to ubiquitin-mediated proteasomal degradation. Additionally, overexpression of Mdm2 may result in subsequent inactivation of p53 [48, 49]. Similarly, CDK1 modifications are also very often in human tumors. Apart from this, lower expression levels of p27 are found in aggressive breast cancers [50, 51], which may be more susceptible to oncogene-dependent transformation [52]. Similarly, lower expression levels of p27 are found in human bladder cancer [53]. Furthermore, either deletion or epigenetic modification, viz. methylation of p15 and p16 is related to human melanomas, lymphomas, and many other cancers [45]. Similarly, lower expression levels of p57 are associated with human bladder cancers [53] and epigenetic modification, viz. methylation of p15 and p16 or their deletion is linked with human mesotheliomas, melanomas, lymphomas, and pancreatic cancers [45].

Alteration in Spindle Fiber Checkpoint

The development of a plethora of human cancers is also linked to modulation in spindle checkpoints. For example, mutations in BUB1 have been identified and linked with the development of human colon cancer [54] which promotes the tumorigenic transformation of cells lacking BRCA2 breast cancer susceptibility gene [55]. Previously it has also been reported that MAD2 haploinsufficiency results in premature anaphase and chromosome instability in mammalian cells, resulting in increased incidences of lung cancer development [56]. Hence, alteration in either of the spindle fiber checkpoint components may manifest in the development of cancerous growth.

Alteration in DNA Repair System

Mutations in the components of the DNA repair pathway may also lead to the development of tumors due to sustained DNA damage. For instance, in ataxia-telangiectasia, a familial disease, ATM mutations are manifested with increased chances of lymphomas, breast cancers, and leukemias [57].

3.5 Therapeutic Approaches to Curb Cell Cycle in Cancer

It is clear that even subtle alterations in the cell cycle result in the development of a plethora of human cancers. Moreover, pieces of evidence have also supported the fact that cells with defective checkpoint functions are more prone to develop cancer. Fortunately, it also provides the opportunity to the scientific community to develop effective therapeutic regimens against carcinogenesis. Hence, the research is always focused on the development of alternative approaches to deal with cancer. The efforts against cancer are focused on the identification of novel, efficient, and potent drug molecules which have potential to target cell cycle checkpoints by considering (1) the use of high-throughput screening of anticancer lead molecules (2) the use of structure-based rational drug designing strategies for the development of small molecules against cancer, and (3) the use of genetics, proteomics, and metabolomics to identify potent anticancer therapeutics. The following few sections are focused on such approaches in a battle against cancer.

Screening of Novel Anticancer Molecules

Strategies involving the search for novel molecules have been employed to identify anticancer compounds against cancer. Previously, the National Cancer Institute (NCI) examined the inhibitory activity of about 70,000 small molecules against 60 different cells of human cancer origin [58]. Similarly, a group of authors also used NCI cell lines to examine the transcriptional levels of genes involved in cell cycle arrest and correlated the outcomes with standard anticancer chemotherapeutics [59]. Previously, it has been seen that the p53 status of cells is a crucial determinant of chemosensitivity since cells with mutant p53 are less responsive towards chemotherapeutic agents as compared to wild type cells [60]. Similarly, cDNA microarray studies have also been used earlier to examine the gene expression status of cell lines responding to the treatment with chemotherapeutic agents. Such evidence provide a valuable and definitive link between chemosensitivity and gene expression [61].

Apart from this, high-throughput screening has also been implemented in order to identify potent small molecules against cell cycle checkpoint components. For instance, breast cancer cells expressing mutant p53 were used in one of such studies where the G2 phase arrest of the cell cycle was induced by radiations. The cells were then co-treated with nocodazole, a microtubule inhibitor, and extracts from marine invertebrates. Consequently, isogranulatimide was identified as a novel inhibitor of

the G2 phase working in synergism with ionizing radiations [62]. Similarly, eight novel molecules with potent anti-mitotic efficacy were identified from 24,000 extracts from marine invertebrates and plants [63].

Genomic Approaches

Genetic approaches to counter cancer primarily depend on (1) conservation of cellular checkpoint pathways and (2) ease of manipulation in the genome of the organism under investigation. Therefore, *Saccharomyces cerevisiae* provides an excellent choice to be considered as a system to encounter against cancer [64]. Previously, anticancer drugs were screened on several strains of *S. cerevisiae* containing known mutations in cellular checkpoint pathways Notably, the toxicity profiles of ionizing radiations and chemopreventive therapeutic regimens were different from one another in several strains with defined mutations indicating the importance of particular mutation in cell cycle checkpoint and DNA repair pathways and thus giving a clue for deciding the therapeutic regimen [65]. Similarly, to identify selective peptide inhibitors and to identify novel cellular therapeutic candidates for anticancer drugs, *Schizosaccharomyces pombe* has also been used [66]. Additionally, the benefits can be taken from yeast genome which can be combined with cDNA microarrays to examine the changes in expression patterns of genes involved in cell cycle checkpoints after treatment with anticancer therapeutics [67]. Indeed, this approach has been used to generate a database of several cell cycle mutants of *S. cerevisiae* to screen novel anticancer molecules and ionizing radiations [68] and fortunately, the analysis of their profiles has demonstrated novel candidates in cell cycle regulatory pathways.

Chemical Approaches

Since the activity of cell cycle components such as CDKs is often deregulated in cancer, inhibitors of CDKs may be effective anticancer agents. For instance, Flavopiridol arrests the cell cycle in G1/S and G2/M phases by acting as CDKI and inhibiting CDK1, 2, and 4. Flavopiridol also acts synergistically with other anticancer drugs and has potent anticancer efficacy in human cancer cells and several in vivo xenograft tumor studies with mice [69]. Additionally, a number of phase 1 studies and phase 2 studies conducted on subjects with lung, renal, colorectal, and esophageal cancers have demonstrated the anticancer potential of Flavopiridol. Furthermore, several anticancer studies with breast and prostate cancer and non-Hodgkin's lymphoma are in process with Flavopiridol [45]. Furthermore, chemopreventive potency of several agents such as ionizing radiations can be enhanced by therapeutic agents such as caffeine or pentoxifylline which disturb G2 checkpoints [70, 71]. Similarly, UCN01 has also demonstrated anticancer activities against a variety of in vitro and in vivo cancer models by acting as a potent inhibitor of several kinases including Akt, protein kinase C, CDKs, and PDK 1. The

anticancer properties of UCN01 involve a variety of cellular pathways including prevention of nucleotide excision DNA repair, inhibition of G2 checkpoint kinase Chk1 thereby arresting the cells in G1/S phase followed by apoptosis [72–77]. Similarly, histone deacetylase inhibitors including FR901228 and MS27275 have shown promising anticancer activity in vitro [78], in vivo [79], and in clinical studies [80]. Therefore, a huge number of plant derived active pharmaceutical ingredients such as curcumin, quercetin, isothiocyanates, gambogic acid, carnosol, and many others are involved in cancer chemoprevention by targeting cell cycle as a preferable anticancer therapy [81–85].

3.6 Experiences from Clinical Studies

From the above discussion it is clear that arresting the cell cycle can be an impending strategy to curb the progression of cancer. Moreover, several clinical studies have also supported a positive correlation between cell cycle arrest and cancer prevention. Inhibition of CDK4/6, aurora kinase, Wee1 kinase, spindle proteins, viz. Kinesin, and microtubules have been seen as some of potent therapies against cancer in a variety of clinical studies [5, 86]. Recently, Mills et al. [87] have reviewed a number of clinical studies justifying the involvement of cell cycle arrest as a potent therapeutic anticancer strategy [87]. Furthermore, some of the completed clinical studies are enlisted below in Table 3.2.

3.7 Conclusion and Future Perspectives

For sustained development of novel and effective anticancer therapeutics, it is necessary that therapeutic agents must have the ability to identify the molecular differences between healthy and cancerous cells. Thereafter, therapeutic agents should selectively target tumor cells keeping the healthy cells intact and alive. Hence, the cytotoxic efficacy of such agents should be at par or well enough to affect cancer cells only. Unfortunately, with partial success in hand, the desired treatment of cancer is not possible. This is further aided by a poor prognosis of cancers in initial stages. However, mechanism-based approaches such as the use of proteomics and genomics have provided enormous opportunities to the scientific community and clinicians, to come up with effective treatment regimens against cancer. Although to fulfill the lacunae in existing treatment approaches, there is a consistent need to develop technologies with enough potential to identify the cell cycle checkpoint components with extreme precision. Additionally, advanced drug-delivery strategies, for instance, nano-encapsulation, may also aid up in present-day treatment approaches giving more effective therapeutic outcomes against cancer. The scientific community should also focus on exploiting the novel, in-depth, and mechanistic approaches to meet the need for early diagnosis and effective anticancer treatment.

Table 3.2 Different clinical trials considering cell cycle arrest as a potent anticancer therapy (Data obtained from: www.clinicaltrials.gov)

Sr. no.	Trial Id	Type of cancer/study involved	Drug molecule under test	Phase	Status	Sponsor name	No of subjects enrolled
1	NCT 00141297	Neoplasms, lymphoma, non-Hodgkin	PD-0332991	I	Completed	Pfizer	74
2	NCT 00840190	Solid tumors, hematologic malignancy	P1446A-05	I	Completed	Piramal Enterprises Limited	29
3	NCT 00407498	Neoplasm	P276-00	I	Completed	Piramal Enterprises Limited	50
4	NCT 00772876	Advanced refractory malignancies	P1446A-05	I	Completed	Piramal Enterprises Limited	39
5	NCT 00292864	Solid tumors	SNS-032 injection	I	Completed	Sunesis Pharmaceuticals	25
6	NCT 00446342	B-lymphoid malignancies, chronic lymphocytic leukemia, mantle cell lymphoma, multiple myeloma	SNS-032 injection	I	Completed	Sunesis Pharmaceuticals	21
7	NCT 01335256	Neoplasms	BAY1000394	I	Completed	Bayer	10
8	NCT 02540876	Metastatic malignant neoplasm, solid neoplasm, unresectable malignant neoplasm	Ilorasertib	I	Completed	University of Chicago, National Cancer Institute	12
9	NCT 00899054	Squamous cell carcinoma of head and neck	P276-00, radiation: External beam radiotherapy (EBRT)	I/II	Completed	Piramal Enterprises Limited	23
10	NCT 01291017	Non-small cell lung cancer	PD0332991	II	Completed	University of Florida	19
11	NCT 01096342	Refractory multiple myeloma	Dinaciclib	II	Completed	National Cancer Institute	16

(continued)

Table 3.2 (continued)

Sr. no.	Trial Id	Type of cancer/study involved	Drug molecule under test	Phase	Status	Sponsor name	No of subjects enrolled
12	NCT 01624441	Estrogen receptor negative HER2/Neu negative male breast carcinoma, progesterone receptor negative recurrent breast carcinoma, stage IV breast cancer AJCC v6 and v7, triple-negative breast carcinoma	Dinaciclib, Epirubicin hydrochloride	I	Completed	National Cancer Institute	40
13	NCT 01684215	Neoplasms, breast neoplasms	PD-0332991, Letrozole	II	Completed	Pfizer	61
14	NCT 02457351	Medical oncology	BAY 1000394, Itraconazole (Sporanox)	I	Completed	Bayer	14
15	NCT 01711528	Recurrent plasma cell myeloma	Bortezomib, Dexamethasone, Dinaciclib	I	Completed	National Cancer Institute	41
16	NCT 02047890	Neoplasms	BAY 1000394 BAY 1000394	I	Completed	Bayer	12
17	NCT 00824343	Squamous cell carcinoma of head and neck	P276-00	II	Completed	Piramal Enterprises Limited	86
18	NCT 00871910	Solid Tumors, lymphoma, non-Hodgkin, multiple myeloma	SCH 727965, Aprepitant, Ondansetron, Dexamethasone	I	Completed	Merck Sharp & Dohme Corp.	81
19	NCT 00871663	Solid tumors, lymphoma, non-Hodgkin, multiple myeloma, leukemia, lymphocytic chronic. B-cell	SCH 727965	I	Completed	Merck Sharp & Dohme Corp.	123
20	NCT 02441946	Breast cancer, hormone receptor positive tumor, early-stage breast carcinoma	Abemaciclib, Loperamide, Anastrozole	II	Completed	Eli Lilly and Company	224

21	NCT 01652144	Mantle cell lymphoma	AT7519M	II	Completed	NCIC Clinical Trials Group, Astex Pharmaceuticals, Inc., Canadian Cancer Trials Group	12
22	NCT 01627054	Chronic lymphocytic leukemia	AT7519M	II	Completed	NCIC Clinical Trials Group, Astex Pharmaceuticals, Inc., Canadian Cancer Trials Group	7
23	NCT 01188252	Neoplasms	BAY 1000394	I	Completed	Bayer	112
24	NCT 01515176	Chronic lymphocytic leukemia, prolymphocytic leukemia, recurrent small lymphocytic lymphoma, refractory chronic lymphocytic leukemia	Dinaciclib, biological: Ofatumumab	I/II	Completed	National Cancer Institute	36
25	NCT 01546038	Acute myeloid leukemia	PF-04449913, low dose ARA-C (LDAC), Decitabine, Daunorubicin, Cytarabine	II	Completed	Pfizer	255

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Apoptotic Cell Death: Important Cellular Process as Chemotherapeutic Target

4

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Abstract

Apoptosis is a biological feature, which causes programmed cell death. It consists of two pathways, namely extrinsic and intrinsic, and mitochondria are the site of apoptotic process completion. An abnormality in the apoptotic process can make cells immoral, which is one of the major characteristics of cancer cell formation and cancer development. Chemotherapeutic molecules, which have been used as anticancer drugs, or drugs under investigations, have mostly designed in a way that they can revert apoptotic abnormalities or induce apoptosis. This book chapter discusses the apoptotic process and its abnormalities in cancer cells, and how chemotherapeutic drugs can induce apoptosis, with most advanced and updated findings on mechanisms of action.

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Keywords

Apoptosis · Intrinsic pathways · Cytochrome C · Extrinsic pathways · Death ligand · Caspase

4.1 Introduction

To grow and eliminate unnecessary or toxic materials, our body takes the support of apoptosis. Apoptosis is a type of cell death known as biological programmed cell death (PCD) in a controlled manner. The principal of apoptosis was first introduced in 1842 by Karl Vogt, a German scientist, which was later explained in detail by Walther Flemming in 1885 [1]. The number of cells is controlled by the contribution of both cell division and cell death. Intracellular cell death is activated when particular cells become useless. This technique is, therefore, referred to as programmed cell death, even though it is more commonly known as "apoptosis", a Greek word meaning "falling off." Billions of cells die in the bone marrow and intestine every hour in a healthy adult human [2]. Development of mouse paws, tadpole to frog, finger and toe formation of the fetus are all about apoptosis. If this were not so, the tissue would go through excess expansion and shrinkage, affected by antigen or limitless cell proliferation. Mainly there are two pathways in apoptosis: extrinsic pathway (via death receptor) is activated by extracellular pro-apoptotic stimuli; intrinsic pathway (mitochondrial) is initiated following mechanisms ingrained to the cell by itself. Stimulation of the caspases is the result of apoptotic pathways, which is crucial for this process [3]. The caspases change from inactive zymogen to active component during apoptosis [4].

Genome integrity and cellular homeostasis are processed through a complex system that proceeds following DNA damage, stimulating checkpoints of cell cycle and promoting DNA repair, or removing injured cells from the proliferation. Moreover, cell death regulates cell proliferation, such as the number of nerve cells to match the number of target cells entailed for innervations. Basically cell death controls cell division. So any stunt in the pathway can lead to heart failures, neurodegenerative diseases, immune-deficiencies, and more to say cancer, that is, uncontrolled cell proliferation [5, 6]. Accelerating apoptosis approach has been a novel way in the history of cancer treatment by the fact that abnormal cell death has seen to be the mainstay of tumor growth and anticancer drug resistance. The most effective anticancer drugs thus might target apoptosis pathway.

4.2 Basic Mechanism of Apoptosis

Approximately 50 to 70 billion cells go through apoptosis in adult people per day [7]. PCD, or more specifically, apoptosis, is a unique strategy for protecting a host from every possible pathogen. The apoptosis process is characterized by the accumulation of nuclear chromatin, condensation of cytoplasm, DNA damaging, formation of blebs, and dissolution of cell into small apoptotic bodies consumed by lysosomes of surrounding cells [8]. This PCD is stimulated by active caspase (cysteine-aspartic acid-specific proteases) protein, following intrinsic or extrinsic route. Extrinsic pathway worked by activating cell surface death receptor, while intrinsic pathway took place in mitochondria impairing the cytoskeletal protein and nuclear proteins which are crucial for cell surveillance [9]. Generally, the caspases remain as inactive zymogen form which develop into their active heterotetrameric forms in a consecutive proteolytic apoptotic stimulation process.

Mitochondrial proteins are involved in intrinsic pathways of apoptosis (Fig. 4.1). Cells with damaged DNA and/or overexpressed oncogenes influence this pathway. The overall pathway is governed by the B-cell lymphoma 2 (Bcl-2) family proteins [9]. The upregulation of Bcl-2 Homology 3 (BH3)-only proteins activates both Bcl-2 Associated X (BAX) and Bcl-2 antagonist/killer (BAK) [10]. BAX is regulated by tumor suppressor p53 [11]. BAK and BAX oligomerization results in forming mitochondrial outer membrane permeabilization (MOMP) after activation. MOMP is the significant event of intrinsic apoptosis and is taken as the point of no return

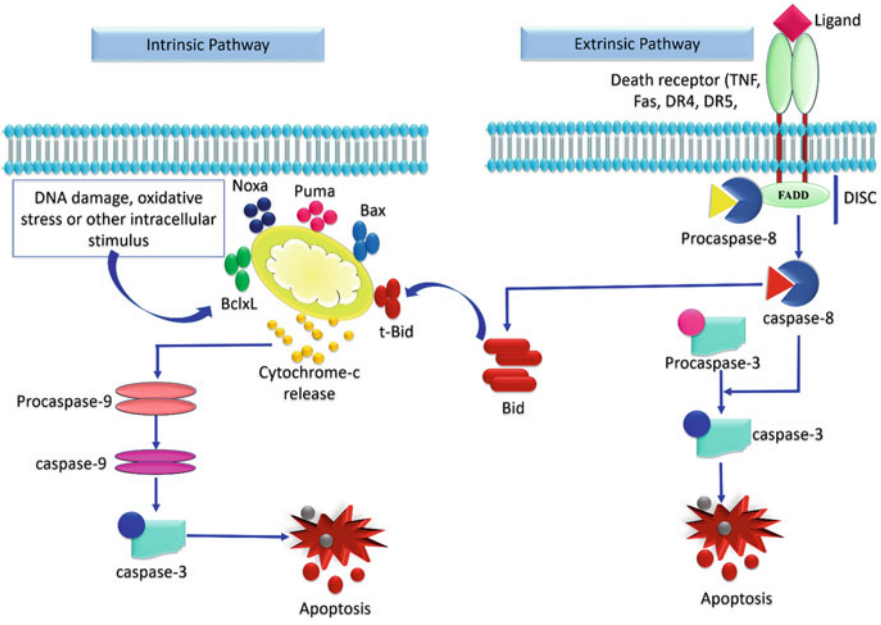

Fig. 4.1 Basic mechanism of apoptosis

[12]. Eventually upon the release of intermembrane protein cytochrome c, apoptosome forms, and apoptotic protease-activating factor-1 (APAF-1), deoxyadenosine triphosphate (dATP) activate procaspase-9 [13]. After that procaspase-9 is activated into caspase-9 that activates killer protein caspases-3 and -7 [14]. The executioner caspases immediately start to cleave proteins that leads to cell death. Additionally p53 has been demonstrated as crucial for the induction of apoptosis enabling activation of cell cycle checkpoints and DNA damage surveillance and p21 has appeared as down-regulator of p53, resulted in controlling apoptosis and cell cycle progression [6].

The extrinsic or death receptor pathway is mediated by death receptors (DR) activated by ligand binding (Fig. 4.1). DRs belong to tumor necrosis factor (TNF) receptor super family. Some death ligands possess TNF, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (Fas-L) [15]. The perforin/granzyme pathway is also involved in apoptosis, but mostly unclear. In this pathway, apoptosis is programmed via any of granzyme A or B. All these three apoptotic pathways coincide in the same terminal cellular pathway [15]. After ligand binding to receptor, intracellular death domain of DRs binds with some specific protein motifs like Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD). These certain proteins are connected with other protein interaction domain, named death effector domain (DED). Pro-caspase-8 also has DED that is stimulated upon interaction with the DED of FADD [16]. At this phase, a death inducing signaling complex (DISC) is formed. This signal triggers auto-catalytic activation of procaspase-8 [17]. The active caspase-8 then activates effector caspases, which performs the execution of destruction. Moreover, there are other pathways of caspase activation too, including a principle role of caspase-2 or caspase-12 in apoptosis activation by endoplasmic reticulum (ER) stress [18]. Several of the inhibitor of apoptotic protein (IAP) family members have also been found to take part in pathological conditions, particularly neurodegenerative disorders and cancer by upsurging proliferation protein [19].

4.3 Apoptosis Dysregulation in Cancer Cells

Abnormal apoptosis has been found to be associated with human diseases whereas extreme apoptosis causes degenerative disorders, and inadequate apoptosis results in neoplastic diseases. Cancer involves the anomalous growth of cells due to the loss of balance between apoptosis and proliferation. The ratio of pro-apoptotic and anti-apoptotic proteins plays an important role in apoptosis regulation. In this respect, cancer cells evade apoptosis by deactivating the machinery of cell death through different mechanisms such as overexpression of Bcl-2 family proteins or inhibition of pro-apoptotic Bcl-2 proteins, thus acquisition of a higher survival benefit. Moreover, another well-known mechanism of cancer cell survival is tumor suppressor p53 inactivation [20]. Usually, cancer cells evade this apoptosis by following mechanisms (1) disruption of pro-apoptotic and anti-apoptotic protein balance

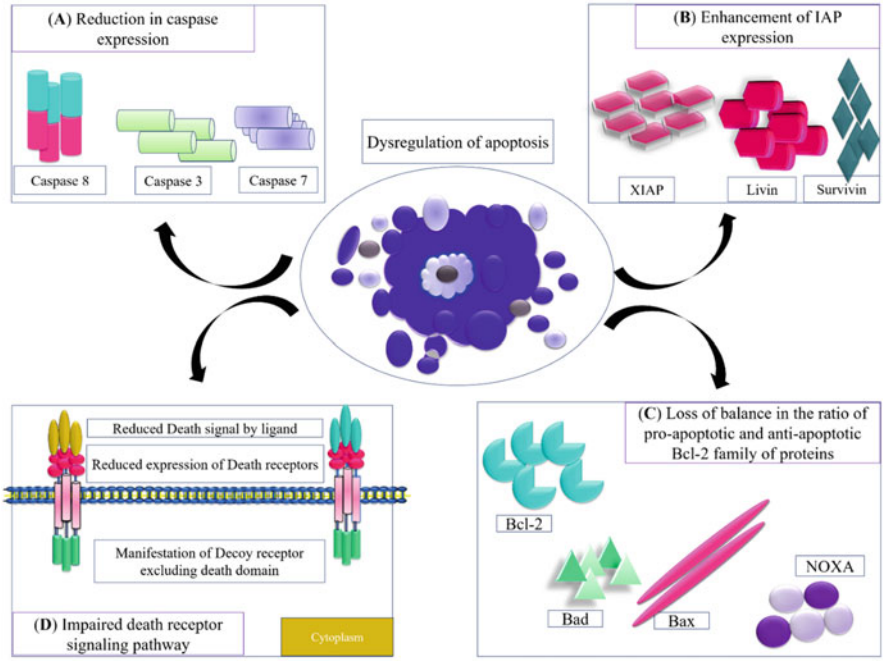

Fig. 4.2 Deregulation of apoptosis due to (**a**) Reduction in caspase activation; (**b**) Enhancement of IAP expression; (**c**) Imbalance in pro- and anti-apoptotic Bcl-2 ratio; (**d**) Impairment in death receptor signaling pathway mediated by reduced death signal, reduced death receptor expression, and decoy receptor expression without death domain

(2) Enhancement of IAP expression (3) inhibition of function of caspases, and (4) compromised signaling in DRs (Fig. 4.2).

Disruption of Pro-apoptotic and Anti-apoptotic Protein Balance

The Bcl-2 family of proteins are anti-apoptotic and pro-apoptotic, and they are involved in apoptosis regulation, particularly through the intrinsic pathway of caspase activation as they exist in upstream of cellular damage (irreversible) and function primarily in mitochondria. Based on the function and Bcl-2 homology (BH) domains, Bcl-2 family proteins are of three groups. (1) The anti-apoptotic proteins Bcl-2, Bcl-xtra large (Bcl-xL), myeloid cell leukemia 1 (Mcl-1), Bcl-w, A1/Bfl-1, and Bcl-B/Bcl-2-like protein 10 (Bcl-B/Bcl2L10) that comprise all of the four BH domains, and they defend cells from apoptotic signals. (2) The second group involves BH-3 proteins including Bcl-2 associated agonist of cell death (Bad), Bcl-2-modifying factor (Bmf), BH3 interacting domain death agonist (Bid), Noxa, Bcl-2-like protein 11 (Bim), BCL2 interacting killer (Bik), p53 upregulated modulator of apoptosis (Puma) and Harakiri, Bcl-2 interacting protein (Hrk).

These pro-apoptotic proteins being the initiator of apoptosis, become activated in response to deprivation of growth factors, DNA damage, and ER stress (3) A third group protein members including Bak, Bax, and Bcl-2 related ovarian killer/Mtd (Bok/Mtd) that contain all four BH domains, and they are pro-apoptotic too [21]. If there is an imbalance in the balance between pro-apoptotic and anti-apoptotic Bcl-2 family of proteins, the outcome is dysregulation in apoptosis process in the damaged cells.

Enhancement of IAP Expression

Apoptosis inhibitor c-IAP1 (BIRC2), NAIP (BIRC1), X-linked inhibitor of apoptosis protein (XIAP, BIRC4), IAP-like protein 2 (BIRC3), c-IAP2 (BIRC8), Apollon (BRUCE, BIRC6), Survivin (BIRC5), and Livin/MLIAP (BIRC7) are a group of functionally and structurally similar proteins, which regulate signal transduction, cytokinesis, and apoptosis. These inhibitors contain a characteristic baculovirus IAP repeat (BIR) protein domain and reduce the activity of caspase via binding BIR domain to caspase active site. IAPs promote degradation of active caspases by this mechanism or by keeping away the caspases from their target, thereby inhibit apoptosis [22].

Reduced Caspase Activity

The cellular machinery that mediates apoptosis includes a cysteine proteases family termed caspases. Therefore, it is rational to consider that Mammalian caspases are divided into 3 clusters functionally: initiator (caspase 2, 8, 9, and 10), executioner (caspase 3, 6, and 7), and inflammatory (caspase 1, 4, 5, 11, and 12) [23]. The binding of a death ligand to a DR initiates the extrinsic pathway of apoptosis, which then recruits, dimerizes, and activates the caspase-8 via TRADD/FADD adapter proteins. Activated caspase-8 later either stimulates apoptosis by cleaving directly and in that way activates the executioner caspases (3, 6, and 7), or stimulates intrinsic pathway of apoptosis via BID cleavage to persuade effective cell death. The mitochondrial or intrinsic or apoptosis pathway can be initiated through different cellular stresses that trigger to the freeing of cytochrome c from mitochondria, and apoptosome formation, consisted of apoptotic protease-activating factor 1 (APAF1), caspase-9, and cytochrome c, consequently activate caspase-9. Later the activated caspase-9 stimulates apoptosis by cleaving and activating executioner caspases [24]. Caspases become one of the key proteins in apoptosis initiation and execution. That is why, a reduced level of caspases or dysfunction of caspases is linked to decrease of apoptosis or cancer progression.

Impaired Death Receptor Signaling

DRs and DR-associated ligands are essential elements in extrinsic apoptotic pathway. DRs which are involved in this pathway are TNFR1 (also called DR 1), Fas (also known as APO-1 or DR2 or CD95), DR3 (also known as APO-3), DR4 (also known as TRAIL-1 or APO-2), DR5 (also known as TRAIL-2), DR 6, nerve growth factor receptor (NGFR) and ectodysplasin A receptor (EDAR). These receptors contain a death domain and triggered by death signaling, death domain attracted by numerous molecules that result in signaling cascade activation. But, when death ligands bind to decoy DRs excluding a death domain, it fails to generate signaling complexes, consequently fail to initiate signaling cascade. Different anomalies in this pathway, leading to avoidance of extrinsic apoptotic pathway have been characterized, for example, receptor downregulation or destruction of its function, as well as a reduction in death signal levels, which play role in the impairment of signaling and henceforth reduce apoptosis [25].

4.4 Chemotherapeutic Drugs and Apoptosis

Researchers developed numerous chemotherapeutics by targeting the intrinsic and extrinsic pathway regulating proteins of apoptosis. Fas and TRAIL induce the extrinsic pathway, and caspase 9 activation by MOMP and blocking of XIAP by second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis protein binding protein with a low isoelectric point (SMAC/DIABLO) play role in the initiation of intrinsic apoptotic pathway [4].

Chemotherapeutics Targeting the Extrinsic Apoptotic Pathway

Pro-apoptotic Receptor Agonists (PARAs)

Activation of TRAIL stimulates apoptosis in cancer cells via TRAIL-R1 and TRAIL-R2 DRs. It is pre-clinically evident that agonistic antibodies against TRAIL-Rs induce apoptosis in different cancer types without affecting normal tissues, that made it an appropriate approach in targeting cancer [4].

Pan Recombinant Human TRAIL (rh-TRAIL) Antibodies: Dulanermin

Both of TRAIL-R1 and TRAIL-R2 are targeted by rh-TRAIL. In cancer cells, Dulanermin selectively induces apoptosis by activating caspase and leading to consequential cell death [26]. A number of studies reported its apoptotic function as a single chemotherapeutic agent or in combination with other agents in hematological cancer and solid tumor [4].

TRAIL-R1 Agonistic Monoclonal Antibodies: Mapatumumab

Mapatumumab, a human immunoglobulin G1 lambda (IgG1λ) targets TRAIL-R1. A number of studies (mainly pre-clinical) revealed that mapatumumab inhibits tumor

progression in mice indicating established human tumor xenografts expressing TRAIL-R1. Mapatumumab is competent to improve the anticancer potential of cytotoxic compounds in numerous cancer cell lines as a single agent, with those resilient to chemotherapy [27]. Its activity also evaluated in combination with other chemotherapeutics by many studies. A phase I clinical trial investigated mapatumumab activity with paclitaxel and carboplatin in advanced solid tumor patients, where 44% of patients acquired stable disease (SD) [28]. Again, mapatumumab was used in combination with gemcitabine and cisplatin, and 25 gained SD with an average length of 6 months [29]. Another study combined mapatumumab and sorafenib in patients with progressive hepatocellular carcinoma (HCC), and reported a PR in 2 patients out of 19, with 4 SD patients [30].

TRAIL-R2 Agonistic Monoclonal Antibodies

Lexatumumab Lexatumumab is a fully recombinant human IgG1λ mAb, which efficiently binds with and triggers TRAIL-R2. Its activity against ovarian, breast, renal, colorectal cancer (CRC), and hematological cells and animal model by activating caspase 8 and caspase 9 is well-evident [31].

Conatumumab Conatumumab (AMG 655), another mAb found to stimulate the caspases in human cancers by targeting specifically TRAIL-R2 [32]. Though there is no data of overall survival (OS) or progression free survival (PFS) advantage with doxorubicin in refractory soft tissue sarcoma or carboplatin and paclitaxel in non-small-cell lung carcinoma (NSCLC) [33, 34], in combination with gemcitabine in randomized phase II study resulted in a non-significant upgrading [35].

Other Agonistic TRAIL-R2 Antibodies: Tigatuzumab, Drozitumab, and LBY135

Tigatuzumab, drozitumab, and LBY135 are agonist antibodies to TRAIL-R2, which have been tested in phase I/II trials. During the study, minor responses were found for drozitumab in 3 patients suffered from CRC, chondrosarcoma, and granulosa cell tumor, whereas 14 patients out of 41 got SD [36]. In case of tigatuzumab phase I trial, 7 patients out of 17 got SD [37]. LBY135 testing reports revealed that clinical activity was restricted to SD, when used as single agent, though 2 PRs (CRC, breast) were attained in combination with capecitabine [38].

Chemotherapeutics Targeting the Intrinsic Apoptotic Pathway

Bcl-2 Inhibitors

Anti-apoptotic Bcl-2 proteins, named Bcl-XL, Bcl-2, Mcl-1, and Bcl-w are overexpressed in different cancers, including hematological malignancies, small-cell lung cancer (SCLC) and B-cell lymphoma [39]. Inhibitors are of different types as follows:

Antisense Oligonucleotides as Bcl-2 Inhibitors: Oblimersen Sodium The 18-antisense oligonucleotide "oblimersen sodium" (Genasense, G3139) targets Bcl-2 m RNA of intrinsic pathway. G3139 exerts pro-apoptotic effects by increasing Bax, discharging cytochrome c from mitochondria to stimulate caspases, and eventually releasing Smac/DIABLO to suppress IAPs, which causes caspase 3 and 9 activation, triggering the initiation of apoptosis [40]. Also, Bcl-2 downregulated by oblimersen in the non-apoptotic pathway where stimulation by Bcl-2 caused the release of Beclin-1 to mediate cell death by autophagy [41]. Furthermore, oblimersen has been found to boost tumor immunity via triggering polyclonal antibody production, and stimulating dendritic cell maturation [42].

Small Molecule Downregulating Bcl-2 Gene or Protein Expression Several small molecules are established for regulating upstream factors of anti-apoptotic Bcl-2 proteins that caused their reduced expression [43]. Sodium butyrate (NaB), Depsipeptide and Vorinostat are the inhibitors of class-I histone deacetylase (HDAC), which expression is positively correlated with Bcl-2 expression. Inhibition of HDAC1 causes the downregulation of the Bcl-2, Bcl-XL, and Mcl-1 in multiple myeloma (MM) and mesothelioma cells [44].

Synthetic Retinoid Synthetic retinoids were documented to decline the expression of Mcl-1 through phosphorylating the c-Jun kinase (JNK) in malignant cells without affecting non-cancerous cells [45]. The upregulation of Mcl-1 is generally linked with several antitumor drugs resistance, so Mcl-1reduced expression should augment cytotoxicity of the cancer cell targeting drugs.

BH3 Mimetics Targeting BH3 Domain of Bcl-2

BH3 mimetics small molecules can target BH3 domain of Bcl-2. These BH3 mimetics make interaction with anti-apoptotic Bcl-2 proteins via binding to their BH-3 binding groove. Some of the BH3 mimetics are discussed below:

Gossypol Gossypol (AT-101, Ascenta) isolated from cotton seeds and roots. This BH3 mimetic natural polyphenolic compound suppressed Bcl-2 by disrupting the Bcl-2 and pro-apoptotic protein hetero dimerization [46]. Levo gossypol with higher affinity binds with hydrophobic groove of Bcl-2, Bcl-XL, and Mcl-1 and mediates apoptosis more competently compared to dextro gossypol [47]. It can also bind to Bak directly, consequently form oligomer by activating the Bak [48]. Moreover, levo gossypol also upsurges the sensitivity of chemotherapy and radiation therapy via activating the signaling pathway of stress-activated protein kinases (SAPK/JNK) that is regulating mitochondrial pro- and anti-apoptotic proteins [47]. Subsequently, levo gossypol is verified in a clinical trial in combination with other chemotherapeutic agents, such as with docetaxel in hormone refractory prostate cancer and with rituximab in treating chronic lymphocytic leukemia (CLL) [43].

Obatoclax Obatoclax (also identified as GX15-070) is an indole bi pyrrole small molecule that can inhibit Bcl-2. It prevents BAK to bind with MCL-1 and upregulates BIM expression [49].

ABT-263 (Navitoclax) and ABT-737 (A-779024) ABT-737 (A-779024) mimics BH3 domain of BAD protein and specifically binds with higher affinity to Bcl-XL, Bcl-2, and Bcl-w, but not to Bcl-B, Mcl-1, and A1 proteins [50]. ABT-263 (navitoclax) shows parallel anti-Bcl-2 activity with its antecedent, and reveals higher affinity for Bcl-2, Bcl-w, and Bcl-XL, but not for protein A1 or Mcl-1 [51]. ABT-737 displays strong antitumor activity as single agent in vitro against small-cell carcinoma cells and lymphoma, and similarly in mouse xenograft models with elevated upregulation of Bcl-XL or Bcl-2 [52]. Phase I and II clinical trials disclosed that both ABT-737 and ABT-263 were efficient in SCLC and CLL. Besides their activity as single agent, ABT-737 and ABT-263 have noteworthy effects in triggering apoptosis as combination therapy with other anticancer drugs. ABT-263 has been found to increase the effectiveness of chemotherapy and radiation therapy for CLL, SCLC, follicular lymphoma, and so on [51], while ABT-737 prompts sensitization of cancer cells to arsenic trioxide, flavopiridol, or fenretinide [53]. Further studies exhibited that ABT-263 promotes sensitization of many solid tumors to conventional agents, such as cyclophosphamide, fludarabine, and rituximab [51, 54]. Nevertheless, both ABT-263 and ABT-737 can decrease platelet for pointing Bcl-xl, which is essential in upholding the life expectancy of circulating platelet, demanding the improvement of Bcl-2 inhibitors selectively [52]. Several other BH3-mimetic compounds developed, that shares similar features like ABT-263 and ABT-737's inhibiting Bcl-xl and Bcl-2; these compounds include S44563, BM-1198, AZD4320, and Bcl2-32 [3].

ABT-199 (Venetoclax) ABT-199 (GDC-0199) showed its inhibitory effect against Non-Hodgkin's lymphoma (NHL) cell lines, comprising those resultant from follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), or mantle cell lymphoma (MCL), along with its activity in clinical trials against acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) cell lines [55]. Due to its specific inhibitory function to Bcl-2, it was approved to treat CLL by FDA in 2015 [56]. ABT-199 was designed to circumvent the nonselective interaction of ABT-263 with Bcl-xl inducing the antagonistic effect of thrombocytopenia [57]. Research studies also exposed that ABT-199 had a substantial sensitizing role in combination therapy with other anticancer drugs, like obinutuzumab, rituximab, in AML and CLL patients [52, 58, 59].

S55746 (Bcl201, Servier-1) This orally available chemotherapeutic agent showed effective killing of cancer cells overexpressing Bcl-2 in vitro and in vivo, and it was tested in refractory CLL patients in a phase I trial. S55746 also tested as a sensitizing agent in combination with phosphoinositide 3-kinase delta (PI3Kδ) inhibitor in follicular lymphoma (FL) and mantle cell lymphoma [52].

Selective Inhibitors Targeting Bcl-XL Agent (A-1155463, A-1331852, and WEHI-539) These therapeutic agents mimic BH3-only proteins and do not bind to Bcl-2, instead they bind strongly at p4 and p2 hotspots of Bcl-XL [60]. In colorectal cancer, Bcl-XL plays vital role, and study showed that these inhibitors are efficient against solid tumors. WEHI-539 was developed based on A-1155463 or A-1331852 and possesses the greatest selectivity for Bcl-XL, signifying its promising role as a single agent for some solid tumors [61].

Selective Anti-Mcl-1 Agents (UMI-177, A-1210477, and AMG176) UMI-77 precludes Mcl-1from binding with Bak and Bax, which stimulate apoptosis for many tumor cells. Though, UMI-77 had a rational selectivity for Mcl-1, demanding additional optimization. Consequently, A-1210477 was created and revealed high selectivity and binding affinity for p3 and p4 hotspots of Mcl-1. Through a sub nanomolar affinity, A-1210477 can be employed as a single agent and could also combine with ABT-263 to kill more cell lines [62].

AMG176, the recognized Mcl-1 inhibitor, also tested for clinical acceptability, antitumor response, pharmacokinetics in combination therapy for refractory multiple myeloma, Burkitt Lymphoma (BL), and AML where it induces apoptosis by altering the expression of anti-apoptotic and pro-apoptotic Bcl-2 proteins [52, 63, 64].

Maritoclax Maritoclax (also called marinopyrrole A) was isolated from marine-dwelling *Streptomyces* species that can directly target MCL-1, and marks it for proteasomal degradation; thus effectively mediating apoptosis. Also it can stimulate apoptosis in MM cell lines through interfering with MCL-1 [3].

ML311/EU-5346, S63845, S64315 (MIK665) ML311/EU-5346 has optimal strength for MCL-1 suppression in MCL-1 dependent cell lines. A threefold to fourfold lower efficacy for Bcl-2 inhibition and negligible effect on BCL-XL inhibition [3]. S63845 revealed effectiveness against MCL-1 reliant cell lines equally in vitro and in vivo, which were resilient to both venetoclax and navitoclax, as like A1210477, but S63845's effectiveness against MCL-1-reliant cell lines was above 1000 times superior. S64315 (MIK665) was derived from S63845, and is currently employing patients for two phase I studies: in myelodysplastic syndrome and refractory/relapsed AML (clinical trial ref.#NCT02979366), and another in patients with lymphoma or relapsed/refractory MM (clinical trial ref.# NCT02992483) [3].

AZD5991 AZD5991 is comparatively newly described. It is macrocyclic structurally and lucidly designed compound demonstrating higher selectivity for MCL-1. It binds directly to MCL-1, promptly enabling the detachment of BAK from the BAK/MCL-1 [3].

Targeting Inhibitors of Apoptosis (IAPs) by SMCS

Smac-Mimetic Compounds (SMCS) [SH-130, JP1201, Compound A (CA), AT-406, LCL-161, GDC-0152, Birinapant, HGS-1029, BV6 XIAP]

A Smac-mimetic SH-130 compound, as a radio sensitizer has revealed activity in prostate cancer cells. JP1201 was found effective against pancreatic cancer model. An unique and smac-mimetic molecule, "compound A" (CA), was found synergistically effective with TRAIL in primary CLL cells as an inhibitor of XIAP to promote effective apoptosis [4].

AT-406, another inhibitor of cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, XIAP play inhibitory role towards solid tumors. It is also utilized synergistically with Carboplatin, cisplatin, Bcl-2, paclitaxel, radiation therapy, TRAIL, and BRAF inhibitors [65]. LCL-161 destroys cIAP1 and cIAP2 and has potential action against solid tumors, multiple myelofibrosis, esophageal squamous cell carcinoma, and NSCLC. It is used in combination with TNF-α/TRAIL, paclitaxel, and radiation therapy [65, 66]. GDC-0152 is an inhibitor of cIAP1, cIAP2, XIAP and ML-lAP, and it has been used against breast cancer and glioblastoma [65, 67].

Birinapant was found to degrade cIAP1 and cIAP2 in solid tumors and melanoma. It is used in combination therapy by combining with Carboplatin, TRAIL, TNF-α [65, 68]. HGS-1029 causes XIAP inhibition, and loss of cIAP expression [69] in colon cancer and adenocarcinoma [65]. BV6 XIAP, degrade cIAP1 and cIAP2 [70] playing role against breast cancer, AML, and childhood ALL in combination with different chemotherapeutics, such as Drozitumab, 5-azacytidine, and dexamethasone [65]. Table 4.1, represents a bird eye view of various chemotherapeutic agents that are known to target apoptotic cell death of cancer.

Targeting Survivin and XIAP

Upregulation of XIAP via apoptotic stimuli is associated with tumor cell death resistance [77]. Some agents targeting XIAP and survivin are discussed below.

AEG35156 This has been tested in early phase clinical trials. Pre-clinical studies displayed the efficacy of AEG35156 in triggering XIAP downregulation and therefore boost apoptosis [71].

YM155 This small imadazolium-based YM155 (sepantronium bromide) compound was recognized against anti-apoptotic protein survivin. YM155 showed pre-clinical success regarding survivin inhibition at both of mRNA and protein levels [72].

LY2181308 This molecule can bind to survivin complementarily and suppress its expression in cancerous cells. As a radio sensitizer, it showed potential effect in cancer cell lines with an inhibition of survivin expression [73, 78], along with substantial suppression of human xenograft growth while directed intravenously.

Table 4.1 Apoptosis inducing chemotherapeutics in pre-clinical and clinical trial and their mode of action for triggering apoptosis

Drug inducing apoptosis	Molecular mechanism	References
Dulanermin	Caspase activation	[31]
Mapatumumab	Enhance the anticancer activities of cytotoxic compounds	[31]
Lexatumumab	Activating caspase 8 and caspase 9	[31]
Conatumumab	Activating intracellular caspases by stimulating DR5	[32]
Drozitumab	Stimulate death receptor DR5	[36]
Tigatuzumab	Stimulate death receptor DR5	[37]
LBY135	Stimulate death receptor DR5	[38]
Oblimersen sodium (Genasense, G3139)	Increasing Bax, discharging cytochrome c from mitochondria to stimulate caspases and eventually releasing Smac/DIABLO to suppress IAPs, and activation of caspase-3 and caspase-9	[40]
Sodium butyrate (NaB), Depsipeptide, and Vorinostat	Downregulation of the anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1	[44]
Synthetic retinoid	Reduce the expression of Mcl-1 through phosphorylating the c-Jun kinase (JNK)	[45]
Gossypol	Suppressed Bcl-2 by disrupting the Bcl-2 and pro-apoptotic proteins hetero dimerization, activate the Bak,	[46, 48]
Obatoclax	Prevents the binding of BAK to MCL-1, and increases BIM expression	[49]
ABT-199, ABT-263, and ABT-737 (navitoclax)	Inhibit Bcl-2, Bcl-XL proteins; but not of BCL-w protein	[50, 51, 55, 56]
S55746 (Bcl201, Servier-1)	Inhibit anti-apoptotic Bcl-2	[52]
A-1155463, A-1331852, and WEHI-539	Inhibit anti-apoptotic Bcl-XL	[60, 61]
UMI-177	Precludes Mcl-1 from binding with Bak and Bax, which stimulate apoptosis	[52, 62–64]
A-1210477 and AMG176	Inhibit anti-apoptotic Mcl-1	[52]
Maritoclax (marinopyrrole A)	Binds to Mcl-1 and induces proteasomal degradation	[3]
ML311/EU-5346, S63845, S64315 (MIK665)	Inhibit anti-apoptotic Mcl-1	[3]
AZD5991	Inhibit anti-apoptotic Mcl-1	[3]
SH-130 compound	Enhance radiation-induced activation of caspase and induction of apoptosis	[4]
JP1201	Inhibit IAPs	[4]
Compound A (CA)	Inhibit XIAP	[4]
AT-406	Inhibit cIAP1, XIAP, cIAP2	[65]
LCL-161	Destroys cIAP1 and cIAP2	[66, 65]
GDC-0152	Inhibit XIAP, cIAP1, cIAP2, and ML-lAP	[65, 67]
Birinapant	Degrade cIAP1 and cIAP2	[65, 68]

(continued)

Table 4.1 (continued)

Drug inducing apoptosis	Molecular mechanism	References
HGS-1029	Inhibition of XIAP inhibition, and loss of cIAP expression	[65, 69]
BV6 XIAP	Degrade cIAP1 and cIAP2	[65, 70]
AEG35156	Down regulation of XIAP	[71]
YM155	Inhibit survivin	[72]
LY2181308	Inhibit survivin	[73]
Thymoquinone	Regulation of p53 pathway, generation of ROS, and interference with NF-κB pathway	[74]
Cordycepin	Increased ROS generation	[75]
Resveratrol	Upregulation of the expression and enzymatic activity of SOD, CAT, and GAP	[76]

LY2181308 also made tumor susceptible to cytotoxics such as paclitaxel, gemcitabine, and docetaxel [73].

Other Molecules

Thymoquinone Thymoquinone (TQ), a compound from black cumin was found to induce apoptosis in cervical cancer cells (CaSki and SiHa). In those cell lines, not by affecting the expression of poly A polymerase (PARP), Bcl-2, Bax, caspase 3 and 9, indicating other possible mechanisms involved in apoptosis induction, such as regulation of p53 pathway, NF-κB pathway, reactive oxygen species (ROS) generation, etc. [74].

Cordycepin Cordycepin treatment was found to enhance apoptotic cell death in SiHa and HeLa cervical cancer cell lines. Its mode of action indicated that apoptotic activity was might be due to the increased ROS generation in the tested cancer cell lines as no remarkable changes were detected for anti-apoptotic or pro-apoptotic proteins [75].

Resveratrol Resveratrol treatment in a low concentration remarkably elevated the activity of superoxide dismutase (SOD) in PC-3, MCF-7, and HepG-2 cells, and upregulated the expression of SOD, Catalase, and glutathione peroxidase disproportionally in cancer cells that leads to H_2O_2 accumulation in mitochondria, which in turn stimulated apoptotic death of cancer cells [76].

Role of Redox Potential of Anticancer Molecules in Apoptosis Induction

ROSs are reactive biochemical components, for example, superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), or nitroperoxide (ONOOH). Upon produced by eukaryotic cells cellular aerobic metabolism plays major role in signaling pathway and apoptosis. Oxidative stress by ROS and associated signaling pathways offer a critical challenge towards anticancer therapies because of its both pro- and antitumor dual roles. Cancer cell requires moderate oxidative stress for its proliferation and invasion, whereas increased oxidative exposure to cancer cell could induce its apoptosis. Highly effective redox system makes cancer cell resistant to oxidative stress. Thus targeting the redox system in cancer cells by using oxidants or antioxidants is an important approach in current cancer therapeutic research [79, 80].

Antioxidant Enzymes: Regulator of Apoptosis

SOD, catalase, glutathione peroxidase (GPx), and thioredoxin reductases (Trx) are important antioxidant enzyme systems. These enzymatic antioxidants possess the ability to destroy ROS that provide highly effective protection against vigorous and substantial oxidative damage.

Studies corroborated that the mitochondria are the key generators of ROS as well as the leading target of generated ROS. Enormous accumulation of ROSs in mitochondria triggers Mn-SOD overexpression to suppress oxidative injury in mitochondria. Besides, this accumulated ROS in mitochondria can promote the transition of mitochondrial permeability, hence distort the stability of mitochondrial membrane. Mitochondrial outer membrane damage eventually causes the cytochrome c release along with pro-apoptotic factors, namely apoptosis inducing factor (AIF), OMI/HtrA2, Smac/Diablo, and endonuclease G, finally prompts caspase activation and apoptosis [81]. GSH used as reductant by GPx to catalyze the conversion of organic hydroperoxides or H_2O_2 into water or the analogous alcohols. GPxs members have anti-oxidative role at diverse cellular organelles, such as cytosol and mitochondria (GPx1), cytosol and nucleus (GPx2), plasma (GPx3), and in membrane (GPx4). The endogenous Trx antioxidant system includes NADPH and Trx, which play very significant role against oxidative insults. These antioxidants repair DNA and protein via reducing methionine sulfoxide reductases and ribonucleotide reductase. Trx antioxidants and its binding proteins (TBP2 and ASK1) regulate apoptosis or metabolism of lipids and carbohydrates. Both Trx and GSH system can defend oxidative attack by removing different ROS effectively [81, 82].

For example, resveratrol, a natural anticancer polyphenol mediates the accumulation of H_2O_2 in mitochondria through antioxidant enzymes regulation, which in turn, stimulated apoptosis in different cancer cells [76]. Resveratrol also plays suppressive role in colorectal cancer in rats by inhibiting oxidative stress. Investigational results demonstrated that resveratrol supplementation (entire-period) considerably elevated the enzymatic (SOD, glutathione reductase, catalase, GST, and GPx)

and non-enzymatic (decreased vitamin C, beta-carotene, vitamin E, and glutathione) antioxidant status along with a concomitant alleviation in the level of lipid peroxidation markers. Taurine upsurges the expression of catalase, SOD, and GPx gene and hence, it was found potent against melanoma [80].

ROS Trigger Apoptosis by Modulating Different Cellular Pathways

Initiation of cell apoptosis originates from intracellular and extracellular signals by the DRs and the mitochondria-mediated extrinsic and intrinsic pathways. After the initiation of cellular apoptosis, disruption of the homeostasis of intracellular redox system and consistent oxidative alterations of DNA, lipid, and protein enhance ROS concentration that influences oxidative stress mediated signaling of apoptosis. ROS stimulate the cancer cell apoptosis through TRAIL, and increase CD95 expression and TRAIL DRs via instigating NF-κB [83]. Further, ROS-induced activation of JNK plays an important role in mitochondrial dysfunction with consecutive apoptosis initiation. Instigation of ROS/JNK can also uplift and withstand p53 activity that further leads to robust apoptotic effect in cancer cells [84]. The mitogen-activated protein kinase (MAPK) that is sensitive to redox and the apoptosis signal-regulating kinase 1 (ASK1) are the upstream proteins of ROS/JNK. The activity of ASK1 is inhibited due to its interactions with redox proteins (Trx1 and Grx). ROS induce the dissociation of Trx1 from the Trx1-ASK1 complex, and also recruit tumor necrosis factor receptor-associated factors (TRAF2/TRAF6) to the Trx1-ASK1 complex. Stimulated ASK1 later provide signals to activate JNK, and persuades apoptosis either by signaling to mitochondria or by AP-1-dependent pro-apoptotic gene transcription. Moreover, ROS-induced distraction of the Trx2/ASK1/ASK2 complex of the mitochondria mediates cytochrome c release. ROS can also be increased due to the ER stress and stimulate the adjacent mitochondria for initiating the intrinsic apoptosis signaling pathway [85].

Anticancer molecules found to play significant role in ROS mediated apoptosis by activating different molecular pathways. Evidence have shown that thymoquinone mediates apoptosis by ROS generation through various molecular signaling pathways, like inducing Akt activation and stimulating Bax protein's conformational changes that eventually leads to the damage of membrane potential of mitochondria and cytochrome c release and next, initiation of the caspase-dependent apoptotic pathway. Also, ginsenosides apply their anticancer potentials through ROS mediated signaling cascades [86]. Figure 4.3 presents a simplified diagram showing ROS mediated apoptotic mechanisms.

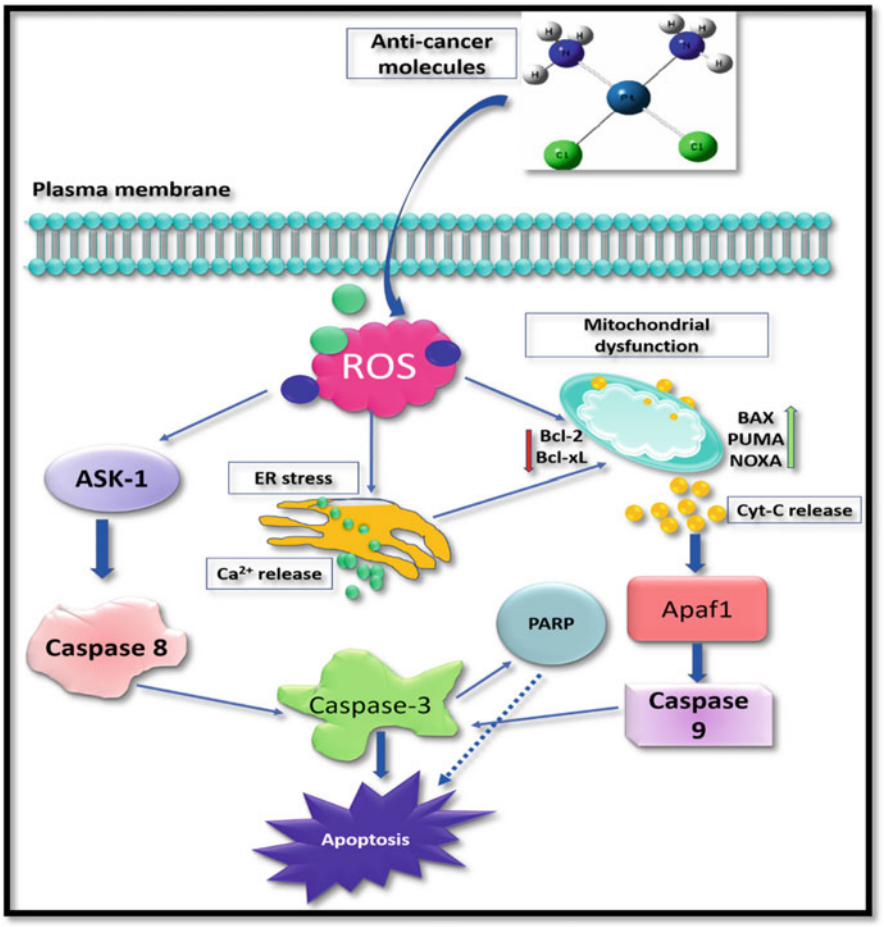

Fig. 4.3 ROS mediated signaling of apoptosis through caspase activation via the release of cytochrome c and ASK-1 activation

Mechanism of Balancing Antioxidant/Oxidant Mechanism by Chemotherapeutic Molecules to Protect Cells and Induce Apoptosis

Cancer cells are capable of adopting to new environments easily because of their highly compatible redox mechanisms that allow them to mediate a new redox balance for promoting cancer cell's growth.

There are different anticancer molecules mimicking antioxidant enzymes, targeting anti-apoptotic Bcl-2 proteins, caspase activation, and IAP. Mangafodipir is a potent SOD mimic possessing a combination of catalase-, SOD, and glutathione reductase-like functions. Hence, it can modulate different ROS cascade steps by neutralizing H_2O_2, $O2^{\bullet-}$ and by reestablishing GSH enzymes actions [87]. Niclosamide has proved as a powerful radiosensitizer that sensitize cells to

H_2O_2, via activating p38 MAPK-c-Jun axis, thus increasing apoptosis [88]. Organotellurides are well designated catalyst of redox with unique prooxidative role. Tellurium and selenium-based compounds convert the oxidizing redox milieu (existed in particular cancer cells) into a deadly accumulation of ROS that force these cells towards an acute redox threshold, and finally destroy these cells via apoptosis [89]. Allicin from garlic is a reactive sulfur species that has oxidizing properties, and is capable to oxidize thiols groups in cells, for example, cysteine residues in glutathione. This organosulfur stimulates apoptosis by elevating the cytochrome c level of mitochondria and release of Bax [90]. Quercetin provides anti-oxidant activity as metal chelator and ROS scavenger. It also exerted anticancer functions in cancer cells mainly via activating apoptosis [91].

4.5 Limitation of Apoptosis Targeting Chemotherapeutics

Chemotherapeutic Dulanermin did not show any maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) in patients. Again, phase I studies reported that Mapatumumab is safe. However, the most recurrent side effects were nausea, hypotension, fatigue, transaminitis, pyrexia, thrombocytopenia, and neutropenic fever found for mapatumumab. In case of Lexatumumab, the DLTs were transaminitis, hyperamylasaemia, and hyperbilirubinaemia. Phase I clinical study of AMG655/Conatumumab displayed fatigue, and elevated lipase level in patients. Study of antisense oligonucleotide Oblimersen revealed fatigue, and LFTs elevation. ABT-263 caused nausea, thrombocytopenia, fatigue, and elevated ALT, grade 4 thrombocytopenia, and bronchitis as dose-limiting toxicity (DLT). Grade III thrombocytopenia in some patients was observed by ABT-199, tumor lysis syndrome (TLS), neutropenia, or infections as adverse effects in patients [92]. Obatoclax showed neurological symptoms including dizziness, gait disturbance, somnolence, euphoric mood, QTc prolongation. AEG35156 showed DLT such as hypophosphatemia, asymptomatic reversible transaminitis, and thrombocytopenia. Another apoptosis inducing therapeutic YM155 showed nausea, stomatitis, pyrexia, and thrombocytopenia. LY2181308 showed flu-like symptoms, prolonged prothrombin time, thrombocytopenia, fatigue, and grade III transaminitis [4]. One of the established chemotherapeutic Levo gossypol affects male reproduction, causes fatigue, diarrhea, lymphopenia, neutropenia, hypophosphatemia, and mediates gastrointestinal (GI) toxicity in patients [93], necessitating the improvement of analogs with less toxicity. This caused the current advancement of apogossypol, which does not possess two reactive aldehydes that have been recommended to be accountable for the levo gossypol toxicity [4]. Conversely, for AZD4320, BCL2-32, BM-1197, S44563, WEHI-539, A-1155463, A-1331852, A1210477, Maritoclax, ML311/EU5346, S63845, and UMI-77, no pre-clinical or active clinical trial done and not assessed in humans for toxicity. Furthermore, S55746 (BCL201, Servier-1), S64315/MIK665, AZD5991, and AMG176 are in clinical trial but no adverse effect has been reported yet [3]. Figure 4.4 summarizes the adverse effects of apoptosis.

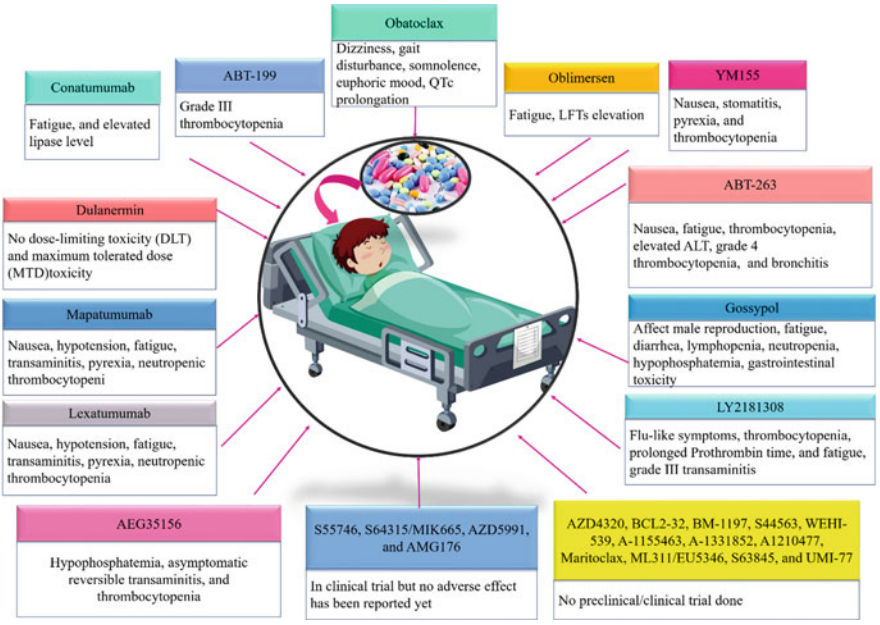

Fig. 4.4 Adverse effect of apoptosis inducing chemotherapeutics in patients

4.6 Conclusion

Apoptosis is one of the vital biological processes of life, and lack of cellular apoptosis is one of the major events in carcinogenesis Targeting the defective regulatory system of apoptosis is thus one of the most important approaches in chemotherapies. Drugs inducing apoptosis by targeting its different events have always received special consideration, and there are ongoing processes in scientific research to develop cancer treatments, especially chemotherapeutics on the basis of targeting apoptosis.

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Regulatory Roles of Autophagy in Cancer

5

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Abstract

Autophagy is an intracellular degradation and recycling system that aids in maintaining the cellular metabolism and homeostasis. Various cellular stresses, including organelle damage, deprivation of nutrients, and accumulation of damaged proteins lead to autophagy that can be associated with cell survival or cell death. Autophagy is initiated with the formation of autophagosome which is a double membrane vesicle. Autophagosome fuses with the lysosome to form autolysosome and deliver cytoplasmic contents that can be degraded or recycled to adapt cellular stressful conditions. Autophagy acts in playing dual roles in tumor suppression and tumor promotion. In addition, autophagy is involved in the

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maintenance of stemness and homeostasis in cancer stem cells, cancer metastases, and development of resistance to anti-cancer reagents by regulating the expression of many autophagy associated genes. Autophagy modulators such as chloroquine, rapamycin, and their derivatives are used against many cancers, and are in clinical trials. The complete understanding of mechanisms that link autophagy with cancer growth and suppression may aid in the development of promising therapeutics against cancer.

Keywords

Autophagy · Cancer · Anti-cancer therapy · Tumor microenvironment · Tumor cells

5.1 Introduction

Autophagy is a process of "self-eating" and degradation of cellular contents such as damaged organelles and misfolded proteins generated under cellular stress including starvation, cell death, tumor development, or tumor suppression. The process of autophagy is initiated with the formation of autophagosome and ends with the degradation or recycling of cellular contents in autolysosomes that are formed when autophagosomes fuse with lysosomes. This helps in protecting the cells from abnormal proteins and toxins, and to maintain cellular homeostasis and metabolism, which aids in the cell survival [1, 2]. Autophagy can be selective or non-selective depending upon the cellular needs of nutrients. In selective autophagy specific targets, viz. damaged organelles, misfolded protein aggregates, and intracellular pathogens are recognized and degraded or recycled. On the contrary, in non-selective autophagy cytoplasmic contents are packed into autophagosomes and supplied to lysosomes for degradation. Increasing evidence have reported that defects in autophagy are associated with metabolic stress, genomic damage, and oncogenesis [3]. Additionally, autophagy has been associated with cancer initiation and cancer suppression [4, 5]. Studies have shown that autophagy may modulate the expression of many oncogenes and tumor suppressor genes [6, 7]. In this chapter, we discuss the role of autophagy in cancer and highlight the recent advances employed in understanding the mechanism of interactions between tumor microenvironment, and autophagy (Fig. 5.1).

5.2 Molecular Mechanism of Autophagy

Autophagy is a naturally conserved cellular degradation and intracellular recycling system that aids in maintaining the cellular metabolism and homeostasis. A number of cellular stresses including organelle damage, deprivation of nutrients, and

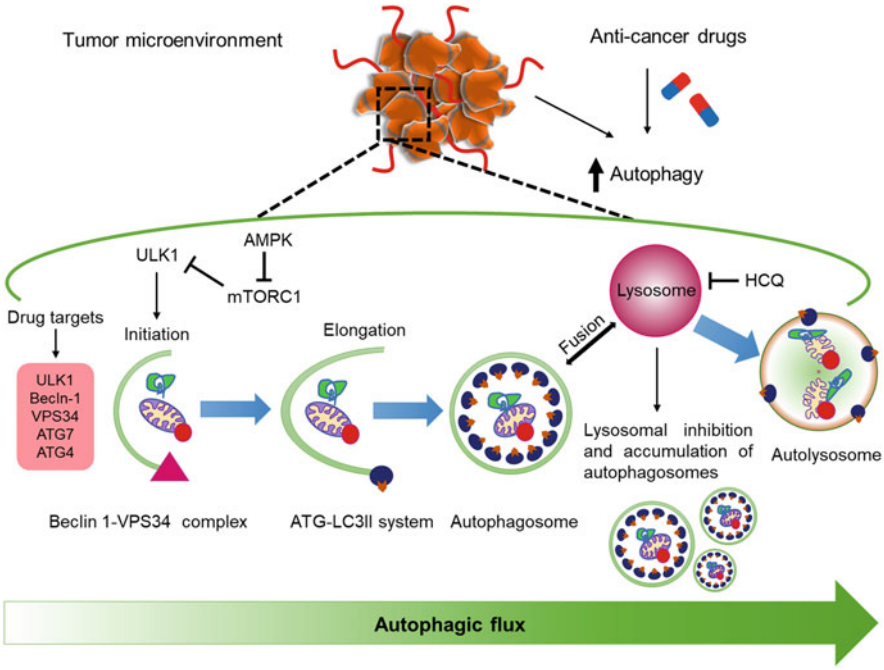

Fig. 5.1 Regulation of autophagy in cancer: Increasing demands of nutrients in tumor microenvironment and chemotherapeutic treatment upregulate the autophagy. Autophagy is initiated with the formation of autophagosome which fuses with lysosome to form autolysosome where cellular contents are degraded or recycled. Targeting the genes associated with autophagosome (ULK1, Beclin-1, ATGs, VPS34) decreases the formation of autophagosome, thus inhibits autophagy and blocking autolysosome formation leads to the accumulation of ineffective autophagosome and cell death

accumulation of damaged proteins lead to autophagy that can be associated with cell survival or cell death [8, 9].

In the normal cells, autophagy is utilized at basal levels for maintaining cellular homeostasis, biological functions, removal of damaged organelles and abnormal proteins, and quality control of cellular content [2, 10]. While in cancer cells, autophagy inhibits the tumor growth by inhibiting the cell survival, and also promotes tumorigenesis by facilitating the tumor cell proliferation [11, 12]. Several proteins control the mechanisms associated with autophagy. For example, mammalian target of rapamycin (mTOR) is one of such proteins which act as a key player of signaling events associated with stress, cell proliferation, and tumor progression. Each complex in mTOR, namely, mTORC1 and mTORC2, exhibits different cellular localization and functions [13–15]. The activity of mTORC1 is regulated by AMP-activated protein kinase (AMPK). Activated mTORC1 phosphorylates autophagy-related protein 13 (ATG13) and prevents it to form a Unc-51-like autophagy-activating kinase (ULK1) complex consisting of ATG1, ATG17, and ATG101. This event blocks the recruitment of this complex to pre-autophagosomes

at the plasma membrane, and inhibits autophagy. Inhibition of mTORC1 activity by various means, such as organelle damage and starvation, leads to the induction of autophagy [16, 17]. Although, the exact mechanism of mTORC1 in the induction of autophagy is not known [18], however, it has been reported that inhibition of mTORC1 dephosphorylates and activates the ULK1 [16]. These activated ULK complexes then lead to the autophagosome nucleation and elongation, a crucial step in the onset of autophagy.

Further maturation and elongation of autophagosome require Beclin1, ATGs and VPS34, microtubule-associated protein 1 light chain 3 (LC3) [19, 20]. ATG5, ATG12/ATG16L complexes recruit LC3 and promote autophagosome elongation. Consequently, ATG4B converts inactive isoform of LC3 to active cytosolic isoform, LC-I. Thereafter, phosphatidylethanolamine (PE), ATG3, and ATG7 drive the conversion of LC3-I to LC3-II, a marker for autophagosome, followed by loading of LC3-II over the inner and outer membrane of autophagosome. After maturation, the autophagosomes fuse with lysosomes and form autolysosomes, exposing their content to hydrolases that catalyze the removal of unwanted proteins and damaged organelles (Fig. 5.1) [21].

5.3 Autophagy and Tumor Suppression

Initially, autophagy was considered as a phenomenon of tumor suppression, as the basal level of autophagy leads to tumor suppression by removing damaged organelles and abnormal proteins to maintain homeostasis. Depletion of Beclin 1 in various cancers such as breast, prostate, hepatocellular, cervical, squamous cell carcinoma, and ovarian cancers results in the inhibition of autophagy, suggesting the role of Beclin 1 gene as a tumor suppressor [22–24]. Furuya et al. showed that MKN28 human gastric cancer cell line with overexpressed Beclin 1 displayed increased apoptosis towards chemotherapeutic drug cis-diamminedichloroplatinum [25]. Similarly, Beclin 1 reduced the cell proliferation and increased the apoptosis induced by paclitaxel in CaSki cervical cancer cell line [26]. A range of proteins such as Bax interacting factor-1 (Bif-1) and UV radiation resistance-associated gene (UVRAG) is important for maintaining the function of Beclin 1 as a tumor suppressor protein, and thereby regulates the autophagy positively [27]. The depletion in Bif-1 and UVRAG proteins decreased autophagosome formation and autophagy, resulting in tumor progression in the prostate, colon, gastric, and breast cancer. Various in vivo studies with mice have shown that depletion of autophagy regulators, viz. ATG4, ATG5, ATG7, ATG3, ATG5, and ATG9 is associated with the development of cancer [28–30]. For instance, in ATG4 null mice, exposure of chemical carcinogens increased the susceptibility to generate fibrosarcoma [31].

In addition, autophagy plays an important role in tumor suppression via regulating reactive oxygen species (ROS) and any damage in mitochondria increases the ROS production and resulting in tumorigenesis [32]. In addition to this, studies with autophagy receptor P62 provided a potential link between autophagy and tumor suppression. Loss of autophagy leads to the accumulation of P62 which can

contribute to tumorigenesis [33]. Overexpression of P62 promotes the oxidative stress and tumor growth in renal cell carcinoma suggesting that autophagy suppresses the tumor growth through the elimination of P62 and any defect in the autophagy leads to the oncogenesis [34]. Therefore, the studies mentioned in this section confirm that autophagy is the crucial event in the regulation of tumor suppression and any impairment in the autophagy leads manifest with the oncogenesis.

5.4 Autophagy and Tumor Promotion

Increasing evidence have shown the involvement of autophagy in tumor initiation and promotion. During tumorigenesis, cells are exposed to various stressful conditions such as nutrient deprivation and hypoxia [35, 36]. During cancer, cellular metabolism is altered to meet the increasing demands of energy and nutrients; that are replenished by autophagy which recycles the important metabolic substrates to proliferative cancer cells, thus help the tumor to grow by increasing stress tolerance. Activating mutations in the Ras gene have been reported in many cancers including colon, lung, and pancreatic cancer. Ras is a GTPase essential for maintaining cell proliferation and survival [37]. It has been observed that activation of Ras in cancer cells increases autophagy, thus leading to increased tumor survival and growth [38]. Similarly, autophagy promotes the growth of BRAF-driven melanoma and lung cancers [39, 40]. Additionally, inactivation of ATG17/FIP200 inhibited the growth of breast cancer in mice suggesting the association of autophagy in tumor promotion [41]. However, the detailed mechanism and the genetic context that lead to autophagy dependency in cancer remain poorly understood and require further investigations. Figure 5.2 describes the role of autophagy in tumor progression and tumor suppression.

5.5 Autophagy and Tumor Microenvironment

The tumor microenvironment consists of many factors associated with inflammation, hypoxia, and immune response. A high demand for nutrients and energy in the tumor microenvironment is fulfilled by autophagy by supplying metabolic substrates [42]. Cancer cells exhibit high hypoxic conditions in the tumor microenvironment, which activates the stress-related signaling pathways, viz. hypoxia-inducible factor-1 alpha (HIF-1α), affecting autophagy pathway to enable cancer cells survival and progression in low oxygen conditions [43–45]. HIF-1α affects tumor growth by regulating many cancer-related genes and autophagy pathways through an increase in glucose metabolism [46]. Factors contributing inflammations are over-activated in the tumor microenvironment and contribute to tumor progression by enhancing the accumulation of ROS in the cancer cells and immune cells, which secretes immune-regulatory cytokines such as transforming growth factor-β, interleukin-6, interleukin-10, and tumor necrosis factor-α in the tumor microenvironment. These

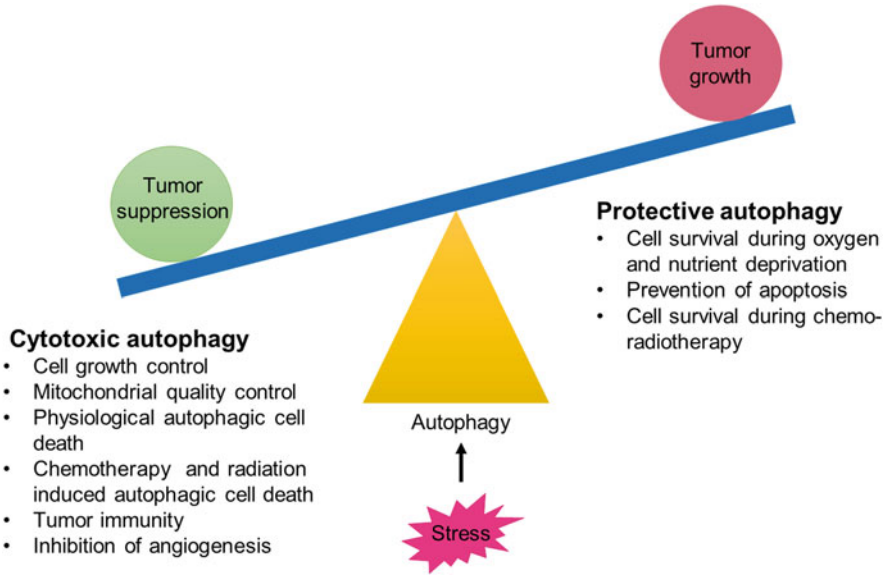

Fig. 5.2 A schematic diagram of dual role of autophagy in cancer

molecules induce chronic inflammatory responses and mediate tumorigenic effects [44]. Thus, inflammation induced by autophagy in the tumor microenvironment and nearby cells results in tumor progression, suggesting the possible role of autophagy in modulating tumor microenvironment.

5.6 Autophagy and Cancer Metastasis

Cancer cells can invade the surrounding tissues and migrate to distant organs through lymphatic and vascular systems. Autophagy displayed both pro- and anti-metastatic effects in many studies. In the early stages of metastasis, autophagy acts in anti-metastatic manner by limiting chronic inflammation and cell death, thereby reducing migration and invasion of cancer cells. Moreover, in advanced metastasis, autophagy promotes metastasis by enhancing cancer cell survival and migration to distant sites [47–49]. In contrast to the tumor suppressive role of Beclin 1 gene, study has shown that inhibition of Beclin 1 and LC3 autophagy genes inhibited breast cancer proliferation, migration, and invasion [50]. However, the relationship between Beclin 1 gene expression and tumor progression is not yet conclusive. In addition to this, reduction in ATG5 expression decreased overall survival in melanoma patients [51]. In another study, inhibition of mTOR signaling inhibited metastasis and induced autophagy mediated cell death in gastric cancer [52]. During apoptotic death, cancer cells get detached from the extracellular matrix (ECM), a process known as anoikis [53]. It is suggested that inhibition of autophagy not only

blocks the lung metastasis but also decreases the anoikis resistance in hepatocellular carcinoma [54].

Epithelial-mesenchymal transition (EMT) is essential for cancer metastasis leading to inhibition in cell–cell adhesion, change in cell polarity to increase cell motility and invasion [55, 56]. During EMT, cells undergo a transition from epithelial to mesenchymal phenotype. Moreover, EMT is essential for embryonic development and plays an important role in wound healing [57]. Few studies have reported the association between EMT and autophagy in cancer. Autophagy is enhanced in cancer cells undergoing EMT transition in response to cellular stress and inhibition of autophagy associated proteins such as Beclin 1, LC3, ATG5, and ATG7 increases the EMT transition in glioblastoma cells, thus leading to migration and invasion [58].

5.7 Autophagy as Drug-Resistant Factor in Tumors

Increasing evidence have shown that upregulation of autophagy leads to the resistance against anti-cancer drugs [59, 60]. Chemotherapy is a commonly used therapeutic strategy in cancer treatment; the success rate of chemotherapy is usually limited due to the development of resistance towards chemotherapeutic drugs [42, 61, 62]. Autophagy acts as one of the protective measures in cancer cells undergoing treatment with chemotherapeutic drugs and the induction of protective autophagy is a major cancer treatment. For example, autophagy is often associated with the development of chemoresistance against 5-Fluorouracil (5FU); an anti-cancer drug which blocks DNA synthesis by inhibiting thymidylate synthase [63, 64]. The activation of Beclin 1 and LC3I to LC3II conversion lead to the induction of protective autophagy followed by the activation of JNK and BCL2, which increase the autophagic flux and thereby leads to the chemoresistance [64]. In addition to 5FU, cisplatin is also used as a primary treatment drug in various solid cancers such as breast, pancreatic, and colon cancers, but treatment efficacy of cisplatin is also restricted due to the development of chemoresistance [64–66]. Studies on ovarian cancer have shown that autophagy contributed to the cisplatin-mediated resistance via activation of the Beclin 1 and ERK pathway [67, 68]. Another study in esophageal cancer has shown that cisplatin treatment enhances autophagy via the overexpression of Beclin 1, ATG7, and LC3I to LC3II conversion [69]. Moreover, the inhibition of autophagy in combination with cisplatin treatment significantly enhanced the cell death in esophageal cancer [69–71]. Autophagy mediated therapeutic resistance has also been shown with targeted agents such as AKT inhibitors, histone deacetylase inhibitors, and tyrosine kinase inhibitors, such as imatinib [72]. On contrary, there are many situations whereby depletion of autophagy decreased the efficacy of a specific therapy [73]. Therefore, further work is necessary in order to understand the mechanism of autophagy activation or suppression in response to cancer-directed therapies.

5.8 Autophagy as a Modulator of Immune Response in Cancer

In addition to conferring chemoresistance in cancer cells, autophagy also regulates the immune response towards anti-cancer therapy. In anti-tumor immunity, tumor cells release the tumor-antigens in the surrounding environment, which are taken up by antigen-presentation cells, and are presented to T-cells for the activation of T cell-mediated cytotoxicity against tumor cells [74]. Other immune cells such as dendritic cells and B-cells eliminate the cancer cells by releasing chemokines and cytokines. Knockdown of Beclin 1 and ATG7 inhibits autophagy and impairs the ability of T-cell to survive by mitochondrial dysfunction and ROS production [75, 76]. Therefore, autophagy is required for T-cells survival. Nevertheless, hypoxia-mediated autophagy leads to the activation of STAT3 and thereby, renders the cancer cells to be killed by T-cell mediated cytotoxicity. Moreover, reduced expression of Beclin 1 and ATG5 inhibits the autophagy and inactivates STAT3, thus sensitize the cancer cells to T-cell mediated cytotoxicity [77]. In conclusion, abnormal activation of STAT3 reduced the sensitivity of the immune response.

5.9 Autophagy in Cancer Stem Cells (CSCs)

Cancer stem cells (CSCs) represent a small amount of population of cells having the ability of self-renewal and differentiation. CSCs can induce tumor initiation, proliferation, and metastasis, thereby contribute to chemoresistance [78, 79]. Various studies have reported the role of autophagy in the maintenance of stemness and homeostasis in these CSCs [78, 79]. For instance, the inhibition of autophagy reduces the differentiation, while its enhancements increase the differentiation in glioma cells [80–83]. Reduction in the expression of Beclin 1 and LC3II is associated with the development of astrocytic cancers [84]. Autophagy contributes to mesenchymal-like properties in breast cancer stem cells and reduction in expression of LC3II and ATG12 proteins decreases cancer stem-like phenotypes [85].

Sharif et al. have shown that the knockdown of ATG7 and ATG5 blocks autophagy and decreases stemness associated markers, viz. SOX2, Oct4, and Nanog. In view of this, reduction in autophagy suppressed tumor cell proliferation and increased the cell death in colorectal CSCs [82]. In another study with colorectal CSCs, induction of autophagy leads to the resistance to anti-cancer drugs and maintained stem cell like phenomenon [86]. All these findings suggested that autophagy is essential for regulating pluripotency and maintaining therapeutic resistance in CSCs.

5.10 Targeting Autophagy in Cancer Therapy

Association of autophagy with tumor progression or suppression makes it one of the potential targets to increase cancer therapy. Various drugs targeting different stages of autophagy starting from autophagosome formation to auto lysosome have been

identified previously [87–89]. For instance, an autophagy inhibitor; chloroquine (CQ) can potentiate the effects of photososan-II-mediated photodynamic therapy and enhance the apoptotic cell death in colorectal cancer cells [90]. Various studies have supported that autophagy acts as a promising and potential therapeutic target. Many autophagy regulators, viz. CQ, hydroxychloroquine (HCQ), rapamycin and their derivatives; temsirolimus and everolimus are currently in use against cancer. Temsirolimus and everolimus are Food and Drug Administration (FDA) approved autophagy regulators [91]. Both inhibitors induce autophagy by targeting mTORC1 [14]. Everolimus is used against breast cancer and neuroendocrine tumors of pancreatic origin, while temsirolimus is used in curbing mantle-cell lymphoma [92, 93]. Both CQ and HCQ are anti-malaria drugs that block autophagy by altering the lysosomal pH and inhibition of autolysosome formation; the last step in autophagy process [94–96]. Preclinical studies with CQ or HCQ in bladder and pancreatic cancer have shown promising results. Treatment with CQ or HCQ inhibited autophagy and induced cell death and apoptosis in bladder cancer. Similarly, treatment with CQ induced autophagic cell death in metastatic pancreatic adenocarcinoma [97, 98]. Therefore, these reagents can potentiate the therapeutic effects of chemotherapy in many cancers. Various natural and synthetic autophagy inhibitors and their mode of actions are listed in Table 5.1 and Table 5.2 indicates the ongoing clinical trials with autophagy inhibitors in different cancer. Information given in Table 5.2 is retrieved from *clinicaltrials.gov*.

5.11 Conclusions

Autophagy plays a very complex and distinct role in cancer either by supporting tumor progression or inhibiting tumor growth. Autophagy promotes tumor progression by supplying essential nutrients to the cells and by maintaining the levels of ROS production. Additionally, autophagy promotes resistance in many cancers to targeted anti-cancer drugs and chemotherapy. Despite this information, there are many unanswered questions with respect to autophagy that need further investigation. For example, it is important to study the molecular events that tumor cells harness to switch the basal level of autophagy to a higher level. It is also not clear whether there are specific cargos or events to be degraded selectively in cancer cells to promote growth. Studying the interaction between tumor microenvironment and autophagy activity will provide the better insights on the dependency of tumor on autophagy. More studies are required in this context to further understand the subsets of tumor benefiting from autophagy inhibition and development of biomarkers of basal level autophagy in tumors that may be useful to develop better cancer therapeutics.

Table 5.1 Natural and synthetic compound targeting autophagy in different cancers

Compound name	Type of cancer	Mode of action	References
Artemisinin	Lung cancer, Esophageal cancer. Pancreatic cancer, ovarian cancer and glioblastoma	Induction of apoptosis in cancer cells in synergism with chloroquine Induction of autophagy by suppressing activation of NF-κB and the ROS accumulation Autophagy and apoptosis in cancer cells through inhibition of mTOR kinase	[99–102]
Artesunate, (semisynthetic derivative of artemisinin)	Colorectal cancer, breast cancer and glioblastoma	Activation of Beclin-1, LC3-I/LC3-II, and caspase-3 Increase sensitivity of epirubicin, a chemotherapeutic agent in breast cancer cells via autophagy	[103–106]
Curcumin	Colon cancer, malignant glioma uterine leiomyosarcoma Mesothelioma and chronic myelogenous leukemia	Decrease activation of AKT/mTOR/p70S6 kinase signaling pathway Induction of autophagy mediated apoptosis by regulating PI3K/AKT/mTOR and NF-κB signaling pathways	[107–112]
Celastrol	Gastric cancer, osteosarcoma glioblastoma and pancreatic cancer	Induction of autophagy mediated apoptosis Promoted proteotoxic stress	[113–117]
Paclitaxel	Breast and prostate cancer	Accumulation of LC3B-II proteins and induction of autophagosomes induced miR-101 and autophagy mediated apoptosis	[118–121]
Resveratrol	Lung cancer, ovarian cancer, myeloma, hepatocellular cancer, cervical cancer, oral cancer, glioblastoma, breast cancer, cervical cancer, Promyelocytic leukemia, prostate cancer, chronic myelogenous leukemia, skin cancer and renal cancer	Induction of LC3-II proteins and autophagy mediated apoptosis formation of acidic vesicular organelles Suppression of Wnt/β-catenin pathway Inhibition of NF-κB pathway and AKT/mTOR pathway	[122–126]
γ-Tocotrienol	Breast cancer	Induction of LC3-II proteins and autophagy mediated apoptosis	[127, 128]

(continued)

Table 5.1 (continued)

Compound name	Type of cancer	Mode of action	References
Palm-mixed tocotrienol complex	Breast and prostate cancer	Induction of autophagy mediated apoptosis Accumulation of dihydroceramide and dihydrosphingosine	[129, 130]
Thymoquinone	Glioblastoma and head and neck squamous cell cancer	Activation of LC3-II and p62 proteins and induction of autophagosomes	[131–133]
Ursolic acid	Cervical cancer, breast cancer, glioblastoma, prostate cancer, Colon cancer and osteosarcoma	Activation of LC3-II and p62 proteins, autophagosome formation, and autophagy mediated apoptosis. Endoplasmic reticulum stress induction; activation of glycolytic and PI3K/AKT-mediated autophagy pathway Modulation of Beclin-1 and Akt/mTOR pathways and JNK pathway	[134–139]
Synthetic ursolic acid	Lung cancer	Activation and accumulation of Beclin-1 and LC3A/B-II	[140]
Chloroquine and hydroxychloroquine	Bladder cancer, melanoma and pancreatic cancer	Activation of LC3-II and p62 proteins, autophagosome formation, and autophagy mediated apoptosis	[97, 98, 141]
Quinacrine	Colon cancer	p53 and p21-dependent regulation of autophagy	[142, 143]
Temsirolimus	Adenoid cystic carcinoma	Activation and accumulation of Beclin-1 and LC3A/B-II inactivation of mTOR Induction of autophagy mediated apoptosis	[144]
Everolimus	Breast cancer	Induces autophagic cell death in aromatase inhibitor-resistant breast cancer by targeting estrogen receptor	[145]

Table 5.2 Detail of ongoing clinical trials with drugs targeting autophagy in various cancers

NCT number	Conditions	Interventions	Phases
NCT03037437	Hepatocellular cancer	Sorafenib, hydroxychloroquine	Phase 2
NCT03774472	Breast cancer	Hydroxychloroquine, letrozole, palbociclib	Phase 1/phase 2
NCT04132505	Pancreatic adenocarcinoma	Binimetinib, hydroxychloroquine	Phase 1
NCT03047837	Colon cancer	Aspirin, metformin	Phase 2
NCT03377179	Cholangiocarcinoma	ABC294640, hydroxychloroquine	Phase 2
NCT04163107	Multiple myeloma	Hydroxychloroquine, carfilzomib, dexamethasone	Phase 1
NCT03243461	Glioblastoma	Temozolomide, chloroquine	Phase 3
NCT03754179	Melanoma	Dabrafenib, trametinib, hydroxychloroquine	Phase 1/phase 2
NCT03598595	Osteosarcoma	Docetaxel, gemcitabine, hydroxychloroquine	Phase 1/phase 2
NCT03972592	Lymphatic malformation	Topical 0.1% Sirolimus	Phase 2
NCT04201457	Glioma	Dabrafenib, trametinib, hydroxychloroquine	Phase 1/phase 2

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ROS and Oxidative Stress in Cancer: Recent Advances

6

Mehmet Varol

Abstract

Reactive oxygen species (ROS) have long been considered as one of the major regulatory factors for the intracellular and intercellular signaling cascades. The sensitive redox balance that is controlled through an improved antioxidant system along with the enzymatic and non-enzymatic ROS production pathways sustains physiological functions in the healthy cells. During the course of cancer, a progressive deterioration of the redox balance can be followed via the overproduction of ROS, and results in the formation of malignant phenotype through induction of cancer hallmarks, including death evasion, uncontrolled proliferation, deregulating the cellular energetics, evading the immune response, provoking inflammation, inducing genome instability and mutations, developing drug resistance, angiogenesis, invasiveness, and metastasis. Apart from the carcinogenic roles of ROS, they have been employed as a target, mediator, and weapon in cancer treatment modalities because of the characteristic features considered as a double-edged sword. This chapter has consequently purposed to indicate the sophisticated roles, contributions, activities, and importance of ROS in the progression of cancer and cancer treatment strategies, and drawn the attention of scientists more to enhance the research on the complicated and versatile relationship between ROS and cancer.

Keywords

Reactive oxygen species · Cancer hallmarks · Carcinogenesis · Cell death pathways · Drug resistance

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6.1 Introduction

Although a mass effort of scientists and the considerable research budgets have been drained to find the convenient cure, drugs, and treatment strategies for cancer diseases, cancer is still ranked as the second leading cause of death and considered as a major public health problem worldwide [1]. The most recent global cancer statics showed that there was an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018, and this bitter truth reminds us once again the importance of the scientific research on the mechanisms of carcinogenesis, cancer treatment strategies, and drugs [2]. It is well known that carcinogenesis is a prolonged, complicated, and multi-stage process that can be induced by the harmful environmental factors along with the genetic predispositions [3–5]. Because of the stress occurring in microenvironment, carcinogenesis begins with malignant transformation of some cells in the organism, and the malign transformation of these cells is followed by hyperproliferation, insensitivity to the growth suppressing factors (evasion), resistance to the programmed cell death (apoptosis), invasiveness, production of angiogenic factors to induce the formation of new capillary vessels from the existing ones (angiogenesis), and finally gaining metastatic ability, which is defined as the ability to reach different parts of the organism through veins [6, 7]. Additionally, the irregularities in the cellular energetics and the escape from the immune system are also considered as the substantial parts of carcinogenesis [6, 8]. Although the process of carcinogenesis is common for almost all cancer diseases, it has been demonstrated that there are more than 200 types of cancer, and a tumor tissue exhibits a morphologically and functionally heterogeneous structure that consists of various cancer cells with different physiological characters, mutations, and epigenetic profiles [9–12]. Thus, understanding the underlying mechanisms of cancer hallmarks has a great importance to find the convenient cure, drugs, and treatment strategies for cancer diseases. It is well known that the cellular levels of oxygen molecules, reactive oxygen species (ROS), and antioxidants have a balance in the healthy cells located in a homeostatic microenvironment [13–15]. However, this balance observed in the homeostatic microenvironment can be disrupted in the progress of some diseases such as cancer, diabetes, neurodegenerative diseases, premature aging, and obesity [16–19]. The disruption of the balance between ROS and antioxidant molecules is considered as a leading factor for many intracellular and intercellular problems such as the disruption of mitochondrial metabolism and cellular energetics, the occurrence of the unstable and hypoxic microenvironment, and the alteration of molecular pathways [20–22]. Moreover, it is well known that reactive oxygen species and oxidative stress play key roles in the progress of carcinogenesis and effect the all cancer hallmarks [23]. Therefore, understanding the complicated and sophisticated roles of oxidative stress and reactive oxygen species has an exclusive place in cancer biology and anticancer therapy [24–26].

6.2 Cellular Sources and Regulation of ROS

Although all living aerobic organisms need molecular oxygen vitally for their cellular respiration as a central molecule, the oxygen-containing free radicals were determined as toxic compounds for aerobic organisms by Gerschman and coworkers [27, 28]. It is well known today that the increased levels of reactive oxygen species (ROS) take disruptive effects on the function, homeostasis, and structure of cells by inducing oxidative stress and lead to the development of various pathologies such as inflammatory, cardiovascular, and neurodegenerative diseases, age-related disorders, and cancer [22, 29]. Cancer cells are characterized by the overproduction of ROS both in the various cellular compartments and in the cancer cell microenvironment, and this overproduction can alter the genetic stability of cells along with many cellular processes [30–32]. Although there is a certain balance between ROS and antioxidant factors in the healthy cells, the balance can be disrupted by endogenous and exogenous ROS generators leading to the excessive ROS production or the antioxidant defenses limitation [33, 34]. The endogenous ROS generators can be listed as mitochondria, peroxisomes, endoplasmic reticulum, transition metal ions, lipoxygenases, cytochrome P450, and NADPH oxidase, though the exogenous ROS generators are ionizing radiation, ultraviolet rays, chemotherapeutics, environmental toxins, and inflammatory cytokines [29, 35]. Reactive oxygen species can be observed as radicals that have at least one unpaired electron, and chemically reactive non-radical species without unpaired electron [36]. The non-radical species such as singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) can be converted to radical ones, though the short-lived and highly electrophilic radicals such as hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), and peroxyl ($RO_2^{\bullet}$) molecules show substantial cytotoxic activity by oxidizing proteins, lipids, nucleic acids, and other cellular molecules [37–39]. The generation of ROS in biological systems can be eventuated by enzymatic and non-enzymatic reactions, and the enzymatic generation of ROS can be achieved by the contribution of the cytochrome P450 enzymes, arachidonic acid, cyclooxygenase (COX), lipoxygenase (LOX), xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS), and NADPH oxidases (NOXs) [39–41]. Superoxide anion radicals that are considered as the primary reactive oxygen species are formed by transferring one electron to the molecular oxygen (O_2), and so the further interaction to generate other reactive oxygen species can be occurred, such as the formation of hydrogen peroxide (H_2O_2), which can be generated spontaneously or by the effect of the superoxide dismutase enzyme (SOD). Moreover, hydrogen peroxide can be converted to the highly toxic hydroxyl radicals ($OH^{\bullet}$) through the iron-catalyzed Fenton reaction, and leads to the cellular damage and genomic instability due to the formation of oxidized proteins, lipids, and nucleic acids [37, 42]. Apart from the enzymatic ROS generation, ROS can be non-enzymatically generated by the mitochondrial respiratory chain [43]. During the aerobic respiration, the oxygen molecules are reduced to water in the electron transport chain by cytochrome-c oxidase, though approximately 1–2% of the oxygen molecules are reduced to superoxide ($O_2^{\bullet -}$) because of the electron leakage from the electron transport steps of ATP production [29]. Then, the formed

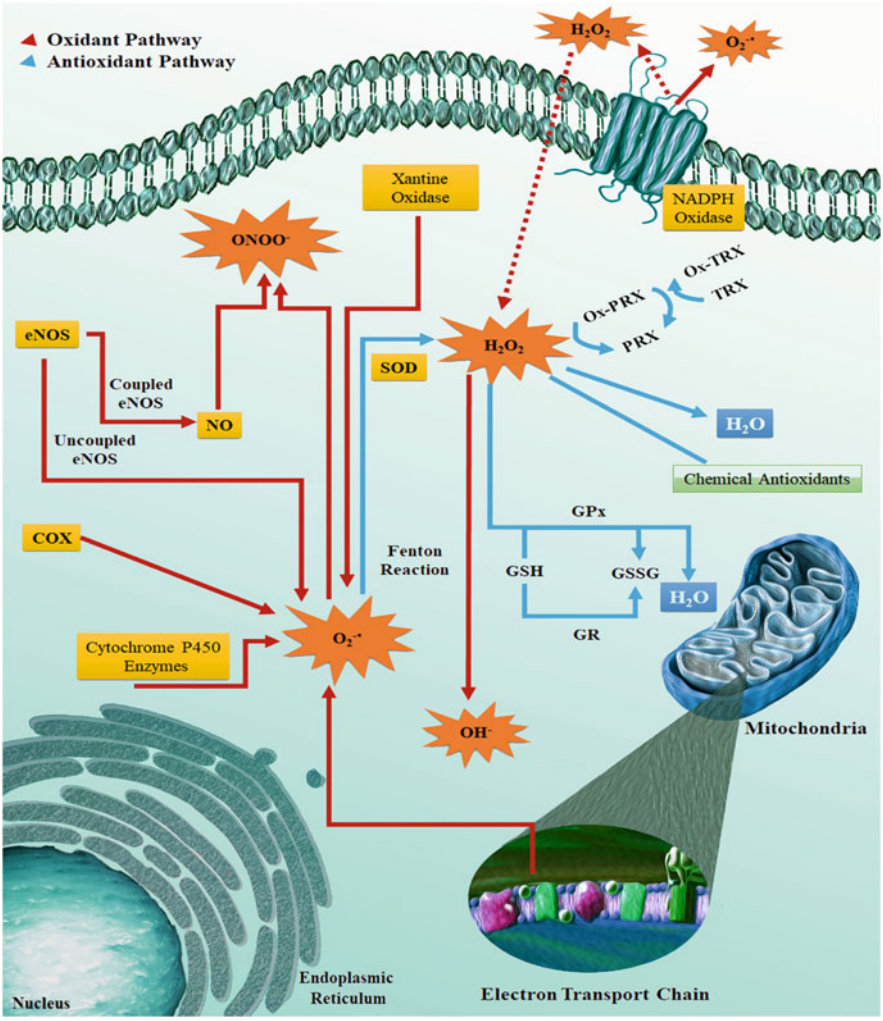

Fig. 6.1 Oxidant and antioxidant pathways for ROS homeostasis. $O_2^{-\bullet}$ (superoxide radical), $OH^{\bullet}$ (hydroxyl radical), $ONOO^-$ (peroxynitrite), H_2O_2 (hydrogen peroxide), H_2O (water), COX (cyclo-oxygenase), eNOS (endothelial nitric oxide synthase), NO (nitric oxide), SOD (superoxide dismutase), PRX (peroxiredoxins), TRX (thioredoxin reductase), and GPx (glutathione peroxidases)

superoxide radicals can be converted by the effect of SOD enzymes to the hydrogen peroxide molecules, which can be further converted to the hydroxyl radicals (OH•) through the Fe^{2+} or Cu^{2+} ions-catalyzed Fenton reactions (Fig. 6.1) [31, 36, 44]. Although mitochondria is widely considered as the major source of ROS, mitochondria-generated ROS production may have been overestimated due to the generation of functional damages during the mitochondrial isolation procedures, and

performing the new techniques showed that there are much lesser amounts of mitochondria-generated ROS than the previously estimated amounts [36, 45].

The membrane-bound NADPH oxidases (NOXs) are also considered as another major source of superoxide radicals [46, 47]. NOXs can be found on the membranes of plasma, nucleus, mitochondria, and endoplasmic reticulum, and so NOXs-catalyzed reduction of oxygen molecules into superoxide radicals can be observed where these membranes are located, and NOX-derived superoxide radical that could not diffuse across membranes can be further converted by SODs into hydrogen peroxides, which have an ability to diffuse across membranes as redox signaling molecules (Fig. 6.1) [31, 37, 48]. Peroxisome organelles are recognized as an another prominent source of ROS by generating superoxide radical, hydrogen peroxide, hydroxyl radical, nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^{-}$) through the reduced catalase (CAT) activity, which has been reported in many cancers such as hepatocellular carcinoma, prostate, lung, colon, and kidney cancers [29]. Another organelle, which is ROS source, is endoplasmic reticulum that has many cellular functions including calcium storage, lipid metabolism and the synthesis, folding, posttranslational modifications, and transport of proteins [49]. Along with the NOX-derived ROS production over the membrane of the endoplasmic reticulum, the accumulation of unfolded and misfolded proteins in the lumen of endoplasmic reticulum due to the alterations in the protein folding pathways may lead to endoplasmic reticulum stress that triggers the ROS production, and the increased levels of ROS in endoplasmic reticulum induce endoplasmic reticulum stress [50, 51]. Although the endogenous and exogenous ROS generators increase the ROS levels in cells, the antioxidant defense factors maintain the ROS homeostasis [25]. The antioxidant defense components include antioxidant enzymes (e.g., catalase (CAT), glutathione peroxidases (GPXs), glutathione reductase (Gr), peroxiredoxins (PRXs), superoxide dismutase (SOD), and thioredoxin reductase (TRX)), antioxidant molecules (e.g., alpha-lipoic acid, bilirubin, coenzyme Q, ferritin, glutathione, l-carnitine, metallothionein, melatonin, and uric acid), dietary natural products (e.g., ascorbic acid, β-carotene, polyphenol metabolites, selenium, and tocopherol), and synthetic products (e.g., butylated hydroxytoluene, N-acetyl cysteine (NAC), and tiron) [36].

6.3 ROS in Cancer Cell Proliferation and Survival

It has been well established that ROS play a key role in mitogenic signaling cascades by prolonging activation of growth factors and boosting levels of cellular signaling factors [52–54]. The proliferation of many cancers such as lung, liver, and breast cancers can be enhanced by the increased ROS level though the proliferation of these cancers can be alleviated by the administration of antioxidants [55]. The metabolism of cancer cells is commonly very active because of the oncogenic signals such as Bcr-Abl, c-Myc, and Ras oncogenes-related signals, and these oncogenic signals can also increase endogenous ROS generation without the induction of apoptosis [26, 56, 57]. Oncogenic Ras mutations, for instance, induce ROS generation through

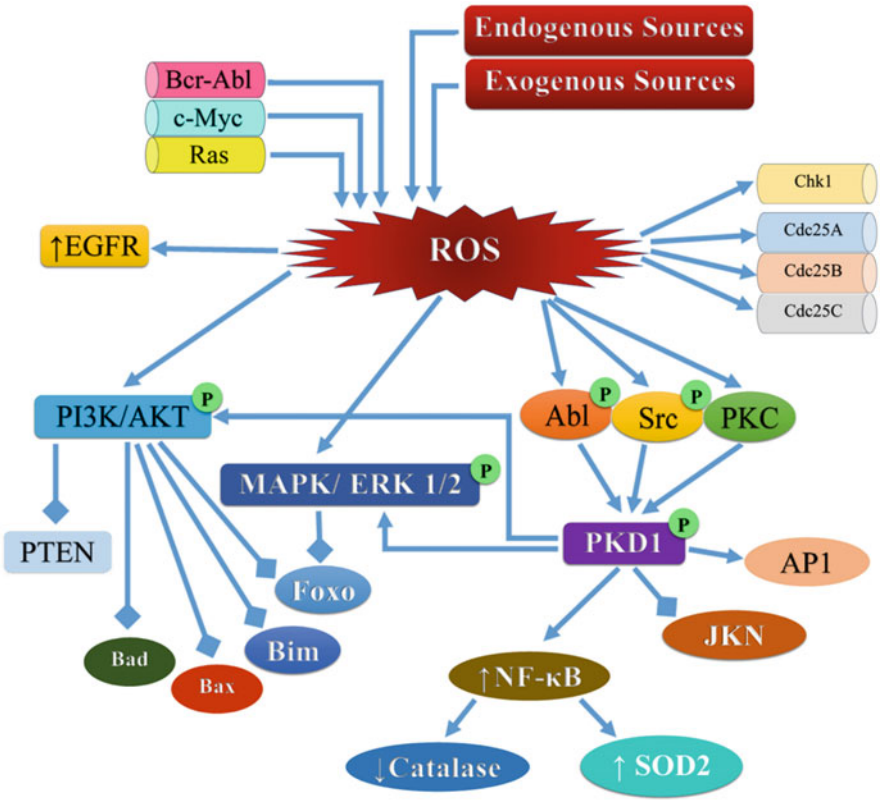

Fig. 6.2 ROS-mediated cell proliferation and cell cycle arrest signaling in cancer

NOX isoform (NOX4) that improves cell proliferation, and K-Ras oncoprotein upregulates the pro-proliferative signal epidermal growth factor receptor (EGFR) by elevating mitochondrial ROS production [31, 58]. Thus, it is widely considered that the oncogene-induced ROS generation positively regulates cancer cell proliferation by promoting mitogenic signaling cascades such as 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 (Fig. 6.2) [52]. For example, increased ROS level inhibits MAPK by oxidation of cysteine residues in the active site and the degradation of MAPK phosphatase 3 (MPK3) prominently reduces ERK 1/2 activity [59]. Similar to the inhibition of MAPK phosphatases, the protein tyrosine phosphatase 1B (PTP1B), phosphatase and tensin homolog (PTEN) protein, and ubiquitin ligase are negatively regulated by the increased level of ROS via oxidation of cysteine residues in the active sites of these proteins [53, 60, 61].

Moreover, the elevated level of ROS activates the cell proliferation-related transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1

(AP1) that upregulate the cancer cell proliferation (Fig. 6.2) [53]. Interestingly, mitochondria-generated ROS can induce both cell proliferation and cell quiescence by playing a dual role in cell cycle. The increased level of mitochondria-generated ROS that mostly formed by superoxide ($O2^{\bullet-}$) induces cell proliferation as well as superoxide dismutase (SOD) antioxidant defense system (Fig. 6.1), which converts the superoxide to the hydrogen peroxide (H_2O_2), and the increased hydrogen peroxide drives proliferating cells into quiescence [52, 62, 63]. Moreover, it is well known that ROS can induce DNA damage such as double-strand breaks and the DNA damage results in cell cycle arrest thanks to the cell cycle checkpoints (Fig. 6.2) [64]. For example, it has been reported that the increased ROS can result in a p53 independent G2/M arrest in colorectal cancer cells by activation of checkpoint kinase 1 (Chk1) [65]. Additionally, the phosphatase inhibition activity of ROS also induces cell cycle arrest by effecting on the cell division cycle 25 (Cdc25) protein phosphatase family consisted of Cdc25A, Cdc25B, and Cdc25C proteins that have substantial roles in the progression of the various cell cycle stages such as synthesis (S) and mitosis (M) phases [66]. For example, it has been reported that the ROS-decreased Cdc25C level leads to G2/M cell cycle arrest and the elevated ROS dramatically decrease Cdc25A level and its phosphatase activity [64, 67–69]. On the other hand, ROS accumulation can also predictably take an important role in cancer cell survival as well as cell proliferation and cell cycle arrest because of the common signaling factors such as PTEN, PI3K, PKD, Akt, ERK 1/2, and NF-κB (Fig. 6.2) [26, 31, 52]. For example, increased generation of hydrogen peroxide leads to the oxidation of cysteine thiol groups of PTEN, PTP1B, and PP2 (protein phosphatase 2) and inactivation of these phosphatases promote cell survival by negatively regulation of PI3K/Akt signaling [31, 70, 71]. It has been reported that this kind of phosphatases' inactivation can be observed in many types of cancer such as breast, prostate, ovarian and endometrial cancers, glioblastomas, and melanomas [72, 73]. Ras activation along with growth factor signaling can be also induced by hydrogen peroxide, and this activation leads to blocking the PTEN signaling cascades and induction of PI3K/Akt/mTOR and MAPK/ERK 1/2 cell survival pathways [31, 58]. Moreover, these cell survival pathways can be regulated by ROS-induced inactivation of their downstream pro-apoptotic targets such as Bad, Bax, Bim, Foxo [52, 74–76]. Apart from the Ras oncogene, the cell survival can be also regulated by the other oncogenes such as c-Myc oncogene that induce hMre11 signals and improve the cell survival in many cancers such as cervical carcinoma, colon cancer, leukemia, lymphoma, and testicular cancer (Fig. 6.2) [77, 78].

6.4 ROS and Endogenous Signaling Molecules

It is widely known that there is a tight relationship between the ROS generation and the endogenous signaling molecules such as the growth factors and cytokines, which regulate the molecular mechanisms of many cellular phenomena such as proliferation, growth, invasion, healing, differentiation, metastasis, etc., by involving the intracellular and intercellular signaling pathways [53, 79]. Although the ROS

production can be induced by the intracellular growth factors and cytokines such as epidermal growth factor (EGF), endothelial cell growth factor (ECGF), transforming growth factor beta 1 (TGF-β1), and hepatocyte growth factor (HGF), the elevated ROS in turn can stimulate the multiple growth factors and cytokines that play crucial roles in carcinogenesis by binding to the cell membrane receptors such as receptor serine/threonine kinases, G protein-coupled receptors, receptor tyrosine kinases, and cytokine receptors [32, 73, 79–81]. This phenomenon between the endogenous signaling molecules and ROS display the existence of a positive feedback loop [32]. For example, it has been reported that ROS production in several culture systems may be elevated by TGF-β1, which plays substantial roles in growth regulation and tumor cell progression as a multipotent cytokine [80–82]. Similarly, the tight relationship has been shown between ROS production and HGF, which is known as a prognostic marker for hepatocellular carcinoma, ROS can mediate the HGF receptor and c-met signaling [83–85]. Moreover, the superoxide level in a cell can be elevated by the stimulation of angiotensin, epidermal growth factor (EGF), lysophosphatidic acid, platelet-derived growth factor (PDGF), and tumor necrosis factor-α (TNF-α) though the oncogenic mutation of RhoGTPase K-ras has been reported to be related with the elevation of superoxide level and the incidences of several cancers [30, 53, 86–91]. As well as the oncogenic mutation of RhoGTPase K-ras, the major ones of growth factors and cytokines including HGF, PDGF, vascular endothelial growth factor (VEGF), and TNF-α increase the production of ROS through NADPH oxidases or mitochondrial electron transport chain system depending on the cellular environment [32, 92, 93]. Although the structures of NADPH oxidases are similar to each other, their regulatory subunits and activation mechanisms are different from each other. For example, p22phox is a necessity for the activation of NOX4 though the other NADPH oxidases do not need it [94, 95]. Moreover, NOX4 can be activated by the influences of various growth factors and receptors such as TGF, bone morphogeneticprotein-2 (BMP-2), insulin like growth factor-I (IGF-I) and toll like receptor 4 (TLR4), and the activated NOX4 plays role in the ROS generation [96–98]. As the effects of NOX-generated ROS production, the relationship between endogenous signaling molecules and ROS generation generally affects the fate of cancer hallmark such as cancer cell proliferation and cell survival, angiogenesis, invasion, metastasis, and increased genomic instability by the altering and blocking of related signaling cascades [52, 99].

6.5 ROS and Emerging Hallmarks of Cancer

As widely known, Hanahan and Weinberg published an influential paper in the year 2000 that describes the hallmark of cancer, including six major traits, and they updated the described cancer hallmarks in 2011 by adding two emerging and two enabling traits of cancer [6, 7]. The emerging hallmarks of cancer have been described as deregulating the cellular energetics and evading the immune response [6]. It is not surprising that there is a strict relationship between the emerging hallmarks of cancer and the intracellular accumulation of ROS, and the elevated

metabolic activity, oncogenic signals, and genetic changes in cancer cells induce an increased ROS production along with the adaptation to the antioxidant system and the compensation for the oxidative damages [31, 100]. Thus, alterations occur in the redox homeostasis and cellular signaling pathways, and cancer cell metabolism is reprogrammed [101]. Cancer cells acquire adaptations to survive under hypoxic conditions and utilize alternative metabolic pathways because of their higher metabolism than the normal cells [23, 101]. This alteration in the energy metabolism of cancer cells was firstly discovered in 1924 by Otto Warburg, who reported that cancer cells convert glucose to lactate using glycolytic pathway instead of pyruvate regardless of the presence of oxygen [102]. This phenomenon has been known as Warburg effect, which contributes an aggressive cancer phonotype because a prolonged survive under hypoxic condition leads to a series of alterations in genetic stability, metabolic pathways, organelles, etc., though the hypoxic condition leads to cell death in normal cells [52, 103]. It can be clearly seen that there is a reciprocal crosstalk between the redox balance and metabolic pathways such as glycolysis, the pentose phosphate pathway, one-carbon metabolism, fatty acid oxidation, and glutaminolysis [31, 104]. For example, redox homeostasis can be regulated by glycolysis through shuttling of the pentose phosphate pathway-generated intermediate nicotinamide adenine dinucleotide phosphate (NADPH) and glutaminolysis-generated intermediate glutathione (GSH) [52]. Although it is expected that the glucose-deprivation causes cell death by the accumulation of hydrogen peroxide, the Warburg effect provides the cancer cells to acquire adaptation of the glucose-deprivation by exaggeratedly using glycolysis pathway to prevent hydrogen peroxide-induced cell death [105, 106]. Targeting glycolysis and lactate dehydrogenase enzyme is therefore considered a successful strategy to prevent the cancer cell progression by inducing oxidative stress and decreasing the production of the intracellular ATP [31, 107–109]. For example, let-7a that is an early-discovered microRNA was used as a therapeutic enhancer because let-7a elevates the ROS generation and downregulates some enzymes involved in glycolysis such as glucose 6-phosphate dehydrogenase (G6PD) and inosine monophosphate dehydrogenase (IMPDH) [110]. Pyruvate kinase muscle isoenzyme 2 (PKM2), the isoenzyme of the rate-limiting glycolysis enzyme named pyruvate kinase, plays a crucial role in reprogramming cancer metabolism, but the ectopic expression of microRNA-1 and microRNA-133b inhibits PKM2 through silencing polypyrimidine tract-binding protein 1 (PTBP1), which can convert the active PKM2 to the inactive PKM1 [111, 112]. On the other hand, it is well documented that cancer cells produce an elevated level of ribose 5-phosphate by employing pentose phosphate pathway that is considered a key feature for many cancers, and regulate the ROS homeostasis through NOXs and replenishing the decreased GSH and TRX [31, 113]. As previously mentioned, mitochondria is considered as one of the major sources of ROS because they are inevitably generated in oxidative phosphorylation as the byproducts [114]. The elevated ROS accumulation because of hypoxia causes oxidative stress and consequently results in damages of organelles and the other cellular components such as lipids, proteins, metabolites, etc. [115, 116]. Moreover, the structure, morphology, and dynamics of mitochondria are considered linking with the

accumulated amounts of ROS, i.e., there is a mutual interaction between mitochondria and ROS [117]. The overproduction of ROS induces mitochondrial damages and these damages result in the elevated ROS production, so this phenomenon is called as ROS-induced ROS release [115, 118, 119]. For example, mitochondria induce elevated production of ROS under hypoxic condition and the hypoxia-induced ROS production can cause the mitochondrial fragmentation though the mitochondrial fusion is considered as a cellular adaptation process for the alterations in the surrounding environment, and which can prevent the elevated production of ROS [120, 121]. One of the most prominent transcription factors for the cellular adaptation to the hypoxic conditions is hypoxia inducible factor-1 (HIF-1) that is a heterodimer consisted of two subunits HIF-1α and HIF-1β, and it is well known that ROS play a key role in the accumulation of HIF-1 [52, 122, 123]. The increased levels of antioxidants reduce the accumulation of HIF-1 though the increased levels of hydrogen peroxide and superoxide elevate the accumulation of HIF-1 [63, 124, 125]. Apart from the endogenous ROS, the exogenous ROS can alter mitochondrial dynamics by inhibiting mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) and inducing the depolarization of mitochondrial membrane potential, which trigger the mitochondrial fission along with the overproduction of ROS [115, 126]. Moreover, the oxidative stress-induced mitochondrial fission and fusion influence on the mitochondrial metabolism and function because of the dramatic changes in mitochondrial DNA (mtDNA), ribosomes, proteins, metabolites, etc., that lead to many diseases including cancers, cardiometabolic diseases, neuropathies, and neurodegenerative diseases [127–129].

As previously mentioned, the second emerging hallmark has been described as evading the immune response, and ROS have been identified as immunosuppressive factors in the cancer microenvironment to facilitate the other cancer hallmarks such as growth, invasion, and metastasis [6, 31, 130]. Apart from the pathological conditions such as cancer, ROS play key roles in the regulation of immune responses and serve as central mediators of immune cells [131]. For example, dendritic cells (DC) that have a substantial role in antigen specific immune response as the major antigen-presenting cells (APC) are activated by hydrogen peroxide, which can be produced in a large quantity by phagocytic cells [132]. On the other hand, the pathological conditions such as cancer or chronic inflammatory diseases can alleviate the function of natural killer cells (NK cells) and the effector T cells depending on the macrophages- and granulocytes-generated ROS levels [133–135]. Although NK cells increase the ROS production in the early stage of the encounter with cancer cells to mediate cytolysis, it has been reported that monocyte-generated ROS production in cancer patients inhibits the interferon gamma (IFN-γ) production, proliferation, activation, and cytotoxicity of NK cells along with the induction of NK cells' apoptosis [136, 137]. The differences of NK cells responses to the ROS generation are regulated by the $CD56^{bright}$ and $CD56^{dim}$ that are the NK cells antigen subsets [138]. Monocyte-derived ROS direct $CD56^{dim}$ NK cells to apoptosis though the $CD56^{bright}$ NK cells display a significant resistance to the ROS-induced functional inhibition and apoptosis because of their stronger antioxidant capacity than $CD56^{dim}$ NK cells [139–141]. Thus, the resistance of $CD56^{bright}$ NK cells provides

the cancer cells to evade immune system because the ROS-sensitive $CD56^{dim}$ NK cells have higher cytotoxic activity than the ROS-resistant $CD56^{bright}$ NK cells [136]. Similarly, the oxidative stress in the cancer microenvironment provides cancer cells to evade immune system by regulating the accumulation of different subsets of T cells because the conventional T cells are more sensitive to the hydrogen peroxide-induced cell death than the regulatory T cells (T_{regs}) that have an ability to inhibit the functions of other infiltrating immune cells [142–144]. Moreover, either the functions of T cells can be suppressed or the apoptosis of T cells can be induced via Jak3/STAT5 signaling pathway, which is regulated through the inducible nitric oxide synthase (iNOS)-generated nitric oxide (NO) production by myeloid-derived suppressor cells (MDSCs) [136, 145–147].

6.6 ROS and Enabling Hallmarks of Cancer

In the paper published by Hanahan and Weinberg [6], two enabling hallmarks of cancer have been added to the previously described six hallmarks along with the two emerging hallmarks, and these two enabling hallmarks have been described as the tumor-promoting inflammation, and the genome instability and mutation [6, 7]. Actually, the relationship between inflammation and carcinogenesis has been known far before the paper of Hanahan and Weinberg [6]. In 1863, Rudolf Virchow reported that the "lymphoreticular infiltrate" reflected the origin of cancer at the locations of chronic inflammation by observing white blood cells or leukocytes in neoplastic tissues [23, 148–150]. The currently known data obtained from the numerous studies performed after the Virchow's hypothesis clearly indicate that there is a tight and intricate relationship between the cancer progression and the promotion of inflammation coordinated by the level of inflammatory cytokines (TNF, interleukin-1 (IL-1), and IL-6), chemokines (CXC chemokine receptor 4 (CXCR4) and IL-8), and inflammation-related factors, especially located in the tumor microenvironment [151–153]. Moreover, it is well known that ROS predictably take important roles in the regulation of the sophisticated interaction between the course of cancer and the promotion of inflammation by effecting the presences, levels, and types of the inflammatory cytokines, chemokines, and inflammation-modulating factors such as activator protein 1 (AP-1), HIF-1α, specificity protein 1 (Sp1), β-catenin, wingless-type MMTV integration site family (Wnt), HIF-1α, NF-κB, peroxisome proliferator-activated receptors-gamma (PPAR-γ), p53, signal transducer and activator of transcription 1 (STAT1)/STAT3, and nuclear factor erythroid 2-related factor 2 (Nrf2) [154–157]. A series of signal transduction cascades can be activated by the accumulation of inflammatory cells in the tumor microenvironment, which provokes the further recruiting of inflammatory cells by producing cytokines and chemokines, and a massive ROS production is therefore occurred by the activation of the oxidation-related enzymes such as iNOS, NOX, XO, and myeloperoxidase (MPO), and the upregulation of the expression of COX2 and LOXs [156, 158]. The massively produced ROS leads to significant oxidative damages in genetic materials, macromolecules, and organelles, which support the progression of carcinogenesis

and overpowered production of supplemental ROS, and this excessively produced ROS activate again the inflammatory cytokines, chemokines, and inflammation-modulating factors. This phenomenon may be named as "inflammatory response-mediated ROS-induced ROS release" by considering its similarity with "ROS-induced ROS release" because ROS-induced inflammatory responses release excessive ROS that induce again inflammatory responses [154, 155, 159, 160].

The other enabling hallmark of cancer has been described as the genome instability and mutation, the main cause of the genetic diversity in many cancers and the cancer cell heterogeneity within the tumor tissue, and the overproduced ROS are known as the prominent factors leading the oxidative DNA damages, including base damages and modifications, deletions and insertions in DNA sequence, DNA miscoding lesions, DNA single-strand and double-strand breaks, gene amplification, and the activation of oncogenes, which contribute in cancer initiation and progression [26, 31, 161–164]. It can be possible to say that there is a cycle between the overproduction of ROS and the oxidative DNA damages. As previously mentioned, the elevated ROS level can activate the oncogenes such as Bcr-Abl, c-Myc, and Ras, which can play substantial roles in the regulation of tumor suppressor genes, cancer cell proliferation, mitochondrial dysfunction, angiogenesis, and metastasis, and the oncogene activation is known as the main cause of the overproduction of ROS that leads to the formation of replication stress [165–167]. For example, it has been reported that the replication fork velocity can be reduced because the polymerase activity is affected by the occurrences of ROS-oxidized deoxyribonucleotide triphosphates (dNTPs) [168, 169]. The progression of replication fork can be regulated by ROS by dissociation of peroxiredoxin2 oligomers (PRDX2), and the fork accelerator named TIMELESS can be inhibited by a replisome associated ROS sensor formed PRDX2 [170]. Thus, the replication fork speed can be reduced through the dissociation of PRDX2 and TIMELESS, which is regulated by the overproduced ROS [170]. Moreover, the replication forks can be prevented physically due to the occurrences of oxidized bases, and this phenomenon can cause the breakdown of replication forks at fragile sites across the genetic material along with the under-replicated or over-replicated DNA [64, 171]. On the other hand, the highly accumulated ROS may directly effect on DNA through reacting with purines, pyrimidines, and chromatin proteins, and causing the DNA single-strand and double-strand breaks [172, 173]. A point mutation, for example, can be formed because of the production of 8-hydroxy-2′-deoxyadenosine (8-OH-dAdo) or 8-hydroxy-2′-deoxyguanosine (8-OHdG) that are the widely known oxidative DNA damage markers, which can be occurred through the reaction of hydroxyl radicals with adenine or guanine nucleotides, respectively [173–176]. Additionally, it should be noted that quinine is considered as the most sensitive nucleobase to oxidation than other nucleotides, and so 8-OHdG emerges as the most common oxidized nucleobase [177]. Apart from the 8-OHdG, there are some other oxidative DNA damage markers such as 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-oxo-7,8-dihydroadenine (8-oxoAde), 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG), 5,6-dihydroxy-5,6-dihydrothymine, 2,6-diamino-4-hydroxy-5-formamido-

pyrimidine, and 4,6-diamino-5-formamido-pyrimidine [29, 172, 178]. ROS-induced production of oxidized nucleobases generally leads to further mutations and DNA damages along with the accumulation on some specific location such as telomere sites, which are less efficiently repaired than the other genomic sites [179]. 8-oxoGua can be, for example, accumulated on the telomere sites, and behaves as a blockage for telomerase activity through reducing the binding potential of telomeric proteins, disrupting telomere length, and precluding of chromosomal-end capping, and this phenomenon can result in cell death, aging, carcinogenesis, chromosome instability, and genotoxic formations such as nuclear buds (NBUDs), nucleoplasmic bridges (NPBs), and micronuclei (MN) [179–181].

6.7 ROS and Angiogenesis

Angiogenesis can be considered as one of the most important cancer hallmarks because cancer cells rapidly proliferate to form and expand the tumor tissue, but the tumor tissue expansion increases the distance between cells and capillary vessels [12]. However, the appropriate distance between the cells and capillary vessels is restricted to 100–200 μm to maintain the balanced composition of oxygen, carbon dioxide, nutrient substances, and metabolic wastes [182, 183]. Additionally, the tumor tissue enlargement provides a hypoxic, hypoglycemic, hypoferric, and acidified microenvironment along with the occurrence of an intolerable mechanical stress on the cancer cells, and so the cancer cells are driven to migrate, invade, and metastasize [12]. The cancer cells induce therefore angiogenesis to form new capillary vessels originated from the existing vessels, run away from the stressed microenvironment by participating in the circulatory system, and sustain the course of carcinogenesis [184]. Angiogenesis is regulated via an angiogenic switch, which can be opened and closed by variation of the balance between angiogenesis promoting (angiogenic) and suppression (anti-angiogenic) factors [185]. The formation of ROS and the occurrence of oxidative stress within the cells and microenvironment predictably regulate the direction of the angiogenic switch along with the activation of angiogenic or anti-angiogenic factors through the regulation of transcriptional factors, releasing of some growth factors, and alteration of the cellular signaling cascades (Fig. 6.3) [186, 187]. For example, the cancer cells in a hypoxic microenvironment can induce the releasing of proangiogenic growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGFB) and the increased production of the other angiogenic proteins such as angiopoietin-1, leptin, endoglin, prominin-1, transforming growth factor beta (TGF-beta), integrins, and matrix metalloproteinase (MMP) enzymes [12, 188–190]. After the opening of angiogenic switch and the formation of new capillaries surrounding the tumor tissue, the tumor cells and their microenvironments are re-oxygenated. Contrary to the expectations, the tumor-induced angiogenesis and re-oxygenation of tumor cells lead to larger problems instead of solving the problem of cancer cells [187].

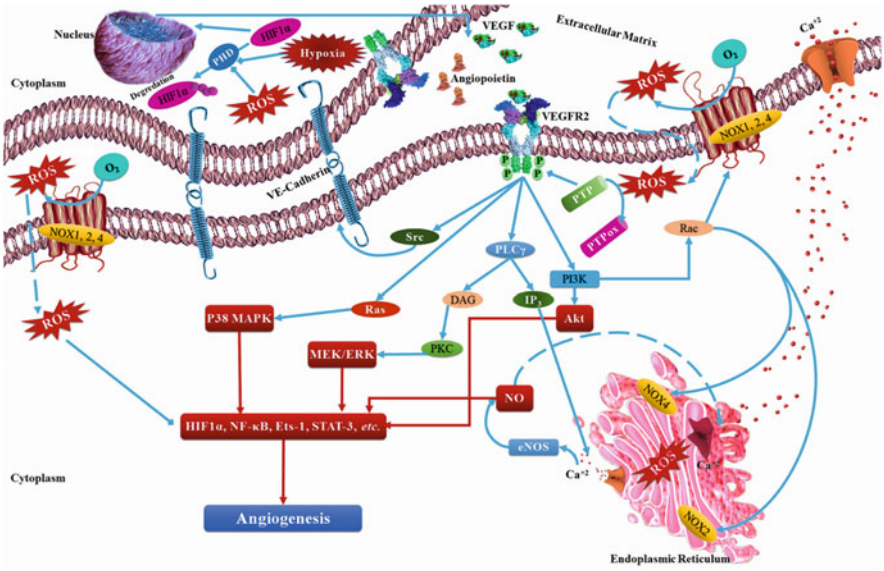

Fig. 6.3 ROS and VEGF-mediated angiogenesis. Hypoxic environment and elevated ROS increase the production of angiogenic factors such as VEGF and angiopoietin, and activate a series of signaling cascades to regulate angiogenesis

Although the hypoxic microenvironment induces an overproduction of ROS by disrupting the mitochondrial metabolism and the electron transport system, an oxygen abundance occurs because of the angiogenesis-motivated re-oxygenation phase, which results in 100 times higher ROS production than hypoxic state [191–193]. This phenomenon is named as "cyclic hypoxia" because hypoxia-induced angiogenesis leads to an excessive production of ROS and these ROS induce again angiogenesis through the direct activation of HIF-1α, VEGF, and VEGFR2, and the oxidation of lipids that stimulate NF-κB pathway-mediated angiogenesis [187]. ROS-induced equilibrium corruptions in the angiogenic switch can lead to many structural and functional abnormalities within the newly formed capillaries surrounding tumor tissue, and these abnormalities result in hyperpermeability, hypoglycemia, hypoxia, abnormal blood flow, and increased pressure, which also increase the ROS production [187, 194]. Additionally, ROS such as superoxide anion and hydrogen peroxide molecules have a special importance for the vascular cells because they can regulate the fate of these cells depending on the concentrations [195]. For example, the low concentrations of hydrogen peroxide such as 0.1–10 μM induce the capillary tube-like formation of endothelial cells though its high concentrations (>125 μM) induce lethal damages [187, 195]. Consequently, ROS have a substantial role in the regulation of angiogenesis though the tumor-induced angiogenesis is one of the major causes of the excessive ROS production, and it is widely considered that the main source of ROS caused by tumor-induced angiogenesis is mitochondria and electron transport system because

of the hypoxic condition and the cyclic hypoxia-induced oxygen abundance [12, 187].

6.8 ROS in Cancer Cell Invasion and Metastasis

Cancer cell invasion and metastasis are commonly considered as the carcinogenesis processes that can be possible depending on the formation of angiogenesis though the metastasis can be observed in many solid tumors regardless of the early or late stages of the carcinogenesis [6, 187, 196]. Besides the similarities of the underlying reasons of the tumor-induced angiogenesis and metastasis, the close interaction between angiogenesis and metastasis has been known since the first observations of Judah Folkman (1971) and Pietro Gullino (1978) [189, 197–199]. As previously mentioned, the uncontrolled expansion of tumor tissue results in an unsuited microenvironment qualified with the hypoxic, hypoglycemic, hypoferric, and acidified features along with the mechanical stress, and so the cells forming tumor tissue would like to escape from this microenvironment by inducing angiogenesis and operating the complex processes of metastasis [12, 187]. Metastasis can be occurred employing a series of cellular phenomena, including the degradation of extracellular matrix (ECM), losing the cellular polarity and detaching from the ECM, cancer cell invasion along with the amoeboid or mesenchymal migration, accessing to the capillary vessels, intravasation, sustaining the anchorage-independent growth and survival by evading anoikis (anchorage-dependent apoptosis), bypassing the immune surveillance, extravasation, adhesion, proliferation, and colonization within the secondary tumor site [200–203]. Numerous papers have revealed that ROS have substantial regulative roles in the complex processes of metastasis as well as in the angiogenesis, and many clinical and experimental data have suggested that the level of ROS is changed during the metastasis [204]. For example, several studies reported that the overproduced ROS induce the epithelial to mesenchymal transition (EMT), a biological phenomenon that acts on the metastasis-related cellular functions such as the cell–cell and cell–matrix interactions along with the cellular motility and migration, and EMT can be regulated by various cytokines such as TGF-β1 and EGF, transcription factors including Twist, Snai1, Slug and ZEB1/2 (zinc-finger E-box-binding homeobox), and signaling pathways such as the inhibitory kappa B kinase (IKK)/NF-kB, MAPK, Notch, PI3K/Akt, TGF-b/Smad, and Wnt/b-catenin signaling pathways [205–208]. Although the cell invasion is facilitated by the elevated expression of urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), the cell–cell and cell–matrix adhesions are emaciated by decreasing the epithelial markers and tight junction proteins such as occludin, claudin, and e-cadherin, and increasing the mesenchymal markers such as fibronectin, vimentin, and n-cadherin [205, 208]. The overproduced ROS are commonly considered as the prominent regulator for the processes of EMT and metastasis, and ROS-induced cancer cell metastasis by affecting the molecular pathways, transcription factor, cytokines, and growth factor have been extensively reviewed by many scientists [204, 205, 209–212]. For example, one of the prominent inducer of EMT named

TGF-β1 can be regulated by ROS-dependent pathway; the Rac1-NOXs-ROS-dependent activation of NF-κB pathway mediates the TGF-β1-regulated uPA and MMP9 activities on cell migration and invasion [210, 213]. Additionally, the Rac-dependent ROS production has been suggested to be related to the activities of MMPs (e.g., MMP2, MMP3, and MMP9) and the transduction of mechanical perturbations into a pro-invasive gene expression [209, 214–216]. Moreover, the loss of TGF-β1-activated kinase 1 (TAK1) can lead to the integrin-Ras-induced ROS production that activates the EMT signaling cascade [217]. Pelicano and coworkers reported that mitochondria-derived ROS production leads to the AP-1 signaling pathway-mediated upregulation of C-X-C motif chemokine 14 (CXCL14) expression and the boost in cell motility by increasing the amount of cytosolic Ca^{2+} levels [218]. On the other hand, the evading anoikis (anchorage-dependent apoptosis), which is the most important part of metastasis is succeeded by ROS-dependent mechanisms. Anoikis resistance of cancer cells can be conferred through NOX4-induced ROS-activated the epidermal growth factor receptors (EGFR) and angiopoietin-like 4 (ANGPTL4)-integrin complex-induced ROS-activated PI3K/Akt and ERK pathway [219–221].

6.9 ROS and Cancer Cell Death Pathways

Although the overproduced ROS are well known as a key factor in the initiation and development of cancer through the disrupting effects on the genetic materials, cellular macromolecules, organelles, signaling cascades, components, and homeostatic balances along with the significant contribution in the cancer cell survival, the disproportionately increased ROS emerge as a substantial approach for the cancer treatment strategies because of the cell death provoking activity [23, 52]. Apart from the non-inflammatory, caspase-independent, and ROS-sensitive special cell death pathway named "oxeiptosis," there are well-described ROS-induced cell death pathways such as caspase-dependent apoptosis, caspase-independent ferroptosis, and necroptosis, inflammasome-driven pyroptosis, and autophagic cell death (Fig. 6.4) [222–227].

Kelch-like ECH-associated protein 1 (Keap1) is known as a main sensor to monitor oxidative and electrophilic stress, and regulates the expression of cytoprotective molecules by ubiquitination and degradation of Nrf2 under the physiological conditions, though the overproduced ROS-oxidized Keap1 leads to insufficient expression of cytoprotective molecules and highly expressed antioxidant factors, viz., NAD(P)H quinone dehydrogenase 1 (NQO1), homeobox protein 1 (Hox1), and Thioredoxin (Txn) because of the highly accumulated Nrf2 [227, 228]. Besides the accumulation and translocation of Nrf2, the oxidized Keap1 could not interact with the phosphoglycerate mutase 5 (PGAM5) that is known as a common factor for many caspase-independent cell death pathways, and so the released PGAM5 dephosphorylates the apoptosis inducing factor mitochondria associated 1 (AIFM1) at Ser116 [227, 229]. Thus, AIFM1-deficient cells undergo to the oxeiptosis by ROS-induced cell death pathway that includes KEAP1, PGAM5 and AIFM1 [227].

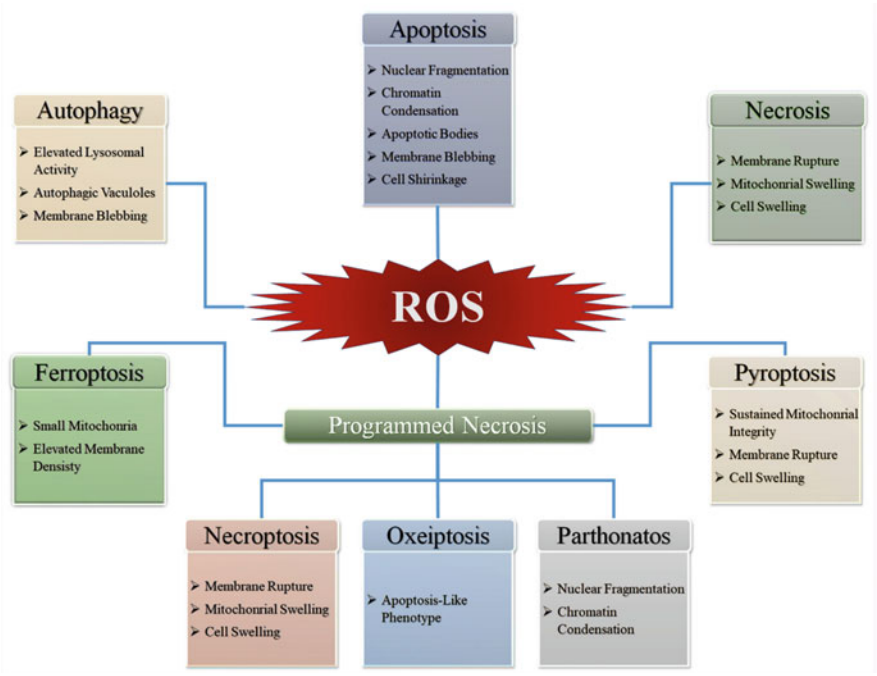

Fig. 6.4 ROS-induced cell death pathways

On the other hand, the ROS-induced caspase-dependent cell death pathways are well known and the cellular pathways have been broadly described in many papers [222–227]. For example, cytochrome-c is released from mitochondria because of the ROS-induced mitochondrial abnormalities and dysfunctions, and so apoptosome complex can be formed by the incorporation of the released cytochrome-c, Apaf-1 (apoptotic peptidase activating factor 1), and procaspase-9 to activate effector caspases, e.g., caspase-3, which leads to the cleavage of cellular proteins and apoptosis [23, 230]. Additionally, the intracellular accumulation of ROS regulates the expression of the pro-apoptotic (Bad, Bak, Bax, Bid, and Bim) and anti-apoptotic (Bcl-2, Bcl-w, and Bcl-xL) members of the Bcl-2 family via their phosphorylation and ubiquitination, and the Bcl-2 family proteins play key roles in the regulation of the mitochondrial membrane permeabilization and apoptotic signaling [52, 231, 232]. The other well-known ROS-induced cell death is autophagy that can be regulated by several kinase cascades such as the most familiar mammalian target of rapamycin complex1 (mTORC1), which can be regulated by PTEN/PI3K/AKT signaling pathway [233]. The overproduced ROS-induced autophagy results in degradation of the mitochondria that excessively produce ROS, and so this kind of autophagy is called as mitophagy that leads to the reduced ROS levels as a result of the NIX/BNIP3L and PARKIN/PTEN induced putative kinase 1 (PINK1) molecular pathways [52, 234–236]. Moreover, the ROS-induced autophagy can be occurred through Nrf2/Keap1 pathway by preventing degradation of Nrf2 as well as the

ROS-induced oxeiptosis [237]. The attentions of many scientists seem to be focused on the ROS-dependent cell death pathways because there is certainly a complex relationship between the intracellular ROS and the cell death pathways, and the ROS-mediated anticancer drugs and treatment strategies are commonly considered as the beneficial treatment modalities.

6.10 ROS and Anticancer Treatment Strategies

Although unexpected and long-term changes in the intracellular ROS level are considered as the main factors for the occurrence of extremely complex cellular processes that induce carcinogenesis, numerous studies have shown that cancer cells are more susceptible to the changes in intracellular ROS accumulations and more dependent on the antioxidant systems than their healthy counterparts [39]. The exogenous ROS generation is therefore considered as a promising option for the anticancer treatment strategies because the vulnerability of cancer cells towards oxidative stress provides a therapeutic selectivity in anticancer therapies [238]. ROS-dependent treatment strategies are generally based on three different approaches such as directing cancer cells to the cell death pathways by promoting an excessive ROS generation, activating ROS-dependent cancer cell death by blocking the antioxidant systems, and inhibiting carcinogenesis by reducing ROS generation via activating antioxidant systems and employing antioxidant molecules. There are many chemotherapeutic agents that increase ROS generation to selectively induce cancer cell death because of the ROS-induced irreparable damages [54]. Examples of these chemotherapeutics include, but not limited to the arsenic trioxide, anthracyclines (e.g., daunorubicin, doxorubicin, epirubicin, and idarubicin), bleomycin, β-lapachone, cisplatin, elesclomol, and sulindac [54, 238–240]. These drugs can induce ROS generation by using different cellular mechanisms. For example, doxorubicin that is a topoisomerase inhibitor, DNA intercalation agent, and also one of the most known chemotherapeutics employed in the treatment of many cancers, including bile duct, breast, endometrium, esophagus, gastric, pancreatic and liver cancers, osteosarcoma, Kaposi's sarcoma and soft tissue sarcomas, Hodgins and non-Hodgins lymphomas, induces intracellular ROS generation by reacting with flavoprotein reductases, intracellular chelation of iron, which respectively result in apoptosis and ferroptosis [52, 241, 242]. Apart from the application of chemotherapy-induced ROS generation, there are different cancer treatment strategies that induce intracellular ROS generation, such as photodynamic cancer therapy (PDT) and sonodynamic cancer therapy (SDT) [243, 244]. PDT is a non-invasive and clinically approved treatment method that induces excessive ROS generation in the presence of molecular oxygen thanks to the synergic interactions of a non-thermal light source and a nontoxic photosensitizer molecule to induce apoptosis by damaging the cellular components of target cells [244, 245]. Similar to the PDT, the ultrasound-mediated cancer therapy (SDT) induces apoptosis in the target cells through the production of ultrasonic cavitation, sonochemical bubble collapse, and finally free radicals and ROS generation

[243, 246]. The second ROS-dependent cancer treatment approach emerges as the suppressing cellular antioxidant systems (e.g., glutathione and thioredoxin) resulting in the overproduced ROS-dependent activation of cell death pathways, and examples of these chemotherapeutics include 2-methoxyestradiol, buthionine sulfoximine, phenylethyl isothiocyanate, imexon, mangafodipir, and tetrathiomolybdate [39]. For example, buthionine sulfoximine, phenylethyl isothiocyanate, and imexon lead to the increased accumulation of intracellular ROS by reducing the intracellular GSH level [247–249]. Moreover, it has been thought that the redox adaptation mechanisms can be evaded by combining the first and second ROS-depended anticancer treatment approaches, viz., the promoting an excessive ROS generation and the suppressing cellular antioxidant systems [39]. The last one of the ROS-dependent treatment approaches is known as the targeting ROS production by using antioxidant molecules that can be employed as a cancer preventive therapy by using daily dietary compounds such as green tea-derived epigallocatechin-3-gallate (EGCG), carotenes vitamin C and vitamin D [250, 251]. However, it should be noted that there are also some reports indicating that some antioxidants such as carotene, vitamin A, and vitamin E can be effective on the elevated risk of cancer [252–254]. The substantial contributions of ROS into the drug resistance development processes and further progression of carcinogenesis should be also noted, because the overproduced ROS are well known as the prominent factors for the oncogenic signaling, genetic instability, and DNA damages along with the metabolic adaptations, enhanced proliferation, and survival [255]. Consequently, it should be thoroughly considered the advantages, disadvantages, and the exact activity mechanisms of the ROS-dependent anticancer therapies because it is clear that targeting redox homeostasis of cells may lead to the unexpected and unwanted consequences as well as the expected and wanted outcomes.

6.11 Concluding Remarks and Future Prospects

As can be clearly seen in the previous parts of the chapter, ROS play substantial roles in the regulation of physiological homeostasis such as the controlling of cellular signaling cascades via low-level productions and the provoking of cell death pathways via overproductions. Although numerous studies have shown that more than 150 human disorders are related to the disruption of redox homeostasis, the bulk of ROS-mediated intracellular signaling pathways and the consequences remain unknown. It is well known that cancer cells induce the overproduction of ROS and the elevated ROS production contributes in the progression of carcinogenesis through provoking the DNA damage and genetic instability, cancer cell proliferation and survival, metabolic adaptations, and drug resistance. Interestingly, ROS-induced cell deaths in a cancer tissue can result in a more aggressive and chemotherapy resistant cancer tissue in some cases because the elevated ROS can kill the sensitive cancer cells though the aggressive ones can cope with the same amount of ROS. Conversely, the combination therapy that is employed by using redox-active

molecules and conventional treatment strategies is considered as a rational option to overcome chemotherapy resistance. Many anticancer drugs, for example, kill the cancer cells by activation of ROS-dependent cell death pathways though the cancer cells develop resistance towards them by activating the antioxidant systems. The antioxidant system inhibitors can be therefore employed to evade the cancer cell resistance. It should be also noted that the anticancer drugs that kill the cancer cells by ROS-dependent cell death pathways are preferred more to perform a selective treatment because the cancer cells are regarded as more sensitive that health counterparts due to the lack of redox homeostasis. On the other hand, the antioxidant dietary substances are generally considered as beneficial to preclude carcinogenesis and many scientists have recommended people to include these substances in their daily diets to keep them away the cancer risks. However, some papers have displayed the link between the increased cancer risks and some dietary antioxidants such as carotene, vitamin A, and vitamin E. Thus, the activity mechanisms of the dietary antioxidants need to be extensively investigated to understand well their benefits and harms, and give recommendations to the people who would like to keep themselves healthy. Although the consequences of the enhanced ROS production in the cells seem to be not predictable because of the dependence on many different factors, the ROS-mediated treatment strategies such as photodynamic therapy and sonodynamic therapy seem to be promising because of their non-invasive features. The relationship between microRNAs and ROS was not extensively discussed to keep concise in this chapter, but this relationship seems to be substantial for the regulation of many intracellular signals and epigenetic changes. Moreover, the extensive investigations of ROS-mediated effects of drugs on epigenetic mechanisms seem to be quite beneficial because ROS are known as the effective factors on epigenetic regulations and aberrations that play crucial roles in cancer heterogeneity and carcinogenesis. Consequently, ROS are considered as a double-edged sword and the effects of the edges should be therefore extensively investigated by developing interdisciplinary projects and collaborations to understand well and employ efficiently this sword as a weapon, target, or mediator in the cancer treatment modalities.

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Inflammatory Mediators: Potential Drug Targets in Cancer

7

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Abstract

Inflammation or allergic responses are bio-physiological processes that are known to be associated with the progression of disorders such as neurodegeneration and cancer. Previously it has been seen that inflammatory markers such as cytokines and chemokines are elevated several times in cancer patients. Majorly, NF-kβ is one among the other inflammation-inducing pathways, which have been found to be inhibited by various chemotherapeutic drugs. They are also found to induce inhibition of inflammation via p38/MAPK and PI3K/Akt and COX-2 activity. In the present chapter, the advancements in understanding the inflammation-mediated cancer progression and associated preclinical/clinical studies will be discussed.

Keywords

Inflammation · Cytokines · Chemokines · Tumor microenvironment · Cross-talk

7.1 Introduction

In recent years, mounting evidence have underlined the link between inflammation and cancer. Many types of cancers stem from sites of infection, chronic irritation, and inflammation (Table 7.1). Patients suffering from chronic inflammatory

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Table 7.1 Examples of inflammation- and infection-associated cancers

Etiologic agent or condition	Inflammation or Infection	Cancer	References
Asbestos fibers	Asbestosis	Mesothelioma	[1]
Tobacco smoke exposure	Chronic obstructive pulmonary disease	Lung cancer	[2]
Excessive alcohol use history, mutations	Pancreatitis	Pancreatic cancer	[3]
Possible genetic and environmental factors	Inflammatory bowel disease	Colorectal cancer	[4]
Helicobacter pylori	Gastritis, ulcer	Gastric adenocarcinoma	[5]
Hepatitis B And/or C virus	Hepatitis	Hepatocellular carcinoma	[6]
Different etiological factors including infections	Prostatitis	Prostate cancer	[7]
Human papillomavirus	Cervicitis	Cervical cancer	[8]
Bacterial infection in prostate	Prostatitis	Atypical prostate hyperplasia and dysplasia	[9]
Several etiological factors	Thyroiditis	Papillary thyroid carcinoma	[10]

disorders are more likely to have cancer, which has been also well-listed by Kumar Kundu et al. [11]. With the extensive knowledge about the tumor microenvironment, orchestrated by inflammatory cells via chemical mediators, has proved that inflammation is an inevitable participant in the neoplastic progress [12] and anti-inflammatory drugs reduces this risk by prevention and also important for therapy [13, 14].

The response of the body to a cancer has many relationships with inflammation. Recurrent or persistent inflammation has been associated with the induction or promotion processes or it may affect the susceptibility to carcinogenesis. In 1863, Virchow hypothesized that chronic inflammation was at sites of origin of cancer, and according to the hypothesis, several irritants may enhance cell proliferation as a result of the tissue injury and ensuing inflammation [15]. Since the Virchow's early observation regarding the relationship of inflammation and cancer, as also evident from the increasing number of published materials in this field (Fig. 7.1), accumulating data have underlined the fact that tumors may originate at the sites of chronic inflammation or infection [16].

Inflammation is the body's natural defense mechanism against cell injury or tissue damage. Upon tissue damage, mast cells and macrophages secrete molecules that regulate the migration of leucocytes and inflammatory cells to the site of damage. In general, acute inflammation is followed by rapid resolution where irritants are cleared from the host. However, as depicted in Fig. 7.2, when resolution fails, a state of chronic inflammation ensues owing to excess production of cytokines, chemokines, and growth factors that inevitably lead to uncontrolled inflammatory reactions which leads to cancer progress [17].

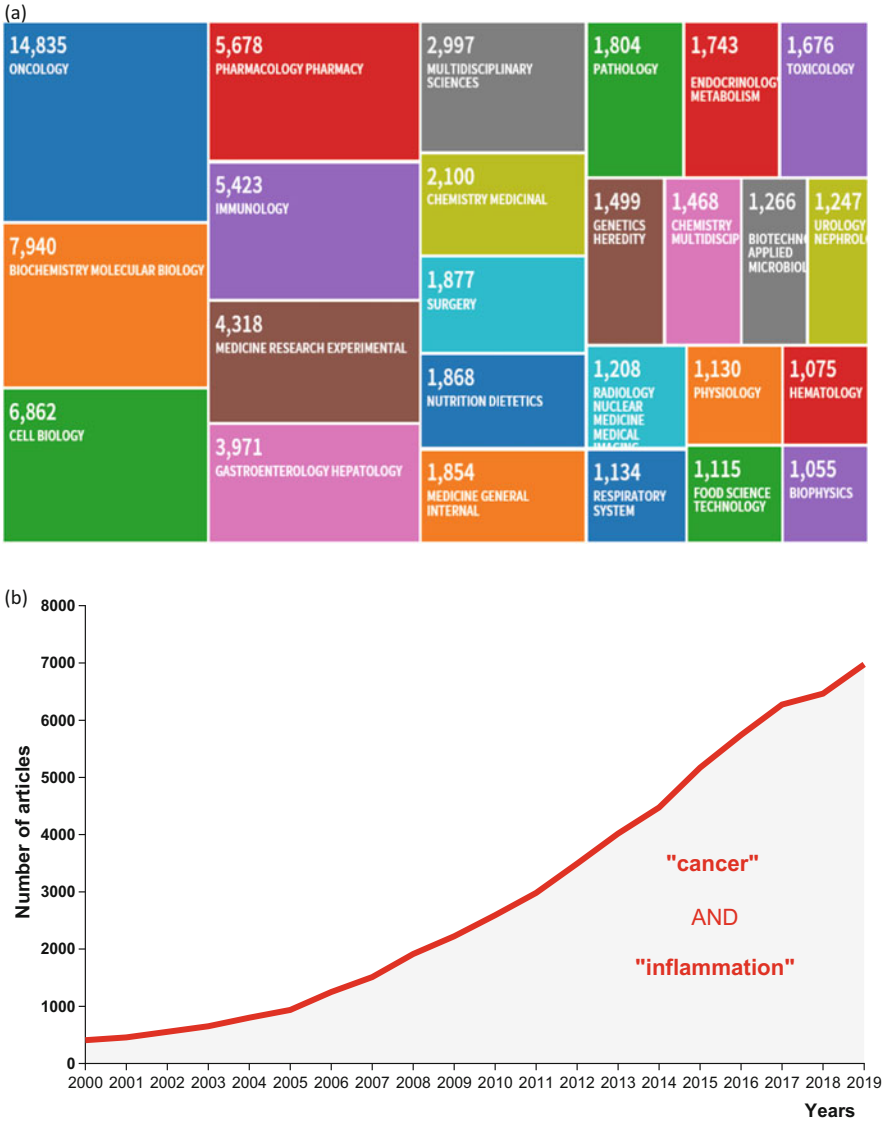

Fig. 7.1 (**a**) Distribution of the top-25 "Web of Science Categories" within the published items regarding the terms "cancer" and "inflammation" (last accessed: June 6th 2020). The distribution of "Web of Science Categories" within the published items on the topics "cancer" and "inflammation" reveals 63,169 records (as of June 6th 2020), while primarily related with "Oncology" as expected (~23.5%), other fields including "Biochemistry Molecular Biology," "Cell Biology," and "Pharmacology Pharmacy" also appear in the tree map. (**b**) The number of publications in the "Web of Science" between 2000 and 2019 per year related to search terms "cancer" and "inflammation." The search of this combination hits 2603 publications in 2020 (last accession: June 6th 2020)

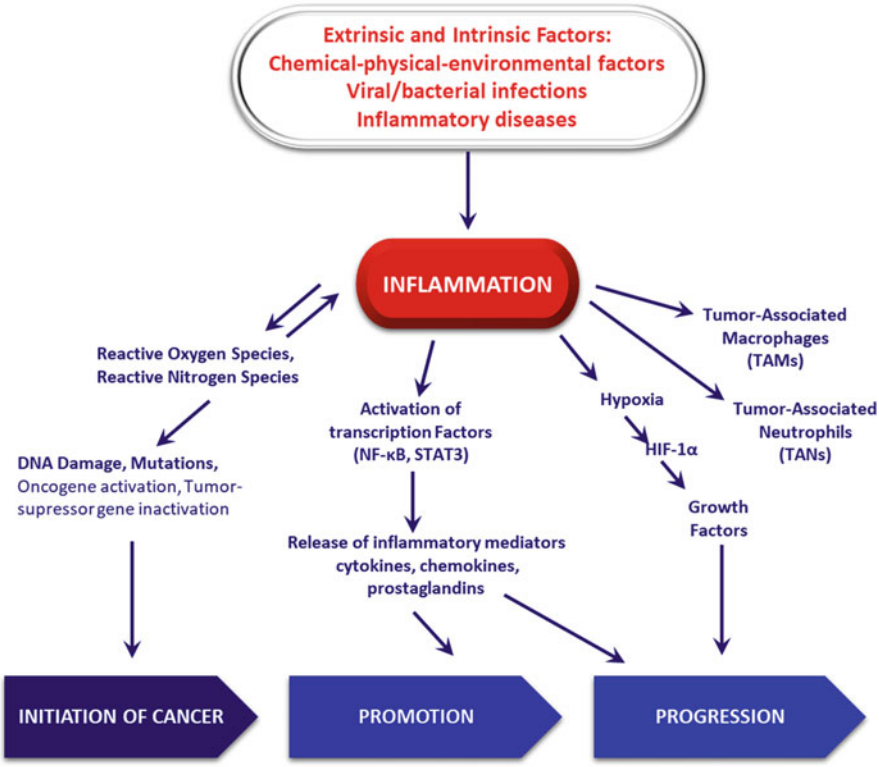

Fig. 7.2 The potential interplay between inflammation and cancer

There are two different paradigms to the link between inflammation and cancer: (1) the intrinsic pathway, and (2) the extrinsic pathway [18]. In the intrinsic pathway, different classes of oncogenes are activated leading to the expression of inflammation-associated programs and cause an inflammatory microenvironment. Therefore, DNA damage, chromosomal instability, and epigenetic alterations that consequently lead to inappropriate gene expression are the key components of the intrinsic pathway. In the extrinsic pathway, inflammatory signals from infections and autoimmune diseases play a crucial role and these inflammatory conditions promote cancer development. Both pathways activate various important transcription factors [including nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3)] that are key inducers of the inflammatory cascade [19–22].

The cross-talk between the pro-inflammatory and tumorigenic mediators (e.g., cytokines, chemokines, oncogenes, transcription factors, immune cells, etc.) retards the efforts to clarify the molecular mechanism(s) that cause formation of the inflammatory-tumor microenvironment. The activation and/or deactivation of these molecular mediators, as delicate key points between inflammation and cancer, are

influenced by intrinsic (i.e., hereditary) and extrinsic (i.e., environmental and lifestyle) factors.

7.2 ROS and RNS and Their Role in Inflammation and Cancer

A possible mechanism by which chronic inflammation can trigger tumorigenesis is the generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) in the inflamed tissue and subsequent DNA damage which results in the activation of oncogenes and inactivation of tumor suppressor genes [14]. Cancer encompasses the initiation, promotion, and progression stages where oxidative stress may affect any of them (Fig. 7.2). In the initiation stage, it causes mutational changes in DNA. In promotion it blocks cell-to-cell communication, changes secondary messenger systems and these lead cancer cells to proliferate faster and lose the apoptosis ability. In progression phase, DNA is changed much more which makes the cancer cells become chemo-resistant and migrate to other tissues [23].

Endogenous or exogenous sources such as alcohol, radiation, pesticides, diet, smoking, developmental life, infections, obesity, generation of ROS, and reactive nitrogen species (RNS) may trigger the cancer progress [24]. Examples for endogenous sources include cytochrome P450 metabolism, peroxisomes, mitochondrial oxidative phosphorylation, activation of inflammatory cells such as macrophages and neutrophils. During mitochondrial respiratory process, it is assumed that 1–2% of molecular oxygen is converted to ROS including superoxide, hydrogen peroxide, hydroxyl and peroxynitrite radicals, through one to three electron reductions in the electron transport chain. These ROS are not stable and may damage the key components of the cell such as lipids, proteins, and DNA. On the other hand, DNA damage is not always necessary for tumor formation and epigenetic alterations is another factor. Epigenetic changes such as DNA methylation, acetylation, deacetylation can be thought as non-genotoxic mechanisms for tumor formation. According to the studies, oxidative stress may cause formation of single-stranded DNA which leads to DNA methylation. DNA methylation, which occurs in the promoter region of genes, causes gene silencing and this contributes to the process of carcinogenesis. In this regard, oxidative stress can trigger cancer formation through genetic and epigenetic mechanisms [25].

Mutation is one of the ROS-related genes which can lead to carcinogenesis. ROS mediated DNA oxidation generates a by-product, named 8-hydroxy-2-′-deoxyguanosine (8-OHdG). This molecule is highly mutagenic for DNA which enhances carcinogenesis. The damage is not observed only in DNA. Also, proteins are affected and they lose their function as a consequence of oxidative stress. Loss of protein function may be associated with many diseases. Cell membrane is rich in polyunsaturated lipids and they are very sensitive to oxidation by ROS which causes lipid peroxidation. This alters the permeability of cell membrane that could lead to cell death [26].

Ras pathway is one of the most critical pathways that is related to oxidative stress, inflammation, and cancer. According to the studies if there is a mutant Ras, it leads to

an increase in ROS levels leading to DNA damage [26]. Almost 25% of all malignancies has a link with a Ras mutation leading to promotion of cell proliferation, tumor growth, and angiogenesis. Ras increases the expression of some inflammatory genes such as IL-1, IL-6, IL-11, and IL-8 [27].

Down-regulation of the antioxidant defense mechanisms by a serine threonine kinase Akt might contribute to the survival of tumor cells. Akt that is activated by ROS via inhibition of phosphatase can disable proapoptotic molecules including caspase 9, Bcl-2 and trigger the NF-κB and inhibit apoptosis. Mild oxidative stress may trigger NF-κB activation while excess oxidative stress lead to inhibition of NF-κB. The activation is dose dependent; however, antioxidants including N-acetylcysteine, thiols, polyphenols, and vitamin E can block NF-κB stimuli [27].

Breast cancer type 1 susceptibility protein 1 (BRCA1) that is responsible for DNA repair has been mutated in 40–50% of hereditary breast cancer patients. BRCA1 can upregulate the genes which are involved in antioxidant response because it can control transcription factors such as nuclear factor erythroid 2-related factor (Nrf2) which induces Glutathione-S-Transferase (GST) and Glutathione Peroxidase (GPx) in order to fight against oxidative stress. It is thought that Nrf2 is the master regulator of antioxidant response BRCA1 also reduces RNS-based protein nitration in cells and enhances DNA repair mechanisms [26]. People who have mutations in BRCA1 gene cannot produce functional protein properly and they are at risk for cancer development.

There are so many signaling pathways that are related with ROS, inflammation, and cancer, some of which are listed in Table 7.2.

ROS-related cancer formation can be divided into different progression categories, including (a) cell proliferation (via ERK1/2), (b) evasion of apoptosis (via PI3K, Src, NF-κB), (c) tissue invasion and metastasis (matrix metalloproteinases; MMPs), and (d) angiogenesis (VEGF) [28, 29]. Under hypoxic conditions, the tissue environment becomes more hypoxic because cancer cells have high proliferation rates. To overcome with this situation, cancer cells upregulate the angiogenesis genes because angiogenesis means new blood and oxygen supply for them. Furthermore, excessive ROS may regulate the metastasis via upregulation of metastasis-linked genes and by induction of enhanced glycolysis with the help of mitochondrial DNA encoding [30].

Tumors have many angiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), angiopoietin-1, fibroblast growth factor (FGF), interleukin-8, platelet derived growth factor (PDGF), and tumor growth factor (TGF). VEGF is highly expressed in most human cancer cells and it is accepted as the rate limiting factor for the regulation of angiogenesis. VEGF, the major factor in angiogenesis, is upregulated in cancer cells by hypoxic conditions, ROS production, growth factors, and cytokines [31]. This factor stimulates endothelial cell proliferation and migration through binding the receptor tyrosine kinase VEGF receptor 2. Binding to the receptor kinase insert domain receptor (KDR) is phosphorylated ends up with the activation of some downstream enzymes such as ERK1/2, Akt, as well as endothelial nitric oxide synthase (eNOS), contributing to

Table 7.2 Signaling pathways linked to ROS and inflammation in cancer

AHR	c-Myc	eNOS	iNOS	mTor	Protein kinase C
AP-1	CREB	ERK	Integrin	NAD[P]H quinone oxidoreductase 1	PPAR-γ
ATM	Cyclins and cell cycle regulation	Fas	Interferon	NF-κB	PTEN
cAMP	Cytokine network	FOXO	JAK/ STAT	Nrf2	Protein tyrosine phosphatases/ protein tyrosine kinases PTPs/ PTKs
cAMP-dependent protein kinase A	DNA methylation	HIF-1α	JNK	PI3K/Akt	Sp1
Cdk5	DNA repair mechanism	Heme oxygenase-1	MAPK	p38	TNF
Chemokines	Epidermal growth factor	IL-10	Mismatch repair	p53	VEGF

angiogenesis of the vessels to carry more oxygen and glucose to the tumor microenvironment.

7.3 Role of Hypoxia and (HIF-1α) in Inflammation and Cancer

Normally at the initiation stage of cancer, cells proliferate so fast and the environment becomes hypoxic. Under these circumstances, signaling pathways are activated to regulate proliferation, angiogenesis, and death but these mechanisms are not effective on cancer cells because they develop adaptive counter mechanisms to survive and proliferate more even under hypoxic conditions. Cells shifts from aerobic to anaerobic metabolism and hypoxia-inducible factor-1a (HIF-1α) like pathways are activated (Fig. 7.2). Prolonged hypoxia causes cell death but new vessels which are formed near cancerous tissue supplies low oxygen at the beginning but cancer cells are adaptive to proliferate under hypoxic condition. After the vessels are grown, they supply enough oxygen for cells to help them proliferate more [32].

7.4 Role of Chemical Mediators of Cancer Microenvironment and Transcription Factors

During chronic inflammation, numerous intracellular signaling pathways are deregulated. For example, inflammation-driven deregulation of kinases such as Janus kinase (JAK) and mitogen-activated protein kinases (MAPKs) leads to transmission of growth signals that permit cellular acquisition of a malignant phenotype. Additionally, inflammation-induced aberrant activation of several transcription factors such as STAT3, NF-κB, and HIF-1α (hypoxia-inducible factor-1α) has often been implicated in oncogenesis [17, 18, 20, 33]. The well described arachidonic acid pathway in cancer progress is depicted in Fig. 7.3.

Some pro-inflammatory gene products have been described that mediate an important role in the suppression of proliferation, apoptosis, invasion, metastasis, and angiogenesis. Some of these products are tumor necrosis factor (TNF) and members of its superfamily, IL-1α, IL-1β, IL-6, IL-8, IL-18, chemokines, MMP-9, VEGF, Cyclooxygenase 2 (COX-2), and Lipoxygenase (5-LOX). The expression of all these genes are mainly organized by a transcription factor known as NF-κB, which is constitutively produced in most tumors and its production is increased by carcinogenic viral proteins (HIV-tat, HIV-nef, HIV-vpr, KHSV, EBV-LMP1, carcinogens (such as tobacco smoke), tumor promoters, chemotherapeutic agents, HTLV1-tax, HPV, HCV, and HBV), and γ-irradiation [34].

Inflammation can contribute to carcinogenesis through potential mechanisms that cover induction of genomic instability, changes in epigenetic events resulting in

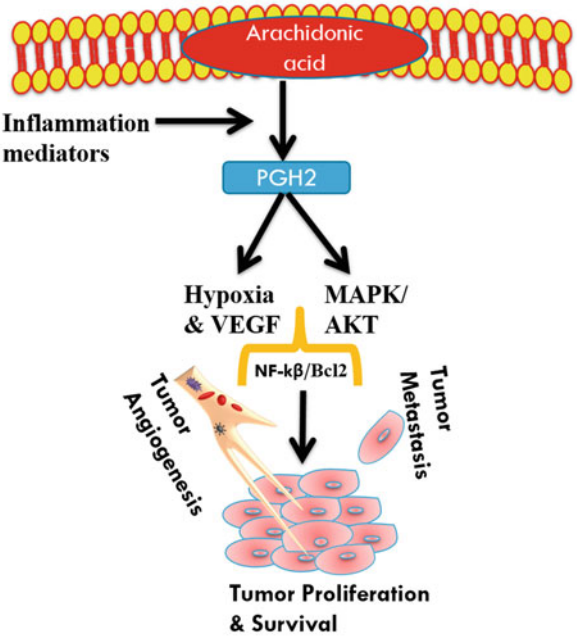

Fig. 7.3 Arachidonic acid pathway in cancer progress

improper gene expression, aggressive tumor neovascularization, resistance to apoptosis, enhanced proliferation of initiated cells, invasion via tumor-associated basement membrane and metastasis, etc. [11].

Many of pro-inflammatory mediators, especially prostaglandins, cytokines, and chemokines turn on the angiogenic switches mainly regulated by VEGF, thereby causing inflammatory angiogenesis and tumor cell-stroma communication.

NF-κB enables a mechanistic linkage between inflammation and cancer, thus provides an important mediator for the control of the ability of pre-neoplastic and malignant cells to fight against apoptosis-based tumor-surveillance mechanisms. In addition, the fact that NF-κB controls tumor angiogenesis and invasiveness, and the signaling pathways that mediate its own activation, provides attractive targets for novel prophylactic and therapeutic approaches [19]. Key mediators at the intersection of the extrinsic and intrinsic pathway include cytokines (e.g., TNF), transcription factors (e.g., NF-κB, STAT3, HIF-1α), and chemokines. Signal transducer and activator of transcription 3 (STAT3) that acts as a point of convergence for various oncogenic signaling pathways is triggered in tumor cells and in immune cells available in the tumor microenvironment.

Constitutively activated STAT3 on the one hand decreases the expression of mediators to be used in immune response against tumor cells, on the other hand increases the production of immunosuppressive mediators that turn on STAT3 in various immune-cell subsets, altering gene-expression programs and, inhibiting anti-tumor immune responses [21] (Table 7.3).

7.5 Role of Inflammatory Cells: TAMs and TANs

Neutrophils act as the first recruited cells to an acute inflammatory response. Next, monocytes differentiate into macrophages, while they are guided to the tissue injury site via chemotactic factors called chemokines. Macrophages, as the major source of growth factors and cytokines, completely affect the endothelial, epithelial, and mesenchymal cell proliferation in the microenvironment. Besides, mast cells take part in the acute inflammation due to the release of inflammatory mediators, namely histamine, cytokines, and lipid mediators leading to large number of cell migration to the local microenvironment of the inflamed tissue [12].

Regarding neutrophils, it has been suggested that a four-step mechanism controls the recruitment of these cells to injury sites as well as to the extracellular matrix including activation of the selectin family of adhesion molecules (L-, P-, and E-selectin).

This process in the microenvironment of the inflammation comprises; (1) facilitation of transport on the vascular endothelium; (2) triggering the signaling pathways related with the activation and upregulation of leukocyte integrins mediated by cytokines and leukocyte-activating molecules; (3) immobilization of neutrophils on vascular endothelium through the medium of tight adhesion via $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins binding to endothelial vascular cell-adhesion molecule-1 (VCAM-1) and

Table 7.3 The role of signaling molecules in inflammation and cancer (a compilation of Refs. [11, 12, 34–61])

Signaling molecules	Role in inflammation-associated cancer	References
Pro-inflammatory cytokines	Over-expression in inflamed, hyperplastic, metaplastic tissues, and adenocarcinomas	[35]
	DNA damage induction	[36]
	Stimulation of inflammatory angiogenesis	[37]
	Activation of pro-inflammatory signaling via JAK-STAT and NF-κB	[38]
	Stimulation of cell proliferation and inhibition of apoptosis	[39, 40]
Chemokines	Attraction of inflammatory and immune cells to the microenvironment	[41]
	Promotion of tumor cell migration	[42]
	Enhancement of extravasation of tumor cells through stromal tissue	[43]
	Stimulation of inflammatory angiogenesis through upregulation of proangiogenic factors (e.g., VEGF and MMP)	[12]
COX-2	Catalyzing the biosynthesis of lipid mediators related with inflammation	[34]
	Contributing to the maintenance of a persistent inflammatory state in the premalignant and malignant lesions	[44]
	Over-expression in cancers related with inflammation	[42]
	Promotion of cell proliferation and apoptosis blockage	[45]
	Acceleration of angiogenesis via triggering PGE_2 signaling and expression of VEGF and stabilization of HIF-1α	[46]
PGE_2	Promotion of tumorigenesis in animal models	[47]
	Excessive production in inflamed, hyperplastic, and dysplastic tissues, and carcinomas	[48]
	Augmentation of cell proliferation, suppression of apoptosis	[49]
	Induction of proangiogenic factors (e.g., VEGF)	[34]
	Activation of pro-inflammatory signaling pathways within the tumor microenvironment	[50]
iNOS	Elevation in tumoral lesions	[51]
	Induction of ROS and RNS associated with DNA damage	[52]
	Production of pro-inflammatory mediators such as nitric oxide (NO)	[53]
	Acting as a downstream effector of NF-κB and inflammatory cytokine-mediated signaling	[53]
NO	Promotion of tumor growth via cell proliferation	[54]
	Leading to *S*-nitrosylation of pivotal proteins related with inflammation and cancer	[55]
	Nitrosative stress resulting in DNA damage	[56–58]
NF-κB	Increasing the expression/production of pro-inflammatory mediators	[59]
	Augmentation of the antiapoptotic proteins expression	[60]
	Promotion of invasion and metastasis	[61]

MadCAM-1, respectively, (4) transmigration through the endothelium to injury sites, with the help of extracellular proteases (e.g., MMPs) [12, 62].

Although the infiltration of leukocytes to the neoplastic tissue appears to provide anti-tumor effect; expanding evidence underlines the fact that the infiltrate of activated macrophages and lymphocytes recruited from the microcirculation is a critical source of pro-inflammatory cytokines, chemokines, growth factors, and angiogenic factors in the neoplastic tissue microenvironment [16, 63].

At the site of injury, chemokines and cytokines play a pivotal role in the recruitment of appropriate subsets of leucocytes to initiate and maintain the inflammatory response. Macrophages are differentiated cells of circulating peripheral-blood monocytes, which migrate into tissues both at steady state and/or in response to inflammation [64–66].

Numerous reports highlight the direct link between tumor-associated macrophage (TAM) density and tumor progress (Table 7.4). Moreover, by regulating activation and/or deactivation of numerous kinases, transcription factors and molecular mediators, TAMs consistently mediate the switch from chronic inflammation to tumorigenesis. An increase in TAM numbers correlates with an increase in tumor angiogenesis. By expressing mediators such as transforming growth factor β (TGFβ), VEGF, PDGF, MMPs, thymidine phosphorylase (TP) and various chemokines, TAMs either directly or indirectly influence the angiogenic process [77, 78].

7.6 A Summary of Inflammation and Its Pro's and Con's in Cancer Progress

As a summary, inflammation is a well-defined mechanism lying under carcinogenesis and uses many common pathways both in the cancer and wound healing progress. In the inflamed environment as a reaction to tissue injury, a multifactorial network of chemical signals is triggered to maintain a host response dedicated to "recover" or "repair" the impacted site which is orchestrated with many cells and the factors released from these cells. For instance, chemotactic factors such as TGF-β and PDGF, derived from activated platelets, induce and activate the proteolytic enzymes crucial for remodeling of the extracellular matrix. Epithelial and stromal cell types engage in a reciprocal signaling dialogue to facilitate healing [12].

The cells related to inflammation play different roles in cancer progress. Early in the neoplastic process, these cells are critical tumor promoters that produce an attractive environment for tumor growth, facilitate genomic instability, and promote angiogenesis. Afterwards, during tumorigenic process, neoplastic cells divert inflammatory mechanisms (e.g., selectin–ligand interactions, MMP production, and chemokine functions) to favor proliferation and metastasis [19–22]. During the inflammation, neutrophil chemotaxis is activated by various components (e.g., circulating complement factor 5, leukotriene B4, kallikrein, endotoxins and factors released from platelets such as PDGF, TGF-β, platelet-activating factor, and platelet factor-4). In wound healing process in the inflamed tissue, these phagocytic cells

Table 7.4 Role of TAM in several cancer types

Type of tumor	Details or outcomes of the study	References
Bladder	A significantly higher TAM count was detected in invasive bladder cancers as compared to superficial cancers. A high TAM count was associated with higher cystectomy rates, distant metastasis and vascular invasion; moreover, these patients had a lower 5-year survival rate. In this regard, evaluation of TAM count in these tissue samples has been suggested to predict the prognosis, as well as a tool for selection of the convenient treatment	[67]
Breast	CD68, CD163, and MMP-9 were co-localized, displaying higher expression in ER- breast cancers. In consideration of the association between higher CD163 protein expression in TAMs with augmented overall survival in ER-cases but not in ER+ cancers, the authors have suggested that triple negative breast cancer may benefit from an analysis of CD163 for a diagnostic and/or macrophage-targeted therapeutic intent	[68]
Breast	Evaluation of the VEGF protein expression in primary breast carcinomas and its association with focal macrophage infiltration (macrophage index) revealed a significant inverse correlation between VEGF and EGFR (high VEGF expression—low EGFR levels). The authors reported two types of macrophage infiltrates in breast cancers: (1) EGFR-positive and low VEGF expression in tumor, (2) EGFR-negative tumors with high VEGF expression. In EGFR+ tumors, macrophage counts were higher, while they found no associations between VEGF expression and microvessel-density increase. On the other hand, VEGF expression and macrophage index were positively associated in the EGFR- group	[69]
Cervix	IL-8 levels and TAM numbers were significantly correlated, thus IL-8 content may be of use as a prognostic indicator as an angiogenic factor originating from TAMs. While the prognosis of patients with high IL-8 was poor, the 24-months survival rate was recorded as 67% in patients with low IL-8	[70]
Colon	This study, enrolling two independent large cohorts, has shown that CD206/CD68 ratio that was significantly associated with poor disease-free survival and overall survival may serve as a prognostic and predictive marker of postoperative adjuvant chemotherapy for stage II colon cancer	[71]
Colorectal	An association between high intra-epithelial CD68+ macrophage density and poor overall survival or progression-free survival was of note. In colorectal cancer, the density of intratumoral macrophages may be utilized as a prognostic indicator in order to further stratify the T cell populations	[72]
Esophageal	The infiltration of CD68(+) macrophages and CD163(+) macrophages have been evaluated in patients with	[73]

(continued)

Table 7.4 (continued)

Type of tumor	Details or outcomes of the study	References
	esophageal cancer. Results have depicted that high CD68 (+) and CD163(+) macrophage infiltration correlated with poor response to chemotherapy, both clinically and pathologically. In the patients undergoing neoadjuvant chemotherapy, CD163(+) as the marker for M2 macrophages, has been suggested as an independent factor for prognosis	
Hodgkin's lymphoma	An increased number of TAMs (CD68+) was strongly correlated with shorter progression-free survival along with a tendency for relapse after autologous hematopoietic stem-cell transplantation that leads to shorter disease-specific survival in patients with classic Hodgkin's lymphoma	[74]
Lung (non-small cell lung cancer)	High levels of M1, CD204 + M2, and CD68 macrophages have been suggested as independent prognostic indicators of prolonged survival in patients. A pathological stage-related evaluation is provided	[75]
Prostate	The odds ratio for lethal prostate cancer has been estimated as 1.93 (95%CI: 1.23–3.03) for patients with high infiltration of CD163-positive M2 macrophages versus low infiltration. Additionally, higher CD163 positive macrophages in patients with an uncertain outcome has been shown to predict a poorer prognosis	[76]

initiate tissue repair by providing the early response pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) [79], and interleukin (IL)-1α and IL-1β [80]. These cytokines play role in the leukocyte adherence to vascular endothelium, to initiate repair by inducing expression of MMPs and keratinocyte growth factor (KGF/fibroblast growth factor (FGF-7)) by fibroblasts [81]. All these factors are key components and suitable targets for cancer therapy.

After the deployment of monocytes/macrophages to the inflammation site, they differentiate into mature macrophages or immature dendritic cells [69]. Endothelial, epithelial, mesenchymal or neuroendocrine cells in their local microenvironment are profoundly affected by macrophage products in cancer site after this complex inflammation process. Macrophages serve in the regulation of the local tissue remodeling: Briefly, they induce ECM components, stimulate production of MMPs, and other proteolytic enzymes as well as urokinase-type plasminogen activator, clear apoptotic and necrotic cells, and modulate angiogenesis via local production of thrombospondin-1 [12, 82].

In conclusion, there are many mediators orchestrated with the cells and the cell end-products released in the tumor microenvironment those have been shown to be related in both cancer and inflammation revealing the strong relationship between them. Taking this into account, many potential drug targets have been identified in this aspect, and in this chapter, we tried to summarize these factors which might be potential drug targets in several cancer types.

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Pharmacologic Modulation of the Immune Response Against Tumours in the Elderly

8

Juan Bautista De Sanctis

Abstract

Despite the high incidence of cancer in the elderly, little is known about the protective immune response against cancer and the treatment of other comorbidities. Inflammaging has been defined to explain a protective inflammatory response in the elderly. New subpopulations of stem cell memory T cells seem to be responsible for a quick memory response to antigens and probably against tumours. Biological immune therapy with anti-checkpoint inhibitors could be an essential tool to treat patients; however, adverse or toxic events are often observed in elderly patients. Several medications used in the elderly, metformin and valproic acid, have been shown to have anti-neoplastic effects. These effects suggest that therapeutic approaches in the elderly should be carefully analysed. Clinical trials are required to assess the exact role of immune response and therapy in tumour incidence and survival in the elderly.

Keywords

Immune response · Elderly · Checkpoint inhibitors · PD-1 · CTLA-4 · Adverse reactions · Metformin · Valproic acid · Comorbidities

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8.1 Introduction

The incidence of cancer in the elderly has always been a matter of discussion [1, 2] (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence). The documented decrease in immune vigilance, along with the increase of inflammation markers, has generated interest in the field [3–5]. Modulation of immune response by vaccination has been considered appropriate therapy for rescuing memory against infectious diseases [6]; however, in cancer, more sophisticated strategies have to be analysed [7]. The increased susceptibility to infections in the elderly is a clear indication of an impaired innate immunity which, as a consequence, leads to a decreased response of adaptive immunity [3–5]. It is expected that ageing will predispose individuals to a less anabolic and catabolic activity which would limit the response of cells and tissue to injuries. One biological mechanism that partially compensates this phenomenon is inflammaging [3–5]. Inflammaging is defined as a dynamic protective response in which pro-inflammatory mediators and circulating primed cells are increased without generating a clinically perceptible inflammation [3–5, 7, 8]. This pro-inflammatory response is a quick adaptive response observed in healthy elderly individuals. It is often underestimated, it could be modified by therapy, and it partially protects from tumour growth [7, 8]. Thus, the immune response of the elderly should be considered different from healthy adults and infants.

Cancer is frequently diagnosed in the elderly, with approximately 50% of patients being over 70 years of age [1, 2] (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence). According to the British cancer organisation, female rates of cancer are lower than male after 75 years, and there is a drop in cancer incidence after 85 years (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence). Tumour screening is either decreased after 85 years or healthy elderly individuals that have an efficient immune response live longer and dye of other natural causes.

In solid tumours, one of the most common cancers in males is prostate cancer and in women breast cancer; however, in both genders, the second most common is lung cancer [1, 2] (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence). Leukaemias and lymphomas are also prevalent in the elderly population [1, 2] (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence). One of the hypotheses in geriatric oncology is that continuous replacement of circulating T cells from the bone marrow, impaired genetic control mechanisms, and the absence of thymic selection increases the probability of generating tumour cells (lymphoma). Patients with mild immune deficiencies and some with acquired immunodeficiencies are prone to develop B cell lymphoma. Others in minor extent develop monocytic leukaemia. Nonetheless, patients that had an incipient or surgical removed tumour may present new tumour growth in the same organ or other organs due to the reactivation of dormant metastatic cells which have not been contained by the immune system [9, 10]. This late group is now carefully monitored by the oncologists due to the marked increase in documented cases [1, 2] (https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence).

Usually, studies that involve tumour therapy or immune tumour therapy do not include elderly individuals [11–15]. It is assumed that most of the elderly individuals have comorbidities; however, there is a group of healthy individuals with an adequate response to pathogens and tumours who have been overlooked [7, 8]. This group may provide new pieces of evidence for immune modulation, which can be useful for the treatment of elderly patients with incipient tumours and tumour survivors with a high risk of metastatic tumours. Due to the marked increase in the elderly population, pharmaceutical companies have started programs to monitor different treatment options.

8.2 Protective Immune Cell Populations in Healthy Elderly

Most of the innate immune response in elderly individuals is partially unresponsive to stimuli [3, 4]. The unresponsiveness is generally due to a decrease in signal pathway activation and a reduction in cytokine secretion [3, 4]. The vigilant tissue immune cells in ageing are slow in the generation of resolution mediators which paradoxically provide a mild advantage on alert immune responses [3–5]. Due to the reduced innate response, adaptive responses, based on memory, take charge of the immune response to many know antigens [7, 8]. Nonetheless, the immune challenge with vaccines has proven a useful stimulation of innate immunity providing a more sustained and effective memory response [6, 16].

One of the hallmarks of healthy ageing is the increase in CD4 cells, the decrease in CD8 cells, and an increase in T reg cells with an increase in PD1 [3–5, 7, 8]. The markers of senescence are expressed predominantly in the CD8 population suggesting that crucial antiviral and antitumour response is partially impaired [7, 8]. Several reports in mice and humans have indicated that this decrease in T cell population is assumed by NK cells, NKT cells, or Tγδ cells although this point is still under discussion [3–5, 7, 8, 16].

In healthy elderly individuals, antigen responsive T cells are composed of central memory T (TCM) cells (CD45RO + CCR7+), effector memory T (TEM) cells (CD45RO + CCR7−), and effector T (TEF) cells (CD45RO-CCR7−) [8]. After continuous antigen stimulation with age, a shift in the T cell subset distribution from naïve T cells to TCM, TEM, and TEF. This process is characterised by the loss of expression CD27 and CD28, which may be accompanied to a higher risk of infections, chronic diseases, and cancer [7, 8]. However, a cell population co-expresses CD28, CD95, CD45RO+, and CCR7+ and has been defined as stem cell memory T cells (TSCM) respond quickly to antigen, generating an active immune response [8]. This population seems to originate from the follicular compartment, and they are released to compensate for a decreased number and function of T cells [17]. Thus, effective T cell responses in healthy ageing can be observed and do not represent the majority of the circulating T cells encountered.

Endogenous glucocorticoids produced in stress conditions and ageing induce a decreased immune response in the elderly [18]. Predisposition to chronic diseases or inflammation along with the lack of exercise, non-proper nutrition, and dysbiosis

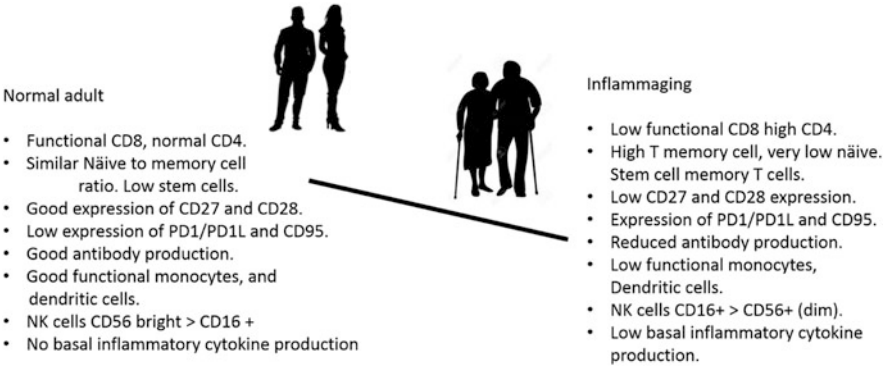

Fig. 8.1 Differences in the immune response between healthy normal adults and elderly individuals

may generate this increase [18]. Behavioural changes can affect the production of glucocorticoids. In mice, unaligned chronic circadian rhythm expedites immune senescence suggesting that simple changes in behaviour may alter immune response which increases the susceptibility to lack of immune response which, in turn, would predispose to more probability of tumour growth [19].

One of the most common infections observed in the elderly population is cytomegalovirus infection (CMV). The infection induces a decrease in the expression of CD28 and NK cell activity making the patient more susceptible to other viral infections and the development of tumours [20]. Different T cell responses are also impaired [21, 22]. However, in some elderly individuals, the immune response is restored, which suggests that genetics plays a significant role in the process [23]. Challenging the immune system with vaccination could provide valuable clinical evidence to assess the individual capacity to respond to pathogens and tumours. One could envision that those elderly patients, survivors of cancers that do not respond to vaccines, tumour reappraisal may occur. Vaccines are then an indirect but essential tool for clinicians to assess effective immune responses. Figure 8.1 represents the main differences between the typical healthy adult immune response and inflammaging observed in the elderly population.

8.3 Chemotherapy and Toxicity

Toxicity due to chemotherapy is frequent in elderly patients [14, 15]. Cytokine storm can be generated in these patients due to a marked increase in cell death. This uncontrolled amount of cytokines can be avoided by treating the patients with steroids or additional immunosuppressants to decrease the inflammatory burden. Adding steroids to the treatment jeopardises the protective immune response, moreover, if the patient has comorbidities, it may aggravate them [14, 15]. This

stress-induced response prolongs hospitalisation, deteriorates the immune response, and the patient is more susceptible to infections. Elderly patients are very labile.

Biological therapy against PD1 and CTLA-4 in elderly patients may not be as effective as in other ages [13, 14]. There are no general guidelines for elderly patients [14, 15]. As aforementioned on T cell populations, the amount of highly active stem T cells in the elderly may be pushed to apoptosis with the anti-checkpoint inhibitors. Thus, checkpoint inhibitors may generate highly toxic side effects in elderly patients by enhancing cytokine storm [13]. These adverse events have been identified as immune-related adverse events (irAEs). The report from the European Society for Medical Oncology (ESMO) differentiates side effect of checkpoint inhibitors, grade 1 and 2 toxicities [24]. The recommendation is to suspend the treatment and monitor the events or start symptomatic or local therapy. The majority of symptoms appear after 4 h. of the initial treatment; however, the manifestations can occur during treatment and be maintained after several months after the treatment has been stopped [13]. Since there may appear skin manifestations, most clinicians would prescribe antihistaminics. In some cases, antihistamines in the elderly give more side effects affecting consciousness and fluid retention deteriorating the patient [25]. If the adverse effects escalate, corticosteroid therapy is recommended (some grade 2 and grade 3 and 4 toxicities). If there is no improvement, more aggressive immunosuppressive therapy is used [24]. In conclusion, biological therapy should be carefully managed in elderly patients.

One of the proposed options in elderly patients is to the use of JAK inhibitors for tumour treatment. However, as suggested after the COVID 19 outbreak, the use of JAK inhibitors could be more detrimental than effective since they would inhibit immune response [26].

8.4 Other Medications that May Affect Tumour Growth and Immune Response

Recently, commonly used drugs in the elderly have been used to treat cancer since some important mechanisms of the compounds have been studied in more detail.

It is well known that hyperglycemic states reduce immune response, and glycemic control restores the effectiveness of the immune system. Metformin is an old drug that has been used in patients with increased insulin resistance and type 2 diabetes for glucose control [27]. The rationale of using metformin in cancer is to decrease the uptake of glucose by the tumour, inhibit rapamycin, enhance mitochondrial control of cell cycle, and eventually induce death by inhibiting autophagy and enhancing apoptosis [28]. Tseng et al. demonstrated that diabetic patients that use metformin had a better survival of lung cancer than their counterparts [29]. On the contrary, diabetic patients without strict glycemic control are prone to have higher tumour growth. Insulin, a known modulator of the immune response, is able to restore immune response at the concentrations normally used to control glycaemia [30, 31]. Hypoglycaemia in the elderly, it is a very dangerous condition, and in patients with cancer with controlled insulin levels is considered a bad prognosis.

Since cholesterol synthesis has been related to tumour growth, treatment with statins was proposed as adjuvant therapy [32]. Perhaps due to the complex metabolism of tumours, no major direct effect has been described. Simvastatin has been studied for breast cancer as an inhibitor of signal pathways related to triple-negative breast tumours [33]. However, the most striking responses on statins and cancer come from trials with diabetic patients in which the use of statins, the best rosuvastatin, seem to enhance a protective immune response increasing patient survival [34]. One may conclude that clinical trials related to the use of statin in elderly patients with cancer should be performed in order to ascertain the effectiveness of these drugs as adjuvant therapy.

Valproic acid (VA), a known anti-epileptic drug also used to treat bipolar disorder, has been shown to a potent demethylating agent useful in cancer therapy [35]. In principle, VA was shown to decrease monocyte to dendritic cell maturation and affect some of the macrophage and NK cytotoxic responses; however, VA increases NK cytotoxic receptors enhancing a specific antitumour response [35]. Most probably these contradictions arise from the in vitro assays as compared to the in vivo assays. The slower clearance of the drug in the elderly [36] suggests that lower doses of the compound would be more therapeutic than higher does which in fact would decrease immune response efficiency.

There are other medications usually used by elderly patients; however, the lack of relevant data prompted us not to comment on it.

8.5 Conclusions

There is general consent that tumours are frequent in the elderly and that elderly individuals have an impaired immune response. These comparisons are usually performed comparing average young and middle-age individuals to the elderly. However, only a few researchers have compared healthy elderly individuals with aged patients with cancer and elderly cancer patients with comorbidities [37]. In general, healthy old individuals have an excellent protective immune response mostly dependent upon pro-inflammatory cells and mediators, which are clinically silent. A good memory response may protect these individuals from tumour appraisal or reappraisal.

On the contrary, in elderly patients with comorbidities, the protective response may be impaired, and tumour appearance and reappraisal increase dramatically. Up-to-date, it is difficult to distinguish if the group with comorbidities is more susceptible to develop tumours and why. Pharmacological therapy can play a role in increasing the risk to develop cancer.

Most of the clinical trials with different therapeutic schemes are usually not performed in elderly individuals. The pharmacokinetics and pharmacodynamics of many compounds are calculated in clinical trials that usually includes young and middle-aged people. Then, the recommended doses may produce toxic effects in the elderly. Besides the fact that unadjusted drug concentrations can be detrimental cell

metabolism or to immune response, drug interactions may not be appropriately addressed.

The use of chemotherapy and checkpoint inhibitor therapy should be strictly monitored in the elderly population, especially in the presence of comorbidities. The addition of coadjuvant therapy should be carefully analysed depending on the individual. Finally, more research is required on the field in order to provide the critical guidelines required. In the recent COVID 19 outbreak, we have learned how many elderly people different countries have and how susceptible elderly populations are to infections. However, many people have not understood that this population is increasing rapidly. It represents a challenge that must be resolved.

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Angiogenesis: A Therapeutic Target for Cancer

9

Neha Atale and Vibha Rani

Abstract

The proliferation and metastatic spread of tumor cells depend on the newly developed blood vessels. Vasculature not only provides an adequate supply of oxygen and nutrients but also removes waste products or gas exchange. The process of angiogenesis is controlled by various transcriptional factors and growth factors. It has been observed that the discovery of angiogenic inhibitors can help to reduce carcinomas growth. Presently, chemotherapeutic drugs mediated inhibition of hypoxia-inducible factor (HIF-1), which initiates neovascularization under hypoxic conditions in the tumor, is being investigated. Vascular endothelial growth factor (VEGF) and receptor VEGFR mediated activation of endothelial cells are also inhibited by chemotherapeutic drugs. Furthermore, chemotherapeutic drugs inhibit the PI3K/AKT/mTOR signaling pathways mediated growth of new blood vessels. The aim of this chapter would be to highlight the role of angiogenesis in cancer progression. Furthermore, various anti-cancer therapeutic strategies/trials based upon inhibition of blood vessels would also be discussed.

Keywords

Tumor angiogenesis · Endothelial cell · Vascular endothelial growth factor · Hypoxia-inducible factor · Anti-angiogenesis drugs

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9.1 Introduction

Angiogenesis is the process of branching out existing blood vessels into new ones. This is highly controlled phenomenon, and has potential role in progression of various pathological conditions, especially cancer [1, 2]. Oxygen and nutrients are prime elements in angiogenesis for the development of blood vessels. Every tissue which requires supply of oxygen and nutrients is found to have blood capillaries within few 100 μm around it. Capillaries are required for exchanging nutrients, metabolites, and gases to tissues and removal of waste products.

Decades ago, Dr. Folkman gave hypotheses towards a therapeutic approach that could stop tumor growth by blocking angiogenesis in tumor cells [3]. The idea behind these was that when the tumor growth starts, the initial tumor cell population is dormant and does not require vasculature for oxygen supply until the size of the tumor reaches 1–2 mm in diameter. Once the cells reach this size, they can recruit surrounding blood vessels to trigger angiogenesis leading to formation of new ones. Angiogenesis triggers the tumor growth and metastasis followed by chemical signals releasing from cancer cells [4]. Physiological factors are also important to strengthen the vascular networks and vessel walls.

Neovascularization is the process of creation of new blood vessels from matured ones following endothelial proliferation and migration leading to angiogenesis. When cancer cells go rogue and start to proliferate, they use blood capillaries around them to access oxygen and nutrients through diffusion which works to range of 100–200 μm allowing the tumor to grow up to 1–2 mm. Beyond this size, the diffusion process is not enough to sustain the growth of the tumor cells and it becomes dormant. To sustain growth beyond this size, new blood vessels need to be created (neo-vascularization) around the tumor [5, 6]. As cells, either tumor or healthy grow and oxygen supply reduces, the cells go into hypoxic stress, which is detected by factors called hypoxia-inducible factor 1 (HIF-1). The activation of HIF-1 leads to formation of angiogenic proteins mainly VEGFs (vascular endothelial growth factors). Various growth factors including VEGFs/VEGFRs, platelet-derived growth factors (PDGFs/PDGFRs), fibroblast growth factors (FGFs), and angiopoietin/tie receptors [7] activate the process of angiogenesis and stimulate hypervascularization [8]. VEGF is considered as the most important regulator of angiogenesis in early embryonic and adult cells [9]. Therefore, angiogenesis is a prime factor for the growth of cancer.

The understanding on the angiogenic tumor progression and its treatment has rapidly developed over the past decades. Clinical evidences have also suggested the effectiveness of angiogenic inhibitors for the prevention of tumor establishment and growth. During tumor formation, there is an imbalance occurring between endogenous stimulator and inhibitor levels, leading to an "angiogenic switch" [10]. It is very necessary to maintain activators and inhibitors equilibrium for vascular homeostasis.

A marking effect of angiogenic cancer therapy was primarily exemplified when Avastin (bevacizumab) was authorized by Food and Drug Administration (FDA) against metastatic colorectal cancer [11]. Therefore, it is essential to explore the mechanisms of tumor angiogenesis for the identification of new therapeutic targets.

Traditional therapies like chemotherapy complemented by anti-angiogenic drugs and nanotechnology was found to be successful in cancer patients. The following chapter gives a broad overview of the mechanisms and growth factors involved in tumor angiogenesis, and also showed chemotherapeutic drugs mediated inhibition of angiogenesis in cancer.

9.2 Significance of Angiogenesis in Cancer

The formation of new blood vessels occurs from pre-existing vessels by the "sprouting" of endothelial cells, which further enlarge the vascular tree [12]. There are few fundamental steps of angiogenesis: (1) protease production, (2) endothelial cell migration and proliferation, (3) vascular tube formation and their conjugation, (4) basement membrane formation and integration of smooth muscle cells (Fig. 9.1).

Previous studies reported that endothelial and smooth muscle cells function using oxygen-sensitive NADPH oxidases, endothelial nitric oxide synthases, and heme-oxygenases [13]. Various cellular activities towards hypoxic conditions are regulated by hypoxia inducible factors (HIFs). All the isoforms of HIFα (HIF-1–3) may form a transcriptional complex by heterodimerizing with the aryl hydrocarbon receptor nuclear translocator (HIFβ/ARNT) subunit that begins the expression of

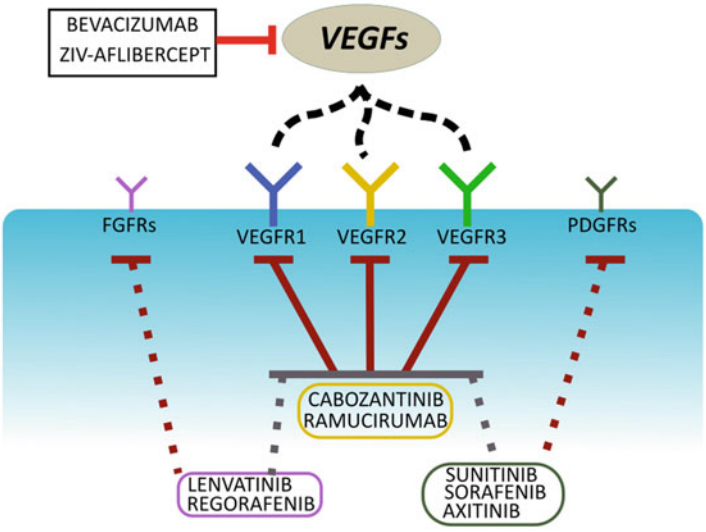

Fig. 9.1 Major events in the formation blood vessels during angiogenesis: Neo-vascularization occurs through sprouting of vessels occurring in multiple stages. Dormant tumor cells detect hypoxia and release growth factors, such as VEGF. These stimulate nearby endothelial cells to migrate towards the tumor by creating a chain from blood vessels. As the ECs reach the tumor, they mature to create a tube where the blood starts to enter. The tubes sprout further to create capillaries engulfing the tumor and creating stable vasculature

various genes regulating cell survival and angiogenesis [14]. HIFs also allow growth of vasculature in healthy tissues where the vessels cannot provide enough oxygen for the growing cells to survive. Cancer cells also need oxygen and nutrients to grow and metastasize which can only be provided by sufficient blood supply. In developing cancers, endothelial cells are very active due to the secretion of IL-8, prostaglandin E1 and E2, TNF-α, VEGF, bFGF that can induce endothelial cell maturation when the anti-angiogenic factors generation is decreased [15]. This allows for the tumor cells to grow continuously by increasing the vasculature around them. Thus it is important to understand mechanism of vascularization in tumor cells to produce efficient anti-angiogenesis drugs.

9.3 Factors Involved in Angiogenesis

Angiogenesis is regulated by different transcriptional factors and growth factors, responsible for the proliferation and migration of endothelial cells in vivo. The following section shows the major factors which trigger the process of angiogenesis.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a vascular permeability factor and a prime agent of angiogenesis. It is an important pro-angiogenic factor in the skin and existing at higher levels in wounds, keratinocytes and fibroblasts [9]. Being a special mitogen for endothelial cells, it triggers endothelial cell functions leading to new capillaries formation, such as proliferation, differentiation, migration, and survival [16]. VEGF-A is a 45 kDa protein, along with the other major members of the family including VEGF-B, VEGF-C, VEGF-D, and PlGF. VEGF-A is produced by cancer cells and is correlated with tumor growth and metastasis. VEGF produces its various isoforms by alternative splicing [17]. VEGF-A binds with VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), while VEGFR-2 is found to be crucial of the two receptors for regulating endothelial cell function by activating downstream signaling cascades. Phosphorylation of tyrosine residues occurs when it binds to VEGF at VEGF receptor and promotes activation of protein kinase B. The binding also stimulates the mitogen-activated protein kinase (MAPK) pathway which is known to stimulate proliferation in endothelial cells. VEGF-A isoforms have also been found to bind with neuropilins (NRPs). These single-pass transmembrane proteins are also known to bind to semaphorins. NRPs work to enhance the activity of VEGFRs and serve as coreceptors for VEGF [18].

During tumor growth, VEGF triggers endothelial cell proliferation by ERK and PI3K/AKT pathways [19]. VEGF based cell invasion also stimulates the production of MT-MMPS, MMP-2, MMP-9 and plasminogen activators. Since VEGF triggers angiogenesis, it has been considered as an important target in anti-angiogenesis mechanisms to stop tumor growth. Many chemotherapy drugs use VEGF/VEGFR

antibodies and inhibitors for tyrosine kinase receptors. But VEGF also plays important role in vasculature in healthy cells and a generic non-targeted approach for VEGF inhibition can lead to many side effects such as related to gastric and neurotoxicity. Thus extensive research is required to understand the VEGF inhibitor concentrations needed to carefully block angiogenesis in tumor cells while reducing the potential side effects due to excessive or poor targeted dosages.

Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) is also an important factor in angiogenesis. PDGF, a 30 kDa dimer, binds with receptors α (PDGFRα) or β (PDGFRβ) to induce proliferation, migration, and differentiation in various cell types. It is comprised of four genes: PDGF-A, PDGF-B, PDGF-C, and PDGF-D [20]. All four PDGF chains are assembled into five isoforms named as—PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD [21]. PDGF A, B, and C have stronger affinity towards PDGFRα, while PDGF-B and D show higher affinity towards PDGFRβ. The binding of these ligands leads to dimerization, which activates the tyrosine kinase pathways and subsequent recruitment of SH-2-domain-containing signaling proteins. Activation of these pathways leads to cellular processes such as proliferation and migration.

PDGF is majorly involved in the process of angiogenesis, cell migration, and proliferation, and also plays some role in tumor growth and development of lesions in inflammatory diseases. The process of PDGF activating tyrosine kinase receptors and the binding of PDGFRα and PDGFRβ is known to upregulate VEGF factors which in turn can induce angiogenesis and modulate proliferation and recruitment of perivascular cells [22]. PDGF may not only lead to VEGF-A production but it has been found that PDGF-B stimulation can induce increased endothelial cells lineage commitment and differentiation of hematopoietic precursors [23]. In knockout models, PDGF-B and PDGFRβ signaling have been found to create functional blood vessels by recruiting and stabilization of perivascular cells. PDGF-B has also been found to trigger production of transcription factor E26 transformation specific sequence-1 (Ets-1) [24]. In endothelial cells, Ets-1 is also elicited by stimulation with PDGF-B. PDGF-D has shown to play some role in the migration, proliferation, and tube formation of endothelial progenitor cells (EPCs) and enhancement of angiogenic capacity of EPCs. PDGF-D also stimulates phosphorylation of many signaling molecules, such as AKT, STAT3, ERK1/2, and mTOR indicating its pivotal role in angiogenesis [25]. PDGF therefore is another critical factor that requires further research for developing safe new anti-angiogenic therapies.

Fibroblast Growth Factor (FGF)

Fibroblast growth factors (FGFs) are pro-angiogenic factors that mainly interact with heparan-sulfate proteoglycans, tyrosine kinase receptors, and integrin. FGFs are involved in organ development and angiogenesis leading to cancer. Specifically, FGF-2 binds with receptor FGFR-1 may allow tumor growth. Gene silencing by targeting FGF2 and FGFR-1 has shown to significantly reduce size of tumor in human melanoma [26]. FGF2 induces VEGF expression in endothelial cells showing its angiogenic response. A novel strategy by combining anti-VEGF treatment along with FGF-ligand trap showed suppression of angiogenesis and reduction in size of the tumor [27].

Angiopoietin (Ang)

In endothelial cells, angiopoietin (Ang) is an important growth factor in angiogenesis by expressing receptor Tie2. There are mainly four major angiopoietins: Ang1, Ang2, Ang3, Ang4, in which Ang 1 and 2 have prime role in tumor development. Ang 2 inhibits Ang1 stimulated Tie2 signaling thus working as an antagonist to Ang1, which is important for stabilization of blood vessels. On the one hand, Ang2 suppressed VEGF expression, thereby inhibiting proliferation of endothelial cells and impairing pericyte coverage of tumor vasculature, further leading to reduction in tumor growth [28]. It has also been reported that specific induction of Ang2 in gliomas, certain mammary and lung carcinomas inhibited tumor growth and metastasis [29]. However few other reports stated that Ang2 concentrations may be correlated with malignancy of certain cancer types. Overexpression of Ang2 showed increased tumor angiogenesis in mice.

9.4 Chemotherapeutic Drugs as Angiogenesis Inhibitors

There are various chemotherapeutic agents/drugs available for inhibition of angiogenesis by targeting VEGF, PDGF, bFGF, and other growth factors (Fig. 9.2). Although monotherapy and combination therapy with such inhibitors have been applied in preclinical and clinical trials in various cancer types, but advanced therapies with better efficacy and less drug resistance are still highly required. In the following section, we have listed the drugs showing anti-angiogenesis effect by targeting different molecules in the molecular pathway of angiogenesis. We have also summarized the drugs along with their targets and therapeutics against cancer in Table 9.1.

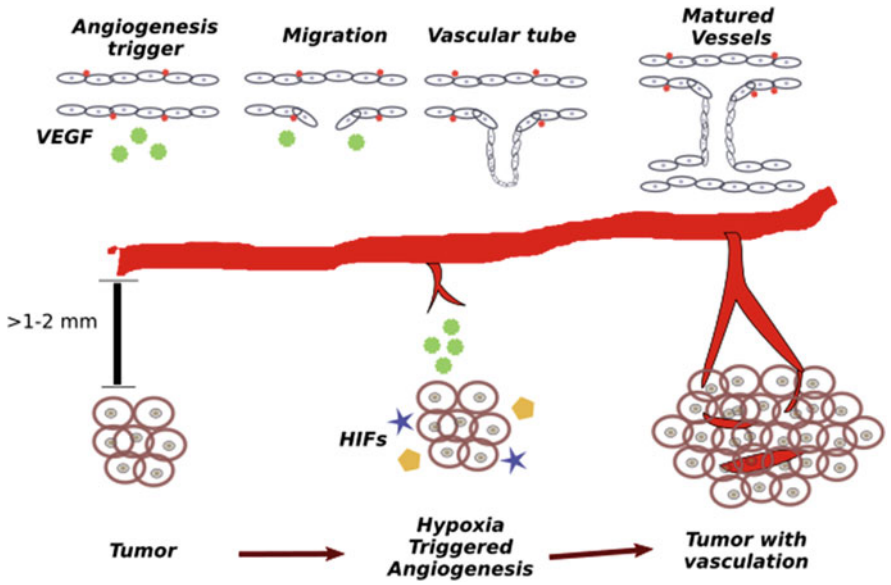

Fig. 9.2 VEGFs targeting Drugs: Anti-angiogenic drugs bind with VEGFs/PDGFs/FGFs and block their activities

Bevacizumab

Bevacizumab is known to be the first U.S. FDA-approved anti-angiogenesis drug. It is known to significantly increase the survival rates in patients having colorectal and various cancer types when administered along with conventional chemotherapy. It is a recombinant monoclonal antibody synthesized against VEGF, and after binding with soluble VEGF, inhibits endothelial cell proliferation and vessel formation [30]. Clinical studies have shown that treatment with bevacizumab alone or in combination with a cytotoxic agent reduces tumor growth. This is used along with paclitaxel and cisplatin for the treatment of cervical, colorectal, and lung cancer. This is also used with interferon alpha in case of renal cell carcinoma.

Cabozantinib

Cabozantinib is used for the treatment of multiple cancer types such as thyroid cancer, hepatocellular and renal cell carcinoma. It is found in the USA under the brand names Cabometyx and Cometriq. This drug mainly targets VEGF receptors (VEGFRs), AXL and MET, which are responsible for angiogenesis and metastasis [31]. Treatment with cabozantinib prevented MET and VEGFR2 phosphorylation in vivo and in vitro tumor models and reduced cell invasion in vitro [32].

Table 9.1 List of anti-angiogenic drugs targeting various factors (VEGFs/PDGFs) and PI3K/AKT/mTOR pathway

S. no.	Drugs	Drug target	Cancer treatment	References
1	Bevacizumab	VEGF	Cervical colorectal, lung cancer, renal cell carcinoma	[30]
2	Cabozantinib	VEGFR2	Thyroid cancer, hepatocellular and renal cell carcinoma	[31, 32]
3	Axitinib	VEGF, AKT	Kidney cancer	[33]
4	Lenvatinib	VEGFR1-3, FGFR1-4, PDGF	Endometrial and hepatocellular carcinoma, thyroid cancer	[34]
5	Ramucirumab	VEGFR-2	Gastric cancer	[35]
6	Regorafenib	VEGFR1/3, PDGFR-β and FGFR1	Metastatic colorectal cancer, gastrointestinal stromal tumor	[36]
7	Sorafenib	VEGFR-2 and PDGFR	Hepatocellular carcinoma, renal cell carcinoma, thyroid cancer	[37]
8	Sunitinib	VEGF, PDGF	Pancreatic cancer, gastrointestinal stromal tumor	[38]
9	Ziv-Aflibercept	VEGF-A, VEGFR1/2	Metastatic colorectal cancer	[39]
10	Leflunomide	Ephrin-A1/EphA2	Breast cancer	[40]
11	LY294002	PI3K	Pancreatic cancer	[41]
12	PX-866	PI3K	Prostate cancer, colorectal, non-small cell lung cancer	[42]
13	Buparlisib	PI3K	Prostate cancer, breast cancer, non-small cell lung cancer	[43]
14	Pilaralisib	PI3K	Solid cancers, breast cancer, gastric cancer, non-small cell lung cancer	[43]
15	Pictilisib	PI3K	Solid cancers, breast cancer, gastric cancer, non-small cell lung cancer	[44]
16	Taselisib	PI3K	Solid cancers, breast cancer, gastric cancer, non-small cell lung cancer	[45]
17	Idelalisib	PI3K	Multiple myelomas, chronic lymphocytic leukemia	[46]
18	Perifosine	AKT	Breast cancer, ovarian cancer, non-small cell lung cancer, breast cancer, multiple myeloma, leukemia	[47]
19	GSK-690693	AKT	Lymphoblastic leukemia	[48]
20	Rapamycin	mTORC1	Melanoma, glioblastoma	[49]
21	Everolimus	mTOR	Metastatic renal cell carcinoma, breast cancer, melanoma, ovarian cancer, neuroendocrine tumors	[50]
22	Temsirolimus	mTOR	Hepatocellular carcinoma, metastatic renal cell carcinoma	[51]
23	Ridaforolimus	mTOR	Endometrial cancer, sarcoma, hematological malignancies	[52]

Axitinib

Axitinib inhibits VEGF-associated endothelial cell migration and adhesion on matrix proteins and promotes early apoptosis. It also blocks protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and mitogen-activated protein kinases (ERK 1/2) phosphorylation [33]. Clinical studies using axitinib in combination with avelumab and pembrolizumab showed significantly longer survival rates in patients with kidney cancer.

Lenvatinib

Lenvatinib is a multi-tyrosine kinase inhibitor which inhibits the VEGF family (VEGFR1–3) along with fibroblast growth factors (FGFR1–4), PDGF receptor (PDGFRα), tyrosine-kinase receptor (KIT) and rearranged during transfection receptor (RET). It inhibits the growth of new vessels and reduces vascular permeability close to the tumor to halt oxygen and nutrient exchange. It is available under the brand name Lenvima and used alone or in combination with pembrolizumab for the treatment of endometrial and hepatocellular carcinoma and thyroid cancer [34].

Ramucirumab

Ramucirumab is the first FDA approved drug against gastric cancer along with chemotherapy [35]. Ramucirumab is a monoclonal antibody that binds to VEGF-R2 and inhibits its activation. This in fact binds to the extracellular VEGF-binding site with high affinity and inhibits VEGFR2 activity. It is mainly used in patients along with docetaxel, for the treatment against adenocarcinoma.

Regorafenib

Regorafenib, a kinase inhibitor, potentially inhibits endothelial cell kinases such as angiogenic kinases (VEGFR1/3, PDGFR-β, and FGFR1). Chemical structure of Regorafenib, or Stivarga®, is similar to sorafenib, however presence of fluorine in phenyl group denotes its higher activity against receptor tyrosine kinases and intracellular signaling kinases, than that of sorafenib. Regorafenib is used for the treatment of gastrointestinal stromal tumors and metastatic colorectal cancer [36].

Sorafenib

Sorafenib is an angiogenic inhibitor and significantly inhibits the stimulation of endothelial cell based VEGFR-2 and PDGFR-h tyrosine kinases, showing its anti-

angiogenic characteristics [37]. It plays a major role in the cure of hepatocellular carcinoma, renal cell carcinoma, and thyroid cancer.

Sunitinib

Sunitinib checks various tyrosine kinases, including VEGF, PDGF, and proto-oncogene cKIT [38]. Sunitinib malate is sold under the brand name Sutent. This has also been concerned in enhanced cancer growth and metastasis. It is used for the treatment against gastrointestinal stromal tumor and pancreatic cancer.

Ziv-Aflibercept

Ziv-aflibercept is available under the brand Zaltrap. It is a high-affinity blocker of VEGF-A and showed better therapeutic efficacy against colorectal cancer. Ziv-aflibercept also found to be an effective inhibitor of VEGFR-1 or VEGFR-2 stimulation [39].

Leflunomide (LFN)

Leflunomide (LFN) is an inhibitor of the mitochondrial enzyme dihydroorotate dehydrogenase, which plays a central role in the *de novo* pyrimidine synthesis pathway. It is recently found that LFN can produce anti-angiogenic effect in breast cancer cells by inhibiting the angiogenic soluble Ephrin-A1/EphA2 system [40]. However, the role of LFN in anti-angiogenesis needs to be studied further.

9.5 Drugs Targeting PI3Kinase/AKT/mTOR

The PI3K/AKT pathway plays an important role in blood vessels formation during angiogenesis. Studies have shown that p110α catalytic subunit of PI3K is very crucial for endothelial cell migration and angiogenesis [53] and defect in its function leads to dysregulation in vascular permeability. When VEGF binds to its receptor on normal endothelial cells, RAS and PI3K pathways are activated. Pharmacological inhibition of PI3K (α/β) suppressed both RAS or VEGF mediated vascular response and survival of primary endothelial cells. There are various inhibitors targeting the PI3K/AKT pathway have been developed and some of them are currently in clinical trials (Fig. 9.3).

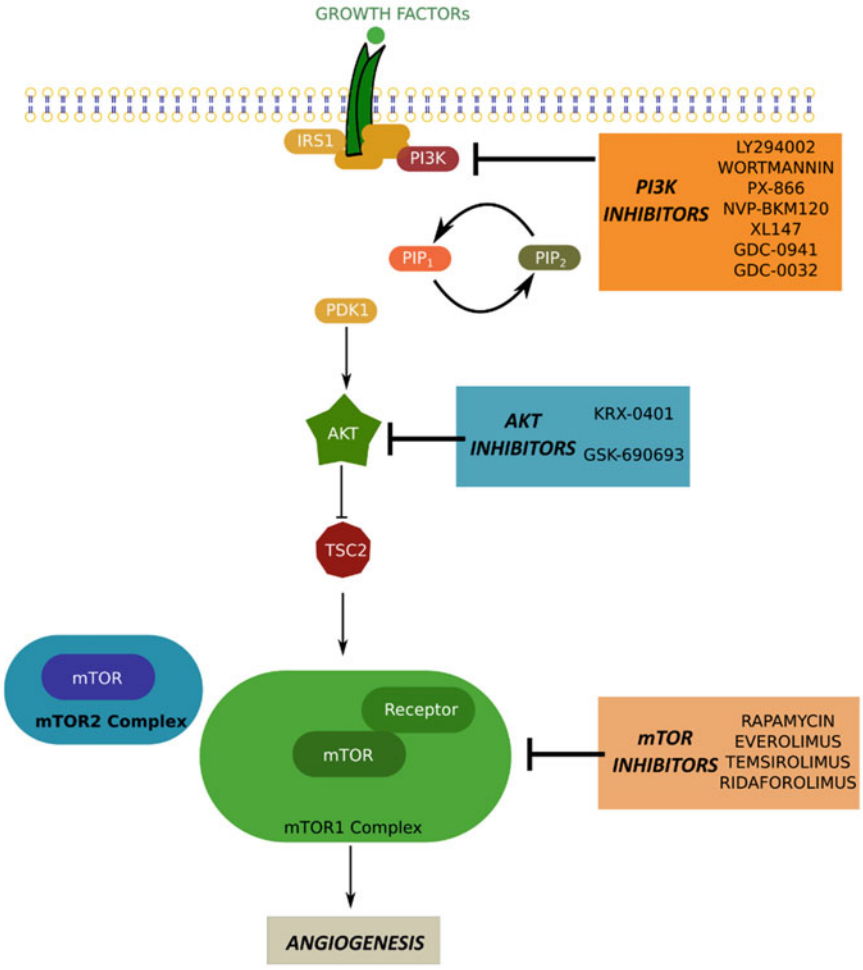

Fig. 9.3 Drugs targeting Pl3K/AKT/mTOR signaling pathway: Various inhibitors bind with Pl3K/AKT/mTOR individually and inhibit their activities, and finally hinder the process of angiogenesis. The pathway is regulated by binding of PI3K to receptor tyrosine kinases, leading to initiate the cascade of events. The phosphorylation and activation of AKT associated with mTORC activation, which results in different cellular processes

PI3K Inhibitors

LY294002 and Wortmannin are ATP binding PI3K inhibitors, and have been used broadly in preclinical models of cancer [41]. Treatment of LY294002 with gemcitabine is found to be effective against pancreatic cancer. PX-866, also a pan-PI3K inhibitor, is used for treatment against prostate, colorectal, and non-small cell lung cancer [42]. Buparlisib (NVP-BKM120) and Pilaralisib (XL147) are other pan-PI3K inhibitors that inhibit the activity of p110-α-γ enzymes

[43]. Clinical studies showed their safe effect in gastric and colorectal carcinomas. Pictilisib (GDC-0941) a selective, orally bioavailable inhibitor of pan-class I PI3K blocks the activity of p110-α//δ, further regulating the process of angiogenesis. It is used to treat solid cancer, breast cancer, gastric cancer, non-small cell lung cancer [44]. Taselisib (GDC-0032) is a PI3K inhibitor with higher attraction for mutated PI3Kα and decreased suppression against PI3Kβ [45]. Another drug, idelalisib has been used to treat certain relapses in chronic lymphocytic leukemia. Along with rituximab, it also shows therapeutic effects in follicular B-cell non-Hodgkin's lymphoma and on its own against small lymphocytic lymphoma [46].

AKT Inhibitors

These are majorly classified as ATP-competitive inhibitors, phosphatidylinositol (PI) analogs, and allosteric inhibitors. Perifosine (KRX-0401), a lipid-based inhibitor, that prevents the translocation of AKT to plasma membrane, required for pathway activation [47]. It is useful against various cancers such as breast, ovarian, multiple myeloma, leukemia, and osteosarcoma [54]. In vitro studies have shown that perifosine allows better therapeutic effect when given with cisplatin and paclitaxel in ovarian cancer [55]. Another new ATP-competitive AKT inhibitor that has to have selectivity for all three AKT isoforms is the GSK-690693. In vitro and in vivo studies in multiple cancer types have found GSK-690693 to suppress proliferation of cancer cells [48]. The compound was under phase I clinical trials but was withdrawn prior to enrollment.

mTOR Inhibitors

Sirolimus (rapamycin; Rapamune®), a well-known chemotherapeutic drug, having anti-angiogenic activity promotes apoptosis by inhibiting the mammalian target of rapamycin (mTOR) pathway [49]. Rapamycin and its analogs (rapalogs) along with FKBP12 (FK506-binding 12 kDa protein) bind to mTOR via its FRB site and inhibit specific cite on mTORC1; however, rapamycin resistance has been discovered in some epitopes phosphorylated by mTORC1. Some ATP-competitive inhibitors of mTOR directly target the kinase part of mTOR [56]. Unlike rapalogs, these can block both mTORC1 and mTORC2 completely. Some of these inhibitors also block PI3K along with the mTOR and are therefore categorize as dual PI3K/mTOR inhibitors.

Two rapamycin analogs, everolimus and temsirolimus (CCI-779; Torisel®), which inhibit cytostatic tumor growths and decrease capillary perfusion have showed some promising results in preclinical trials [50, 51]. Unfortunately, everolimus did not show significant efficacy in phase II clinical study but it still showed some anti-angiogenic properties that could allow for a potential use in combination therapy. Temsirolimus, on the other hand, along with chemotherapeutic agent, temozolomide, resulted in reduction of tumor growth and increased apoptotic

death in melanoma cells that had become resistant to BRAF inhibitor vemurafenib [57]. In phase I clinical trials, a combination of temsirolimus and hydroxychloroquine (an autophagy inhibitor) showed promising results with increased cell death in melanoma [58].

Ridaforolimus (AP23573;MK8669) a derivative of rapamycin has recently gained some attention. In preclinical studies, ridaforolimus when used alone reduced tumor growth up to 67% in leiomyosarcoma xenografts, however its use with other traditional drugs like with doxorubicin, carboplatin, or paclitaxel gave promising results in endometrial and sarcoma cells. In several phase II studies it has shown promising results in one endometrial cancer and sarcomas trial and in another trial stabilizing hematologic malignancies in 40% patients, showing strong response while 10% patients having partial stabilization of the cancer [52]. The drug has recently entered phase III clinical trial for sarcoma.

9.6 Drugs Targeting Hypoxia-Inducible Factor-1

An increasing number of chemotherapeutic drugs have been shown to inhibit tumor growth and suppress HIF action by reducing HIF-1α mRNA levels and protein synthesis as well as HIF subunit heterodimerization and transcriptional activity. Many of the following drugs that are used for the treatment of cancer or other diseases are given below.

Hycamtin (Topotecan), known as a topoisomerase I inhibitor, inhibits hypoxia-inducible factor (HIF)-1α protein aggregation. Drugs that suppress topoisomerase I and II levels are also able to reduce HIF-1α levels [59]. GL331, a podophyllotoxin derivative, and also topoisomerase II inhibitor reduced HIF-1α mRNA as well as protein levels [60]. DX-2-1, a carbomycin derived compound functions as a HIF-1 inhibitor, along with the other transcription factors. Vorinostat inhibits HDACs and HIF and regulates the release of growth factors, invasion and metastatic markers, and cytokines in cutaneous T cell lymphoma (CTCL) [61].

9.7 Advances in Clinical Trials and Drug Discovery for Anti-Angiogenesis

There are various clinical trials on the horizon for further analyzing anti-angiogenic therapy. Different combinations of drugs are tested for their efficacy and reproducibility. Paclitaxel in combination with nivolumab and ramucirumab are currently being under test as secondary chemotherapeutic agents in a phase II clinical study (UMIN000025947). These drugs show dramatic improvements and reduced side effects when associated with anti-angiogenic drugs. Garcimultiflorone K, polyphenol compound extracted from *Garcinia multiflora* stems directly act on the AKT/mTOR/p70S6K and AKT/eNOS pathways which leads to significant reduction of angiogenesis in zebrafish models by inhibiting proliferation, migration, and tube generation in EPC cells [62].

One of the recent studies combined anti-CD40 immunotherapy in combination with dual Ang2 and VEGFA blockade to achieve signification tumor regression. Interestingly, anti-CD40 alone or in combination with VEGFA blockade could not achieve the same results. This indicates a possible strategy to use Ang2 inhibition as an anti-angiogenic method along with T cell-targeting immunotherapies [63].

CIGB-247 vaccine used for VEGF suppression is another promising drug for tumor reduction. Recent phase I clinical trials of the vaccine showed that the cancer patients had more VEGF in their platelets, which were then reduced to the range observed in healthy control. This results shows that the CIGB-247 can normalize VEGF levels in platelets of the patients showing more promising possibilities ahead for this vaccine [64].

Another possible anti-angiogenesis compound that has recently gained interest is Celecoxib (CXB) [65]. CXB is believed to have many different antitumor mechanisms. These include proliferation inhibition, triggering of apoptosis, immunoregulation, and importantly some anti-angiogenic effects. Many clinical trials are currently looking into effectiveness of CXB as anti-tumor agent [66].

Interestingly, ibuprofen (IBP) is also looked into as a possible anti-angiogenesis drug. It has been found to decrease mitosis rate and trigger inhibition of proliferation of several types of cancer cells [67]. Recent in vitro experiments have suggested that IBP can induce anti-angiogenesis, apoptosis, and altered expression of Akt, p53, proliferating cell nuclear antigen, Bax and Bcl2 [68]. Radiation therapy in presence of the above-mentioned anti-angiogenic agents can greatly increase the effectiveness of cancer treatments, killing both cancer and endothelial cells at the same time.

9.8 Nanoparticle for Targeted Anti-Angiogenesis

Current status of anti-angiogenic therapy needs improvement as new studies show that tumor cells can use multiple pathways to achieve angiogenesis therefore becoming resistant against specific treatments. Further, evaluating the optimal dose calculation of angiogenic drugs, especially in presence of other chemotherapeutic agents, is very challenging. An interesting new approach in anti-angiogenesis is using nanoparticles (NPs) to target specific ligands to deliver drugs. This also helps in reduction in side effects and toxicity, further improving the overall efficacy of chemotherapy. Lipid-based Nps showed highly efficient delivery of VEGF siRNA in human lung samples resulting in inhibiting angiogenesis [69]. Sorafenib, an otherwise effective anti-angiogenic but with poor targeting capabilities showed high efficiency, when encapsulated with lipid NPs in treating glioblastoma by inhibiting CD31 [70]. Similarly, rapamycin and other equivalent drugs loaded onto lipid-based NPs proved to have strong targeted anti-angiogenic effects [71, 72]. Polymer NPs such as water-soluble TNP-470 conjugated 2-Hydroxypropyl methacrylamide (HPMA) copolymer and nanopolymeric micelles (Lodamin) have shown to provide some crucial benefits such as allowing for better targeting, controlled drug release and being orally delivered without toxic side effects. The NP targeted drug has been shown to inhibit A2058 human melanoma

and Lewis lung carcinoma (LLC) tumor growth [73]. Nanopolymer was also successful in delivering several anti-angiogenic drugs using LyP-1 peptide as a targeting ligand [74].

Inorganic NPs such as AuNPs inhibit VEGF165 leading to anti-angiogenesis. Quercetin when delivered with gold nanoparticles has been shown to effectively inhibit tumor angiogenesis, epithelial-mesenchymal transition, and metastasis by blocking EGFR/VEGFR2 controlled pathway in in vitro and in vivo breast cancer [75]. In in vivo mice model, use of nanoceria (NCe) NPs showed promising inhibition of ovarian cancer causing activation of MMPs and inhibition of vascular endothelial cell migration and proliferation [76]. Protein based NPs are another possible nanoparticle family ideal for drug delivery due to their highly biodegradable nature. An albumin-based NP encapsulating paclitaxel and 4-HPR (angioprevention vitamin A analog) showed excellent anti-glioma efficacy in mouse model by inhibiting angiogenesis, and inducing apoptosis [77]. Rapamycin's targeting efficiency can also be improved when combined with albumin-based NP in breast cancer xenograft models.

9.9 Conclusion

Angiogenesis is found be a significant process during tumor progression. Effective inhibition of angiogenesis may control the process of tumor growth but would not eliminate the tumor completely, especially with alone anti-angiogenic agent. Therefore a combination of various anti-angiogenesis agents may prove to be significant prospective for anti-cancer therapy. Novel multi-schema strategies, involving traditional chemotherapeutic approach with anti-angiogenic drugs can prove to be highly effective in reducing tumors and inhibiting them from metastasizing. Moreover, nanotechnology has shown promising results in providing better targeting and drug delivery to prevent angiogenesis within tumors while avoiding side effects. However, extensive studies are necessary in order to measure the potential relevance of nanoparticles-based strategies for clinical studies.

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Metastasis: A Major Driver of Cancer Pathogenesis

10

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Abstract

Cancer is a multifactorial condition that originates from genomic alterations in the cells, which confer them the ability to evade various cellular regulations and proliferate incessantly. Furthermore, the accumulation of these mutations confers metastatic abilities to the tumor cells, which help them in contriving various features essential for invasion of the host tissues and evading immune surveillance and thus spreading to distant sites. Metastasis is a key phenomenon in cancer pathogenesis, which involves invasion of host tissue, escape into the blood vascular system, survival within the circulation, extravasation into the secondary sites, establishment of micrometastasis, and colonization. The tumor cells utilize various host cells and pathways to reach the pre-destined sites, also known as pre-metastatic niches (PMNs). The primary tumor is known to secrete various factors, which render the secondary metastatic sites hospitable for the arriving tumor cells. These tumor cells, in turn, invade the PMNs and either undergo dormancy or outgrow to develop secondary metastases. Since metastasis involves

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a cascade of events, it also offers attractive targets for therapeutic intervention. This chapter elaborates the series of events involved in metastasis initiation and progression along with the role of PMNs and various therapeutic approaches to target metastasis.

Keywords

Metastasis · Pre-metastatic niches · Cancer · Tumor cells · Extracellular matrix · Anti-cancer therapy

10.1 Introduction

The process of movement of primary tumor cells from their original site of growth to other distant sites or organs, where they colonize and establish secondary metastases, was termed as metastasis by Jean Claude Recamier in 1829 [1]. During metastasis, the tumor cells gain the ability to invade neighboring tissue, access the blood supply, and disseminate to distant organs [2, 3]. Today, metastasis is considered to be a major contributor to cancer related deaths. In fact, 90% of the cancer associated mortalities are attributed to metastasis following failure of surgical resection and chemotherapeutic approaches [4, 5]. Metastasis is a multi-step process, occurring in a defined pattern, which involves a variety of steps in a successive manner, including the invasion of the surrounding tissue, intravasation into the blood vessels, survival of cancer cells in the blood circulation, extravasation into the distant sites, adaptation in the new tumor microenvironment, and colonization (Fig. 10.1) [6, 7].

In fact, the formation of PMNs by the primary tumor cells itself lays the foundation for metastatic spread, thus justifying the words said by Paget, "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil" [8]. Thus, the distant organs/sites (soil) which are occupied by the metastatic tumor cells (seed) are primed prior to the arrival of these cells by various factors secreted by the primary tumor itself, which render them conducive for the invading tumor cells to grow and colonize. The steps of a metastatic cascade are sequentially discussed below.

10.2 Invasion of the Surrounding Tissue

Invasion of a tumor into its malignant phenotype is the very fundamental step in metastasis. Normal cells in the body grow in a dynamic environment defined by the extracellular matrix (ECM) surrounding stromal layers. The ECM mainly comprises of collagen, fibronectin, proteoglycans, elastin, and laminins apart from water, proteins, and polysaccharides [9]. Whereas the tumor-associated stroma encompasses a heterogenous population of cells such as endothelial cells (ECs), fibroblasts, myofibroblasts, adipocytes, plethora of bone marrow-derived cells (BMDCs), and several immune cells including macrophages [10]. The ECM

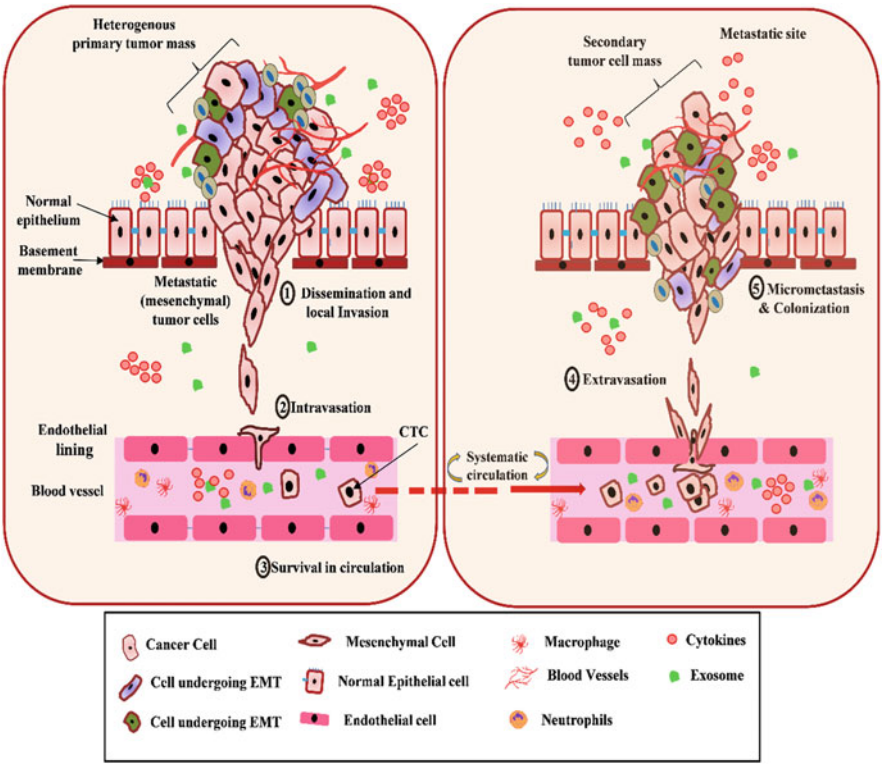

Fig. 10.1 The metastatic cascade: Metastasis encompasses a sequential occurrence of events, which ensues from invasion followed by the intravasation, survival in the circulation, extravasation to the distant metastatic sites, development of micrometastases, and colonization of the occupied sites. The role of various host cells in accomplishing each of these steps of this cascade is imperative

performs a key role in cell growth, morphogenesis, and plasticity of the parenchyma by providing a spatio-temporally regulated scaffold to the epithelial cells, thus maintaining the cell polarity. It is also responsible for providing essential bio-chemical and bio-mechanical signals or cues required for cellular differentiation and homeostasis, alteration of which is known to cause cancer [11–13]. The metastatic process initiates with the acquisition of invasive potential by the primary tumor cells, which then break free from the basement lining and move into the surrounding tissues, a phenomenon known as epithelial to mesenchymal transition (EMT) (Fig. 10.2) [14–16]. Various aspects of EMT are described in subsequent sections below.

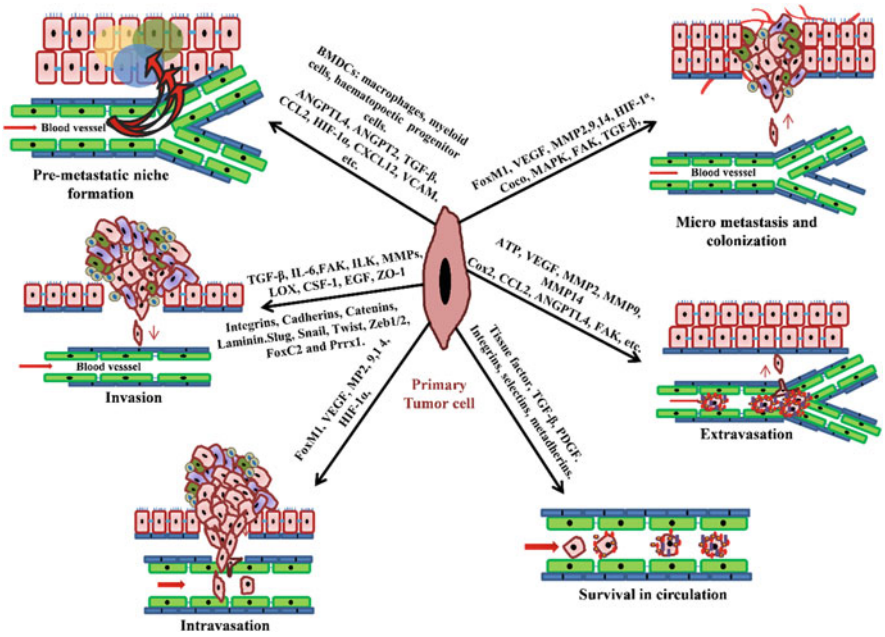

Fig. 10.2 Various factors regulate the metastatic cascade: Metastasis encompasses several sequential steps, which are regulated by the interplay among various signaling molecules released by the primary tumor cells and the host-derived factors. Several factors such as TGF-β, MMPs, etc. exhibit pleiotropic functions in metastasis

10.3 Epithelial to Mesenchymal Transition (EMT)

The tumor-associated stroma consists of a heterotypic population of cells, which resembles the inflammatory stromal configuration and is induced upon wound healing processes under normal physiological conditions. This modulated stroma then releases various signaling molecules such as interleukin-6, transforming growth factor (TGF)-β, WNT, etc. which assist the adjacent carcinoma cells to activate the silent EMT mechanism. EMT involves the conversion of normal epithelial cells to an invasive mesenchymal phenotype by modulating their apical-basal polarity [17–19]. These mesenchymal cells are characterized by enhanced invasive and migratory capabilities and display resistance to apoptosis.

Evasion of apoptosis upon detachment from the anchorage of the basement membrane, i.e. anoikis, is a key feature of invasive cells [20, 21]. Integrins, which mediate the cellular attachment to the ECM, play a major role in escaping anoikis. Among various forms of integrins, upregulation of $\alpha_5\beta_3$ integrin is important in this process [22–24]. It also stimulates the production of matrix metalloproteinase (MMP) 2, thus further enhancing metastasis [25]. Integrin associated signaling pathways subsuming focal adhesion kinase (FAK) and integrin linked kinase

(ILK) are also involved in the obstruction of anoikis [26–28]. Similarly, cadherins contribute critically in mediating cell–cell adhesion by forming intercellular complexes with catenins that link them to the cytoskeletal proteins. Thus, the loss of certain epithelial cell surface markers such as ZO-1, laminin, E-cadherin, which favor homotypic cell adhesion, and the upregulation of N-cadherin, which promotes heterotypic cell adhesion, lead to the dissolution of intercellular junctions favoring the mesenchymal phenotype [29]. This transition is facilitated by activation of various pleiotropic transcription factors, namely Slug, Snail, Twist, Zeb1/2, FoxC2, and Prrx1 [30, 31]. This allows the migrating tumor cells to cross the basement membrane as well as the ECM, and intravasate into the blood or lymphatic vessels either as single entities or as clumps [32].

The migratory process involves the mechanical modulation of ECM by contraction and protrusion of the cells accompanied by degradation of the ECM by various proteases. Although the degradation of the ECM is the most common mode of migration of tumor cells, a protease independent mechanism is also known [33]. This mode involves the formation of invadopodia (actin-rich projections of cancer cells), which utilize protrusive and contractile forces to make their way through the ECM, indeed depending on the plasticity of the ECM components [33–35]. The role of macrophages in the initial stages of metastasis is also noteworthy. They have been shown to assimilate along the endothelium of blood vessels adjacent to the site of inflammation, and these macrophages secrete epidermal growth factor (EGF), which drives the chemotactic movement of tumor cells towards the vasculature as observed in breast cancer models [36]. The tumor cells exhibit EGF receptors on their surface and also secrete colony stimulating factor 1, which draws the macrophages and instigates them to secrete EGF and vice versa, thus forming a closed paracrine loop among themselves. This paracrine signaling results in modulation of the actin cytoskeleton in both tumor cells as well as macrophages, thus leading to the development of invadopodia in the migrating tumor cells and podosomes in macrophages.

The protease dependent mechanism followed by the migrating cells involves secretion of various MMPs responsible for the breakdown of several proteins involved in maintaining the integrity of the basement membrane and associated cellular parenchyma [37, 38]. The MMPs are also called as matrixins, and they belong to the metzincin superfamily of zinc-endopeptidases, which specifically cleave a variety of ECM components by proteolysis. Apart from the MMPs, other prominent members of this superfamily include A Disintegrin and metalloproteinases (ADAMs) and A Disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS). MMPs are further categorized on the basis of their substrates into Collagenases, Gelatinases, Stomelysins, Matrilysins, Membrane-type MMPs, and other MMPs [37]. These MMPs cleave and degrade their respective substrates, thus facilitating the alteration of the ECM.

Moreover, the rapidly proliferating tumor mass also develops hypoxic conditions towards its core due to lack of proper blood supply, thus generating hypoxic conditions. Hypoxia mediated upregulation of lysyl oxidase (LOX) is also known to activate FAK and integrins, which further drive actin polymerization in the

invadopodia, thus enhancing the migration of these cells. The exploration of LOX functioning in breast cancer cells uncovered its essential role in recruiting various MMPs, viz. MMP 2, MMM 9, and MMP 14, thus potentiating the tumor cell motility [39]. Therefore, the cancerous cells invade through the ECM, cross the basement membrane, reach the nearest blood or lymphatic vessel, and proceed to intravasation.

10.4 Intravasation

The process of entering the lymph or blood vessels by the locally invasive cancer cells is known as intravasation, which marks the second step in the metastatic cascade. There are two known modes of dissemination of cancer cells: the hematogenous spread, which occurs via the blood vessels, and the lymphatic spread, which proceeds via the lymphatic system. Hematogenous spread of the carcinogenic cells is the most common mode of transmission in metastasis [4]. In order to intravasate, the presence of blood vessels in proximity to the tumor cells is mandatory. The tumor cells therefore induce neo-angiogenesis by secreting various chemokines, which induce the generation of nascent blood vessels. This vasculature generated by neo-angiogenesis is prone to leakage due to lack of basement membrane and unorganized perivascular layers. These haphazardly formed blood vessels thus lead to the irregular supply of nutrients and oxygen to the rapidly proliferating tumor mass. Additionally, these mal-developed vessels provide various growth factors and cytokines to the tumor-associated matrix but their leakiness also leads to a poor blood supply to the core of the developing tumor, thus rendering it hypoxic. Various transcription factors that are responsive to low availability of oxygen are thus activated, which bestow the tumor cells with the ability to survive these oxygen deficient conditions. One such key protein is the hypoxia inducible factor (HIF1α). HIF1α further activates various subordinate genes involved in angiogenesis and invasion such as Forkhead Box M1 (FOXM1) and vascular endothelial growth factor (VEGF), etc. [40]. FOXM1 is an oncogenic transcription factor that controls the expression of several downstream genes regulating metastasis. In fact, FOXM1 is also known to transcriptionally regulate VEGF, MMP 9, etc. [41]. VEGF is the most potent angiogenic factor involved in the production of new blood vessels [42]. Besides VEGF, activation of MMPs such as MMP 2, 9, and 14 further aggravates the invasive nature of the carcinoma cells [38]. MMP 9 leads to the release of the sequestered VEGF, thus making it available to bind to its receptor VEGF-R, which enhances the generation of defective endothelial blood vessels. Thus, the interplay between these various molecules leads to intravasation of the invasive tumor cells into the blood circulation, resulting in circulating tumor cells (CTCs). These CTCs upon survival within the blood vessels migrate into the distal target organs and form micrometastases (Fig. 10.2).

10.5 Survival in the Circulation

Upon entering the blood circulation, the majority of the CTCs die, either due to the stress of blood flow or due to the immune destruction. Thus, only 0.01% of the circulating cells survive to form secondary metastases even though tumor cells shedding into the vasculature provide an ample number of tumor cells to intravasate [43, 44]. Altogether, they spend a short time in transit through the blood vessels and usually get trapped into the first capillary bed which they encounter [45]. Prior to their entrapment, the CTCs encounter a plethora of cells in the circulation such as platelets, natural killer cells (NK cells), and various bone marrow cells during their travel to secondary metastatic sites. The CTCs are able to undergo remarkable changes in their nuclear and overall shape to fit into the capillaries [46]. They acquire various features that enable survival in the host circulation, such as loss of various immunogenic markers from the cell surface and elevated expression of certain immune-suppressive markers, thus enabling them to evade apoptosis mediated by NK cells and circulating macrophages [47]. The CTCs express tissue factor (TF) as well as P-selectin ligands on their surfaces, which lead to interaction and activation of platelets, respectively, while instigating coagulation as well [48, 49]. Platelets are known to play a critical role in the survival of CTCs in the circulation as their depletion by genetic manipulation or pharmacological inhibition in metastatic tumor models greatly reduces metastasis [50]. Stimulation of platelets by the CTCs also serves as a source of TGF-β, which suppresses the immunolytic ability of NK cells by diminishing the NKG2D receptor. TGF-β is also reported to act in concert with the platelets to induce the activation of nuclear factor kappa B (NF-κB) pathway in the CTCs, thus sustaining their EMT phenotype. The secretion of platelet derived growth factor by platelets is also known to enhance their survival in circulation [51–53]. Apart from this, the interaction of platelets with the CTCs forms a physical shield over them forming tumor-platelet emboli, which helps them escape the immune surveillance. The CTCs draw similar benefits from the neutrophils present in the circulation, for example, the formation of neutrophil extracellular traps (NETs), which are known to entangle the tumor cells in circulation, thus enhancing their survival and providing them apt surface to adhere to the endothelial cells and extravasate [54]. Formation of tumor-host cell emboli mediated via interactions of CTCs and immune cells not only prevents the metastasizing cells from immune destruction but also helps them to reach the destined secondary sites and extravasate. Apart from passive trapping of the tumor emboli into the capillaries, the adherence ability of these complex structures is also found, which enables them to adhere to vessels of larger than the capillary diameter. This active adhesion is mediated by various adherence molecules such as integrins, selectins, and metadherins, which are also contributed by the interacting platelets, leukocytes, and other stromal fibroblasts [55–59]. Therefore, CTCs survive the circulation and get blocked in the capillary beds, where they extravasate into the metastatic site and form micrometastases.

10.6 Extravasation

Following the course of the bloodstream, the CTCs either get arrested in the capillary beds within few minutes after entering the circulation due to the capillary diameter restriction or adhere to the EC surface mediated by various adhesion and cell signaling mechanisms. Extravasation is similar to intravasation, which requires the CTCs to cross the endothelial barrier and this phenomenon is referred to as transendothelial migration (TEM) [60]. Most of these extravasated cells then migrate to the PMNs but only a few survive and proceed to micrometastasis and colonization whereas most of them are destroyed by immune cells. While the tumor cell-platelet emboli arrest at the endothelial lining, the activated platelets release adenine nucleotides (viz. ATP), which interact and activate the P_2Y_2 receptors on the ECs. This interaction leads to downstream activation of protein kinase C and causes unlocking of the endothelial barrier [61]. As mentioned earlier, the interaction of CTCs with various cells in the blood circulation as well as the endothelium leads to the secretion of various other chemokines such as VEGF, MMPs, cyclooxygenase 2 (COX2), and C-C motif ligand 2 (CCL2). These chemokines alter the integrity of the vascular membrane, thus facilitating extravasation [60, 62]. Similarly, the lung tumor and stromal cells secrete CCL2 which recruits CCR2+ monocytes that facilitate extravasation [63, 64]. Furthermore, secretion of TGF-β by the CTCs is also known to stimulate secretion of Angiopoietin-like 4 (ANGPTL4), and promote vascular permeability in breast carcinoma cells [65, 66]. Most of these factors are also implicated in the formation of PMNs as well as facilitation of invasion and intravasation, thus implying the pleiotropic nature of these molecules in metastasis.

The employment of various bone marrow-derived cells (BMDCs) further aids in extravasation by inducing the expression of several cell surface markers on both the ECs as well as the CTCs. For example, the recruited neutrophils are known to induce expression of selectins, integrins, intercellular adhesion molecules (ICAM 1) on the ECs as well as the tumor cells, thus favoring cellular interactions [67]. These interactions, in turn, facilitate the movement of CTCs from the endothelial lining towards the PMNs. In fact, the expression of β1 integrin and FAK helps in forming filopodium like protrusions, which are required for the invasion of vascular endothelium. Apart from the common mechanism of TEM, CTCs have also been reported to skip the conventional mode of extravasation and proliferate in the vascular lumen itself, thus disrupting the endothelial barrier by the shear stress of proliferating tumor mass [55]. Interestingly, in 2016, Strilic et al. reported a previously unknown mechanism of extravasation in lung metastasis, wherein CTCs were shown to elicit controlled necrosis (necroptosis) in the ECs, thus disrupting the endothelial membrane [68].

10.7 Micrometastasis and Colonization

Certain sites in the human body are predisposed to metastatic growth. This predisposition also leads to organotropic metastasis in cancer, for example, the prostate tumor cells metastasize preferably to bone while cancer of breast colonizes bone, liver, brain, and lungs whereas colorectal cancers mostly metastasize to the liver. This propensity of various cancers to disseminate to various distant organs relies on the receptive environment provided by the PMNs.

10.8 Pre-metastatic Niche (PMN)

The primary tumor is known to send off certain chemokines (collectively known as secretome) to induce the formation of pre-metastatic niches at distant sites, thus enabling the disseminated tumor cells to colonize those tissues easily (Fig. 10.2). These factors stimulate the establishment of a suitable microenvironment in distant sites/organs that are amicable to the growth of secondary metastases prior to the arrival of metastasizing cells [8, 69, 70] (Fig. 10.2). This suitable microenvironment is also known as PMN. These PMNs are formed as a consequence of combined systemic efforts of the tumor secretome and extracellular vesicles derived from tumors. These secreted factors support a cascade of events culminating in the establishment of PMNs. Formation of anomalous blood vessels is the foremost event followed by modification of the local cell milieu and recruitment of various other cells such as BMDCs subsuming macrophages, myeloid cells, and hematopoietic progenitor cells to the target site which, in turn, attract the CTCs to the PMNs.

Tumor derived factor such as EGFR ligand epiregulin, COX2, MMP 1, MMP 2, MMP 9, ANGPTL4, VEGF-A, etc. are well observed to aggravate the loss of integrity of blood vessels in breast cancer [71]. These factors lead to the activation of FAK, which leads to disruption of inter-cellular connections among the ECs, thus facilitating the metastasis in breast cancer [62]. In fact, the activation of MMP 9 leads to the release of various sequestered cytokines, such as stromal cell-derived factor 1, which serves as a chemoattractant for CTCs [70]. The secretion of TGF-β is also reported to provoke the expression of ANGPTL4 and angiopoietin 2 in breast and lung tumor cells, respectively, thus increasing the permeability of blood vessels [65, 72]. Moreover, the secretion of chemokines such as CCL2 by both the tumor and stromal components leads to the recruitment of various BMDCs, which assist the CTCs in the process of extravasation as well as the formation of PMNs. CCL2 acts as a powerful chemoattractant for macrophages, NK cells, monocytes, and T-lymphocytes, thus functioning as a primary mediator of PMN formation and the metastatic colonization in various cancers [63, 73–75]. Apart from recruiting these cells, in order to promote an inflammatory environment in the PMNs, CCL2 is also known to suppress the immune ability of NK cells in breast cancer and melanoma models by hampering their maturation, thus shielding the CTCs from NK cell mediated destruction [76]. Another common regulator of inflammatory cues in

PMNs is the S100 family of proteins. They act both intracellularly and extracellularly to mediate the cross-talk between stromal cells and tumor cells during the configuration of PMNs. In the lung PMNs, expression of these S100 proteins on the endothelium layer is known to be instigated by various tumor secreted factors such as TGF-β, VEGF-A, TNF, and CD11b + myeloid cells [73, 77]. Similarly, HIF1 is also a crucial protein involved in the formation of PMN in various cancers [39, 78]. Studies encompassing breast cancer have demonstrated the increment in the shedding of extracellular tumor vesicles in a HIF dependent manner [79].

Apart from the chemokines secreted by tumor cells, extracellular vesicles (EVs) secreted by the tumor cells also play a substantial role in not only the establishment of PMNs but also carrying out metastasis. Tumor secreted EVs have been shown to carry genetic material (DNA and RNA), micro RNAs, proteins, and metabolites (fats and small metabolites), thus promoting PMN formation and disease progression [80, 81]. Surprisingly, tumor cells are known to exhibit amplified ability to secrete EVs, which is, in turn, boosted by hypoxic conditions [69, 79]. Various exosomes derived from the primary tumors display adhesion molecules on their surface such as integrin, which bind to ECM components and lead to the development of organotropic PMNs favoring organ-specific metastasis.

Facilitated by the PMNs, the extravasated cells then enter the secondary site, which is usually distant and has a different microenvironment as compared to the primary tumor site. Most of these cells persist as single disseminated tumor cells (DTCs) in the foreign tissue and either die or enter a state of dormancy, which eventually are either eliminated by the immune system or develop successful metastases [71, 82]. This period of dormancy can last up to days, weeks, or even years depending upon the availability of supportive signals and proliferative microenvironment. The state of dormancy is activated when the disseminated tumor cells fail to adapt to the new microenvironment or by the over-powering anti-proliferative signals in the secondary tissue or even by the failure to induce angiogenesis [83]. The patients who develop such dormant DTCs are designated to have minimal residual disease and are on the verge of greater risk of metastatic relapse. The dormant DTCs instigate certain signaling mechanisms to sustain in a quiescent state, such as the activation of AKT and SRC pathways by secretion of CXCL12 by the stroma in the metastatic niche. Upon metastasis to the bone, breast cancer cells have been shown to set off pro-survival mechanisms in response to CXCL12 secreted by the bone parenchyma [84]. These pro-survival pathways enable the DTCs to evade TRAIL-induced apoptosis as well as resist anoikis by further expressing tyrosine kinase receptor (TrkB) or by stimulating the non-canonical WNT pathway mediated by WNT2 [85]. The failure to interact with the ECM, and thus sensing the mitogenic cues also results in the induction of dormancy. For example, the DTCs undergo dormancy when they fall short to interact with the β1 integrin, which leads to the failure in stimulating the FAK mediated proliferative signaling [86–88]. Various such chemical interactions among the ECM and DTCs are also reported to induce a cell cycle exit into the G_0 phase, thus inducing a state of suspended growth [89]. The emergence of these indolent DTCs definitely requires favorable signals, which is distinct in different cancers. For example, the gain of

VCAM1 expression can activate the metastases of bone, by binding to the $\alpha_4\beta_1$ integrin receptor on the osteoclast progenitor cells, thus initiating colonization [90]. Similarly, the micrometastases in the lungs breakout of dormancy by expressing coco, which is an inhibitor of the bone morphogenetic protein (BMP) signaling thus potentiating metastatic colonization. These gains of function in the dormant metastatic cells indicate a low-level proliferation of the cells, which seems to be inevitable for the survival of DTCs. Acquisition of pro-proliferative signaling, mediated by MAP kinase, FAK, TGF-β, etc. is also known to enhance the colonization process as well [91].

10.9 Targeting Metastasis: Opportunities and Challenges

Metastasis is a highly unpredictable event, almost leading to the culmination of cancerous growth, making it certainly difficult to treat the cancer patients due to widespread mutations acquired by the metastasizing cells [92, 93]. Since metastasis is the major contributor to cancer related mortality, targeting metastasis provides a vast window of possibilities in dealing with cancer. However, by the time metastasis is detected in cancer patients, it has already spread to distant sites, which makes it a daunting target to follow [94]. Moreover, the involvement of various host cells, thus forming a heterogenous population that initiates and sustains metastasis is another major hurdle in pharmacological targeting of the metastatic cascade. Genetic instability forms the basis of neoplastic growth and the accumulation of these mutations with time makes it difficult to control metastasis. Increasing genetic instability confers the tumor cells with unprecedented variations which not only allow them to evade immune checkpoints but also survive under unfavorable conditions. Nevertheless, analysis of the metastatic cell karyotype and single cell studies have shown that these cells can originate from a single tumor cell potentiated by genetic variations [95, 96].

Since metastasis consists of a series of events, blocking the progression of any of these steps can be crucial in stopping it. While dealing with cancer metastasis, the majority of the therapies target the rapidly proliferating cells and associated mechanisms. Various anti-metastatic approaches have been enlisted below in Table 10.1. However, since the DTCs are known to be crucial purveyors of metastatic growth and relapse, specific approaches to target them should also be employed to obtain the recurrence-free survival of cancer patients. Different approaches to target metastasis have been employed, such as the inhibition of invasion promoting MMPs, thus curbing metastasis. The role of platelets in assisting CTCs to survive and extravasate has also garnered attention, thus the drugs targeting platelets have also been utilized against metastasis, although they do not reduce pre-existing lesions [115]. Following the entry into the blood, the CTCs have been proposed as markers of metastasis; however, these cells can also be targeted to prevent the establishment of metastases. With the advent of various techniques for isolating the CTCs from patient blood samples including the FDA approved Cellsearch® platform, various approaches to target them have been deployed

Table 10.1 Various inhibitors targeting different target molecules or pathways being used in treatment of metastatic cancer

S. No.	Name	Target molecule/ pathway	Clinical Status	References
1.	Bevacizumab (monoclonal antibody)	VEGF/angiogenesis	Approved by FDA for resistant ovarian cancer, glioblastoma, cervical cancer, colorectal cancer, metastatic lung cancer, and renal cancer	[97–102]
2.	Denosumab (monoclonal antibody)	Receptor activator of nuclear factor kappa-B ligand/osteoclast activation	Approved by FDA for glioblastoma, metastatic lung cancer, colorectal and renal cancer. Also approved for cervical, colorectal, and resistant ovarian cancer	[103, 104]
3.	Cetuximab (monoclonal antibody)	EGFR	Metastatic colorectal carcinoma, non-small cell lung cancer (NSCLC), and head and neck cancer	[105]
4.	Gefitinib/ Erlotinib (small molecule)	EGFR/downstream receptor tyrosine kinase pathway	Approved by FDA for metastatic NSCLC	[106]
5.	Dasatinib (small molecule)	SRC/ABL kinase	Approved by FDA for chronic myeloid leukemia (CML) and resistant acute leukemia (AL)	[107]
6.	Olaparib (small molecule)	Poly (ADP ribose) polymerase	Approved by FDA for metastatic breast cancer	[108]
7.	Lutetium Lu 177dotate (radioactive compound)	Somatostatin receptor	Approved by FDA for neuroendocrine tumors (GEP-NETs)	[109]
8.	Abiraterone acetate (hormone drug)		Approved by FDA for castration resistant prostate cancer in combination with prednisolone	[110]
9.	Abemaciclib (small molecule)	CDK4/CDK6	Approved by FDA for metastatic breast cancer	[111]
10.	Brentuximab vedotin (antibody drug conjugate)	CD30 antigen	Approved by FDA for classical Hodgkin's lymphoma in combination with chemotherapy	[112]
11.	Osimertinib (small molecule)	EGFR	Approved by FDA for metastatic NSCLC	[113]
12.	Trastuzumab deruxtecan (monoclonal antibody-drug conjugate)	Human epidermal growth factor receptor 2 (HER2)	Approved by FDA for unresectable and metastatic HER2 positive breast cancer	[114]

[116–118]. Since the diagnosis of cancer in its earliest stages is not possible, targeting the formation of PMNs does not sound to be a confident option. Surgical resection of primary tumors definitely reduces the tumor cell load as well as the clonal variants in the host body; however, a holistic approach, which can target multiples facets of metastasis simultaneously, seems to be the best option for now.

10.10 Role of Natural Compounds in Targeting Metastasis

Targeting metastasis in anti-cancer research has proved to be an effective approach to curb cancer. However, the use of anti-metastatic agents is associated with several adverse outcomes. Surgical removal of tumor is also not possible in every carcinogenic scenario, for instance, in leukemia. Similarly, radiotherapy also has its own limitations and cannot be used everywhere as a generalized anti-cancer approach. In such scenario, the use of natural compounds against metastasis has proven itself a boon for cancer patients. Recent years have witnessed a spike in the use of natural compounds in treating cancer, and this is further aided by the fact that utilization of natural compounds is considered safer with no or lesser side effects than any other anti-cancer approach. Therefore, a variety of natural anti-metastatic agents are being currently used against cancer. A few of recently used anti-cancer natural compounds are listed below in Table 10.2.

10.11 Conclusion and Future Perspectives

Metastasis is a life-threatening phenomenon, which is initiated by the primary tumor cells and it subsequently marks distant organs for the development of secondary tumors by forming PMNs. Although this cascade has been acknowledged as the basis of most cancer related deaths for several years, the precise mechanisms and molecules involved in the spatio-temporal regulation of this cascade are still incompletely understood. However, the active involvement of the host cells and chemokines with the tumor cell milieu has garnered considerable attention and appreciation in recent years. The utilization of host-derived factors and cellular components for metastatic dissemination demonstrates a remarkable interaction among the primary tumor cells and the metastatic niches. Additionally, the rebel nature of metastatic cells not only allows them to successfully evade the host immune system but also utilize it for their own propagation and survival. These characteristics also bestow these cells with the ability to resist various therapeutic agents targeting cancer. Thus, metastasis stands as a major challenge for the scientific community today in dealing with cancer and necessitates in-depth research in the coming years. Although a plethora of studies have shed light on various happenings that lead to the origin of primary tumors and subsequent establishment of clinically detectable metastases, still a lot of effort is needed to comprehend the cues leading to the initiation of metastasis and subsequent colonization of distant metastatic sites. Further dissection of the microenvironment alterations and

Table 10.2 Various natural compounds, their chemical class, source, molecular targets, and type of cancer where they are used

S. no.	Compound	Class	Source/Name	Molecular target(s)/	Type of cancer studied	Reference (s)
1	Evodiamine	Alkaloid	Plant/*Evodia rutaecarpa*	NFκB, MMP2, pERK1/2	Breast, colon, lung, melanoma and nasopharyngeal cancer	[119–123]
2	Hirsutine	Alkaloid	Plant/*Uncaria rhynchophylla*	MMP2/9, NFκB, ROCK1/PTEN/PI3K/GSK3β.	Breast cancer, lung cancer	[124–126]
3	Naringenin	Flavonone	Plant/citrus fruits.	VEGF, MMP2/9, AKT, mTOR, TGF-β.	Breast cancer, lung cancer, prostate cancer, pancreatic cancer	[127–131]
4	Genistein	Isoflavonone	Plant/*Genista tinctoria*	FLT4, MMP2/9, FAK, NFκB, ERK/PI3K/AP1	Lung cancer, prostate cancer, hepatocellular carcinoma	[132–135]
5	Myricetin	Flavonoid	Plant/*Myrica nagi*	MMP2/9, STAT3, PIM1/CXCR4, PI3K	Breast, cholangiocarcinoma, colon, esophagus prostate cancer, pancreatic cancer, medulloblastoma	[136–140]
6	Silibinin	Flavonoid	Plant/*Silybum marianum*	MMP2, vimentin, NFκB, Zeb1, SLUG, TGF-β	Lung cancer, prostate cancer, bladder carcinoma	[141–143]
7	Delphinidin	Anthocyanidin	Plant/ pigmented fruits and vegetables	ERK/p38MAPK, MMP9, NFκB, EGFR	Colorectal cancer, osteosarcoma, breast cancer, hepatocellular cancer	[144–147]
8	Shikonin	Naphthoquinone	Plant/ *Lithospermum erythrorhizon*	Integrin b1, ERK1/2, MMP2/9, GSK3β/β-catenin, RIP1/3, SIRT2	Prostate cancer, breast cancer, lung cancer, osteosarcoma, colorectal cancer	[148–151]
9	Sulforaphane	Isothiocyanate	Plant/ cruciferous vegetables	MMP2/9, pERK, MMP9, GSK3β/β-catenin, EGFR, TRAIL, BCl2/X_L and MCL1, COX2/MMP2/9/SNAIL/ZEB1	Lung cancer, prostate cancer, skin cancer, breast cancer, bladder cancer	[152–158]
10	Curcumin	Curcuminoid	Plant/*Curcuma longa*	NFκB, AP1, STAT3, MMP2/9, FAK, HLJ1	Breast cancer, lung cancer, prostate cancer	[159–161]

11	Paclitaxel	Diterpene	Plant/*Taxus brevifolia*	Tubulin, Aurora kinase/cofilin1	Breast cancer, lung cancer, glioblastoma, gastric cancer	[162–165]
12	Camptothecin	Alkaloid	Plant/ *Camptotheca acuminata*	DNA topoisomerase I	Ovarian cancer, colorectal cancer, lung cancer, pancreatic cancer, gastric cancer	[166–169]
13	Actinomycin D	Glycopeptide	Bacteria/ *Streptomyces* sp.	RNA polymerase I	Rhabdomyosarcoma, testicular cancer, Ewing's sarcoma, ovarian cancer, lung cancer, pancreatic cancer	[170–173]
14	Bleomycin	Glycopeptide	Bacteria/ *Streptomyces verticillus*	DNA strands	Squamous cell carcinomas, Hodgkin's lymphomas, and testicular tumor	[174–176]
15	Doxorubicin	Anthracyclines	Bacteria/ *Streptomyces peucetius*	Bcl2/Bax, topoisomerase I and II.	Breast cancer, acute lymphocytic leukemia, Kaposi's sarcoma	[177–180]
16	Vinblastine	Alkaloid	Plant/ *Catharanthus roseus*	Tubulin	Breast cancer, renal cell carcinoma, melanoma, lung cancer	[181–185]
17	Cytarabine	Anti-metabolite	Marine animal/ *Tectitethya crypta*	DNA replication	Acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, non-Hodgkin's lymphoma	[186–190]
18	Trabectedin	Alkylating agent	Marine animal/ *Ecteinascidia turbinata*	FUS-CHOP, IL-6, p-glycoprotein.	Liposarcoma, leiomyosarcoma	[191–193]
19	Brentuximab vedotin	Antibody drug conjugate	Marine/ *Dollabella auricularia*	CD30 antigen	Hodgkin's lymphoma	[194]

(continued)

Table 10.2 (continued)

S. no.	Compound	Class	Source/Name	Molecular target(s)/	Type of cancer studied	Reference (s)
20	Salinosporamide A	Bicyclic g-lactone b-lactam	Bacteria/ *Salinispora tropica*	NFκB, MMP9	Multiple myeloma and mantle cell lymphoma	[195–197]
21	Quercetin	Flavonoid	Plant/ cruciferous vegetables	MMP 2/9	Melanoma, oral cancer	[198]
22.	Carnosol	Polyphenol	Plant/ *Rosmarinus officinalis*	MMP 2/9	Melanoma	[199]
23.	Gambogic acid	Xanthonoid	Plant/*Garcinia hanburyi*	MMP 2/9	Adenocarcinoma, breast cancer	[200]

host-tumor interplay will not only allow us to understand the early events involved in metastasis but also will assist us to formulate specific and better therapeutic modalities against it.

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Designing Personalized and Innovative Novel Drug Therapies for Cancer Treatment

11

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Abstract

Cancer being a multifactorial disease, its genesis and progression are enormously complicated. The classical chemotherapeutics along with recent targeted molecular therapy approach have not been effective in complete eradication of all tumor cells and is often been limited by drug resistance and side effects on normal tissues and cells. With the fast evolving field of genomics and molecular medicine translating into precision medicine, the importance of individualized therapeutic protocols has been realized. For transitioning from surgical treatments to radiotherapy to chemo and immunotherapies, in this fast advancing world, it will not be far away when the personalized medicine will be the choice of treatment for

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one and all. The major challenge in the anticancer drug development is multidrug resistance and relapse. In this chapter, we describe the promising anticancer targets in different phases of drug development in clinical trials along with new drug targets for personalized cancer treatment in near future.

Keywords

Cancer vaccine · Gene therapy · Monoclonal antibody · Gene editing · Nano-delivery

11.1 Introduction

Cancer is the second leading cause of mortality and morbidity, just after the cardiovascular diseases, causing almost ten million deaths each year globally [1, 2]. Current standard treatment modalities include chemotherapy, radiotherapy, and surgical resection [3–6]. Although chemo- and radiation-therapies when applied either alone or in combination are effective in killing a population of malignant cells, however, these therapies cannot eradicate all malignant cells. Consequently, relapse occurs and tumor cells metastasize at distant sites [7]. Moreover, even when tumor cells initially respond to chemotherapeutic agents and irradiation treatment, after a while, they can rapidly develop molecular mechanisms of resistance and continue their growth and spread over the body [8]. Furthermore, cytotoxic chemotherapeutics and radiotherapy are toxic also to healthy cells, destroying normal functioning of several tissues and leading to bone marrow toxicity, hematological toxicity, cardiotoxicity, neurotoxicity, hepatotoxicity, and nephrotoxicity among others [9]. Taking into consideration these bottlenecks in the current cancer treatment methods, there is no doubt that novel, more efficient, and safer strategies are highly needed to reduce the duty that humankind must pay to this frightening disease.

Until the recent years, the same treatment scheme has been prescribed to patients suffering from the same type and stage of tumors. However, individuals with the same malignancies can often react differently toward the same treatment scheme depending on the genetic changes in their tumors [10]. Personalized approach in cancer treatment takes these genetic peculiarities into consideration and administers the most efficient therapy to those patients who gain the maximal benefit from it, sparing others from toxic side effects. The selection criteria for such personalized strategy of drug prescription include the expression of certain targets on the tumoral cells or in the cancer microenvironment (Fig. 11.1) [11–14]. In the current chapter, different approaches of personalized cancer treatment are reviewed according to their molecular targets and cellular mechanisms. It is hoped that combination of these novel modalities with traditional cancer treatment strategies enhances survival rate and improves the quality of life of cancer patients.

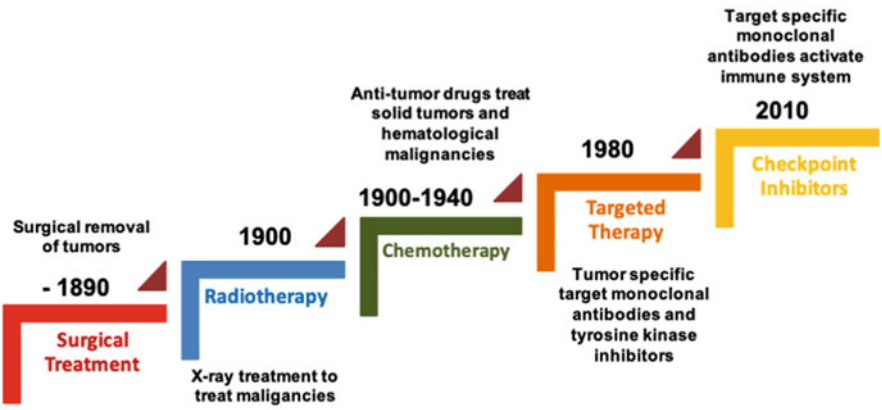

Fig. 11.1 Evolution of cancer therapeutics

11.2 Role of Vaccines in Cancer Therapy

Vaccines are considered as the most safe and economical prophylactic agents against various diseases like smallpox, chickenpox, measles, polio, etc. Though cancer vaccines are difficult and challenging, but with the advancement in the field of molecular biology and a greater understanding of mechanisms to harness the immune system, it has become possible. These advancements have made it possible to develop cancer vaccines which are being used to treat cancer patients via activation of immune system [15]. Vaccines for cancer can be broadly classified as preventive and therapeutic and precisely into categories which encompass genetic (DNA, RNA, and viral) vaccines, protein/peptide vaccines, and cell vaccines (tumor or immune cell) [16]. Research in the last few decades to develop preventative vaccine against different forms of cancer had though resulted in many futile outcomes but recent attempts focused on improving therapeutic cancer vaccines have been found to be encouraging [17]. The preventive vaccines like human papilloma vaccine (HPV) (Cervix [18], Gardasil, and Gardasil-9 [19]) that prevent infection by certain types of HPV and hepatitis B (HBV) vaccine [20] that inhibits Hepatitis B, are commercially available for cervical cancer and liver cancer, respectively. These vaccines are the only vaccines clinically approved for cancer prevention. Keeping in view the immuno-compromised condition and low immunogenicity of cancer patients, more emphasis is given to development of vaccines from therapeutic viewpoint which can improve immune response via increasing antibody production or activation of cytotoxic T cells [21]. Bacillus Calmette–Guérin (BCG) which is basically a tuberculosis vaccine, got its first approval in 1990 from Food and Drug Administration (FDA) and henceforth there has been no looking back with the positive biotherapies for the treatment of early-stage bladder cancer for more than 30 years [22]. Furthermore, researchers were also successful in

identification of some cell proteins that are produced abnormally high by cancer cells and targeted these proteins to develop therapeutic vaccines like Sipuleucel-T vaccine (PROVENGE) which was approved by FDA in the year 2010 and used for the treatment of patients with advanced prostate cancer. This vaccine evokes an immune response against prostatic acid phosphatases (PAP) that is often over expressed by prostate cancer cells. Still research is continuing to further improve efficacy of this very first FDA approved immunotherapy through combination approaches [23]. Moreover, unlike over expressed proteins some specific proteins that arise due to mutations are exclusively expressed by tumor cells. These proteins called as "neoantigens" also exhibit unique targets to develop cancer vaccines and thus can become part of standard cancer therapy and prevention [24]. Earlier, heat shock proteins (HSP)-based vaccines were also intended to be one of the therapeutic approaches for malignancy management as expression of HSP genes is believed to be elevated in tumors. With this purview, HSPPC-96 complex, called Vitespen (formerly Oncophage), a HSPs-based vaccine was formed and has been considerably examined in Phase I and II clinical trials showing activities on different malignancies with admirable effects in melanoma and kidney cancer in Phase III clinical trials [25]. Recently, with the emergence of therapeutic cancer DNA vaccines, unprecedented avenues have opened up to enhance specific and enduring immune response against tumor antigens. These are mainly the bacterial plasmid vaccines which encode antigens and encode immune stimulatory molecules (interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), etc.). However, cancer DNA vaccines established moderate efficacy and thus limiting standard cancer management. Consequently, it was deciphered that combination therapies, i.e. combining DNA vaccines with traditional procedures (chemotherapy, radiotherapy, surgical procedures) can synergistically potentiate immune response, thus leading to effective cancer treatment [15].

In conclusion, different target antigens have been tested for vaccine platforms and the field is still evolving with many vaccines which are still under clinical trials only. In order to have promising cancer treatment with enhanced immune responses and minimal additional toxicity, it is evident that combining immune checkpoint inhibitors with therapeutic vaccines may uphold great potential for effectively modulating the antitumor immune response and thus treating malignancies [17].

11.3 Role of Monoclonal Antibodies for Cancer Treatment

Antibodies are proteins which are heterodimeric in nature and approximately of 150 kDa in size. Antibodies consist of two each identical heavy and light chain which are arranged in a Y shaped conformation joined by disulfide bonding [26]. There are two distinct parts of an antibody, the antigen binding fragment (Fab) and the constant fragment (Fc). The Fab consists of complementary determining region in variable heavy and light chains and particularly responsible for identification and binding to antigen epitope [27]. The Fc domain is responsible for communicating with the effector immune cells through its binding with Fc

gamma receptors (FcγR) and initiating both complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [28, 29].

Antibodies have been segregated into different classes depending on the composition of heavy chain sequences, which are IgD, IgA, IgE, IgM, and IgG. IgG monoclonal antibodies (mAb) are used for clinical therapeutic applications among the mentioned five classes due to ease of production process and increased half-life in circulation. IgG mAb's have been further divided into IgG1, IgG2, IgG3, and IgG4 subtypes. Out of these four subtypes, IgG1 is the preferred mAb for use in cancer therapeutic applications due to its ability to induce ADCC, a desired effector function for cancer treatment [27, 30].

Rituximab was the first mAb approved by FDA for clinical chemotherapeutic application in 1997 [31]. Moreover mAb can be divided into two types based on their origin and function. mAb's can be generated as chimeric (suffix: ximab), humanized (suffix:zumab), and human (suffix: umab) [31–34]. Functionally mAb's can target multiple pathways. First, they can bind the antigen on cancer cells and prime the immune system. Second, mAB target the immune checkpoint regulators which include programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) which up-regulate the immune response. Third, they can bind to growth receptors implicated in cancer and block their activity. Fourth, utilizing conjugation mAb's can be used as delivery vehicles for chemotherapeutic drugs to tumor cells.

11.4 Role of Non-Coding RNA in Cancer Treatment

In 1990s and 2000, the completion of Human Genome Project revealed the number of Protein coding regions genes, i.e. 20,000–25,000 and human genes 35,000 approximately through clone based methods [35–37]. On the other hand, non-coding RNAs in human have also been studied through transcriptome [38–40], which plays an important role in diseases and cellular responses and cancer treatment [41]. In human genome 95% of DNA sequences are non-coding which are further transcribed into non-coding RNAs which contain several kinds of long non-coding RNAs (lncRNAs), small interfering RNAs, microRNAs (miRNAs), and antisense RNAs (asRNAs) [42–45]. Non-coding RNAs are RNA transcript which does not translated into protein and contains diversity in their structure. The therapeutic potential and targets of ncRNAs playing an important role in preclinical studies and clinical trials against cancer and genetic disorders and defects in human. In cancer therapy, the major challenge is to develop the anticancer resistant drugs. Non-coding RNAs and its different types play an important role and regulatory network to overcome in complex mechanism of chemoresistance and chemosensitivity [46, 47]. MiRNA, cirRNA, and lncRNA are ncRNA which plays an important role and studied against variety of cancer drugs, chemoresistance, and sensitivity [48]. The detailed role of ncRNA in cancer cells and its functions on therapeutic resistance and sensitivity is given, playing role in regulating the processes of DNA damage repair, apoptosis, and EMT [49, 50]. In in vivo studies in

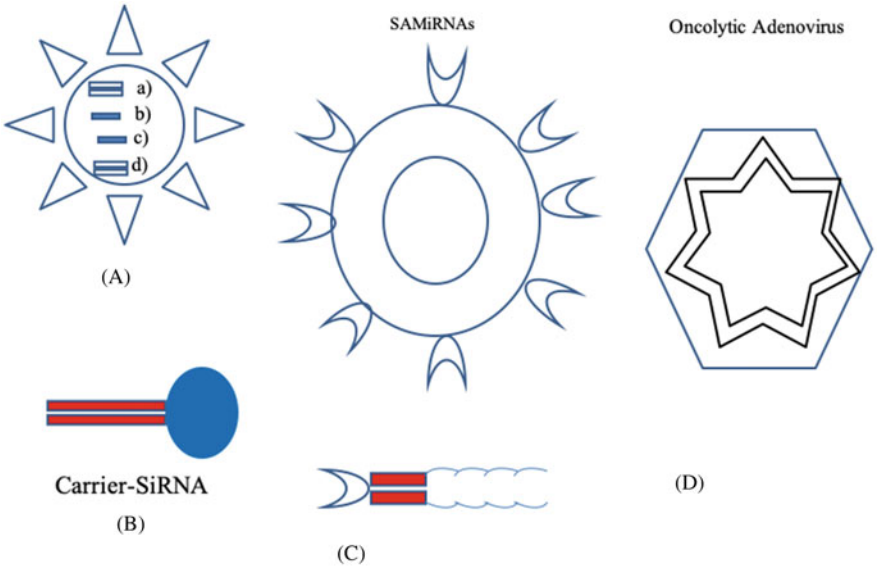

Fig. 11.2 Diagrammatic illustration of different forms of ncRNA in cancer therapy. (A) (a) SiRNA, (b) MiRNA, (c) ASO, (d) SaRNA. (B) SiRNA. (C) PEG-siRNA-lipid siRNA. (D) ShRNAs, sgRNAs

mouse models, doxorubicin and miR-10b have been used for treatment of breast cancer [51]. Additionally, antisense oligonucleotides (ASOs) blocking metastases associated lung adenocarcinoma transcript 1 (MALAT1) [52] and phosphorodiamidate morpholino oligomer that silences miR-487 [53] help in treating the tumor burden and metastasis of cancer cells with no damage to normal cells. A morpholinos based drug named as AVI4126 is used to treat various cancer cells, such as breast, lung, and prostate cancer by inhibiting the expression of c-myc translation sequence by blocking and mis-splicing of its pre-mRNA [54, 55].

Above study proved that the drugs which were based on morpholinos can show promising effect for cancer therapy by targeting oncogenic ncRNAs. Although these ncRNAs have therapeutic potential because of their uniqueness in chemical properties and mechanism and its pharmokinetics trial. There are still more studies which need to carried out to realize and validate its therapeutic potential of ncRNAs in treatment of cancer. The diagrammatic illustration of different forms of ncRNA in cancer therapy is explained in Fig. 11.2A. In lipid nanoparticles (LNP) encapsulated non-coding RNAs (SOs, SiRNA, saRNA, and miRNA) are protected from degradation from biological conditions and are directly delivered to tumor cells. Figure 11.2B illustrates how chemically conjugated SiRNA with carriers forms carrier-siRNA conjugates which are used for cancer treatment. Similarly, SAMiRNA, the self-assembled lipid nanoparticles are formed from modified PEG-siRNAs and lipid molecules as illustrated in Fig. 11.2C. With the help of

oncolytic adenovirus-mediated strategy, Fig. 11.2D ShRNA and sgRNA can hereby accomplish a long-lasting expression of ncRNA in malignant cells [41].

Due to recent studies and progress in biotechnology and pharmaceutical industries, ncRNAs have become the promising players in the fight against cancer. The basic treatment like radio and chemotherapy will remain there as mainstream for cancer patients, the ncRNAs with its conjugate carriers act as mediator in chemo- and radio-resistance which will be in high demand for specific receptor with the help of nanotechnology in drug delivery system.

11.5 Gene Therapy for Cancer Treatment

All over the world gene therapy has been poised as first line of therapy for cancer and it possesses a number of advantages such as low off-target toxicity, high specificity, multiple gene delivery, high potency [56] and have limited side effects [57]. It involves the transfer of genetic material in vivo to the targeted tissues [58]. More than 400 clinical studies have been performed using gene therapy over the past 15 years, out of which 70% were focused on cancer gene therapy [59]. Multiple alterations at genetic levels lead to the development of various cancer and different therapeutic genes have been used to alter the tumoral lesions [60]. Two gene groups, i.e. oncogenes and tumor suppressor genes, counterbalancing each other, play important role in the development of cancer. Cell proliferation is enhanced by oncogenes, whereas apoptosis or programmed cell death is induced by tumor suppressor genes. These both gene groups could be used in cancer treatment. In addition, cancer can be treated by suicide gene strategy which involves the combination of gene therapy and chemotherapy. In this strategy the non-toxic prodrug is converted into active cytotoxic metabolite by a non-mammalian enzyme within the tumor [61, 62]. Gene therapy can also be mediated by using cytokines encoding genes which enhances the immune response against the cancerous cells [63, 64]. Gene therapy can be mediated by DNA vaccines [65, 66] or injecting naked DNA directly into the tumors [64, 67]. Biological systems such as viruses and non-biological agents like liposomes, cationic peptides, and cationic polymers can be used as gene therapy vehicles. Viruses are modified to enhance their efficiency and reduce their pathogenicity. They infect the host cell and release their genetic material into them, but they suffer from limitations such as restricted size of genetic material transferred into host, and they are difficult to produce [68]. These limitations can be prevented by using non-biological agents for gene therapy; however, these agents have limited efficiency. Therefore, it is crucial to modify the biological as well as non-biological agents to achieve desirable characteristics for efficient gene therapy [58].

11.6 Gene Editing for Cancer Treatment (CRISPR)

Our body contain many cells, and each cell harbors a copy of our genome that contains over 20,000 genes and each gene consists of 3 billion letters of DNA consists of two strands twisted into a double helix held together by a simple pairing rule **A** pairs with **T** and **G** pairs with **C**. Due to the tremendous advances in DNA sequencing and advent of next generation technologies, numerous disease specific association of genes have been identified. In the last 20 years, a new method called clustered regularly interspaced short palindromic repeats (CRISPR) method has been introduced which has shown promising results with the technology of editing the DNA of humans and other species as well. CRISPR technology is based on the response mechanism of how bacteria protect itself from viral infection. Upon viral infection, bacteria successfully detect viral DNA which then leads to production of two types of short RNA (one of which contains a sequence matching that of invading virus). A CRISPR associated protein 9 (Cas9) complex is formed with these two RNAs which targets the DNA and disables virus activity.

In the laboratory, RNA oligos (crRNA and tracrRNA) are widely used, since we can design their structures. Once inside the nucleus, Cas9 complexed with tracrRNA, and will lock onto a short of the protospacer-adjacent motif (PAM) sequence [69]. When this happens the cell tries to repair the cut either by homology directed repair (HDR) endogenous repair mechanisms or non-homologous end joining (NHEJ). But the repair process is error-prone leading to mutations (insertion or deletion (indel) mutations) that can disable the gene, allowing researchers to understand its function. Over the past few years, researchers studying the system realize that this could be engineered to cut DNA sequence at a specific location. The CRISPR-Cas9 system has also been successful in generating the generically manipulated mutant mouse models using previous approaches [70]. For instance, microinjection of the Cas9 mRNA and gRNA was used by one group to create a human lateral meningocele syndrome (LMS)-related mutant mouse model of the Notch3 gene [71]. This technology has also been used in several other studies for mouse models of osteoporosis [72–74].

In the context of cancer, CRISPR-Cas9 knock-in mice for genome editing and cancer modeling were widely used. For developing this model, adeno-associated viruses (AAV) vector system was delivered with the gRNA of the top three significantly mutated genes, i.e. GTPase (KRAS), KRAS proto-oncogene, p53, and liver kinase 1 (LKB1) to induce lung adenocarcinoma [75]. Nevertheless, these mutations are random but sometimes researchers have also tried replacing a healthy copy in place of a mutant gene. It has also been emphasized that cellular communication network factor 2/connective tissue growth factor (CCN2/CTGF) leads to over expression of matrix metalloproteinases (MMP) family proteins in tumor cells [76]. Specifically, matrix metalloproteinase 3 (MMP3) has been reported to regulate CCN2/CTGF and knockout of MMP3 by CRISPR/Cas-9 has been showed to inhibit migration and invasion in cancer cells via reduction of promoter activity of CCN2/CTGF [77]. Additionally, it has been noted that the high expression of nuclear factor erythroid 2-related factor 2 (NRF2) is one of the major causal factors of

chemoresistance in cancer cells [78]. Kelly and his colleagues have identified a unique PAM which specifically cleaves *NRF2* in a site specific manner in malignant cells [69] implicating the importance of CRISPR-directed gene editing in solid tumors.

Lastly but not the least, despite that all this CRISPR gene editing can be done in cultured, unlike previous methods, CRISPR can be used to target many genes at once, which gives a big advantage for studying complex diseases which are attributed to mutations in multiple genes acting together. These methods are being improved rapidly and will have many applications in basic research and clinical trials in the anticancer drug development or for treating human patients with cancers due to genetic mutations.

11.7 Targeted Drug Delivery Through Nanotechnology for Cancer Treatment

Chemotherapy in combination with surgery and radiation remains the most successful lines of treatment for malignant growth [79]. However, these medications when applied either separately or in combination have different antagonistic impacts like general distress, neuropathy, cytotoxicity, nausea, myelosuppression, nephrotoxicity, alopecia, cardiotoxicity, and poor solubility of medications [80–82]. Further, high dose of these medications should be directed to accomplish restorative levels, because of which healthy cells are also injured. Further, many a times malignant growth is analyzed in late stages which diminish the general adequacy of these medicines [83]. Another serious issue is that malignant growth cells can become resistant towards chemotherapeutic medications [84].

Nanotechnology utilizes the combination of therapeutics with diagnostics which help in specific drug delivery to disease tissue without influencing ordinary tissues, consequently gaining huge consideration worldwide for malignant growth treatment [85]. In nanomedicine, nanoparticles are used for diagnosis and treatment of cancer. These nanomedicine have high surface to volume proportion which let them being absorbed and pass on to focused site as therapeutic agents with biomolecule like DNA, RNA, medications, and proteins [86]. These drug carriers help in delivering chemotherapeutic agents to tumors, maintaining a strategic distance from normal cells via specific targeting which reduces toxicity to normal cells [87, 88].

To date, different organic (templated, lipid-based, layer-by-layer assembled, and cell-membrane inferred) and inorganic (silver, iron oxide, gold, and silica or silicon) nanoparticles have been synthesized [89–92] and are endorsed for clinical use [93]. Not just these nanoparticles help in decrease of side effects, e.g., decreased nausea/vomiting, hair loss, anemia, and cardio toxicity [94] yet some ongoing clinical preliminaries are indicating guarantee of higher survival benefit when contrasted with standard treatment [95]. The first clinically endorsed nano-based anticancer drug carrier Doxil/Caelyx (PEGylated liposomal doxorubicin) was used for Kaposi's sarcoma treatment [87]. The nanotechnology based medication was

seen as clinically extra convincing and less harmful than the standard blend chemotherapy (bleomycin, doxorubicin, and vincristine) [96].

Another serious issue in viable malignant growth treatment is early stage detection of disease especially before tumor cells metastasize. The majority of the tumors can be dealt with successfully in the event that they are identified at a beginning period. However, diagnosis at early stage still remains a challenge, as clinical symptoms seldom manifest before disease advances to a lethal stage. In recent times, many kind of nanoparticle-based technologies are being created for enhanced imaging for different type of cancers [97, 98]. Nanoparticles, for example, semiconductor quantum spots and iron oxide nanocrystals, have optical, attractive, or auxiliary properties that do not happen in normal particles. Different particles can be used with nanoparticles for targeting cancer cells in particular and include various antitumor agents ranging from different antibodies to peptides, different particles, conjugation with which can be valuable in screening tumor cells and early detection [99]. In view of the promise nanotechnology has presented these nanomaterials have been used to recuperate target-specificity and additionally tissue infiltration of a symptomatic test, consequently permitting prior recognition of threat [100]. These advance and sensitive imaging procedures will permit the prior identification and better prognosis as well as focused delivery of medication will also help eliminate the need for radiation therapy and/or invasive surgery [101, 102]. Hence, the development of highly specific and highly sensitive nanoparticles could revolutionize prevention, diagnosis, and treatment of malignant growth.

So it may be well presumed that malignancy nanomedicines (nanodrugs, nanocarriers, or nanotherapeutics) are miniaturized delivery frameworks, which helps in improving the viability of presently available chemotherapeutic agents. Nanomaterial's in oncology additionally incorporates diagnostics, theranostics, clinical gadgets, and more recently therapeutics for customized medication (Fig. 11.3) [101, 103]. Finally, nanotechnology can help permit real-time tracking of the targeted delivery of therapeutics in cancer patients [104].

11.8 Conclusion and Future Directions in Personalized Medicine

With the fast evolving field of genomics, biotechnology, and molecular medicine, translating into precision medicine, the importance of individualized therapeutic interventions is being considered by the pharmaceutical companies and basic researchers. For transitioning from surgical treatments to radiotherapy to chemo and immunotherapies, in this fast advancing world, it will be not be far away when the personalized medicine will be the choice of treatment for one and all. This is strongly supported by the initial experience in the field of personalized medicine which is directed to the patient at individual level and also decreases the trial-and-error during diagnosis and treatment. The medical fraternity is slowly realizing the importance of genetic and molecular basis of disease specifically in cancer and is at the initial forefronts to adapt molecular screening for assessment of disease

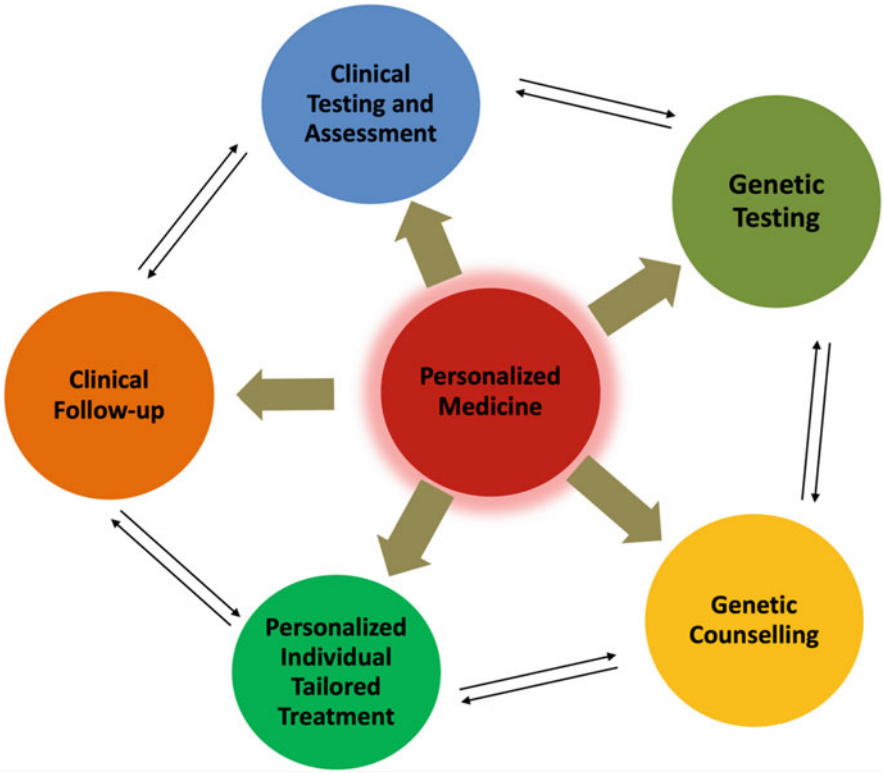

Fig. 11.3 Personalized cancer therapeutics: Current and future

associated risk factors and preventive mechanisms. The personalized medicine is also being closely followed by the government authorities, regulatory authorities, and healthcare agencies for updates on the safety and efficacy for long-term translation of promising era of molecular medicine. In view of the promise personalized medicine holds in transitioning the future from conventional chemotherapy regime to precision medicine, it may not be long when we see translation of personalized medicine to clinics. This translational value of personalized medicine will help manage the diseases at the forefront on individual basis and response to therapy which will be promising in the upcoming era.

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