Chapter 17
Counteracting Hypoxia in Radio-Resistant Metastatic Lesions

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Abstract The identification of hypoxia-regulated genes and proteins, has provided the basis for the generation of new hypoxia-targeted drugs, conceived to re-oxygenate hypoxic tumor areas. In patients with advanced metastasizing prostate cancer (PC), these kinds of drugs are expected to optimize the effect of radiotherapy, reducing also its side effects. Immunohistochemistry, DNA, proteomic and, tissue array profiling, are increasingly providing us with exciting data, that could lead to the formulation of pre-treatment multimarker tests able to identify the individualized tumor response profiles to radiotherapy, basing on the specific cancer tissue hypoxia pattern and degree (Bussink et al., Radiother Oncol 67:3–15, 2003).

As an example, the recent discovery of the role of microRNA in PC tumor genesis points towards (Kulshreshtha et al., Cell Cycle 6(12):1426–1431, 2007) the, Inactivation of miRs affected by hypoxia as a promising synergistic therapeutic strategy for the radiotherapy-refractory subset of metastatic PC (Kulshreshtha et al., Cell Death Differ 15:667–671, 2008).

This chapter aims to give an outlook of the main hot-topics concerning the new trends of hypoxia-targeted molecular therapies for advanced metastasizing prostate cancers.

17.1 Background

Radiation therapy produces, in most cases, a durable disease control of prostate cancer. According to a recent study on 3,546 patients, the 10-year disease-free survival rates of patients treated with radiation is 75 %, similar to that registered for radical prostatectomy, without all the inconveniences and complications linked

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to surgical intervention. However, a subset of prostate cancers can recur, in a great extent (>75 %) in the first 5 years after treatment, and in about 5 % during or even after the 10-year follow-up (Critz et al. 2013).

These recurrent cancers are aggressive, metastasizing and radio-resistant. Ionizing radiations exert their therapeutic effects on tumors mainly via the production of cytotoxic reactive oxygen species (ROS), leading to irreversible DNA damage (Morales et al. 1998), and cell death (Wilson and Hay 2011).

This accounts, for a large extent, for the resistance to radiotherapy showed by metastasizing solid tumors, characterized by the frequent presence of multiple hypoxic areas (Moeller and Dewhirst 2006).

In hypoxic tumors, ROS down-regulate the permeability of the mitochondrial outer membrane, shutting-down the mitochondria-driven apoptosis (Moll et al. 2006; Kroemer 2006).

In addition, ROS activate PI3K (stabilizing HIF-1a and favoring the up-regulation of glycolysis with anaerobic ATP production) (Muzandu et al. 2005; Kaelin 2005).

Tumor hypoxia is due to the presence of dysfunctional, abnormal blood vessels and artero-venous shunts responsible for the overall low and heterogeneous blood-flow. Besides low oxygen tension, cells of malignant tumors have to face also high extracellular hydrostatic pressure and low pH (Helmlinger et al. 1997; Jain 1999). A growing body of data indicates that the correction of these tumor microenvironment alterations may mitigate, or even reverse, the malignant phenotype of cancers (Kenny and Bissell 2003). Successful approaches have been developed to counteract tumor hypoxia, as (Bussink et al. 2003) the use of radio- or chemotherapy combined with hyperbaric oxygen or hypoxic cell sensitizers (Henk 1986; Overgaard et al. 1998; Watson et al. 1978).

Several phase III trials are currently investigating new strategies. However, most of these treatments showed an increase in side-effects. To date, treatments targeting tumor hypoxia, widely accepted in clinical practice, are unavailable. This is particularly challenging for advanced, metastatic prostate cancer (PC) which, as previously outlined, frequently shows a poor response to radiotherapy, with an overall worse outcome for most patients. There is the urgent need to reliably predict the risk of tumor recurrence after radiotherapy, to enable the selection of high-risk PC patients that would be candidates for treatment with novel investigational agents. Recently, it has been shown that PC, in spite of a median blood flow three times higher than in normal prostate (NP), exhibits mean pO2 values fewer than one fourth than in NP, with an extremely heterogeneous distribution of intratumor oxygen, independent from clinical or pathological features (Vaupel and Kelleher 2013).

In solid cancers, when the tumor's bulk exceeds 1 mm^3 in volume, neoplastic cell growth progressively overcomes neo-angiogenesis (Shannon et al. 2003).

Tumor blood vessels, surrounded by a rapidly remodeling connective tissue, become leakier than the vessels of the corresponding healthy tissue, and allow serum proteins to infiltrate extracellular matrix, contributing to the elevation of interstitial pressure (Dvorak 1986). Thus, a hypoxic tumor environment takes place

(Huang et al. 1998b); the intrinsic structural and functional abnormalities lead to repeated shut-down of the tumor microvessels, with a parallel decrease of oxygen gradients and nutrients, and, even, to the local reversion of blood flow (acute hypoxia) (Vaupel et al. 1989, 2001; Dewhirst et al. 1999). At the same time, the increase in diffusion distances results in chronic hypoxia, leaving cells chronically deprived of oxygen and nutrients (Vaupel et al. 2001).

Acute, chronic, and, also, regions of intermediate hypoxia, are common findings in advanced metastasizing cancers. Tumor cells react to the hypoxic environment through the paroxysmal activation of physiological responses: up-regulating the expression of a network of gene products that advantage to tumor growth in this adverse conditions and favour resistance to radiotherapy (Anastasiadis et al. 2003).

The frequent resistance to radiation therapy, of hypoxic cancer cells, is largely thought to be caused by the lack of oxygen as a source of radiation induced radicals. As a rule, in normal cells, the highly reactive free radicals produced by radiation induce cell death through the generation of DNA double-strand breaks. The presence of oxygen stabilise the free radicals, further increasing DNA damage and impairing the DNA repair (Höckel and Vaupel 2001; Horsman and Overgaard 2002; Isa et al. 2006).

Intratumoral oxygen levels are, then, directly correlated with the cytotoxic effects of radiation. By converse, hypoxia induces rapid changes in cancer cells gene expression, altering proliferation kinetics by inhibiting DNA repair and apoptosis. Moreover, it increases anaerobic glycolysis that ensures the fast proliferation of hypoxic tumor cells (Harrison and Blackwell 2004; Wouters et al. 2004).

This has been recently demonstrated in human soft tissue sarcomas, in which the more hypoxic tumors showed the fastest proliferating tumor cells (Nordsmark et al. 1996; Bussink et al. 1999; Kennedy et al. 1997; Ljungkvist et al. 2002; Schmaltz et al. 1998; Webster et al. 1995).

All these changes facilitate the onset of radiation resistance and/or cytotoxic drugs treatment, up to a level three times greater than non-hypoxic cancer cells. Tumor hypoxia, then, represents a major cause of treatment failure and poor outcome of human malignancies and, thus, is to be considered for prognostic evaluation of tumors and therapeutic options for cancer patients (Lundgren et al. 2007; Le and Courter 2008).

The modification of tumor hypoxia significantly improved radiotherapy outcome in several tumor types, as head and neck carcinomas (Overgaard and Horsman 1996).

Based on immunohistochemistry and direct oxygen-electrode measurements, hypoxia may be detected in 30–90 % of prostate cancers. The presence of hypoxic regions has been associated with radio-resistance and poor clinical outcome, of prostate cancer patients, representing a very troublesome concern, for the treatment of this tumor (Thomlinson and Gray 1955; Höckel et al. 1991).

Radiation therapy (RT) is commonly used as a primary treatment for prostate cancer, sometimes combined with neoadjuvant and adjuvant hormone therapy (Srigley et al. 2012).

Patients with locally advanced PC, in fact, frequently develop relapse after RT. In addition, recurrence, morbidity, and toxicity, often, complicate radiotherapy, used either as the primary radical treatment of prostate cancer, or as an adjunctive therapy after radical prostatectomy, or palliative therapy.

Recently, it has been confirmed that hypoxia correlates with tumor stage and long-term biochemical outcome, in prostate cancer patients treated with brachytherapy. Several molecules have been proposed as specific biomarkers of the hypoxia-induced response of prostate cancer cells. Among these, Hypoxia inducible factor-1 (HIF-1), VEGF, and osteopontin are among the most frequently reported as overexpressed in aggressive prostate cancers (Huang et al. 2012)

The pathways activated by ionising radiation and hypoxia, include also several pro-caspases, anti-apoptotic proteins and the transcription factor NFKB (Rugo and Schiestl 2004; Gilbert and Knox 1997; Chresta et al. 1996; Romashkova and Makarov 1999).

However, HIF-1 is the only DNA regulatory element truly regulated by oxygen.

17.2 HIF-1

HIF-1 consists of alpha and beta subunits. This transcription factor becomes activated during alpha/beta dimerisation, bounding to p300 to form a complex. This active complex rapidly targets the so-called "hypoxia- response element (HRE)" of more than 60 hypoxia-inducible genes such as erythropoietin (Epo), VEGF, glucose transporter-1 (GLUT-1) (Semenza et al. 1991; Shweiki et al. 1992; Levy et al. 1996; Bashan et al. 1992), and multidrug resistance (MDR) gene, (Comerford et al. 2002).

In prostate cells, HIF-1 expression has been shown to be induced under normoxic conditions (Park et al. 2007). In normal prostatic tissue, HIF-1 probably acts as an intrinsic defender of prostate cells against a zinc-rich environment. Normal prostate tissue and prostatic fluid are, in fact, extremely rich of zinc (Costello et al. 2005), (1,000–3,000 and 9,000 mol/kg, respectively), and this may explain the ability of prostate cells to stabilize HIFs (Ku et al. 2010).

HIF-1 beta is constitutively expressed in all normal cell types, whereas HIF-1 alpha is rapidly post-transcriptionally hydroxylated in the presence of oxygen (Yasuda 2008), and targeted for ubiquitation, a process directly mediated by the von Hippel-Lindau (VHL) tumor suppressor ubiquitin-ligase protein (Maxwell et al. 1999).

HIF-1α is overexpressed in 70 % of human cancers and their metastases provided the first clinical evidence supporting the role played by HIF-1 in human cancer progression (Zhong et al. 1999).

Expression of HIF-1 alpha, assessed by immunohistochemistry, has been recently shown to predict tumor aggressiveness of several primary malignant tumors, (Koukourakis et al. 2002; Aebersold et al. 2001) including prostate cancer, and has been found overexpressed also in their corresponding metastases (Zhong et al. 1999; Lekas et al. 2006).

Several other reports confirmed the association between the immunohistochemical overexpression of HIF-1alpha, increased prostate cancer patient mortality and radiotherapy failure (Aebersold et al. 2001; Quintero et al. 2004).

Evidences on rat and human prostate cell lines have further supported this. Very intriguingly, knocking down HIF- 1α expression with small interfering RNA (siRNA), sensitizes to radiation the androgen independent, highly metastatic PC3 cell line, enhancing their apoptotic rate. HIF-1α siRNA transfection resulted in a significant decrease in the G0/G1 phase, with an accompanied cell cycle arrest at the proliferative phase. It is common knowledge that cells at the proliferative phase are more sensitive to therapy, including irradiation, than in the resting phase. The increase in both interphase death and reproductive death after irradiation, apoptotic potential, and cell cycle arrest (at the proliferative phase) contribute to its radiosensitizing effect (Huang et al. 2012).

HIF-1α inhibition looks, then, promising as an effective molecular therapy to sensitize PC to RT. Hormone-independent prostate cancers have mutations in a critical regulatory domain of the HIF-1α protein, oxygen-dependent degradation domain, which may have a great relevance for the development of therapeutic androgen blockade (Anastasiadis et al. 2002) resistance. HIF-1α inhibition might have more anti-apoptotic effect in hormonal-independent prostate cancers. This however deserves further confirmatory studies.

17.3 miRNA

The hypoxia-inducible factor-1 seems to be involved also in determining a particular kind of hypoxic signature of prostate cancer cells, constituted by a specific mark on microRNA profiles (hypoxia-regulated microRNAs, HRMs). In eukaryotic cells, microRNAs regulate the expression of most genes, participating in cell differentiation, proliferation, metabolism and death (Bartel 2004; Calin et al. 2004, 2005) through translational repression and/or mRNA degradation (Cheng et al. 2005; Croce and Calin 2005).

Sensible microRNA changes have been described in human cancers, sometimes correlated with the clinico-pathological features of tumors (Iorio et al. 2005; Yanaihara et al. 2006).

To date, the pathogenetic events underlying this phenomenon are largely unknown. However, there are evidence that miRNA take a part in the hypoxia-mediated gene repression, contributing to cell survival in low-oxygen conditions. Specific microRNA patterns are a signature of normal and/or neoplastic hypoxic cells. They include miR-23, -24, -26, -27, -103, -107, -181, -210, and -213; miR-26, -107, and -210 are also overexpressed in a variety of human hypoxic tumors, in which they are thought to have a role in tumor-genesis, via the decrease of proapoptotic signaling (Volinia et al. 2006).

The great number of HRMs that are overexpressed in hypoxic tumors suggests that hypoxia represents a driving force leading to microRNA alterations in cancer.

Besides HIF-1, additional transcription factors responsive to hypoxia/anoxia, such as p53 and NF-kB, may induce the expression of several specific microRNAs (Yanaihara et al. 2006; Zhao et al. 2005).

As well, VEGF is considered a potential target for a series of hypoxia-responsive candidate regulatory microRNAs, as miR-16, miR-20, let-7b, miR-17-5p, miR-27, miR-106, miR-107, miR-193, miR-210, miR-320 and miR-361. The patterns of microRNA alterations reported in cancer versus normal tissues, is likely the consequence of the alteration of complex interacting molecular pathways induced, at least in part, by hypoxia. These different patterns, however, are relatively scarce, if compared with the plethora of genes and proteins commonly altered in tumors. To date, a definitive explanation of this phenomenon does not exist. In the meantime, this could be facilitating the possible future applications of miRNA for cancer therapy. The recent availability of microRNA derivatives with increased half life and binding efficiency, such as AMOs (anti microRNA oligonucleotides), LNAs (locked nucleic acids) and antagomirs represents potentially important developments for such purpose (Kulshreshtha et al. 2007; Weiler et al. 2006; Orom et al. 2006; Krutzfeldt et al. 2007).

It has to be pointed out that, in any case, multiple microRNAs are involved in the hypoxic response, this implying that the various attempts of therapies miRNA-specific should be performed through the simultaneous combination of several selected microRNAs (Bartel 2004).

This strategy could improve the outcome of conventional therapies, as early studies have recently reported. In prostate cancers, as in a large number of other tumors with different histogenesis (breast, lung, colon, stomach), specific alterations of microRNA expression have been identified. Very interesting, in prostate cancer, miR-210 seems to be an interesting marker of chronic hypoxia, irrespective of the androgen dependency and should, therefore, be tested as a prognostic marker in high risk prostate cancer patients (Volinia et al. 2006).

Considering that, recently, miR-210 has been detected in serum of lymphoma patients as well as in sera of patients with solid tumors, the hypothesis of the future development of non-invasive cancer new diagnostic tests utilizing miRNAs, could be considered as feasible (Crosby et al. 2009).

17.4 VEGF

The Vascular Endothelial Growth Factor (VEGF) is a master growth factor driving angiogenesis and tumor cell growth, promoting the increase of blood vessel permeability, endothelial cell growth, proliferation, migration, and differentiation (Senger et al. 1983; Ferrara 1995; Hicklin and Ellis 2005). It is regulated by a plethora of cytokine growth factors (EGF, PDGF, bFGF, TGFalpha). Tumor hypoxia directly up-regulates VEGF transcription through the increase of HIF-1 alpha levels (Pugh and Ratcliffe 2003; Harris 2002).

Recent studies demonstrated that the increasing levels of hypoxia correlated with the highest tumor expression of VEGF (Cvetkovic et al. 2001) and predicted biochemical failure after radiotherapy, showing an independent prognostic value (Movsas et al. 2002; Moeller and Dewhirst 2006).

This implies that the VEGF expression directly reflects tumor hypoxia and reduced radiosensitivity of prostate tumor cells (Gray et al. 1953).

This observation could justify the hypothesis that the assessment of tumor VEGF expression on pretreatment diagnostic biopsies, may identify with reasonable probability patients non-responder to radiotherapy, that need to be treated with more aggressive radiation treatments and/or anti-angiogenic or hypoxia-targeted drugs. Further studies on prostate cancer treated with radiotherapy or radical prostatectomy showed that biochemical failure might be better predicted by the increased expression of both HIF-1 alpha and VEGF, independently of T stage, Gleason score, and PSA levels. HIF-1alpha and VEGF, then, are both to be considered very promising as new therapeutic targets for aggressive prostate cancers (Kimbro and Simons 2006; Semenza 2003; Rohwer and Cramer 2011).

The protein kinases inhibitors (TKIs) are the ideal candidates for this purpose. They block the tyrosine kinase-dependent pathways in a mono-specific manner (one TKI directed against a single type of TK) or can be directed toward several tyrosine kinase receptors, thus being able to inhibit multiple signaling pathways. The use of multikinase inhibitors, like Sunitinib and sorafenib, that interfere with several HIF-1 related signaling pathways (i.e. VEGFR/PDGFR) (Merino et al. 2011; Nilsson et al. 2010), is showing encouraging results, even combined with small molecules targeting HIF-1 (Nordgren and Tavassoli 2011).

As for traditional therapies, also in this case, the combination of molecule targeted toward different targets seems to produce a positive, synergistic, effect in counteracting aggressive cancer cells. Even in localised prostate cancers, the extent of tumor hypoxia (Parker et al. 2004) seems to be correlated with long-term poor outcome of prostate cancer patients (Denhardt and Guo 1993; Shweiki et al. 1992), when the immunohistochemical over-expression of HIF-1 alpha and VEGF is found associated with the hypoxia-induced secreted phosphoglycoprotein osteopontin (Zhong et al. 1999; Strohmeyer et al. 2004; Forootan et al. 2006).

Further investigations are needed to evaluate the better way to therapeutically regulate the multi-layer cross-talks between HIF-1alpha and VEGF pathways in hypoxic prostate cancers. Several aspects of these interactions are still matter of active investigation. Among these, the role of the reciprocal interactions between HIF-1, VEGF and the androgen/androgen receptor axis is of particular relevance. Androgens influence tumor vasculature through several mechanisms, enclosing a paracrine signalling mediated through androgen receptors expressed by endothelial cells (Godoy et al. 2008, 2011).

Hypoxia induces androgen hypersensitivity. The transition from androgen-dependence to androgen-independence is a key event in prostate cancer. Patients with clinically localized prostate cancer showed a reduction in the hypoxic fraction following androgen withdrawal (Milosevic et al. 2007) that appear to correlate

with downregulation of VEGF expression (Aslan et al. 2005), Tumor hypoxia progressively decreases due to the "normalization" of prostate cancer vasculature allowed by the down-regulation of VEGF (Shweiki et al. 1992; Overgaard et al. 2005).

Quantitative dynamic contrast-enhanced magnetic resonance imaging (DCE MRI) detected an increased and highly functional vascular network in experimental prostate tumors after ADT, which was confirmed by quantitative analysis of fluorescent immunohistochemistry qIHC. However, long-term androgen deprivation induces transient acute hypoxia, and this may be involved in the transition to androgen independence (Rothermund et al. 2005; Park et al. 2006).

Anti-androgens bind to AR in the tumor, but have affinity for pituitary and hypothalamus receptors, stimulating over-secretion of androgens; moreover, in recurrent prostate cancers, an increased expression, stability and translocation of the AR takes place, making tumor cells hypersensible to the growth-promoting effect of dehydrotestoterone (DHT) that, in turn, stabilizes HIF-1a, contributing to the hypoxic response (Mabjeesh et al. 2003; Bakin et al. 2003).

The chronic activation of the androgen receptor (AR) has also shown to upregulate HIF-1 alpha and VEGF in prostate cells through the autocrine receptor tyrosine kinase receptor/PIP3K/AKT-1/mTor signaling (Culig and Bartsch 2006). A prolonged androgen withdrawal leads, as side-effect, to the over-activation of Akt signalling, increasing the ultimate apoptosis-resistance of prostate cancer cells. This partially explains why hormone-resistant prostate cancers are also resistant to most other forms of therapy, comprising the inhibition of PI3K-mediated response (Pfeil et al. 2004).

Although apoptosis is not the main biological effect of ionising radiation, apoptosis resistance has been correlated with radiation therapy failure and proposed as an effective marker for the radioresponse of prostate tumors (Szostak and Kyprianou 2000; Zhivotovsky et al. 1999; Wang et al. 2004).

In prostate cancer cells as in radical prostatectomy specimens (as detected by immunochemistry), Bcl-2 overexpression was positively associated with a high risk of biochemical failure in clinically localized prostate cancer, and poor therapeutic response to radiation therapy (Xie et al. 2006; Revelos et al. 2005; Huang et al. 1998a; Scherr et al. 1999).

These findings indicate that AR-induced HIFs-VEGF-overactivation may represent a potential source of pitfall for experimental trials utilizing VEGF sequestrants, as Bevacizumab, in patients with hormone- resistant prostate cancer.

All these aspects have to be considered to achieve a beneficial combination therapy based upon RT, anti-angiogenic/vascular disrupting therapy, and ADT, in advanced PC. Indeed, strategies aimed at restoring apoptosis pathways in prostate tumor cells seem a pivotal feature of new radiotherapy treatment protocols. The ultimate question that still arises after about two decades of intensive research, concerns the nature, hierarchy and timing of the prevalent acquired mechanisms of radio-resistance in metastasizing, hypoxic prostate cancers. Pathway redundancy, molecular crossing-over and the progressive selection of hypoxic-resistant tumor cells are among the most promising pathogenetic factors for this phenomenon.

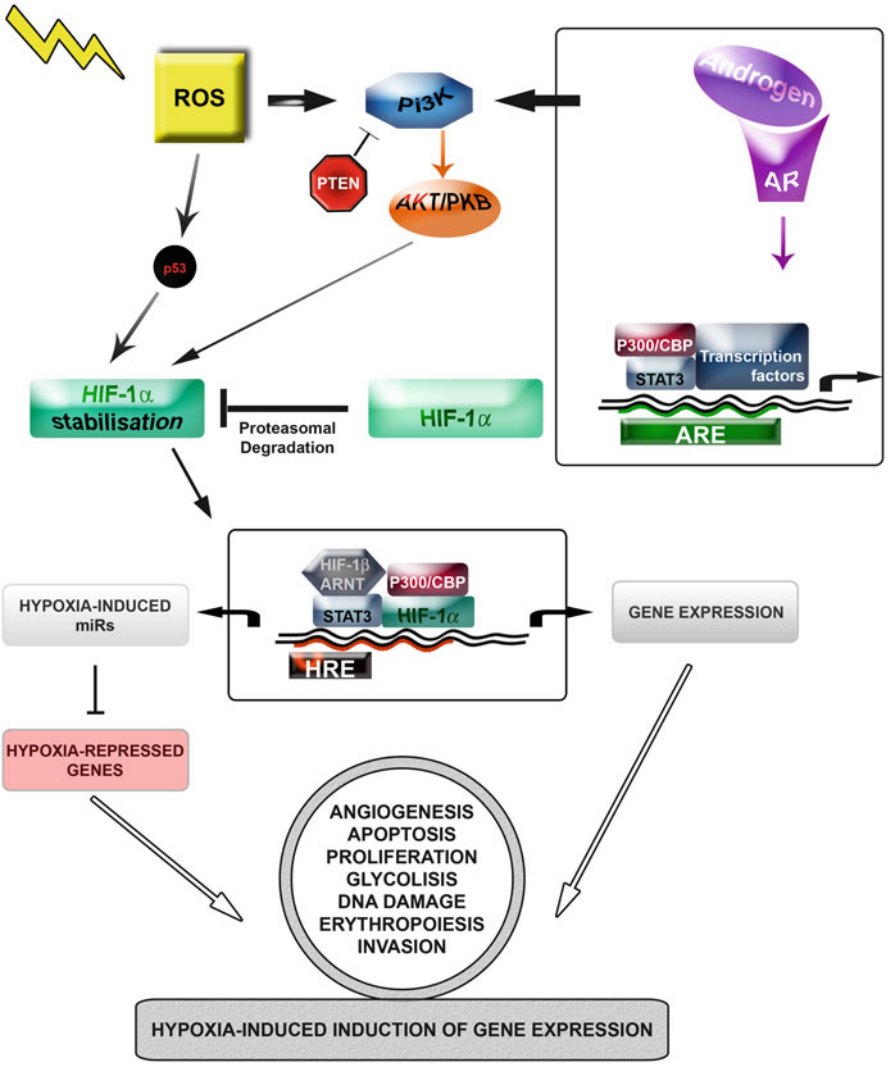

Fig. 17.1 Hypoxia regulated genes and proteins. The main mechanism of action of ionizing radiations in the therapy of tumors is the production of cytotoxic reactive oxygen species (ROS), leading to irreversible DNA damage and cell death. ROS activate PI3K that drives the stabilization of HIF-1a and the up-regulation of glycolysis. Hypoxia modifies gene expression of cancer cells, causing inhibition of DNA repair and apoptosis and increases anaerobic glycolysis which favors the proliferation of hypoxic tumor cells. Moreover, ionising radiation and hypoxia regulate the expression of several pro-caspases and anti-apoptotic proteins. HIF-1 is the most reliable marker of hypoxia. It targets hypoxia-inducible genes and is involved in determining a specific mark on microRNA profiles (hypoxia-regulated microRNAs, HRMs). miRNA take a part in the hypoxia-mediated gene repression, contributing to cell survival in low-oxygen conditions

It seems likely that the plethora of hypoxia-induced transcription factors, operating in hypoxic prostate cancers, may lead to radio-resistance also through the generation or expansion of cancer stem cells.

Moreover, it has recently been shown that, under hypoxic conditions, prostate cancer cell lines interact with p16ink4a via HIF1-alpha, preventing cells entering the senescent state and thereby increasing tumor radioresistance. The existing data are still far to be conclusive. Nevertheless, Hypoxia is now recognized as a major factor driving malignant progression and resistance to treatments of a considerable amount of prostate cancer and the impressive amount of reports in the recent literature support the hypothesis that, counteracting hypoxia through its molecular mediators, is a valid way to fight aggressive prostate cancers. The incomplete comprehension of the molecular events responsible for the cellular reaction to hypoxia, can be reasonable considered as the principal responsible of the variable failure of the majority of the treatment proposed. For instance, still unexploited is the role of a homologous member of the HIF family, : HIF-2. HIF-1 and HIF-2, differ in their transactivation domains, this suggesting that may regulate distinct target genes (Hu et al. 2003; Koukourakis et al. 2006).

As well, the inter-relations between the different members of hypoxia-related pathways should be interpreted further, to optimize the therapeutic approach. As an example, the first attempts of antiangiogenic therapies have produced, as a final effect, an elevated tumor hypoxia with HIF-alpha up-regulation and further gain-of-aggressiveness and radio-resistance of tumors. Furthermore, the use of molecules interacting with most of physiological processes, frequently leads to a relevant toxicity as medium (or long) term side effect. However, the time in which targeting hypoxia will be routinely addressed in the management of aggressive prostate cancer, is rapidly coming in (Fig. 17.1).

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