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Contents

Chapter 1
Mechanisms of Normal Tissue Response

Jolinta Y. Lin, Isabel L. Jackson, and Zeljko Vujaskovic

Abstract Radiation therapy (RT) has been used for decades to treat a wide spectrum of cancers and remains an important tool for both definitive and palliative cancer treatment. Since high doses of irradiation are often required to halt cancer growth and promote tumor cell death, the surrounding normal tissues and organs often impose dose-limiting constraints. Irreparable normal tissue damage can result, leading to persistent toxicity for patients. This chapter focuses on the mechanisms of normal tissue response from RT in four frequently irradiated organs: brain, spinal cord, lung and gastrointestinal tract. A common theme among radiation-induced toxicities is a heightened pro-inflammatory response and inability of these normal tissues to recover to their original states, as vascular changes, fibrosis, and necrosis cause irreparable damage. Understanding the mechanisms of normal tissue response associated with these major toxicities and dose-limiting organs may allow for opportunities to reduce toxicity and improve patients' quality of life by combining more sophisticated treatment delivery technology, such as intensity modulated RT, and therapeutic mitigators that interrupt the altered biological responses.

Keywords Normal tissue injury response • Radiation injury • Inflammation • Fibrosis • Toxicity

Radiation therapy (RT) can be an effective method of cancer treatment, with approximately 47–60 % of cancer patients receiving RT at some point during the course of their disease [1, 2]. A major aim for radiation oncologists is to control tumor growth by eliminating gross and microscopic tumor cell deposits. However, the dose delivered to the tumor is often limited by the proximity of critical organs that may suffer irreversible damage after receiving high radiation doses. Clinically, radiation injury is generally divided into two forms: acute and late. Acute side effects occur during

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RT or within the first 3 months of completing RT, and are generally reversible; although in some cases, the development of acute injury predisposes the patient to late complications [3, 4]. Irreversible late side effects occur several months to years after the end of treatment, and may have more significant impact on a patient's quality of life (QOL).

As cancer detection and management advance, the United States has seen an increase in the number of long-term cancer survivors. The 5-year relative survival rate for all cancers diagnosed in the United States from 1975 to 1977 was 49 %, compared to 68 % from 2004 to 2010 [5]. With more people being cured of cancer, an increased emphasis on QOL issues has surfaced and has led more awareness of the significant side effects from cancer treatment.

An early model for describing the development of acute and late side effects after RT is the target-cell hypothesis. This theory is centered on RT as a direct cause of cell killing in tissues and organs, thus depleting crucial cell populations and resulting in functional deficiency [6]. RT is thought to cause irreversible damage to a proportion of cells, preferentially injuring rapidly proliferating cell populations, such as the epithelial lining of the GI tract, more likely to be in relatively radiosensitive phases of the cell cycle. These damaged cells in turn lose their ability to replicate and induce a regenerative response. The latent period between radiation exposure and repopulation appears to depend on both tissue turnover time and RT dose, as stem cells experience asymmetrical cell divisions and stem cell divisions accelerate in an attempt to counteract RT damage [7]. Both parenchymal cell loss and vascular endothelium damage occur; vascular hyper-permeability and venous exudation follow and have been theorized to contribute to clinically devastating toxicities, such as radiation myelopathy [8–10]. While the target-cell hypothesis may explain some of the mechanisms by which acute side effects occur, it does not appear to fully explain the development of late side effects. Recent research has shown a complex interaction between multiple cell types, leading to persistent overexpression of reactive oxygen species, pro-inflammatory and pro-fibrotic [6, 11].

1.1 Radiation-Induced Brain Injury

Significant attention has been focused on the cognitive impairment that whole brain RT (WBRT) may cause in patients. Several studies, such as the European Organization for Research and Treatment of Cancer (EORTC) 22952 and a study by Chang et al., have found that WBRT patients had more significant cognitive impairment compared to patients who had less volume of brain tissue treated using stereotactic radiosurgery or with surgery alone [12, 13]. Historically, vascular injury to small- and medium-sized blood vessels was thought to contribute to the bulk of injury in the brain, as blood vessels developed a waxy hyaline appearance of fibroid necrosis and endothelial cell proliferation increased; the combination of occluded vessels and decreased blood flow causes ischemia and tissue necrosis [14].

Damage to the vasculature of the brain may contribute to the toxicities of WBRT. Vessel density and length have been noted to decrease in rats 10 weeks post-WBRT, suggesting that the early damage may contribute to vessel rarefaction and lead to late toxicities [15]. Vascular endothelial growth factor (VEGF) is known to initiate endothelial cell proliferation and subsequently promote vasculogenesis and angiogenesis. However, whole brain irradiation has been observed to decrease mRNA and protein expression of VEGF. In addition, WBRT also increases in angiopoietin-2 expression, promotes endothelial cell apoptosis and alters the balance of angiopoietin-2 and VEGF. Such changes are in endothelial cells are thought to initiate vessel rarefaction and possibly contribute to neurotoxicity [16].

Besides the blood vessels, other factors have been identified in contributing to cognitive impairment from WBRT. The white matter of the brain has been known to be the most vulnerable to coagulation necrosis secondary to radiation injury [14]; recent rodent models have shown that brain irradiation decreases neurogenesis, increases neuronal inflammation and leads to progressive cognitive impairment, such as memory impairment, dysphoria, and lethargy [9, 11, 17–20]. Reactive oxygen species may also play a role in the radiation responses of neural precursor cells [21].

Neural progenitor cells in the dentate gyrus are known to be exquisitely radiosensitive; changes in the dentate neurogenesis are associated with altered cognitive function [17]. The dentate subgranular zone (SGZ) of the hippocampus continues to have neurogenesis throughout life as cells migrate to and integrate into the granule cell layer, and develop into granule cells and neuronal markers [18, 22]. Granule cell neurons have enhanced synaptic plasticity and are important in forming new memories [23]. However, decreased neurogenesis in the hippocampus has been identified in rodents that have received brain irradiation (Fig. 1.1) [18, 19]. Rola et al. identified radiosensitive cells in the dentate SGZ of young mice consisting of proliferating cells and immature neurons that experienced acute changes after RT. Irradiated mice had a qualitative and persistent decrease in new neuron production, as well as a chronic inflammatory reaction in conjunction with reduced neurogenesis; with behavioral testing, the authors observed that reduced SGZ neurogenesis correlated in time with deficits in hippocampal-dependent memory retention and differences in the number of immature neurons in irradiated animals relative to unirradiated age-matched controls [18].

Thus, the hippocampus has been identified as a critical structure in preserving cognitive function and has led to interventions in the clinic, such as the use of memantine and hippocampal-sparing RT; these strategies will be discussed later in the chapter. Glutamate is the principal excitatory amino acid neurotransmitter in cortical and hippocampal neurons, and can activate the N-methyl-D-aspartate (NMDA) receptor [24]. The effect of NMDA receptors is paradoxical and can promote neuronal health as well as kill neurons [25]. NR1 and NR2A subunits of the NMDA receptors are recruited to the membrane by tyrosine phosphorylation to enhance NMDA receptor surface localization for participation in neurotransmission [26]. High-frequency stimulation and activation of NMDA receptors are followed by a persistent increase in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responses,

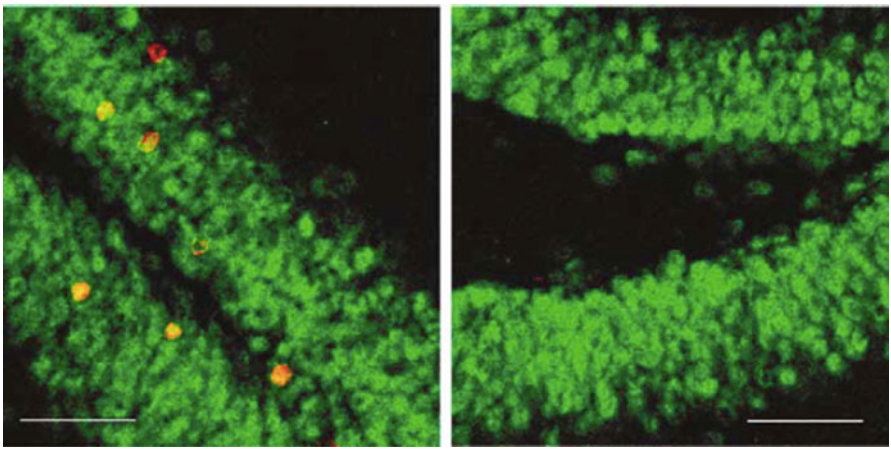

Fig. 1.1 Confocal images of dentate gyrus of mice that received the same dose of BrdU. *Left panel*: A section from a control (non-irradiated) rat. *Right panel*: A section from an irradiated rat. NeuN-labeled cells (*green*) are seen in both sections, whereas BrdU-labeled cells (*red*) are seen only on the left panel. BrdU is a synthetic nucleoside that is similar to thymidine, and is incorporated in replicating cells during DNA replication and serves as a useful indicator of cellular proliferation. Scale bar in both = 50 um. BrdU = 5-bromo-2′-deoxyuridine. NeuN = a neuronal marker protein. Adapted from Madsen TM, et al. Neuroscien 119:635–42, 2003 [19]

also known as long-term potentiation (LTP) [26]. LTP is important in learning and memory [26–28].

Irradiation to brain tissue may acutely decrease the availability of NMDA receptors available for neurotransmission. Rapid internalization of NR1 and NR2A subunits of NMDA receptors was observed after 10 Gy of irradiation to rat hippocampal slices [27]. Irradiated hippocampal slices have demonstrated acute changes with rapid removal of excitatory NMDA receptors and simultaneous increases in surface expression of inhibitory gamma-aminobutyric acid (GABA) receptors in the dentate gyrus [27]. Biochemical assays analyzing the rapid alteration in the trafficking of excitatory NMDA receptors and inhibitory GABA receptors induced by irradiation suggest that the biochemical changes may correspond with impaired synaptic plasticity [27]. Acute decreases in neuronal function after irradiation may lead to a persistent functional reorganization of the synapse, leading neuronal progenitor cells to adopt glial, rather than neuronal fate [27].

However, excessive stimulation of NMDA receptors may also contribute to neuronal cell death, as seen in pathologies like stroke, neurological trauma, and Huntington's disease [25, 29]. Long-term effects of irradiation appear to diminish LTP [28]. Animal studies of fractionated whole brain irradiation of 45 Gy in 9 fractions delivered to adult rats revealed that irradiated rats had impaired Morris water maze performance at 12 months post treatment compared to sham-irradiated mice, as well as increases in levels of NR1 and NR2A subunits in irradiated rats compared to controls [30].

Reducing excessive stimulation of the NMDA receptor with an NMDA receptor anatagonist, such as memantine, has been useful in protecting against further damage

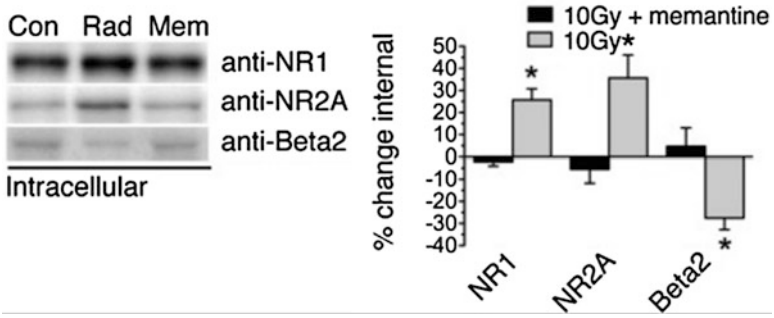

Fig. 1.2 Pre-incubation in the non-competitive NMDAR antagonist memantine (50 μM) prevented both NMDAR internalization and GABA surface retention. Tyrosine phosphorylation of NR2A subunits is thought to promote surface localization of NMDARs and synaptic plasticity (NR1, −2.1 ± 2.0 % and 25.7 ± 5.0 % change internal 10 Gy with drug and 10 Gy respectively, NR2A, −5.4 ± 6.4 % and 35.6 ± 10.5 %, Beta2, 4.7 ± 8.4 % and −27.5 ± 5.3 %, *p ≤ 0.05 two-tailed Student's *t*-tests, n = 4). *NMDAR* N-methyl-D-aspartate receptor. GABA gamma-aminobutyric acid. Adapted from Wu PH, et al. PLoS One 7:e37677, 2012 [27]

in patients with vascular dementia [24]. Memantine has shown positive results in delaying cognitive impairment in preclinical stroke models as well as in phase III multi-institutional studies in treating vascular dementia [27, 31–35]. These findings have led to the study of memantine in delaying or preventing neurocognitive toxicities from whole brain irradiation in both preclinical and clinical studies. Biochemical assays demonstrate that memantine prevented radiation-induced NMDA and GABA receptor alterations in dentate gyrus slices (Fig. 1.2) [27]. Maintaining the NMDA receptors on the surface of the hippocampus with memantine may help preserve neurotransmission in the hippocampus, and thus delay cognitive impairment from insults such as WBRT [27].

With the many preclinical studies demonstrating changes in the NMDA and GABA receptor surface expression, memantine has been introduced into the clinic for mitigating toxicity from WBRT. The Radiation Therapy Oncology Group (RTOG) recently reported their results from study 0614, which was a phase III study of 554 adult patients with brain metastases who were randomized to receive placebo or memantine within 3 days of starting 37.5 Gy of WBRT for 24 weeks. Patients were stratified according to recursive partitioning analysis class and prior surgical therapy. All patients underwent a battery of neuropsychological tests at pre-defined time points. Over time, the patients who received memantine showed better cognitive function over time, and had superior results in executive function, processing speed, and delayed recognition compared to those who received placebo. While the study did not meet its primary endpoint because of loss of statistical power from patient loss (only 149 analyzable patients at 24 weeks), the study shows some benefit in cognitive function with the use of memantine during and after WBRT [24].

An alternative approach to delaying cognitive impairment is to avoid delivering RT to the exquisitely sensitive neural progenitor cells in the hippocampus. Since metastasis to the hippocampal area is relatively rare, hippocampal-sparing WBRT has been pur-

sued. A retrospective study of 371 patients with a total of 1133 brain metastases found no metastasis within the hippocampus, and 8.6 % of the patients had metastasis within 5 mm of the hippocampus, thus giving investigators the ease to spare the hippocampus with WBRT [36]. A multi-institutional phase II clinical trial, RTOG 0933 tested the feasibility of performing hippocampal-sparing WBRT and used the Hopkins Verbal Learning Test-Revised Delay Recall (HVLT-R DR) to test for cognitive decline. Hippocampal sparing WBRT can be performed in clinical practice by contouring the hippocampus as an avoidance structure and using intensity modulated radiation therapy (IMRT) to deliver the WBRT (Fig. 1.3). Central review of the contours was performed and planning requirements mandated that 100 % of the hippocampus could not exceed 9 Gy and maximal hippocampal dose could not exceed 16 Gy. One hundred patients received 30 Gy hippocampal sparing WBRT with IMRT and 42 analyzable patients' HVLT-R DR were compared to historic controls at 4 months post-treatment. The historic control patients who did not have hippocampal avoiding WBRT had a 30 % mean relative decline in the HVLT-R DR from baseline to 4 months post-WBRT, in contrast to the 7 % mean relative decline in the HVLT-R DR seen in patients who received hippocampal sparing WBRT [37]. Thus, Gondi et al. concluded that conformal avoidance of the hippocampus during WBRT was associated with preservation of memory with a decreased mean relative decline in the HVLT-R DR and QOL.

Given the encouraging results of RTOG 0933, hippocampal sparing WBRT is the focus of two ongoing cooperative group studies: NRG (National Surgical Adjuvant Breast and Bowel Project, Radiation Therapy Oncology Group, and Gynecologic Oncology Group) CC (Cancer Control) 003 and NRG-CC001. The NRG-CC003 study is a randomized phase II/III trial of PCI comparing WBRT versus hippocampal-sparing WBRT in patients with small-cell lung cancer who achieve a complete or partial response to chemotherapy, whereas NRG-CC001 is a randomized phase III trial of hippocampal-sparing WBRT plus memantine versus WBRT plus memantine in patients with brain metastases.

The neuroinflammatory response secondary to WBRT is also another area of interest. Mouse models have indicated acute and persistent inflammatory marker elevations after cranial irradiation [38–40]. Mice continued to exhibit increased lev-

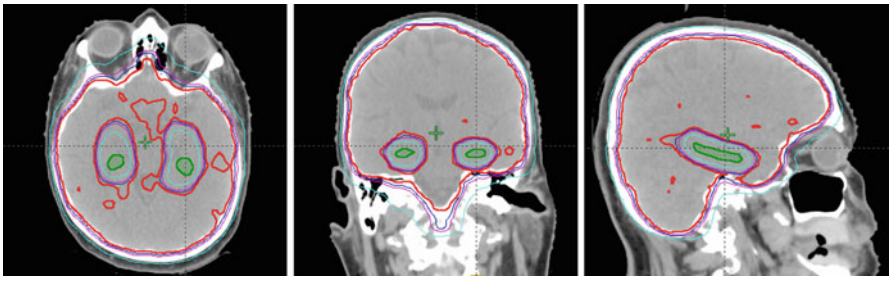

Fig. 1.3 Axial, coronal, and sagittal views of example isodose lines typical of hippocampal sparing whole brain radiation therapy. Isodose lines: red = 100 %, dark blue = 95 %, magenta = 90 %, cyan = 70 %. Green contour and color wash: hippocampus

els of T cells, MHC II-positive cells, and CD11c-positive cells at 1 month with doses ≥15 Gy to the brain; increased levels persisted even 1 year post-RT [38]. CD11c-positive cells were found almost exclusively in white matter and expressed MHC II, suggesting a "mature" dendritic cell phenotype [38].

Pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1) are also being investigated as contributors to neurocognitive impairment from WBRT [41]. In rats that received WBRT, mRNA and protein expression of pro-inflammatory markers in the hippocampus and cortical regions are increased in comparison to those that received sham-irradiation; increases in pro-inflammatory environments may contribute to pathways that lead to radiation-induced toxicities [41].

Another potential source of inflammatory stimuli are microglial cells, which are immune cells that predominate in the grey matter and are found in high concentrations in the hippocampus [42, 43]. If brain injury or immunologic stimuli are present, microglia can be activated through retraction of cell processes, proliferation, and increased production of reactive oxygen species, cytokines, and chemokines that mediate neuroinflammation [42, 44]. WBRT can lead to activation of microglial cells through increases in pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1, with subsequent chronic inflammation and decreased neurogenesis occurring after treatment to the brain [41, 45]. However, the clinical outcome of having increased expression of proinflammatory mediators in the brain after WBRT is unclear.

1.2 Radiation Myelopathy

The spinal cord also contains gray and white matter. Some similarities are seen between the radiation-induced neurotoxicity of the brain and radiation myelitis (Table 1.1). While rare, radiation myelopathy is one of the most feared late complications of RT since interruption or transection to a spinal cord segment can cause dramatic and permanent loss of function [43]. Marcus and Million report a 0.18 % rate of radiation myelitis in 1112 head and neck patients who received a minimum of 30 Gy to at least 2 cm of the cervical spinal cord [46].

Given the severe consequences of radiation myelopathy, most institutions and clinical trials adopt conservative dose constraints for the spinal cord. Since there have been few cases of reported radiation-induced myelopathy, mathematical models have been employed to estimate the probability of severe toxicity from radiation to the spinal cord. Using an $\alpha/\beta = 0.87$ Gy, Schultheiss et al. estimate that the median tolerance dose of the cervical cord was 69.4 Gy (95 % confidence interval, 66.4–72.6), and that the (extrapolated) probability of myelopathy at 45 Gy, 50 Gy and 59.3 Gy is 0.03 %, 0.2 % and 5 %, respectively [47].

Radiation myelopathy can be explained by early white matter necrosis and late vascular damage [8, 48]. White matter lesions are characterized by demyelination, destruction of nerve fibers and loss of axons, focal necrosis, and subsequently lique-

Table 1.1 Comparison of radiation-induced damage to brain and spinal cord

	Brain	Spinal cord
Sensitive regions	• Small- and medium sized vessels	• Vascular damage [43]
	• Neural progenitor cells in the dentate subgranular zone [18, 19]	• White matter changes [8]
Pathologic characteristics	• Waxy hyaline appearance of blood vessels [14]	• Hyalin thickening of vessel walls
	• Increased endothelial proliferation	• Telangiectasia
	• White matter necrosis [119]	• Vascular occlusion
	• Decreased neurogenesis in hippocampus [20]	• Focal hemorrhage and hemorrhagic necrosis [48]
	• Increased apoptosis in neural stem cells [20]	• Demyelination
	• Increase in neuroinflammatory and pro-inflammatory markers [38, 41]	• Destruction of nerve fibers and loss of axons
		• Focal necrosis with subsequent liquefactive necrosis [8]
Recognized dose tolerance	• For fractionated RT with a fraction size of <2.5 Gy, an incidence of radiation necrosis of 5 % predicted to occur at BED 120 Gy (range, 100–140) and 10 % at BED 150 Gy (range, 140–170), using an α/β ratio of 3 [120]	• 50 Gy in 2 Gy per fraction over 5 weeks, BED of 100 Gy_2 using an α/β ratio of 2 for cervical and thoracic cord [53]
Interval and recovery	• Decline in cognitive function peaks ~4 months post treatment, with possible transient recovery over the first year [121]	• Estimates of ~50 % recovery of occult injury induced by 44 Gy at 1 year, ~60 % at 2 years, and ~65–70 % after 3+ years [51]
Potential agents or techniques to decrease toxicity	• Memantine = NMDA receptor antagonist	• SBRT to spinal metastases
	• Hippocampal-sparing intensity modulated RT	
Retreatment interval and dose (conventional fractionation)	• Frequently used retreatment WBRT doses for brain metastases: median first course of 30 Gy, median second course of 20 Gy [122, 123]	• Risk of myelopathy low for total
		• Cumulative ≤135.5 Gy_2 when interval between treatments is no less than 6 months and dose per each treatment course is ≤98 Gy_2 [53]
Retreatment interval and dose (SRS or SBRT and fractionation)	• Retrospective single institution report mild radionecrosis seen in 10 % of patients who received SRS for recurrent high-grade glioma, median SRS dose was 15 Gy (range, 12.5–25 Gy) [124]	• After conventional radiotherapy of an nBED of 30–50 $Gy_{2/2}$, Saghal et al. recommend (1) a thecal sac Pmax total nBED of no more than 70 $Gy_{2/2}$, (2) SBRT thecal sac retreatment dose to the Pmax not exceeding 25 $Gy_{2/2}$, (3) thecal sac SBRT Pmax nBED/total Pmax nBED ratio not exceeding 0.5, and (4) a minimum time interval to reirradiation of at least 5 months [125]

BED biological effective dose, *nBED* normalized BED, *Pmax* maximum point dose, *NMDA* N-methyl-D-aspartate (NMDA) receptor, *SRS* stereotactic radiosurgery, *SBRT* stereotactic body radiotherapy

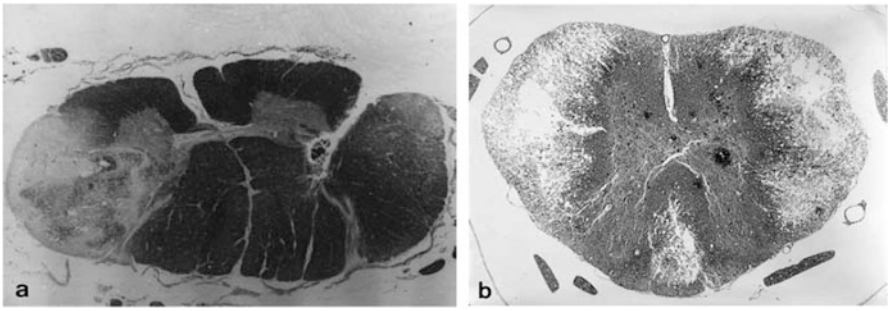

Fig. 1.4 Histologic examples of radiation myelopathy. (**a**) Radiation myelopathy of the sixth cervical spinal cord of a 51-year-old-man with laryngeal cancer (twice irradiation of 71 Gy in 28 fractions over 65 days and 81 Gy of X-ray over 51 days with an interval of 3 years), about 11 months after the last irradiation. (**b**) Bilateral liquefactive white matter necrosis (cystic type) mainly in the lateral funiculus of rat thoracic spinal cord irradiated with carbon ions (17 weeks after single 20 Gy irradiation). From Okada S, et al. Neuropathology 21:247–65, 2001 [8]

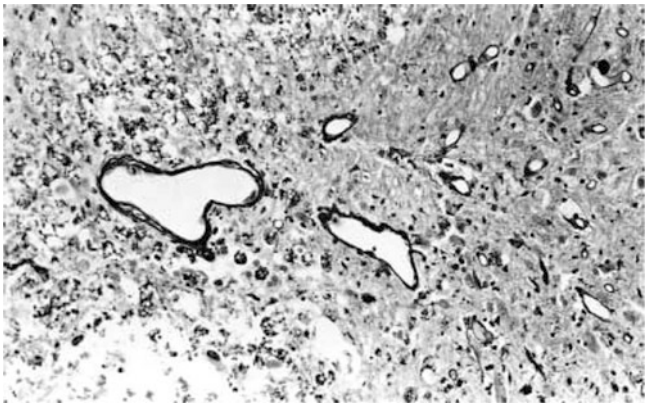

Fig. 1.5 Vascular dilatation around liquefactive white matter necrosis of rat spinal cord irradiated with X-rays (5 months after single 30 Gy irradiation). From Okada S, et al. Neuropathology 21:247–65, 2001 [8]

factive necrosis (Figs. 1.4 and 1.5) [8, 43, 48]. Historic studies of degenerative changes seen in spinal cords of rats that received doses from 1 to 60 Gy demonstrated morphological changes, such as paranodal myelin breakdown and nodal widening, and structural changes in the axons including axonal swelling and axonal degeneration [49]. Radiosensitivity in the cord may also be dependent on the region of irradiation. Bijl et al. used proton irradiation to preferential target different areas of the cervical spinal cord in rats and saw that the central white matter was significantly more radio-resistant than the lateral white matter in terms of extremity paralysis [50]. Notable differences in white matter necrosis were identified in the lateral white matter compared to irradiated central white matter, and no lesions were identified in the gray matter [50].

Thus, white matter demyelination and necrosis appears to be the dominant morphologic features in clinically- and experimentally-induced radiation myelitis [43, 50].

Preclinical models of re-irradiation of the spinal cord have also suggested some tissue recovery with time after initial course of irradiation. A series of non-human primates were followed for 2.5 years after re-irradiation of 57.2 Gy or 66 Gy one or more years from an initial course of 44 Gy, and 4 out of 45 monkeys developed myeloparesis. The symptomatic monkeys had a mixture of white matter necrosis and vascular injury in the spinal cord seen by light microscopy, in contrast to most of the asymptomatic monkeys that did not have overt lesions in their spinal cords (Fig. 1.6). Ang et al. observed that most animals receiving a cumulative dose of 110 Gy in 2.2-Gy fractions did not have morphologically detectable spinal cord lesions, and estimate that there is ~50 % recovery of occult injury induced by 44 Gy at 1 year, ~60 % at 2 years, and ~65–70 % after 3 years or more [51].

Vascular damage, in particular venous damage, may also contribute to radiation myelitis [43]. Vascular damage includes characteristics such as hyalin thickening of vessel walls, telangiectasia, fibrinoid necrosis, vascular occlusion, focal hemorrhage, and hemorrhagic necrosis [48]. Schultheiss et al. categorized three types of radiation myelopathy: type 1 with the predominant features of the spinal cord lesions with demyelination or white matter necrosis; type 2 consisting of telangiectasia, fibrinoid necrosis, thrombosis, or other related vascular damage; and type 3 as having both white matter parenchymal damage and vascular lesions [48]. They found that type 1 lesions were associated with significantly shorter latent periods compared with type 2 vascular lesions [48]. Similarly, patients with type 3 combination lesions had short latent periods comparable to those with type 1 white matter lesions, but had statistically significant shorter latent periods than patients with type 2 vascular lesions [48].

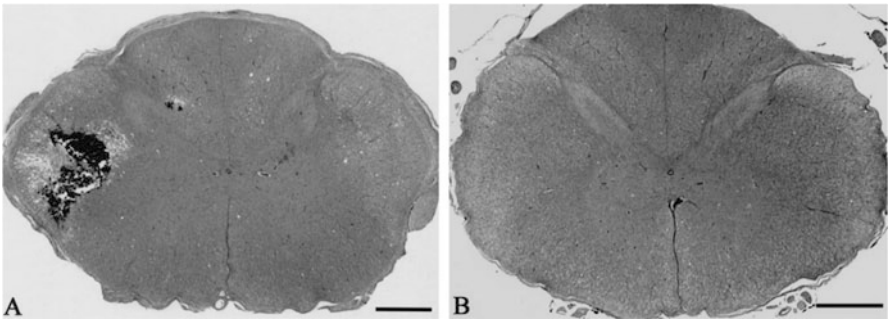

Fig. 1.6 (**a**) Axial cross section of the cervical spinal cord of a symptomatic animal (24 months after receiving 57.2 Gy, 1 year following the initial 44 Gy). Of note are large area of liquefactive malacia surrounding *dark black* mineral in left posterior lateral and lateral funiculus (*long arrow*), small focal area of spongiosis, malacia, and mineralization (*short arrow*), and individual isolated dilated axon sheath (*arrowhead*). (**b**) Axial cross section of the cervical spinal cord of an asymptomatic animal with no overt lesions (24 months after receiving 57.2 Gy 1 year after the initial 44 Gy). H&E, bar 5 100 mm. From Ang KK, et al. Int J Radiat Oncol Biol Phys 50:1013–20, 2001 [51]

Some argue that the glial model of injury is insufficient in fully explaining the pathology of radiation myelopathy, and instead there may be an immune component as patients with inflammatory reactions have shorter latent periods to radiation myelopathy than those who do not have inflammation [48]. More recent attention has been focused on abnormal cytokine production as possible explanation for pathogenesis of radiation myelopathy [14, 43, 52]. Inflammatory markers such as prostaglandins have been noted to sharply increase after irradiation, which may lead to increased vascular permeability. While researchers noted a normalization of prostaglandin levels 14 days out from RT, they also observed persistently elevated prostaglandin levels 120 and 240 days post-RT [52]. While the impact of an inflammatory component is not completely understood in the setting of radiation myelopathy, it likely has a strong impact on some of the other tissue damage as seen in radiation-induced pneumonitis, fibrosis and enteritis [47].

With improvements in imaging modalities and systemic treatment contributing to longer survivorship, increased attention has been placed on re-treatment of the spinal cord. Comparisons of re-irradiated patients who develop radiation myelopathy compared to those who did not develop radiation myelopathy have indicated that the risk of myelopathy is low in patients who have a total cumulative $\leq$135.5 Gy_2, and when the interval between treatments is no less than 6 months and the dose per each treatment course is $\leq$98 Gy_2 [53]. Stereotactic body radiation therapy (SBRT) has also been used to increase dose to the affected vertebral body while sparing more of the spinal cord, particularly after conventional palliative radiation therapy. After conventional radiotherapy of an nBED (normalized biological effective dose) of 30–50 $Gy_{2/2}$, Saghal et al. recommend (1) a thecal sac maximum point dose (Pmax) total nBED of no more than 70 $Gy_{2/2}$, (2) SBRT thecal sac retreatment dose to the Pmax not exceeding 25 $Gy_{2/2}$, (3) thecal sac SBRT Pmax nBED/total Pmax nBED ratio not exceeding 0.5, and (4) a minimum time interval to reirradiation of at least 5 months.

Several groups have also reported their single institutional experience with the safety and efficacy of SBRT for spinal metastases [54, 55]. The NRG is currently accruing patients for a multi-institutional phase II/III trial, RTOG 0631, looking at image-guided radiosurgery/SBRT for localized spinal metastases; thus far, the phase II data indicate good accordance with protocol constraints in a cooperative group setting and has transitioned to the phase III component of the trial [56]. Perhaps conformal and image-guided SBRT may help deliver increased dose of RT to spinal metastasis and decrease the potential for toxicities such as radiation myelitis.

1.3 Radiation-Induced Pneumonitis and Fibrosis

The lung is known to be a radiosensitive organ. Despite the lung's radiosenstivity, approximately 61 % of all lung cancer patients will develop one or more indications for RT at some point in their disease course, with 44.6 % receiving RT as part of their initial treatment, and 16.5 % later in their course for recurrence or progression [57]. In addition, many lung cancer patients have decreased lung function prior to

treatment because of damage from smoking and exposure to carcinogens or occupational hazards. Thus, the potential of radiation-induced lung damage is an important dose-limiting component. Radiation-induced pneumonitis can emerge early, approximately 1–3 months post-treatment, with symptoms such as cough, dyspnea, chest pain, congestion and fever; oftentimes, the symptoms subside with symptomatic management with steroids and occasionally, supplemental oxygen [11, 58].

However, radiation-induced pneumonitis can translate to irreversible fibrosis months to years after treatment. Radiation-induced fibrosis is characterized by vascular damage and collagen deposition (Fig. 1.7) [58]. While radiation-induced fibrosis a radiographic diagnosis, it does not always cause clinical symptoms if only a small volume of the lung becomes fibrosed [11, 58]. Larger volumes of scarring and fibrotic lung can lead to cough, shortness of breath, and decreased diffusion capacity and respiratory volume [58].

Vigorous studies to understand the mechanism of radiation-induced pneumonitis have been undertaken over the last several decades. By causing DNA damage, ionizing radiation can cause change in the microenvironment chemokines, inflammatory cytokines, and fibrotic cytokines, as well as alter cell–cell interactions, elicit an influx of inflammatory cells and perfusion changes [6, 59–62]. The early release of cytokines can cause other cells, including inflammatory, stromal, endothelial, and parenchymal cells to release or activate downstream cytokines, growth factors, and chemokines to further propagate the biological response of inflammation and hypoxia (Fig. 1.8) [6, 59, 60, 62].

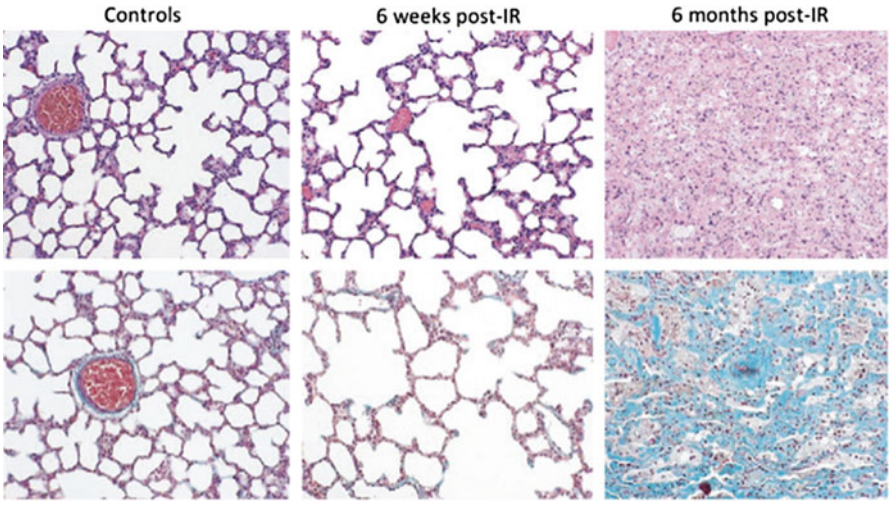

Fig. 1.7 Radiation-induced pulmonary injury at 6 weeks (*middle panels*) and 6 months (*right panels*) after radiation exposure. At 6 weeks the changes are mild with a thickening of the alveolar septae due to the presence of inflammatory cells. Exudative material is present in some alveoli, but the architecture is preserved and there is no fibrosis. At 6 months, there is replacement of the normal alveolar architecture with widespread fibrosis. Far left panels show control tissues. *IR* irradiation. From Stone HB, et al. Lancet Oncol 4:529–36, 2003 [58]

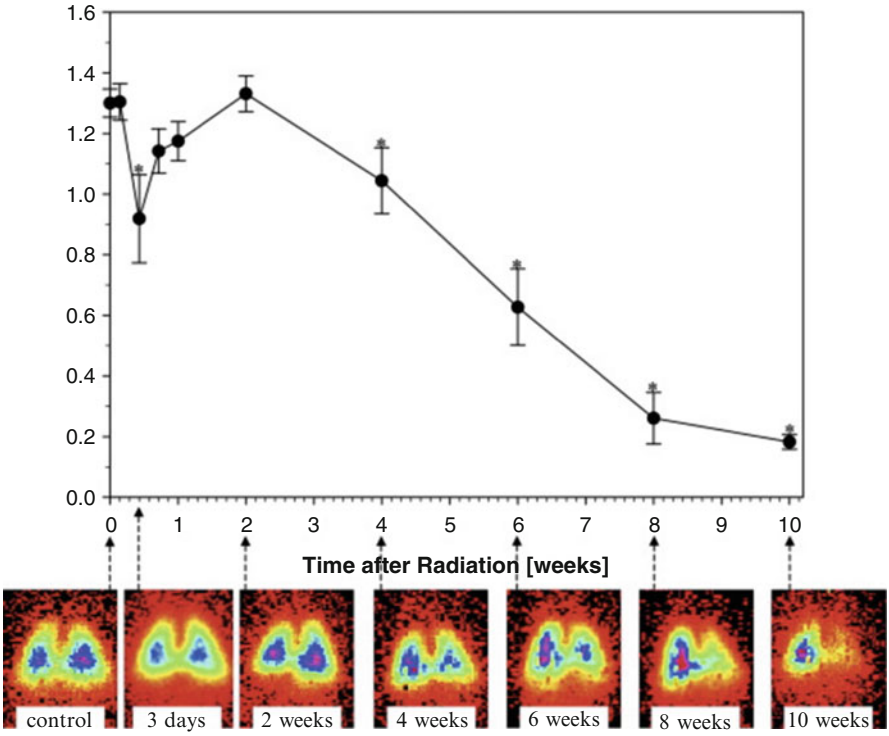

Fig. 1.8 Changes in rat lung perfusion after 28-Gy, single-dose irradiation to the right hemithorax at designated time points. Mean perfusion ratio is defined as a function of time (at pre-irradiation time point, t = 0, for a period of 10 weeks after irradiation). Lung perfusion decreased significantly 3 days after irradiation, followed by a short recovery period and subsequent progressive decline. From Fleckenstein K, et al. Int J Radiat Oncol Biol Phys 68:196–204, 2007 [62]

Transforming Growth Factor β (TGFβ) is a growth factor and cytokine that is strongly profibrotic and causes extracellular matrix and collagen deposition [6, 60, 61]. Dysfunction with TGFβ has been implicated in immunodeficiency, delayed wound healing, fibrotic diseases in the kidney, liver, lung, arteriosclerosis, rheumatoid arthritis, and scleroderma [6, 63]. TGFβ has 3 isoforms, TGFβ1-3, with TGFβ1 as the most frequently implicated in fibrosis, and mediates extracellular matrix synthesis and deposition [6, 63]. TGFβ1 is strongly chemotactic for neutrophils, T cells, monocytes, and fibroblasts, which in turn, begins to secrete fibroblast growth factor, tumor necrosis factor, interleukin (IL)-1, and fibroblasts to synthesize extracellular matrix proteins. In addition, TGFβ1 also appears to be able to cause autoinduction and recruits infiltrating and residents cells to produce more TGFβ1, thus amplifying the pro-inflammatory response and leading to fibrosis [63]. Even very low doses of ionizing radiation, as low as 0.1 Gy, have been shown to quickly induce TGFβ activation (Fig. 1.9) [6, 64].

When TGFβ is activated by extracellular events, the Smad pathway activates additional signaling pathways; an increase in production of various transcriptional

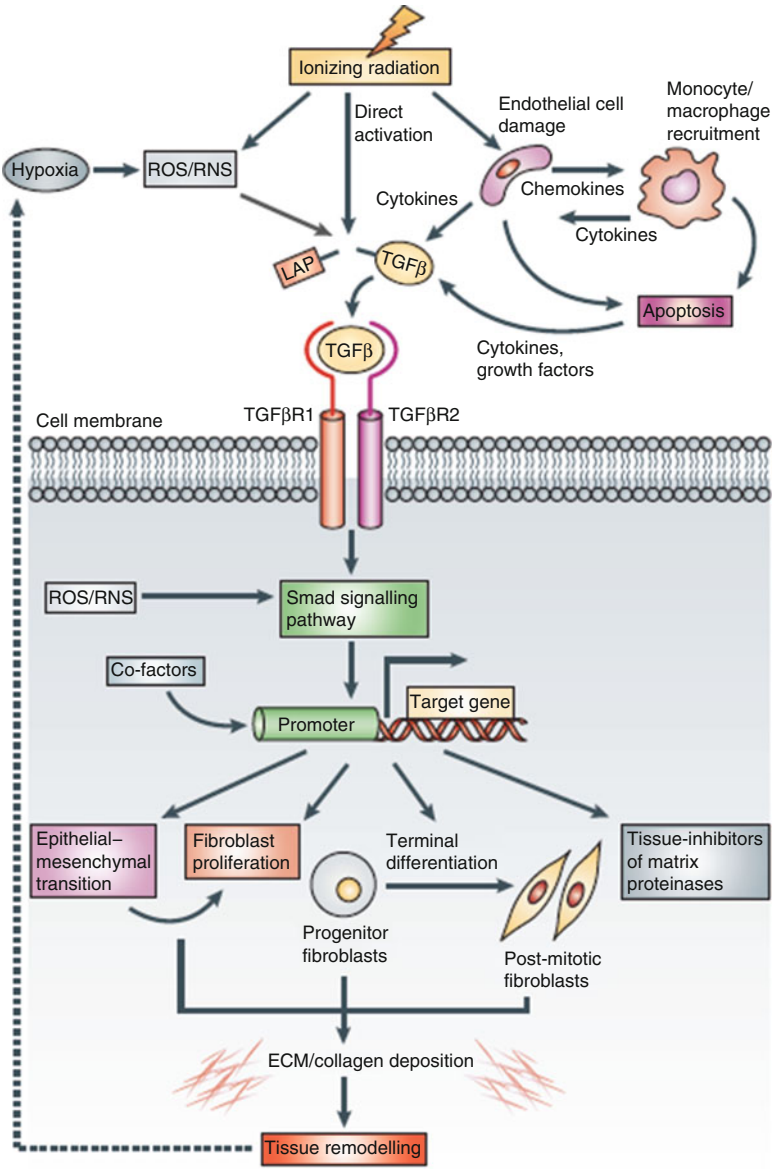

Fig. 1.9 Key processes in radiation fibrogenesis. Ionizing radiation directly activates transforming growth factor-β (TGFβ) through the dissociation of the latency-associated peptide (LAP) from the active mature form of TGFβ. Furthermore, radiation damages endothelial cells, which in turn initiate a cellular response that also leads to the release of pro-fibrotic cytokines, including TGFβ. A third main effect of radiation is that it perturbs the homeostatic control of the reactive oxygen and nitrogen species (ROS and RNS), which again leads to the activation of TGFβ and directly interferes with the Smad signaling pathway. These extracellular events activate the TGFβ signaling pathway, which in turn produces various transcriptional responses, all of which lead to increased extracellular matrix (ECM) and collagen deposition. The radiation-induced vascular damage and uncontrolled tissue remodeling can lead to tissue hypoxia, which could be one of the mechanisms perpetuating the fibrogenic response. From Bentzen SM Nat Rev Cancer 6:702–13, 2006 [6]

responses leads to increased extracellular matrix and collagen deposition [6, 65]. The Smad proteins are a set of cytoplasmic signal-transducing proteins that receive signal from TGFβ signaling to regulate gene activity or modulates other DNA-binding transcription factors' activity and can lead to fibrosis [65]. Mice that lack Smad3 have decreased cutaneous injury and fibrosis from ionizing radiation [65].

Clinical studies looking at patient serum have notable elevations in pro-inflammatory markers such as TGFβ1, IL-1, and IL-6 after exposure to ionizing radiation; persistent elevations of these early markers may correlate with patients who are more likely to experience radiation pneumonitis after receiving thoracic irradiation [66, 67]. Paun et al. have observed that increased pulmonary Th1 and Th17 lymphocytes, and decreased levels of IL-17 and interferon-γ levels were significant predictors of late stage fibrosis in mice exposed to 18 Gy of whole thorax irradiation compared to untreated mice [68]. In the fractionated irradiation setting, rats that received 8 Gy in five fractions to the right hemithorax had elevated and persistent levels of markers for hypoxia, oxidative stress and angiogenesis/capillary proliferation, macrophage activation, and fibrosis [69]. More recent research has been focused on identifying biomarkers that may predict patients who are at risk for developing radiation pneumonitis. The field of radiogenomics has looked at single nucleotide polymorphisms (SNPs) in particular genes, such as TGFβ1, ataxia telangiectasia mutated, P53, and methylene tetrahydrofolate reductase to determine if genetic background may explain some of the differences in toxicity and tissue damage among different individuals. A SNP is defined as an inter-individual variation in the DNA sequence that involves the substitution of a single nucleotide that occurs in more than 1 % of the population [6].

Identifying SNPs that may be involved in genes of carriers who are more prone to radiation-related toxicity may be important in personalizing and recommending treatment options to patients. Genomic DNA samples of patients who have undergone definitive thoracic RT have been used to identify specific SNPs in various genes that may be associated with a higher or lower risk of radiation pneumonitis [70–73]. However, recent meta-analyses have questioned the validity of specific associations of SNPs and the risk of fibrosis in other cancers, such as breast cancer. Thus the significance of specific SNPs in the development of radiation injury is still being determined [74, 75].

Radiation-induced inflammation also generates reactive oxygen and nitrogen species (ROS or RNOS) in cells, and these mediators appear to be increasingly important in normal tissue response to radiation damage [6, 76, 77]. Superoxide dismutase (SOD) catalyzes the reaction of superoxide anion radical to hydrogen peroxide, and is a major controller of the steady-state concentration of superoxide anion radical [77]. Since the elevation of superoxide radicals appears to have a role in activating cytoplasmic signal transduction pathways after irradiation, one potential intervention is to give supplemental SOD to scavenge for and convert superoxide radicals to hydrogen peroxide; several preclinical models have supported the theory that SOD mimetic can decrease radiation-induced lung damage by countering radiation-induced cytokine accumulation [78–82].

The renin-angiotensin system (RAS) may also have an influence on late side effects. Angiotensin-converting enzyme (ACE) inhibitors, are potential modulators in reducing risk of late effects such as pulmonary fibrosis since ACE inhibitors pre-

vent the conversion of biologically inactive angiotensin I to active angiotensin II [6, 83, 84]. Angiotensin II has been highlighted as a key player since angiotensin II appears to have a role in regulating two proteins that contribute to pulmonary fibrosis: TGFβ and α-smooth muscle actin [6, 11]. Thus, angiotensin II receptor blockers may also have similar effects as ACE inhibitors [85, 86].

Several preclinical studies demonstrate a decrease in the inflammatory response in the lung parenchyma, and subsequent reduction in pulmonary fibrosis in animals given ACE inhibitors or angiotensin II receptor blockers [80, 84–86]. Kharofa et al. retrospectively reviewed the charts of 162 patients who were treated with definitive thoracic RT for stage I–III non-small cell lung cancer or small cell lung cancer, and found that rate of grade 2 or higher pneumonitis was lower in ACE inhibitor users vs. nonusers (2 % vs. 11 %, p = 0.032) [87]. Another retrospective analysis consisting of a larger group, 413 patients, did not find that ACE inhibitors were associated with the risk of symptomatic radiation pneumonitis on multivariate analysis, hazard ratio = 0.66 ($P = 0.07$); however, subgroup analysis showed that ACE inhibitors use had a statistically significant protective effect from grade ≥2 radiation pneumonitis among male patients and among patients who received a low (≤20-Gy) mean lung dose [88].

Several components may influence the likelihood of developing radiation fibrosis including imbalance of pro-inflammatory signaling, SNP variation, generation of reactive oxygen and nitrogen species, and activation of the RAS. Understanding the pathophysiology of radiation fibrosis may identify opportunities for intervention and prevention of radiation fibrosis.

1.4 Radiation Enteritis

Bowel symptoms such as diarrhea, bowel urgency, abdominal cramping, nausea, and vomiting can occur frequently in patients who receive abdominal or pelvic RT for gynecologic or gastrointestinal (GI) malignancies. Over 300,000 patients a year are estimated to receive pelvic or abdominal radiation therapy with a 60–80 % incidence of acute bowel toxicity [4]. While GI symptoms following pelvic RT are common, the cause is complex. Predisposing factors such as body mass index, previous abdominal or pelvic surgery, concurrent chemotherapy, and other clinical factors such as pre-existing irritable bowel syndrome or inflammatory bowel disease, may contribute to the acute toxicities. In addition, reasons such as relapsed tumor, confounding GI symptoms unrelated to RT, and previous history of more than one GI disorder may also contribute to persistent GI symptoms after pelvic RT [89].

Nonetheless, patients who receive pelvic RT appear to have more persistent QOL issues than those who do not receive pelvic RT. Two large clinical trials performed by the Post Operative Radiation Therapy in Endometrial Carcinoma (PORTEC) groups demonstrated persistent QOL changes in patients who received pelvic RT even after a median of 13.3 years post-treatment for PORTEC-1 and 7 years post-treatment for PORTEC-2 [3, 90]. Despite the long time lapse from RT, patients who were randomized to pelvic RT report significantly worse QOL scores for diarrhea, fecal urgency, fecal leakage, and limitations of daily activities because of bowel

symptoms compared to those who were randomized to the observation or vaginal brachytherapy groups [3, 90].

The pathology of radiation enteropathy was originally based on the target cell theory, in which the severity of the injury was dependent on the proliferation rate of the fast-recovering epithelial cells versus slowly proliferating cells; however, a multitude of other contributors, such as mucosal atrophy, intestinal wall fibrosis, vascular sclerosis, and functional changes from malabsorption and dysmotility have now been described [91].

The crypt cells in the intestinal epithelium are known to be rapidly proliferating cells and are the first to be affected by irradiation. With ongoing irradiation, crypt cells die and the body is unable to replace the villus epithelium [4, 91]. Increasing dose is known to cause more damage with changes in intestinal structure (Fig. 1.10) [92]. Crypt cell death can also lead to breakdown of the mucosal barrier, mucositis, and prominent compensatory and proliferative reactions [4, 91]. The mucosal barrier is a thick mucus layer that separates the lumen of the intestine from the single layer of epithelial cells, helping to protect and separate the epithelial cells from opportunistic bacteria to prevent attachment and subsequent invasion [4, 93].

Rat models have shown changes in epithelial barrier properties with loss of structural morphology in the ileum and loss of barrier integrity with increasing single doses of radiation [92]. A historic study by Otterson et al. demonstrated significant changes in motility in dogs that had received fractionated 2.5 Gy every other day to 22.5 Gy to the abdomen. While a slowed contractility was observed in the proximal small intestine, the

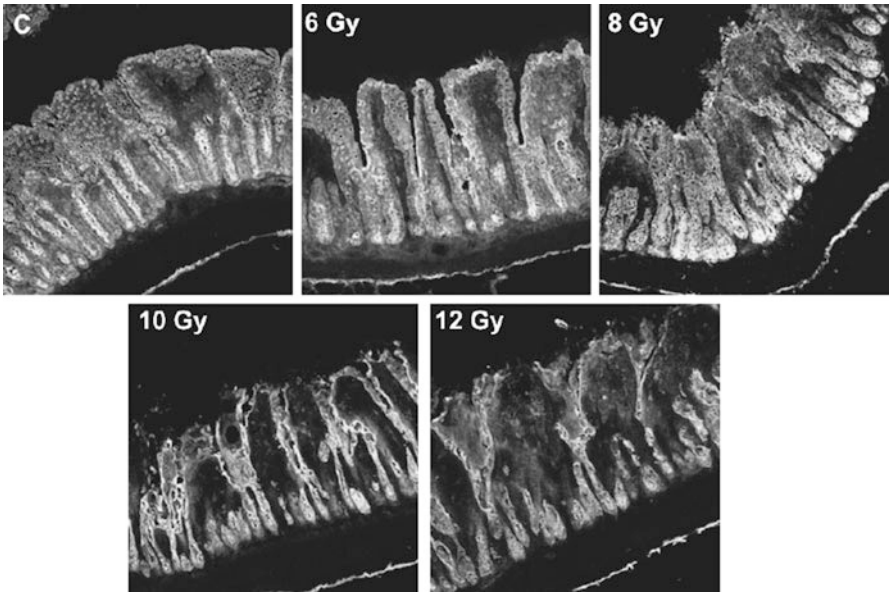

Fig. 1.10 Dose-dependent changes in pan-cytokeratin staining in ileal samples at 3 days after exposure to lower body single fraction irradiation. C = control animal. Objective × 10. From Dublineau L, et al. Can J Physiol Pharmacol 82:84–93, 2004 [92]

giant migrating contractions in the colon would dominate and lead to rapid propulsion of watery diarrhea [94]. With RT, an increase in giant migrating colonic contractions that originated in the ileum was also seen and reported to migrate to the colon [95]. Comparisons of weekly scheduled colonoscopies to pre-radiation biopsies demonstrated that the mucosal integrity architecture remained well-preserved, but there was noticeable increased mucosal friability and increased inflammatory infiltrates in the lamina propria after RT [95]. In the clinical setting, small intestinal transit time was also notably shorter in patients treated with RT compared to healthy human volunteers [96].

While acute side effects usually resolve within 1–3 months after completing RT as the crypt cells recover, another complicating factor is that it has been difficult to differentiate intestinal dysfunction from RT versus other factors. Carratu et al. highlights the complexity of GI symptoms after pelvic RT in 20 patients who were all found to have significantly increased intestinal permeability after 15 days of pelvic RT; however, all of the 20 patients' intestinal permeability returned to normal values by the completion of RT despite all complaining of continued GI symptoms [97]. The development of acute GI symptoms is more complicated than a temporary increase in intestinal permeability. Symptoms may be attributed from clonogenic and apoptotic cell death in the crypt epithelium, shortened villi, mucosal barrier breakdown, mucositis, prominent compensatory and proliferative reactions, impaired motility, and malabsorption of carbohydrates, amino acids and bile acids (Fig. 1.11) [91, 96, 98, 99]. In addition, patients typically receive fractionated course of irradiation considering of 1.8–2 Gy per fraction, for around 5–6.5 weeks. The multiple repetitive injuries from a fractionated course of treatment is likely to recruit inflammatory cells as well as the accumulation of direct tissue injury, thus eliciting a dynamic spectrum of cellular injury, ongoing repair, molecular responses, inflammation, and other pathophysiologic responses [4].

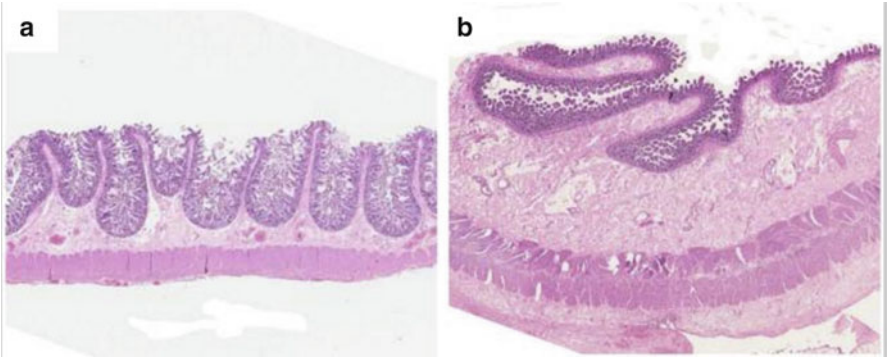

Fig. 1.11 Resection specimens of normal human small bowel and delayed radiation enteropathy. (**a**) Normal intestine (original magnification 0.5X). (**b**) Resected small intestine from a woman with severe delayed radiation enteropathy. Note atrophic mucosa and severe fibrosis in submucosa and subserosa (original magnification 0.5X, same as *Panel A*). From Hauer-Jansen et al. Nat Rev Gastroenterol Hepatol 11:470–9, 2014 [4]

While most patients have improvement in their acute GI symptoms within the first 3 months after completing pelvic RT, the concern is for developing late complications as symptoms can persist in long-term survivors who are otherwise cured of their cancers [3, 90, 96]. Although rare, acute radiation-related symptoms have been associated with the development of subsequent late complications in animals, as well as among patients participating in clinical trials [3, 4, 100–102]. Late GI side effects can be attributed to mucosal dysfunction, intestinal dysmotility with bacterial overgrowth, irreversible mucosal atrophy, intestinal wall fibrosis, and microvascular sclerosis [4, 91]. Mucosal dysfunction may be attributed to a variety of factors such as atrophy, decreased brush border membrane enzyme activity and mucosal barrier effect, reduced mucosal blood flow and lymph drainage [91]. Late effects in the rectums of prostate cancer patients have also been observed and cause symptoms of rectal bleeding, proctitis, and fistula (Fig. 1.12) [4].

As seen in other sites of radiation damage, inflammation has long been suspected to be an instigator of late side effects in radiation enteritis. Rats that have received abdominal irradiation have been noted to have an in imbalance of proinflammatory mediators: IL-1β, TNF-α, and IL-6 levels in the ileal muscularis layer. They increase quickly after irradiation with subsequent neutrophil accumulation as endothelial cells, macrophages, smooth muscle cells, and fibroblasts to secrete neutrophil-attracting cytokine, IL-8 (Fig. 1.13) [103]. TGFβ and its main downstream effector, connective tissue growth factor (CCN2), initiate fibrogenic differentiation of resident mesenchymal cells and lead to radiation fibrosis; however, radiation-induced enteropathy appears to have a slightly different mechanism. Instead of the Smad pathway leading to fibrosis as seen in other normal cell response, chronic radiation enteropathy may be elicited by fibrosis-initiated cells from autoinduction of CCN2 using a Smad3-independent pathway. High levels of TGF-β1 are observed with radiation fibrosis at other organ sites; however, radiation enteritis is known to have low levels of doses TGF-β1. The low levels of doses TGF-β1activates the Rho/ROCK pathway in fibro-

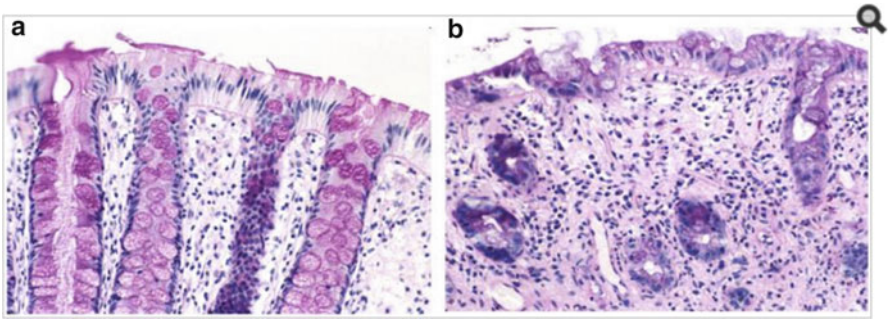

Fig. 1.12 Human endoscopic biopsies of rectal mucosa obtained from patient before and during ongoing radiation therapy of prostate cancer. (**a**) Periodic acid Schiff (PAS)-staining of normal rectal mucosa before start of radiation therapy. Note intact surface epithelium, straight glands, and the many PAS-positive goblet cells (original magnification 20X). (**b**) Glandular atrophy, mucosal inflammation, and loss of PAS-positive goblet cells 2 weeks into the course of radiation therapy (original magnification 20X). From Hauer-Jansen et al. Nat Rev Gastroenterol Hepatol 11:470–9, 2014 [4]

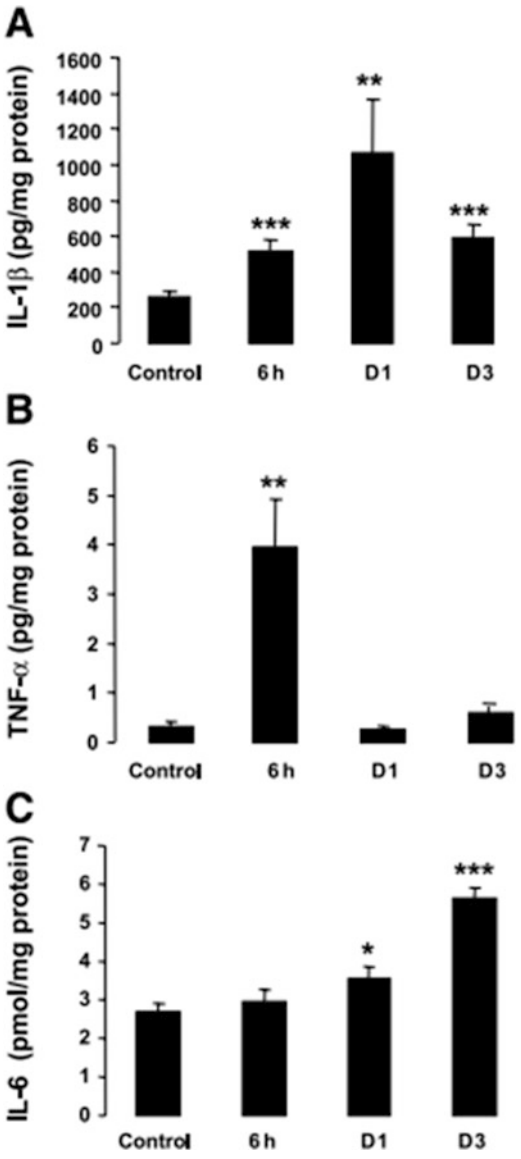

Fig. 1.13 Effect of a 10-Gy abdominal -irradiation on ileal procytokine levels of IL-1, TNF- α, and IL-6 by ELISA in ileal muscularis layers obtained 6 h, 24 h (D1), and 3 days (D3) postirradiation. Values are means SE, n = 5, *P<0.05, **P<0.01, ***P<0.001 irradiated vs. control rats [103]

sis-derived cells, leading to auto-induction of the CCN2 gene and increases fibrosis [104].

Besides damage to the intestinal structure, RT is likely to influence the microbiome, or natural flora, of the intestine. The human GI tract contains 300–500 different species of bacteria, which serve three major roles: (1) the metabolic role aids in digestion, recovers metabolic energy and absorbable substrates for the host, and provides nutrients such as vitamin K, some vitamin B's, folate, short chain fatty acids that stimulate epithelial cell proliferation, and absorb ions; (2) its trophic role controls

epithelial cell proliferation and differentiation and modulate the immune system as the gut-associated lymphoid tissues is the largest pool of immunocompetent cells in the human body; and (3) the protective role enhances the barrier effect and protect against pathogens and bacterial translocation [93]. Three primary mechanisms of bacterial translocation are overgrowth of bacteria in the small intestine, increased permeability of intestinal mucosal barrier, and deficiencies in host immune defenses [93].

The impact of RT on the microflora is an emerging area of interest given the gut's influence on the immune system and on the development of radiation enteropathy [4, 93, 105]. Similarities have been between radiation enteropathy and inflammatory bowel disease since germ-free mice are resistant to both inflammatory colitis and radiation enteropathy development [4]. Comparison of molecular profiles of fecal samples between patients who suffered diarrhea from pelvic RT versus controls indicated differences in microbiota profiles in patients who had GI symptoms; interestingly, patients who had RT but did not suffer diarrhea had maintained similar molecular profiles during their and after RT [105]. Renewed interest in antibiotics and probiotics in mitigating radiation injury has been growing [99]. Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts by aiding in metabolic and nutritional function of commensal microbiota, modulating immune function, and enhancing mucosal barrier function to protect the host from pathogens translocation [106–108]. However, additional research is necessary to understand this complex and intricate relationship between the microflora balance and radiation enteritis. The process of radiation enteropathy is complex with a variety of factors and contributors, such as delayed epithelial injury, mucosal damage, microvasculature damage, imbalance of immune response and intestinal microbiome, as well as changes in the luminal content (Fig. 1.14).

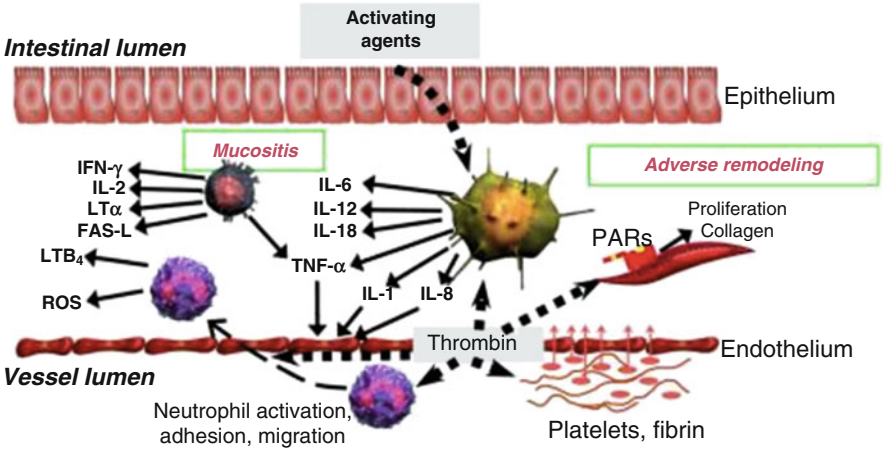

Fig. 1.14 Involvement of the intestinal immune system and microvascular endothelium in the regulation of acute radiation mucositis and subsequent adverse tissue remodeling (intestinal fibrosis). From Hauer-Jensen M, et al. Nat [4]

1.5 Conclusions

RT is an effective method to control tumor growth, but nearby organs and tissue can pose dose-limiting constraints as acute and late side effects may results from high-doses of irradiation. Several theories have been attributed to normal tissue toxicity such as target-cell hypothesis, heightened inflammation with subsequent fibrosis, damage to developing cells, vascular changes, and necrosis have been thought to contribute to toxicities such as radiation-induced brain injury, myelopathy, pulmonary fibrosis, and enteritis. While radiation oncologists have been conscious of keeping doses to normal structures within tolerable thresholds, additional investigations that maximize advanced technology such as IMRT, proton therapy, and therapeutic mitigators are being vigorously investigated to try to reduce toxicities from RT.

As emphasized earlier in the chapter, acute toxicities during treatment may increase the likelihood of late toxicities. Thus, decreasing toxicity during treatment can play an important role in mitigating radiation toxicity. Many exciting research approaches are being addressed in the preclinical setting to decrease fibrosis in radiation enteropathy, such as pravastatin decreasing CCN2 production in human explants and smooth muscle cells isolated via Rho pathway inhibition [109–111].

However, positive clinical trials demonstrating benefit in proposed radiation mitigators have been few in comparison. With the exception of the RTOG 0614 trial demonstrating memantine preserving cognitive function, executive function, processing speed, and delayed recognition in patients treated with WBRT, other clinical trials have been equivocal or negative [24]. A few preclinical studies have demonstrated that *Lactobacillus*-based probiotics may protect against acute intestinal injury [112, 113]. However, the results from several phase III clinical studies using *Lactobacillus*-based probiotics in the setting of pelvic RT have had mixed results [108, 114–118]. Additional studies are needed to improve the therapeutic ratio to decrease toxicity for patients as the number of long-term cancer survivors continues to increase.

Acknowledgments

Conflict of Interest Notification The authors state that an actual or potential conflict of interest does not exist with the submitted manuscript.

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Chapter 2
The Role of Hypoxia in Radiation Response

Monica M. Olcina, Ryan Kim, and Amato J. Giaccia

Abstract Radiation is a very effective form of cancer therapy but its effectiveness is significantly influenced by changes in the levels of oxygen, nutrients and pH in the tumor microenvironment. Radiation dose is also limited by normal tissue toxicity, which can manifest itself both in early effects as well as in late effects. Therefore, approaches aimed at improving the therapeutic window for radiotherapy should consider both the effects of the tumor microenvironment such as hypoxia, and also target pathways that may reduce radiation-induced normal tissue toxicity. With these concepts in mind, we review the biological consequences of tumor hypoxia and the effects of hypoxia/HIF on tumor radiation sensitivity as well as the effects of targeting the HIF/PHD axis for normal tissue radioprotection.

Keywords Hypoxia • Ionizing radiation • Normal tissue toxicity • Tumor response

2.1 Introduction

Radiation is an effective form of cancer treatment used in approximately 50 % of patients. Ionizing events following delivery of ionizing radiation result in damage due to direct interaction with DNA or indirectly through interaction with water and other cellular compartments [1]. Several components of the tumor microenvironment will affect the effectiveness of radiotherapy [2, 3]. One of the best studied is the low oxygen (hypoxic) regions frequently found in tumors [4]. Severely hypoxic regions may require up to 2–3 times higher radiation doses to achieve the same biological effects since oxygen is required for the chemical reactions that lead to the generation of DNA damage following energy absorption from ionizing radiation [5]. The greatest effects of oxygen on radiosensitivity occur as oxygen tensions increase from anoxia to approximately 10 mmHg with minimal changes occurring once oxygen concentrations are in the normal tissue range [6]. The importance of oxygen status on radiation response was already realized in the 1930s by Crabtree

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and Cramer [7]. Later in the 1950s, the studies of Gray and colleagues demonstrated the importance of the "oxygen effect", leading to the conclusion that viable cells under chronic severe hypoxia were present in tumors [5, 6, 8]. Subsequent studies further highlighted the fact that areas of severe hypoxia could also arise due to blood flow fluctuations [9, 10].

Hypoxia results in the induction of a myriad of biological responses typically associated with malignant progression and treatment resistance, which can also hinder radiation effectiveness. These responses are in part driven by the hypoxia-inducible factor (HIF) family of transcription factors. HIF-1α and HIF-2α are the main HIF-α isoforms, and form transcriptionally active heterodimers with HIF-1β to drive the expression of genes that facilitate adaptation and survival in hypoxic conditions [4, 11]. Radiation resistance in hypoxic regions has typically been thought to arise mainly from the effects of reduced oxygen on radiation-induced radical formation. However, more recently, HIF-1-dependent gene expression changes have been proposed to also contribute to radiation resistance [12].

Approaches devised to increase the therapeutic window for radiotherapy aim to decrease radiation-induced normal tissue damage without increasing the radioresistance of the tumor, or ideally while also radiosensitizing the tumor (Fig. 2.1). Several efforts have been undertaken to improve the therapeutic window for radiotherapy including the improvement of treatment planning techniques, resulting in increasingly precise dose delivery to the target with maximal normal tissue sparing [13]. However, despite these advances normal tissue toxicity to certain areas is often unavoidable. For example, areas of the gastrointestinal tract are invariably present in

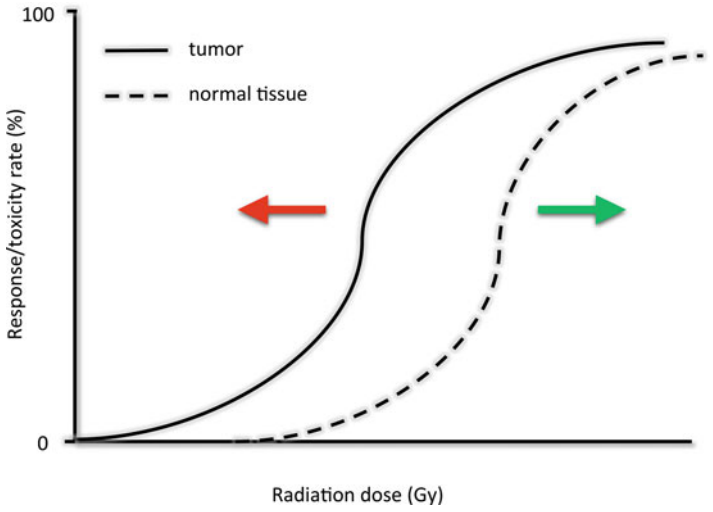

Fig. 2.1 Schematic representation of the tumor and normal tissue toxicity dose–response curve. The therapeutic window represents the 'gap' between the two curves. The ideal radioprotector/radiosentitizer should move the response curves apart reducing normal tissue toxicity while maximizing tumor response for a given radiation dose. This is indicated by the *red* and *green arrows*. Adapted from [90]

the irradiation field when abdominal tumors are irradiated [14]. When thinking about improving the therapeutic window for radiotherapy, it is therefore important to take both the normal tissue as well as tumor sensitivity into consideration. Interestingly, targeting the HIF/PHD axis has recently been proposed as a means of reducing normal tissue toxicity [15]. In this chapter a description of the biological consequences of hypoxia, with a specific focus on HIF-signaling and the effect of hypoxia/HIF on tumor radiation sensitivity will be reviewed. Furthermore, recent findings describing targeting the PHD/HIF axis for normal tissue radioprotection will be discussed.

2.2 Characteristics of the Tumor Microenvironment

The tumor microenvironment consists of the cells surrounding the tumor and components with which the tumor interacts. These include surrounding blood vessels, fibroblasts, immune inflammatory cells, pericytes, stromal stem cells and the extracellular matrix (ECM) [16]. These components are often altered in ways that are conducive to tumor growth and contain properties that lead to the creation of a Darwinian selection for specific oncogenic traits [16]. Hypoxia is a physical feature of the tumor microenvironment. The study of hypoxia and cancer has drawn a substantial amount of attention in recent years due to its association with radioresistant tumors, tumor recurrence after radiotherapy, and poor patient prognosis [17–19]. Tumor hypoxia, which is defined as an inadequate oxygen supply, occurs as a gradient. Depending on the context and tissue type, the hypoxic region can be defined by different internal partial pressures of oxygen (pO_2) ranging from less than 10–15 mmHg, less than 7 mmHg or less than 2.5 mmHg [20–22]. Hypoxia arises as the growth of the solid tumor surpasses the ability of the existing vasculature to supply the tumor with adequate oxygen. As the distance away from the blood supply increases oxygen tension decreases and the hypoxic gradient is formed (Fig. 2.2). The existing blood supply is capable of supporting a tumor size of approximately 1 mm in diameter after which the developing tumors must form their own blood supply network. This is achieved either by utilizing preexisting host vessels or by formation of new vessels via tumor angiogenesis factors [23, 24]. However, the newly formed vessel network possesses structural and functional abnormalities that result in a decreased ability to supply oxygen. These include incomplete, interrupted, or missing endothelial linings and basement membranes, decreased wall contractility, leakiness, existence of arteriovenous shunts, and a distorted architecture. These effects contribute to decreased blood perfusion and irregular blood flow, resulting in a decreased delivery of oxygen. This effect is compounded by the increase in tumor mass and diffusion distance [25].

Hypoxia can occur in two forms: acute and chronic. Acute hypoxia occurs when the structural and functional defects in tumor vasculature result in transient blockage of blood vessels [10]. The random opening and closing of these vessels periodically deprives the downstream cells of oxygen in an episodic manner. Chronic hypoxia occurs as the diffusion distance from the capillaries increases. Tumor proliferation is

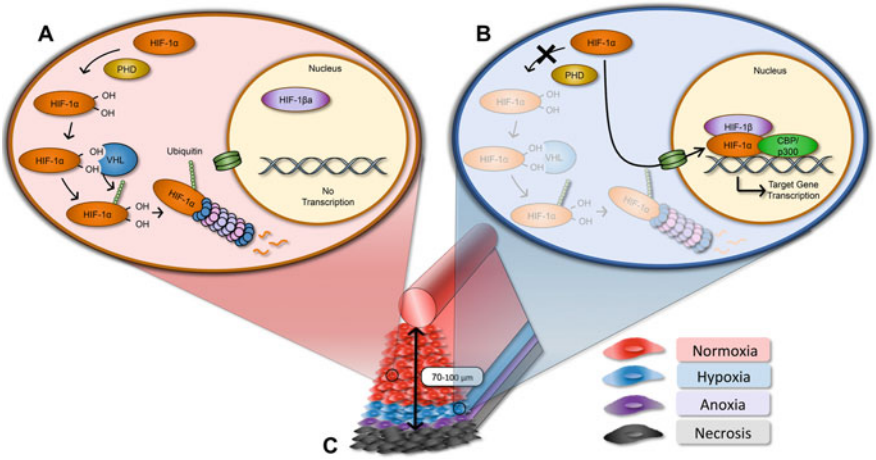

Fig. 2.2 Schematic representation of the canonical model of HIF-1α regulation under normoxia and hypoxia. (**a**) Under normal oxygen concentrations, HIF-1α is hydroxylated by PHDs, which allows VHL to recognize and ubiquitylate HIF-1α. The ubiquitin-tagged HIF-1α is then degraded via the 26S proteasome. HIF-1β is constitutively expressed and localizes to the nucleus. (**b**) Under low oxygen tensions HIF-1α is stabilized because PHDs are unable to hydroxylate the proline residues. HIF-1α translocates to the nucleus where it combines with HIF-1β to form the active transcription factor HIF-1. CBP/p300 is a coactivator that binds to HIF-1 at hypoxic response elements and aids in transcription of target genes. (**c**) Oxygen tension decreases as distance from the blood vessel increases. The hypoxic and anoxic regions lie in between the normoxic and necrotic zones. Cells can survive at a distance of 70–100 μm away from the supplying blood vessel. Adapted from [27, 91]

dependent on the oxygen and nutrient supply from the blood vessel. The zone in which cells are sufficiently supplied can be referred to as the normoxic region [8, 22, 26, 27]. Approximately 100 μm from tumor capillaries cells begin to die from lack of oxygen and nutrients; this is the necrotic region [8, 22, 26, 27]. Between the normoxic and necrotic regions cells are subject to chronic hypoxia. In this region, cells obtain an amount of oxygen adequate for survival but insufficient for proliferation [8, 22, 26, 27].

2.3 HIF-Signaling

The hypoxic environment results in multiple biological changes to adapt to the low oxygen conditions. Many of these changes are regulated by transcription factors such as the HIFs, nuclear factor kB (NF-kB) and AP-1. One of the most widely studied transcription factors in the field is the HIF family [25].

As mentioned above, HIF-1 is a heterodimeric helix-loop-helix transcription factor consisting of one alpha (HIF-α) and one beta subunit (HIF-1β) [28]. HIF-1β is constitutively expressed while HIF-1α is expressed under hypoxia and degraded under normoxia (Fig. 2.2). Canonically, in normoxia HIF-1α is hydroxylated by prolyl-hydroxylase domain containing enzymes (PHDs). This takes place on at least

one of two available conserved proline residues within the oxygen-dependent degradation domain (ODD) of the alpha subunit [29, 30]. The β-domain of the von Hippel-Lindau tumor suppressor protein (pVHL) ubiquitylates the alpha subunit upon recognition of the hydroxylated proline residues, thus marking it for degradation by the 26S proteasome [31]. PHDs require oxygen to carry out the hydroxylation of the alpha subunit and fail to catalyze the reaction under hypoxia. As a result, when oxygen tensions are low HIF-1α fails to be hydroxylated, is not recognized by pVHL and subsequently escapes degradation by the 26S proteasome. HIF-1α is able to then translocate to the nucleus where it heterodimerizes with HIF-1β. The newly formed active HIF-1 protein can then bind to hypoxia response elements (HREs) which consist of a core 5′-[A/G]CGTG-3′ consensus sequence and highly variable bordering sequences in the promoters of HIF-activated genes [21, 32]. Factor inhibiting HIF (FIH) can also hydroxylate HIF-1α under normoxia, at asparagine-803 (at the C-terminal transactivation domain) inhibiting the interaction between HIF-1α and its transcriptional coactivators [21].

Furthermore, regulation of PHDs can occur through a number of additional mechanisms besides oxygen deprivation: PHDs can be inhibited by nitric oxide, several intermediates of the Krebs (TCA) cycle such as succinate and fumarate, and reactive oxygen species (ROS) [21, 33]. Furthermore, the mitochondrial deacetylase sirtuin-3 (SIRT3) inhibits ROS production, thus destabilizing HIF-1α through indirect promotion of PHD activity [34].

Recent studies have further expanded the canonical understanding and elements that influence HIF's activity. For example, it has been shown that HIF-1α stability is dependent on a phosphatidylinositol 3-kinase, Akt, and the mammalian target of rapamycin (mTOR) signaling pathway [22, 35, 36]. Additional levels of regulation of HIF stability are shown schematically in Figs. 2.3 and 2.4.

2.4 Biological Consequences of Tumor Hypoxia and HIF Activation

In an attempt to develop a more effective oxygen delivery, genes such as those encoding erythropoietin, the angiogenic vascular endothelial growth factor (VEGF), and transferrin receptors are upregulated [25]. VEGF, one of the target genes of HIF-1, is involved in the formation of new blood vessels [25, 37, 38]. Additionally, activation of the VEGF receptor (Flt-1) has been shown to stimulate migration of macrophages, which produce several angiogenic factors including VEGF and tumor necrosis factor alpha (TNF-α) [39, 40]. Other factors relating to angiogenesis have also been shown to be regulated by HIF-1, including PDGF-B, VEGFR-1, endothelin-1, inducible nitric oxide synthase (iNOS), monocyte chemotactic protein, adrenomedullin, and EGF [25]. Some of these factors have been shown to modulate vascular tone, indicating that the mechanism by which HIF-1 induction controls blood flow is complex beyond simply angiogenesis. Because well-vascularized tumors have a higher chance of escaping the

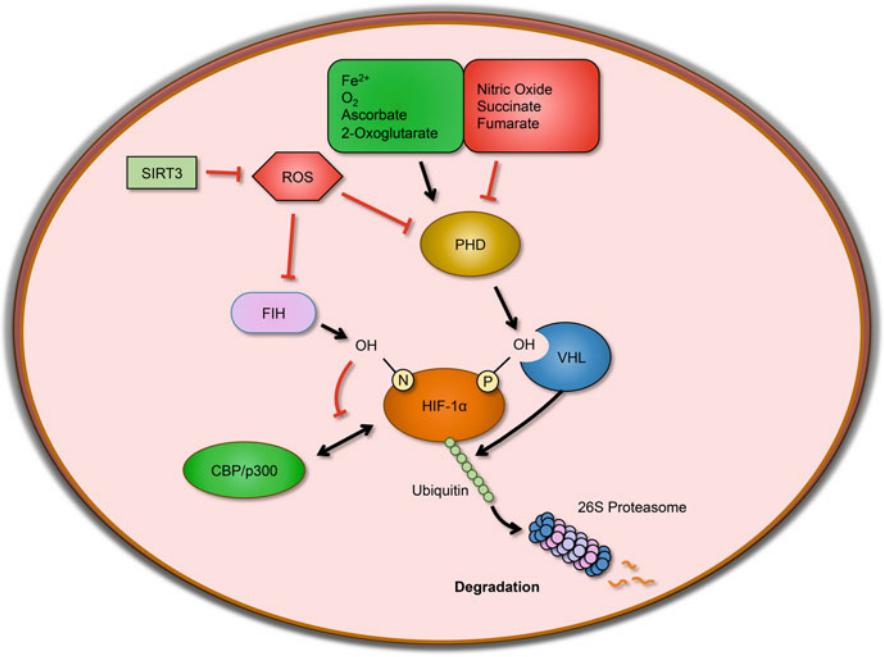

Fig. 2.3 Schematic representation of additional mechanisms of HIF-1α regulation in normoxia*:* In addition to the canonical degradation pathway via hydroxylation followed by 26S proteasome-mediated degradation, HIF-1α can be destabilized by factor inhibiting HIF (FIH) which inhibits interaction of HIF-1α and its coactivator CBP/p300. PHD activity is also regulated by intracellular components including ROS, which in turn is inhibited by SIRT3. Adapted from [21]

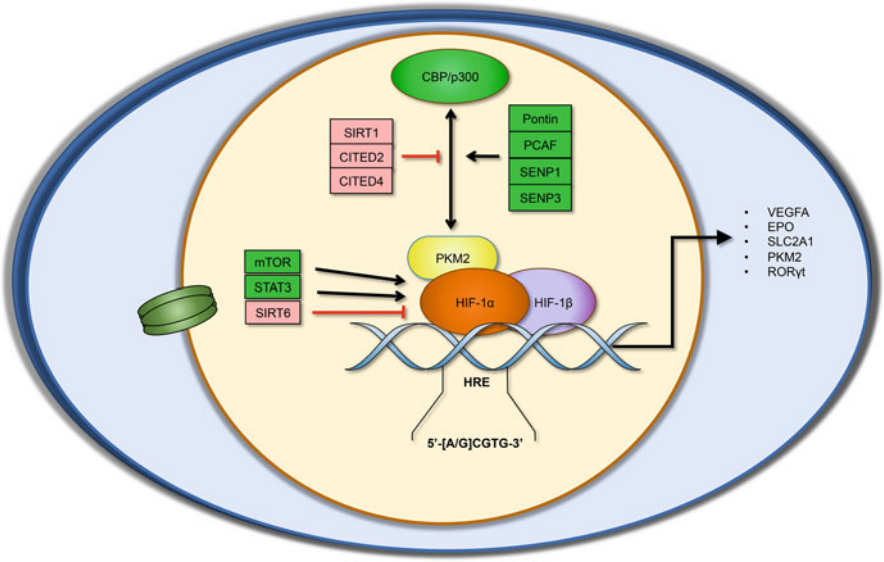

Fig. 2.4 Schematic representation of additional mechanisms of HIF-1α regulation in hypoxia: Once inside the nucleus, HIF-1α can be regulated via its interaction with its coactivator CBP/p300. SIRT6, STAT3, and mTOR modulate HIF-1α mRNA levels. Adapted from [21]

local environment and metastasizing, the ability to induce an angiogenic response has been associated with a more aggressive phenotype in tumors [25, 41].

In response to hypoxia, tumor cells also display an altered metabolic character. HIF-1 appears to drive changes that are conducive to a glycolytically favored metabolism. The upregulation of glucose transporter GLUT1 and hexokinase allows cells to intake and catabolize glucose at higher rates, thereby maintaining sufficient ATP for survival under oxygen deficient conditions [42]. Other enzymes upregulated by HIF-1 include aldolase A, phosphoglycerate kinase 1, and pyruvate kinase M [43]. Consequentially, the production of lactic acid is increased along with the glycolytic rate. This results in a decrease in extracellular pH as H+ ions are exported via a Na^+/H^+ symporter [44].

Studies have also shown the hostile nature of hypoxia on genomic stability through induction of point mutations, gene amplification, and chromosomal rearrangement [45]. This may occur through a combination of mechanisms such as insufficient DNA repair and/or errors in DNA replication. Furthermore, reoxygenation of cells following periods of hypoxia may cause oxidative damage [25, 46–48].

Hypoxia can also induce apoptosis through p53-dependent and independent mechanisms [49, 50]. Severe hypoxia results in the activation of a DNA damage response, including activation of ATM/ATR and phosphorylation of p53 [51–53]. Hypoxia-induced p53 may result in activation of downstream pro-apoptotic effectors such PHLDA3 [54, 55].

2.5 Effects of Hypoxia and HIF-Signaling on Tumor Radiation Response

Both HIF-1α expression and tumor hypoxic fraction have been correlated with poor prognosis following radiotherapy [18, 56, 57]. However, the effects of ionizing radiation on HIF stability as well as the effects that increased HIF-signaling can have on radiation sensitivity appear complex [58, 59]. A number of studies have reported increased HIF stability following irradiation. For example, HIF-1α stability may be increased by ionizing radiation through increased interaction between Hsp90 and HIF-1α. Proteins with a role in the DNA damage response that are induced in response to irradiation (such as ATR/ATM) have also been reported to phosphorylate HIF1α, facilitating its stability and resulting in increased HIF signaling [60–62]. Reoxygenation following irradiation has also been reported to increase nuclear HIF stabilization with subsequent induction of HIF-target genes. Reoxygenation-induced HIF signaling was proposed to result in protection of endothelial cells from radiation-induced apoptosis through increased cytokine production [63]. Indeed vascular damage was associated with HIF-1 inhibition, an effect that was thought to be associated with the increased radiosensitivity [63]. Increased radiation-induced HIF-1α stabilization in reoxygenated areas has also been proposed in a recent study, where the increased HIF-1α expression appeared to increase the movement of cells in these regions towards tumor

blood vessels [64]. Use of a HIF-1 inhibitor was reported to reduce tumor recurrence and the movement of cells towards blood vessels in this study [64].

Targeting the diverse mechanisms resulting in HIF stability could offer a means of improving radiation sensitivity. For instance, disruption of the Hsp90-HIF-1α interaction with the use of an Hsp90 inhibitor resulted in increased radiation sensitivity [65]. ATR inhibition also results in increased radiosensitivity, and reduced HIF signaling in hypoxic conditions. It is important to bear in mind that due to ATR's central role in the DNA damage response, the effects of ATR inhibition on radiosensitivity may primarily result from loss of ATR signaling rather than/in addition to HIF signaling [61].

Despite the numerous studies focusing of HIF-1 and radiation response, there is a much more limited literature describing the role of HIF-2α in this response. HIF-2α deficiency, however, has been proposed to result in increased p53 activity, cell death and radiation sensitivity at least in in vitro studies [66].

While numerous reports have proposed increased radiosensitivity in vitro and in vivo upon HIF-1α loss, others have reported that loss of HIF-1 could contribute to radioresistance [12, 59]. HIF-1-mediated regulation of metabolism, decreased proliferation and increased apoptosis has been reported following HIF-1 loss and irradiation, contributing to radioresistance in certain models. The authors of this study suggested that the tumor microenvironment of each specific tumor, as well as, the timing of radiation should be considered when targeting HIF-1 in the context of radiotherapy highlighting the complexity of this potential therapeutic strategy [58].

Interestingly, pharmacological HIF stabilization can be achieved by a number of compounds currently in clinical trials for anemia in patients with chronic kidney disease [67]. Since PHD inhibition has been proposed as a means of reducing radiation-induced toxicity it will be important to understand the short and long term effects of HIF-stabilization following PHD inhibition and radiotherapy. On a short term level this question has been addressed with the use of xenograft tumor models, where PHD inhibition with DMOG had no significant tumor radioprotective effects [68]. Given the previously established associations between HIF-1α and/or HIF-2α expression with increased vascularization and poor patient prognosis, the effects of HIF activation on future tumor development should be investigated [69, 70].

Besides targeting the HIF pathway several other means of 'targeting hypoxia' have been investigated with the aim of improving radiotherapy outcomes [3, 71]. Erythropoietin administration, red blood cell transfusions and hyperbaric oxygen treatments, for example, have all been proposed as means to improve tumor oxygenation before or during radiotherapy [72–74]. Unfortunately, the inconsistent results achieved in most clinical trials and problems with feasibility of administration of hyperbaric oxygen, have precluded the widespread use of some of these approaches to target tumor hypoxia [3, 75]. Similarly, clinical trials testing the ARCON approach (Accelerated Radiotherapy with Carbogen and Nicotinamide) have so far yielded variable results in local tumor control improvement [76–78].

The use of agents acting as 'oxygen mimetics', including nitroimidazole derivatives, has also been attempted [71]. While dose-limiting toxicities were reported in initial trials using earlier compounds, improved survival outcomes have been reported in several trials with other members of this class of compounds that have

improved toxicity profiles. One of these compounds is nimorazole which has been evaluated in a Phase I/II trial for Non-Small Cell Lung Cancer together with chemoradiotherapy and in Phase III trials with radiotherapy in supraglottic and pharyngeal cancer [79–82]. In fact, nimorazole is used as the standard-of-care in Denmark in combination with radiotherapy [3, 82]. The mechanism of action of nitroimidazoles is also attractive since these compounds undergo specific reduction under hypoxic conditions resulting in cytotoxicity selectively under hypoxic conditions (through enzymatic reduction under hypoxia) [71]. The concept of hypoxia-activated bioreductive prodrugs has further been exploited through other agents also evaluated in several clinical trials (such as tirapazamine and TH-302), allowing the release of cytotoxic compounds specifically under hypoxic conditions [83–85].

2.6 Targeting PHDs for Radioprotection

As mentioned above, an attractive approach to improve the effectiveness of radiation therapy is to improve the therapeutic window by reducing radiation-induced toxicity. Radiation-induced toxicity affects a significant number of cancer patients since it is often difficult to spare areas of healthy tissue in the gastrointestinal tract when several abdominal tumors are irradiated [14, 86].

Radiation protectors and mitigators, which are given either before or shortly after radiation can offer a means of reducing radiation-induced normal tissue toxicity. Radiorpotectors should have a number of favorable properties if they are to be used in the care of cancer patients. Firstly, they must only protect the normal tissue but not the tumor. Ideally they should not be toxic by themselves, and should have pharmacokinetic properties that allow feasible administration schedules [14, 87].

HIF signaling can result in widespread protective effects on cellular compartments affected by radiation-induced damage. For example HIF can regulate epithelial integrity, modulate angiogenesis and immune cell function [68, 88]. Increasing HIF stability pharmacologically by the use of PHD inhibitors has therefore been proposed as a means of reducing radiation-induced normal tissue toxicity [15]. Following irradiation, HIF-1α and 2α expression in normal tissues is detected in colon, liver and kidneys but not lung. Interestingly, genetic knockdown of all three PHDs specifically in the gastrointestinal tract results in abundant HIF expression. Moreover, both triple PHD knockout mice as well as mice treated with the small molecule PHD inhibitor DMOG can significantly improve survival after 18 Gy of total abdominal irradiation (TAI) [68].

Furthermore, at the molecular level PHD inhibition appears to increase crypt regeneration and reduce cell death, resulting in improved crypt survival after TAI. Measurement of apoptosis by transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining also demonstrates reduced staining in the colon and intestine in the DMOG treated group [68].

PHD inhibition is also able to maintain normal gut physiology as assessed by a number of assays, including counting stools and measuring electrolytes in the DMOG and saline control groups following irradiation. While saline control treated

mice had almost no stool formation 5 days post-irradiation and developed hypernatremia and hyperglycemia, DMOG treated mice maintained a significantly increased number of stools and did not develop these electrolyte disturbances. This was despite the fact that both groups maintained similar water intake. DMOG treated mice also maintained their body weight better than saline controls and these mice were also able to regain the weight lost after irradiation, having reached comparable weights to control mice 2 months after 20 Gy. Epithelial integrity was also investigated in this study by assessing FITC (fluorescein isothiocyanate)-dextran uptake into the bloodstream of irradiated treated mice (FITC-dextran will only enter the bloodstream when epithelial barrier is lost). Four times less FITC-dextran was detected in the bloodstream of DMOG treated mice than in saline controls [68].

While clonogenic stem cell death was previously thought to govern severity of radiation-induced damage, it has now been established that changes in the functions of endothelial cells [86, 89], the nervous and immune system also impact radiation-induced damage. Following radiation-induced damage, endothelial cells and intestinal stem cells (Lgr5+ and Bmi1+ cells) have reported roles in facilitating 'regeneration' after radiation-induced damage [89]. Interestingly, expression of HIF-2α specifically in epithelial cells (with the use of Villin-Cre) was sufficient to improve survival post 18 Gy TAI, while expression of HIF1α or HIF2α specifically in the endothelial cells (with the use of tissue specific Tie2-Cre) or in intestinal stem cells (Lgr5+ and Bmi1+ cells) did not improve survival after irradiation with respect to littermate controls [68]. Epithelial specific HIF-2α expression in these mice resulted in increased VEGF expression in the gastrointestinal epithelia and serum. Pharmacologic PHD inhibition also led to increased VEGF expression and correlated with increased microvessel density in jejunum crypts following irradiation [68]. Importantly, inhibition of VEGF function by the use of adenovirus encoding a soluble VEGF receptor (Ad-Flt1) that binds VEGF, abrogated the protective effect conferred by DMOG treatment. Furthermore, the survival advantage conferred by DMOG is lost upon HIF2α deletion supporting the role for HIF2α in radioprotection [68].

2.7 Conclusion

The negative effects of hypoxia on tumor radiation sensitivity have been studied for decades, however the relationship between HIF and tumor radiationsensitivity, still appears complex [5, 7, 59]. Developing pharmacological strategies aimed at improving the therapeutic window for radiotherapy, remains an area of clinical need. When developing these strategies, it will be important to think about the normal tissue toxicity. Targeting the PHD/HIF axis may be a potential future therapeutic strategy for normal tissue radioprotection, however caution must be exercised before this strategy is used clinically since the effects of acute HIF induction on tumor radiation sensitivity and future tumor progression must be first investigated in detail [15].

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Chapter 3
The Role of Cancer Stem Cells in Tumour Radioresponse

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Abstract In order to achieve complete tumour cure after radiotherapy, it is mandatory that all cancer stem cells (CSCs) are being killed. Therefore, new anti-cancer treatments should not only be directed against the bulk of the tumour but also have to target those tumour cells, which are expressing putative CSC markers since they highly determine tumour radioresistance as discussed in this chapter. This is further being influenced by other factors such as the tumour microenvironment, epithelial-mesenchymal transition as well as changes during the course of radio(chemo)therapy. Together with established parameters such as the tumour volume for primary radio(chemo)therapy or the human papilloma virus infection status for head and

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neck squamous cell carcinoma, the utilization of putative CSC markers may help to predict radiotherapy outcome and allow for patient stratification for individualized treatment strategies.

Keywords Biomarker • Cancer stem cells • Prognosis • Prediction • Radiotherapy • Resistance • Treatment individualisation

3.1 Introduction

Cancer stem cells (CSCs) are playing a key role in tumour development, tumour progression and recurrence after anti-cancer treatment. CSCs have been defined as those cells within the tumour, which possess the capacity to self-renew and to cause heterogeneous lineages of cancer cells that compromise the tumour [1]. Per definition, already a single remaining CSC after anti-cancer therapy can already cause a tumour recurrence, i.e. all CSCs have to be killed in order to achieve complete tumour cure. The gold standard to identify CSCs experimentally is the tumour cell transplantation assay, which measures the cell number necessary to be injected into experimental animals, which causes a tumour take in 50 % (TD_{50}) of the cases (1). Recent technological advances also allow the identification and sorting of tumour cells into CSC-high and CSC-low subpopulations, e.g. based on the expression of cell surface markers (19). However, these markers are not exclusive for CSCs and therefore their stemness still has to be validated by functional radiobiological assays as discussed in this chapter.

CSCs have a pivotal impact on permanent tumour cure. Therefore, new anti-cancer treatments should not only be directed against the tumour bulk but also have to target those tumour cells, which are expressing putative CSC markers since they highly determine tumour radioresistance as discussed below. In addition, putative CSC markers can be utilized for patient stratification for individualized treatment strategies.

3.2 Cancer Stem Cell Research in Radiation Oncology

The curative potential of radiation therapy is based on the induction of reproductive cell death as result of intolerable DNA damage, leading to cell death or senescence. A basic tool for the study of the radiation effect on the reproductive potential of single cells is the clonogenic cell survival assay. This method is based on the ability of a single cell (which is called clonogen) to form a colony of greater than 50 cells which is equivalent to 6 cell divisions (2^6) [2]. This assay has been developed by Puck and Marcus in 1956 by plotting the number of cell colonies formed by single HeLa cells (clonogenic survival) as logarithm of the surviving fraction (SF) versus the X-ray dose [3]. Since this seminal radiobiological study was published, this

assay has been extensively used in different studies to detect the cells retaining their clonogenic capacity after genotoxic treatments such as radiation and some chemotherapeutic drugs (e.g. busulfan or doxorubicin), where SF is calculated as follows: $SF = \frac{number\ of\ formed\ colonies}{number\ of\ seeded\ cells\ x\ PE}$. The plating efficacy (PE) is described as:

$$PE = \frac{number\ of\ formed\ colonies}{number\ of\ seeded\ cells} \times 100\%$$

To directly correlate cellular tumorigenicity and radioresistance, Hewitt and Wilson determined the first in vivo survival curve for leukaemia cells in 1959 using the serial dilution assay [4]. They applied whole-body irradiation to CBA mice, which have a low spontaneous incidence of leukaemia. After irradiation, single-cell suspensions of leukaemia cells from the liver of these leukemic mice were prepared and the number of viable leukaemia cells was counted using phase-contrast microscopy, presuming that the radiosensitivity of liver leukaemia cells is representative of the total leukaemia cell population. This study demonstrated an in vivo linear relationship between radiation dose and logarithm of survival rate. The survival rate was calculated as ratio between tumour transplantability defined as TD_{50} (transplantation dose 50 %, i.e. the cell dose necessary to obtain tumour takes in 50 % of the transplanted animals) for cells from untreated mice and TD_{50} for cells from irradiated mice. Further investigations demonstrated that CBA mice can develop leukaemia with a high frequency even after a single, short exposure to X-rays suggesting that this animal model might reflect a competition between inactivation of CSCs by irradiation and radiation-inducing malignant transformation [5].

Nevertheless, the dilution assay established by Hewitt and Wilson was also employed by other radiobiologists to correlate the TD_{50} and in vivo curability by radiation defined as tumour control dose 50 % (TCD_{50}). A study by Hill and Milas examined the proportion of CSCs in 12 different isogenic C_3H mouse models of varying histopathological type by measuring tumour curability by TCD_{50}, transplantability by TD_{50} and the in vitro plating efficacy [6]. They showed a significant, inverse correlation between the TCD_{50} and TD_{50} as well as between the TD_{50} and in vitro plating efficacy. This reflects not only a different CSC content between the experimental tumours but also shows that a higher radiation dose is required to control tumours with a high CSC density [6]. Baumann et al. examined the relationship of clonogenic cells, cellular radiation sensitivity at tumour control doses in vivo and tumour rescuing units at different sizes of the tumours in human squamous cell carcinoma FaDu growing in NMRI nude mice and irradiated under clamp hypoxia as a model system. This study demonstrated a significant correlation between TCD_{50} and tumour volume [7]. Taken together, there is experimental evidence that the CSC content may differ between tumours even of the same histopathological type and that a higher proportion of clonogenic cells is correlated with higher radioresistance [6–8].

In further experiments on human malignant glioma and squamous cell carcinoma xenografts, local tumour control after fractionated radiation did not correlate with the surviving fractions at 2 Gy (SF2) of the same cells in vitro [9]. The results of this study illustrated that the relevance of the in vitro clonogenic cell survival

assay to CSC inactivation remains questionable due to a lack of microenvironmental factors affecting CSC properties such as oxygen tension, nutrient supply, pH, extracellular components and interaction with stroma cells [9, 10]. This might also explain contradictory results of predictive assays for patients' radiation response, which are based on the SF2 values for tumour biopsies treated ex vivo [10–12].

A systematic search for the tumour model, where regeneration of a single CSC can be directly visualized led to the discovery and characterization of AT17 mouse mammary carcinoma. The unique feature of this tumour is that after high dose radiotherapy, only one or few stem cells survive and form in vivo colonies, which can be easily counted after histological staining [13]. The number of these colonies decreases with increasing irradiation dose and can be used to determine the number of surviving CSCs. Analysis of the correlation between local tumour control and number of surviving CSCs in irradiated AT17 tumours revealed that the local tumour control probability of 37 % (TCD_{37}) is correlated with the survival of only a single clonogenic cell per tumour [14, 15].

A first compelling evidence of CSCs was shown in 1997 when Bonnet and Dick demonstrated that they can be isolated by using specific surface proteins in acute myeloid leukaemia. These leukemic CSCs are defined by a CD34+/CD38− phenotype and initiate malignant growth after injection into mice [16]. While the CSC concept for solid tumours was existent already decades before for the field of radiation oncology, the first discovery of marker positive CSCs in solid tumours was made in 2003 by Al-Hajj et al. who characterized tumorigenic CD24−/CD44+ cell population in patient-derived breast tumours [16]. Since then, putative CSC markers have been defined in a broad spectrum of human and mouse solid tumours. These findings opened a new era for the experimental investigation of CSCs and development of CSC-based predictive tests where CSC specific proteins are used as surrogate markers for prospective identification of tumour-initiating cell populations [17, 18]. In retrospective clinical studies for different types of cancer, these predictive tests were applied for estimation of the CSC number in pre-treatment tumour biopsies for prediction of the patients' outcome after radiotherapy. The details for some of these studies are reviewed in other sections of this chapter (vide infra).

3.3 Cancer Stem Cells Determine Tumour Radioresistance

The overall aim of curatively intended radiotherapy is to inactivate all CSCs. The radiation dose to reach permanent local tumour control inversely correlates with the logarithm of the number of CSCs if all other factors are being kept constant. After application of the same radiation dose, tumours of the same size and histology but lower CSC content show a better local control rate compared to tumours with higher CSC content. The absolute number of CSCs has been shown to increase with tumour volume, which mainly accounts for the volume dependence of the tumours' cure [9, 19–21]. The CSC density can differ between tumours and shows an inverse impact on local tumour control after radiotherapy [6, 8, 20, 22]. Therefore, the TCD_{50} may

be used as a parameter to estimate the number of CSCs in the respective tumour in the experimental setting. However, the study by Hill and Milas also demonstrated, that some tumours show a significant difference in their TCD_{50} despite the same TD_{50}. This leads to the conclusion that, in addition to the sole number of CSCs, other radiobiological parameters such as extrinsic microenvironmental stimuli likely affect the radioresistence of CSCs and thereby impact local tumour control after radiotherapy [6, 19, 23].

In the 1970s, radiobiological mechanisms have been formulated as the "4 R's" which all have an impact on the success of radiotherapy [24, 25]. The "4 R's" include the repair of sublethal DNA damage, repopulation between radiotherapy treatment fractions, redistribution of cells in the cell cycle and reoxygenation of hypoxic cells. Importantly, the "4 R's" are referring to both tumour and normal stem cells. The radioresistance of these cells can be impacted by additional factors, therefore intrinsic radiosensitivity was postulated as 5th "R" [26].

Radiotherapy is being biologically effective through its ability to induce DNA damage, which is mediated by the production of water-derived reactive oxygen-species (ROS) or by a direct ionization of DNA molecules [27] and may lead to an impaired DNA metabolism, but can also implicate DNA replication and/or RNA transcription [28]. Dependent on the type and extent of DNA damage, the cells have different possibilities to respond. They may activate DNA repair pathways but can also undergo temporary or permanent cell cycle arrest or cell death. Furthermore, irradiation can also induce mutagenesis in surviving cells, which could lead to the formation of more aggressive cancer (stem) cells or may help CSCs to arise from normal stem cells [29, 30].

Ionizing irradiation induces a plethora of DNA damage including single-strand breaks (SSBs), double-strand breaks (DSBs), and oxidative base damages which all are affecting the integrity of the DNA or can alter its chemical structure (summarised in [31]). In most cases, the DNA damage is sublethal and can be repaired. Accumulating sublethal damages can finally leading to lethal damage and cell death [32]. However, CSCs have been described to dispose a significant enhancement of DNA damage response (DDR) mechanisms. Some of these mechanisms are described in the following (summarised in [33]). The most lethal form of DNA damage caused by irradiation are DSBs. They initiate the genomic DNA repair pathway via recruitment of ATM and ATR complexes, which regulate their downstream checkpoint proteins Chk2 and Chk1 [34–37] or induce irreversible cell cycle which leads to apoptosis, senescence or mitotic catastrophe [38]. ATM activates additional downstream substrates such as H2AX histones via phosphorylation. These histones are known to be an important regulator of the DDR mechanisms and, upon DNA damage, are being phosphorylated and recruited to the sites of the DSBs. Therefore, H2AX is not only a marker for DNA repair and for DNA repair efficiency following DNA damage but can also be used as a surrogate marker for the DNA repair efficiency of CSCs [39]. In addition, the DSB repair mechanism is also cell cycle dependent. Proliferating cells in the G1-phase are using the error-prone non-homologous end joining (NHEJ) repair mechanism whereas dividing cells of the late S-/G2-phase will utilize error-free homology-directed recombination (HR) [40, 41].

An increased DNA repair capability has been described for a number of tumour entities such as breast cancer, glioblastoma as well as lung and prostate cancer [42–45].

Photon-based irradiation mediates its DNA damage only to a small extent by direct DNA damage mechanisms. To a large extent, it is mediated by free radicals produced by toxic and highly reactive oxygen species, which are being generated by ionization of water molecules [46]. They are characterized by a short half-life and are directly interacting with biomolecules such as DNA in the cells. Physiologically, they are involved in many cellular processes such as in differentiation, proliferation, cell- cell adhesion, cell motility and autophagy. Physiological ROS levels are maintained by free radical scavengers, i.e. specific enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase, superoxide reductase, catalase and the DNA repair/redox protein Ape1/Ref1. The quantitative existence of ROS will determine the extent of DNA damage. At low oxygen tensions, glutathione and other thiols have been shown to provide substantial protection against radiation damage [47, 48] and are thereby playing a role in radiosensitivity. An increased ROS production will lead to oxidative stress and finally to cell death. As a defense mechanism, free radical scavengers were found to be up-regulated in CSCs, thereby protecting cells against DNA damage [49]. Down-regulation of ROS has been shown to lead to enhanced CSC self-renewal and to promote CSC aggressiveness [50]. Down-regulation of ROS scavengers in CSCs may be a promising treatment approach, which consequently leads to an up-regulation of their ROS levels and is thereby increasing the radiosensitivity of CSCs [51].

3.4 The Tumour Microenvironment Promotes CSC-Mediated Radioresistance

In addition to the intrinsic mechanisms (vide supra), a plethora of microenvironmental factors are also promoting radioresistance of cancer cells and specifically of CSCs and thereby protecting CSCs from (chemo)radiation-induced cell death. CSCs can reside in anatomically distinct niches within the tumour microenvironment such as hypoxic, invasive and perivascular niches and are thereby protected from the immune system. CSC niches are also maintained by cells of the tumour microenvironment, such as by macrophages, monocytes and cancer-associated fibroblasts [52, 53]. All of these niches are underlying dynamic changes due to fractionated radiotherapy and/or chemotherapy, with interactions between CSCs and the tumour microenvironment and thus, are preserving the phenotypic plasticity of the CSCs [17, 54].

It is well known that hypoxic cells are usually more radioresistant than well-oxygenated cells [55, 56]. Hypoxia is caused by abnormal vasculature, anaemia and increased oxygen consumption by highly proliferating tumour cells [57, 58]. Tumour hypoxia is expected to be present in the vast majority of the tumours, with an inter- and intratumoral heterogeneity. From experimental preclinical as well as clinical data there is ample evidence that local tumour control after radiotherapy inversely correlates with

tumour hypoxia [59–61]. However, anti-hypoxia-treatment, e.g. with hypoxic modifiers, have shown that tumour control is being improved, suggesting a decrease in previously hypoxic-niche protected CSCs [62]. In addition to the direct effect of low oxygen, CSCs may be protected by activation of the hypoxia-inducible factor (HIF) pathway [49, 63, 64]. HIF2-alpha has been shown to regulate CSC function and/or differentiation through activation of the transcription factor Oct-4 [65]. HIF1-alpha, activated by ROS or directly by hypoxia, can directly activate pro-survival pathways such as Notch/WNT and Hedgehog signalling, which are important for CSC maintenance and cell-fate decisions [66–68]. Notch is also required to maintain tumour cells in an undifferentiated state [69] and Notch overexpression has been shown to correlate with aggressive phenotypes [70–72]. Activation of WNT signalling is further promoting an undifferentiated tumour cell state [73]. In addition, HIF1-alpha can also antagonise activation of c-Myc, which decelerates cell-cycle progression and thereby protects CSCs from DNA damage and enhances stemness [74]. Both HIF genes are also inducing angiogenesis via activation of VEGF [75]. Under hypoxic conditions, CSCs may secrete VEGF, which further stimulates tumour angiogenesis [76].

Tumour hypoxia is one of the factors affecting radiation treatment outcome after fractionated therapy [77]. Fractionation of the radiotherapy dose allows reoxygenation of hypoxic niches and thereby may increase the radiosensitivity of residing CSCs [78].

The perivascular niche is another important factor for radiation resistance of CSCs. In primary head and neck squamous cell carcinoma, it has been shown that the majority of CSCs reside within a radius of 100 nm to blood vessels, whereas the selective ablation of tumour-associated endothelial cells results in a decrease of CSCs [79]. Furthermore, secreted cytokines of endothelial cells such as IL-6, CXCL8 and EGF enhance cancer cell survival, migration and protection against programmed cell death induced upon cell detachment from the extracellular matrix (anoikis), through activation of signalling pathways such as STAT3, ERK and AKT pathways [80].

Another important factor of the tumour microenvironment is epithelial-mesenchymal transition (EMT). EMT is a physiological process during embryonic development and wound healing but is also playing an important role in tumour cell invasion and metastasis (summarised in [81]). The mesenchymal-like transformation was first described by Greenburg and colleagues [82]. They demonstrated that well-differentiated epithelial cells from the apical surface are elongating, developing pseudopodia and filopodia characteristics of migratory cells and thus acquire the mesenchymal phenotype after suspension in collagen gels. During EMT, epithelial cellular characteristics are being lost and cells gain mesenchymal characteristics, which enhance migration and invasion of these cells. It has been demonstrated that radioresistant prostate cancer cells show increased colony formation, invasion ability and spheroid formation capability in comparison with their parental cell lines [83]. They further showed an enhanced expression of EMT/CSC-associated markers, activation of the DNA repair checkpoint proteins Chk1 and Chk2 and induction of the PI3K/AKT/mTOR signaling pathway. The application of BEZ235, a dual PI3K/mTOR inhibitor, together with radiotherapy significantly increased radiosensitivity and induced apoptosis of these formerly radioresistant prostate cancer cells

compared to radiotherapy alone. Another in vitro study using non-small cell lung cancer (NSCLC) cells showed that irradiation-surviving cells gain EMT characteristics [84]. Post-irradiation, sphere cells show an increased expression of putative CSC markers such as CD24 and CD44, they express nuclear b-catenin and the EMT markers Snail 1, vimentin and N-cadherin and show an overexpression of PDGFR-beta compared to non-irradiated lung cancer cells. When radiotherapy was combined with the PDFGR inhibitor axitinib or dasatinib, radiation efficacy has been significantly improved [84].

When CSCs of the invasive tumour front are gaining EMT characteristics, tumour cell spread via blood or lymphatic vessels may be supported. There is increasing evidence, that circulating tumour cells (CTCs) are showing EMT as well as CSC-like features [85–87]. Furthermore, the number of CTCs may be indicative for tumour response to therapy and may allow monitoring disease progression [88], and they also have been shown to correlate with lymph node metastases [89]. Recent research by Tinhofer and colleagues [90] demonstrated that CTC detection represents an independent risk factor for tumour progression in patients with locally advanced non-oropharyngeal HNSCC, who received curatively-intended postoperative radio(chemo)therapy.

3.5 Tumour Heterogeneity, Diversity of the CSC State and Changes During Anti-cancer Treatment

Tumour cell heterogeneity arises from continued genetic and epigenetic changes of individual clonogenic cells, when they are exposed to different oxygen tension or tissue pH, nutrient supply and growth factor availability as well as to different cell types of the tumour microenvironment within the CSC niches [91, 92]. This intratumoural heterogeneity has been demonstrated by molecular profiling such as exome sequencing, chromosome aberration analysis or ploidy profiling on spatially separated samples in different tumour entities such as renal cell carcinoma and human ovarian cancer and is very likely to lead to different response to anti-cancer treatment [93, 94]. Tumours of the same entity are being defined by different molecular subtypes, which are showing distinct mutations and thus leading to distinct CSC phenotypes [95, 96].

The intratumoral genetic heterogeneity is likely caused by different CSC subpopulations, which can co-reside within the same malignant tumour [97]. More importantly, these subclones may also change during the course of fractionated radiotherapy and chemotherapy, which accounts for the plasticity of CSCs (vide infra). In terms of estimating the CSC density, the application of biomarker panels may therefore be more suitable than analysing single CSC-associated biomarkers [98].

CSCs within single genetic clones have distinct functions, and the anti-cancer therapy tolerance of these cells of the same clone can be different. Using DNA copy number alteration analysis, deep sequencing and in vivo tracking of 150 single lineages, which were derived from 10 human colorectal cancers, Kreso and colleagues [99] demonstrated that not only genetic variety but also epigenetic changes are con-

tributing to tumour growth and resistance to therapy. Serial transplantation experiments revealed a heterogeneous dynamic repopulation of CSC clones with some CSC clones being stably expressed whereas other clones were only transiently detectable. They further showed that chemotherapy can promote the dominance of previously minor or dormant lineages, whereas other clones are disappearing or even reappearing depicting the plasticity of CSCs [99].

Genetic and epigenetic changes are also caused by the tumour microenvironment. There is increasing evidence that tumour hypoxia can be associated with chromosomal instability and, together with inflammation, might lead to the induction of EMT and associated epigenetic changes [100–102]. Radiotherapy itself can also trigger EMT through mutations or through co-stimulatory signals from tumour-infiltrating, cytokine-producing inflammatory cells such as granulocytes or macrophages and their secreted factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) and TGF-beta [103, 104] leading to treatment-related changes, and may also cause treatment resistance. In a preclinical study, Peitzsch et al. demonstrated that radiotherapy induces CSC marker expression (aldehyde dehydrogenase activity, BMI1, NANOG, Oct-4, ATP-binding cassette sub-family G member 2), and differential regulation of specific pathways such as the phosphatidylinositide 3-kinase PI3K/AKT pathway as well as increased b-catenin and vimentin expression indicating activation of EMT in prostate cancer cell lines [104]. During the course of radiotherapy, an additional phenotypic switch was observed, which was associated with stable genetic and epigenetic changes [104]. Irradiation has also been shown to increase ALDH1A3 expression in HNSCC, negatively impacting tumour radiocurability in vivo [105]. Increased CSC marker expression after radiotherapy may be the result of accumulation of radioresistant CSCs but can also be due to the generation of new CSCs out of non-tumorgenic stem cells [29, 30], e.g. caused by pro-inflammatory signaling [106]. Changes of the tumour microenvironment under radiotherapy are also frequent and have especially been shown for tumour hypoxia [107]. This may also impact the behaviour of CSCs as interactions may appear especially in hypoxic niches (vide supra). Preclinical experiments and clinical data showed that the hypoxic volume is increased after 2 weeks of a 6-weeks course of radiotherapy in comparison with its evaluation prior to radiotherapy [108, 109]. This indicates that the variability of biomarkers such as CSCs has to be considered and evaluated carefully for treatment-related changes since their predictive power may be determined by its dynamics in the early treatment phase [23].

Tumour progression under or after treatment is also due to the fact, that CSCs are not only non-migrating (stationary) cells, but have also the potential to migrate. Stationary CSCs are still embedded within the epithelium, e.g. in the primary tumour, metastases or in benign precursor lesions where they cannot yet disseminate [107]. In contrast, invasive (or also termed “migrating”) CSCs can be found on the invasive tumour front at the tumour-host interface. Derived from stationary CSCs, they can acquire transient EMT in addition to their stemness. These invasive CSCs can now disseminate and cause metastasis, provided that the cells retain their stemness characteristics. Therefore the invasive tumour front is also being called “germ-cell layer” of migrating CSCs, which asymmetrically divide into proliferating and

differentiating malignant tumour cells (summarised in [110]). In order to achieve complete response to radiotherapy, it is important to fully include the invasive tumour front in the clinical target volume (CTV), especially when applying high precision therapy [111] but also to image and target migrating and quiescent CSCs as well as to consider the non-homogeneous distribution of CSCs, e.g. also with the help of CSC imaging [112, 113]. Gaedicke and co-workers demonstrated the non-invasive detection of the AC133 epitope of the putative stem cell marker CD133 by positron emission tomography and near-infrared fluorescence molecular tomography in subcutaneous and orthotopic glioma xenografts using antibody-based tracers [114]. Such imaging approaches provide a promising basis for concepts on optimisation of clinical radiotherapy treatment planning.

3.6 Modern Tools and Models for Cancer Stem Cell Research

Since the first human cell line was established from the cervical carcinoma of Henrietta Lacks in 1951 and the first radiobiological cell survival assay was developed by Marcus and Puck in 1956 till present days, two-dimensional (2D) colony formation assay remains the standard analysis of clonogene survival after therapy [2, 3, 115]. Nevertheless, the 2D cell culture model with its spatial limitation and lack of microenvironmental stimuli, which tumour cells experience in tissues, only roughly resembles clonogenic survival in vivo. Additional problems are arising from the use of the established cell lines. Many common cell lines are derived from metastases and fast growing tumours and often possess multiple genomic alterations arising from their long time adaptation to in vitro growth. As a result, they very roughly resemble the growth kinetics and tumour heterogeneity of the primary tumours [116]. A growing body of evidence demonstrated that cells cultured in three-dimensional (3D) conditions such as spheres or 3D Matrigel colonies are more radioresistant compared to 2D cultures [17, 117]. Growing of the established cells lines or patient-derived biopsies under sphere forming and serum-free conditions was one of the first methods that laid the foundation for the enrichment of CSC populations in vitro. Most of the cells cultured under these conditions undergo anoikis. The surviving cells are enriched for CSCs and undifferentiated progenitor cells compared to the conventional culture of attached cells grown in serum-containing medium. This assay allows investigations of the self-renewal capacity of CSCs by assessing their ability to form spheres in multiple passages. When spheres are dissociated into single cell suspension and passaged few times under sphere forming medium, only self-renewing cells are capable to regrow the spheres in a few generations [118]. When combined with irradiation, this assay allows to measure the clonogenic survival of spherogenic cells and can be employed to analyse the effect of potential radiosensitizers on tumour cell populations enriched for CSCs [119, 120]. Although the sphere formation assay is the most prominent CSC in vitro assay, it is not always suitable for CSC analysis. Culturing cells under these conditions does not always lead to CSC

enrichment, since this is strongly dependent of the cell type and does not recapitulate microenvironmental conditions such as hypoxia. The organoid model for in vitro CSC cultures allows to grow long-term 3D cultures derived from adult stem cells, which can be isolated from different types of normal and cancerous tissues from the gastrointestinal tract as well as from pancreas, prostate and glioblastoma [121–124]. To grow organoid cultures, single adult stem cells are embedded in a 3D matrix and allowed to differentiate and self-organize into epithelial cells of the corresponding organ of origin [125]. This cell culture system can be established from tumour biopsies and surgically resected specimen and is currently used by many laboratories for investigation of an impact of genetic mutations on tumour-drug sensitivity. Importantly, this method allows recapitulating a hypoxic gradient and tumour heterogeneity that is not possible for conventional 2D cultivation of cell lines. Interestingly, a study of Jeremy Rich and co-workers demonstrated that in glioblastoma-derived organoids, CSC populations defined by the Sox^+ phenotype, are more radioresistant compared with adjacent Sox^- non-CSCs [124]. This study demonstrated that tumours formed by orthotopically transplanted patient-derived organoids are more representative of the tumours of origin than those formed by the corresponding patient-derived sphere culture [124]. The important next step is now to investigate whether the closer genetic relation of organoid models to their origin in the patient indeed translate into a closer relation of functional endpoints (i.e. spheroid response parameters including complete inactivation) with the response of patient tumours. Nevertheless, despite its attractiveness as a relatively reasonable and easy to maintain cancer model, which represents the genetic cancer spectrum and possess a high success rate of establishing and biological stability, organoids lack tumour stroma as well as the immune and vascular system [125].

In that respect, xenograft tumour models of human cancer might be more physiologically relevant [126]. The serial transplantation assay is a standard method for CSC analysis, which is used to distinguish tumour cell population with long-term multipotency and self-renewing potential, and to identify the tumour cell-of-origin [91, 127]. For the transplantation assay, tumour cells are fractionated by fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS) based on different CSC-associated features such as cell surface marker expression, enzymatic activity of aldehyde dehydrogenase (ALDH), low 26S proteasome activity, slow proliferation (e.g. by retaining PKH26 fluorescent dye), drug efflux capacity defined by side population or by cell size [17, 128] (Fig. 3.1). Isolated tumour cells are then xenografted into mice at limiting dilutions and with serial transplantation to determine frequency of the marker positive cells in a given tumour, their long-term tumorigenic potential and ability to recapitulate the cellular heterogeneity of the parental tumours [91, 120, 127] (Fig. 3.1).

PDX can be obtained by implanting small pieces of freshly resected tumour tissues or tumour single cell suspensions (alone or with tumour stroma cells) subcutaneously or orthotopically into immunocompromised mice [129]. Interestingly, some cancer cells do not form tumours without co-injection of tumour stroma owing to their dependence on the tumour microenvironment [91]. Patient-derived tumour cells which are injected or transplanted in mice, are exposed to more physiological

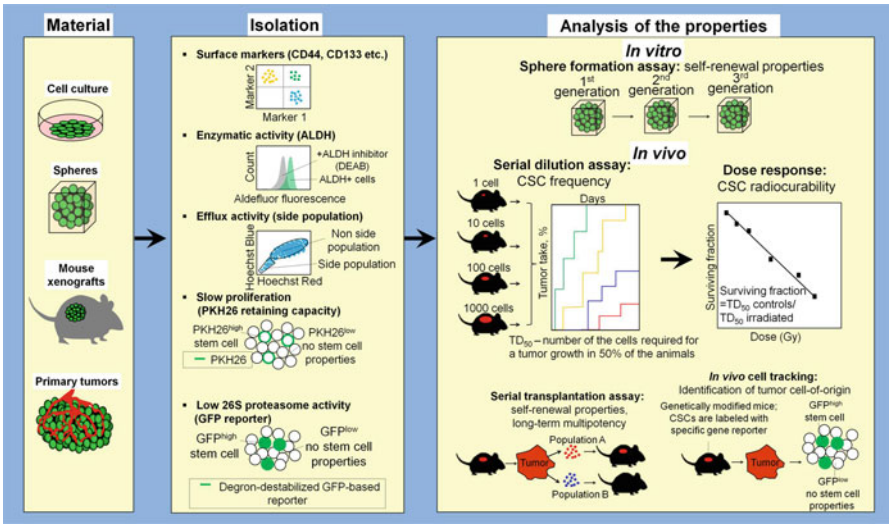

Fig. 3.1 Methods of cancer stem cell isolation and analysis

conditions than in a plastic dish and therefore can better mimic the feature of the original tumour including histological architecture, malignant properties, heterogeneity, gene expression, and treatment response [126, 130]. Importantly, such tumours maintain the morphological characteristics, intra-tumoural heterogeneity and clonal stability through serial tumour transplantations [99, 131]. Taking into account that human tumour cells can be targeted by immune response when transplanted into immunocompetent mice, many spontaneous and transgenic immunodeficient murine strains have been developed for human tumour transplantation. Nude (nu/nu) mice were the first described immunodeficient murine strain with mutation in *Foxn1* gene which is associated with nude phenotype, lack of functional thymus and, consequently, a greatly reduced number of T-cells [132], allowing transplantation of e.g. murine tissue. Although these mice are a commonly accepted model for tumour engraftment, they are characterized by accumulating T-lymphocytes during aging. It has been demonstrated that some of the tested human tumours were immunogenic in nude mice, a factor that can be counteracted by whole body irradiation with 4–6 Gy before tumour transplantation [133–135]. The mice of Scid strains have more severe immunodeficiency than nude mice due to the impaired production of functional NK-, T- and B-cells. These mice carry the mutated *Prkdc* gene encoding for DNA protein kinase (DNA-PK), which is an important regulator of DNA repair. Therefore Scid mice and other derivative strains such as NOD-Scid and NOD-Scid-IL2rγnull are more sensitive to irradiation than nude mice and thus might not serve as suitable model for the radiobiological studies [132, 136].

Different endpoints can be used for the assessment of in vivo tumour response to irradiation, whereas only permanent local tumour control, which is evaluated by e.g. $TCD_{50,}$ reflects CSC inactivation by radiotherapy. This was first illustrated by a linear correlation between TCD_{50} and TD_{50} in the experiments of Hill and Milas [6]. Correlation

between CSC density and tumour control was also supported by recent clinical data [18, 137]. As CSCs constitute a minor population of all tumour cells and because of potential differences in their radiosensitivity, tumour volume-related endpoints are not reflective of CSC survival after treatment. This might explain the discordance between TCD_{50} and tumour growth delay in many experiments [138]. Nevertheless, tumour volume-based assays have their role as screening experiments for new treatments or for evaluation of signalling pathways which can be associated with treatment of the tumours with irradiation or combination modalities [104, 139, 140].

The quantitative transplantation assay remains the "gold standard" for analysis of the radiosensitivity of CSCs since it was established by Hewitt and Wilson in 1959 [141]. Although it was extensively optimised in isogenic tumour mouse models, the data for human cell line xenografts is limited [4, 6, 142] and no data for correlation of TCD_{50} and TD_{50} were reported so far for CSCs in primary patient derived xenografts (PDX). The major obstacles for the performance of this assay are the high labour and animal resources required for these experiments, but also some technical challenges. Engraftment of primary tumour cells requires severe immunosuppression of the recipient mice, which could be difficult to achieve in nude mice taking into account that in vivo growth of some tumour transplants takes a few months, and nude mice might develop anti-tumour immune response within a few weeks even after whole body irradiation [129].

Although PDX are an important preclinical model to test the efficacy of cancer therapy, a profound difference between the murine and human immune system, stromal cells, extracellular matrix, growth factors and tissue architecture even for orthotopically engrafted tumours could be an obstacle for tumour growth and for analysis of tumour response to treatments that are largely dependent on the mentioned factors [118, 143]. For questions directly addressing the CSC- or non-CSC origin of tumour cells in vivo and about the fate of CSCs after cancer treatment, a genetically modified mouse model was established where distinct cell populations e.g. CSCs can be labelled by using different cell-specific gene reporters [91, 127]. Chen and co-workers used a genetically engineered murine model of glioma where quiescent CSCs were positive for expression of GFP under promoter of *Nes* gene encoding for nestin, which is a putative glioma CSC marker. This study demonstrated that CSCs are resistant to the drug temozolomide (TMZ) and lead to tumour growth also after TMZ therapy [144]. Although this model has fundamental differences with human tissues and cannot be used as a tool to predict individual human tumour response to radiotherapy, it might pave the road for unravelling the role of distinct genes for tumour radioresponse and for comparative analysis of radioresistance in the CSC marker-positive cell populations.

The rise of molecular tools which facilitate genome-editing such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and the recently developed clustered regularly interspaced palindromic repeats/CRISPR-associated (CRISPR/Cas9) technologies opened a new era in genetic investigations and might help researchers to reveal the functions of single genes at its endogenous locus both in vitro cellular models and in genetically modified in vivo model organisms to establish a direct correlation between the cellular genotype and functional properties in future [145].

3.7 Treatment Strategies to Overcome Radio(chemo) resistance of CSCs

For effective anti-cancer treatment, it is of utmost importance to target CSCs e.g. by increasing their radiosensitivity and to improve local tumour control. Targeting can be aimed selectively on molecules that are overexpressed in CSCs compared to non-CSCs or non-selectively on signalling pathways that are activated and of higher importance to CSCs than to non-CSCs. To date, most of the pharmacologic developments are performed using the end point tumour growth delay. This endpoint does not necessarily translate into an effect on local tumour control and does not reflect the response of CSCs [15, 19]. To date, a number of clinical trials on targeting CSCs in acute myeloid leukaemia as well as in solid tumours are ongoing.

In a preclinical study, bivatuzumab mersantine, an immunoconjugate of the humanized anti-CD44v6 monoclonal antibody BIWA 4 and the maytansinoid DM1 significantly improved local tumour control by a dose modifying factor of 1.9 after fractionated irradiation with five fractions in a human HNSCC tumour model [146]. This is a good model compared to the majority of other combined treatment approaches [23]. Although this compound is no longer available from the manufacturer for combined use with radiotherapy, it represents a promising approach to selectively target tumour cells [23]. A similar approach has been undertaken by Li and colleagues in a combined clinical and preclinical trial on pancreatic tumours [147]. Patients with high CD44s expression of their tumours showed a significantly reduced overall survival compared to patients with CD44s low expressing tumours. In their translational study, the anti-CD44s antibody did not show a radiosensitising effect in in vitro colony formation assays but the antibody alone had a significant effect on clonogenic tumour cells and reduced the in vitro cell viability and invasion. In a volume-based assay, in vivo tumour growth, metastasis and tumour recurrence of xenograft tumours in mice were inhibited after radiotherapy. Analyses of pancreatic cell lines showed down-regulation of the stem cell self-renewal genes NANOG, Sox-2 and Rex-1 as well as inhibition of STAT3-mediated pro-survival signalling and cell proliferation [147].

A radiosensitising effect of the 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase (HMG CoA reductase) inhibitor simvastatin was demonstrated in mammosphere-initiating cells of a number of inflammatory and triple negative breast cancer cell lines. In two non-inflammatory breast cell lines, a radioprotective effect of simvastatin was observed. Interestingly, the use of statins showed an independent association with local recurrence-free survival in a retrospective study of 519 patients with inflammatory breast cancer [148]. Simvastatin also showed a synergistic effect with irradiation on gastric cancer and colorectal cancer via the inhibition of basal clonogenic survival and proliferation in vitro. In vivo experiments showed the reduction of xenograft tumour growth after combined irradiation combined with simvastatin compared to irradiation alone [149]. These promising data warrant the conduction of an early prospective clinical trial for the subgroup of breast cancer patients who are on a very high risk of recurrence as well as in patients with gastric or colorectal carcinoma [23].

Another promising approach is the application of radiolabelled antibodies, which are being used as theranostics. For example, they can be applied in diagnostics such as in positron emission tomography and in combination with another radionuclide as therapeutics. Radiolabelled anti-EGFR-antibody cetuximab showed statistically significant tumour growth delay and a reduction of the TCD_{50} after combination with external beam irradiation [150]. This approach could potentially also be applied for other targets including CSC-associated proteins which are over-expressed in malignant tumours compared to their surrounding normal tissues [23].

In terms of drug treatment, one has to consider that the tumour vascularisation is playing an important role. Blood perfusion is required to reach the target cells. This is further being complicated by the localisation of CSCs in hypoxic niches with no or very little blood supply. The drug has to possess a high CSC-specificity and a long plasma half-life to reach their target in order to show a similar effect on CSC inactivation as in previous in vitro experiments [23]. From preclinical and clinical studies, there is also evidence that vascularisation is inhibited and endothelial cells are being destroyed by radiotherapy but can be restored by bone-marrow derived macrophages and monocytes [151, 152]. The inhibition of the influx of these cells via blockage of cytokine pathways may be a promising therapeutic strategy.

Targeting tumour hypoxia is a non-specific but very relevant approach to also target CSCs. The application of the hypoxic radiosensitizer nimorazole simultaneously with radiotherapy has been shown to significantly improve loco-regional tumour control of hypoxic HNSCC, whereas patients with "less-hypoxic" tumours did not benefit from nimorazole [153]. Increased tumour oxygenation leads to improved radiosensitivity of cancer cells and CSCs and improves the efficiency of systemic treatment in reaching more cancer cells. Another way to overcome hypoxia-mediated radioresistance is to apply higher irradiation doses specifically to more hypoxic areas, which could be imaged by ^{18}F-misonidazole positron emission tomography. Ongoing developments in imaging and tracking of CSCs may help to identify CTC niches and to further improve the therapeutic potential of radiotherapy in the near future.

Promising developments are also ongoing in stimulating slow growing or quiescent (R0-phase) CSCs into self-renewing cells [154, 155], which then are targetable more effectively by chemotherapy, and in the field of CSC targeted nanomedicine. The latter might provide a tool to deliver small molecule agents, nucleic acids and antibodies to specifically target CSCs, and thereby may lead to an increase in their radiosensitivity and to an improved efficacy of specific anti-CSC therapies by decreasing drug-resistance. Nanocarrier-based therapeutic agents offer new possibilities to penetrate CSC niches, leading to efficient drug accumulation within CSCs [156, 157].

3.8 CSCs in Radiation Treatment Planning

Radio(chemo)resistant CSCs may respond better to charged particles with high linear energy transfer, such as carbon or protons. Proton irradiation results in a more significant increase in the intracellular ROS levels in CSCs compared to photon

Table 3.1 Examples of specific and unspecific targets to overcome CSC-resistance

Examples of specific CSC-targets		
Target/Signaling pathway	Entity	References
ALDH1A1	HNSCC	[191, 192]
AKT/PI3K/mTOR	GBM, medulloblastoma	[193, 194]
ATM/Chk2	GBM, prostate cancer	[43, 195]
ATR/Chk1	HNSCC	[196]
CD44v6	HNSCC	[146]
CD44/ALDH1	HNSCC	[197]
EGFR	HNSCC, NSCLC, Prostate cancer	[198–200]
Glutathione biosynthesis	Breast cancer	[201]
HER2	Breast cancer	[202]
mTOR	Breast cancer	[203, 204]
PDGFR	NSCLC	[84]
Reactive oxygene species	Breast cancer	[120]
VEGFR	HNSCC	[205, 206]
WNT	GBM	[207, 208]
Examples of unspecific CSC-targets		
CXCR4/CXCL12	NSCLC	[209]
Hypoxic cytotoxins	HNSCC	[210]
Hypoxic modifiers	HNSCC	[211]
IL-6	HNSCC	[212]
PD-1/PD-L1 checkpoint	Breast cancer	[213]
VEGF/VEGFR	HNSCC, GBM	[205, 206, 214]

GBM glioblastoma multifome, *HNSCC* head and neck squamous cell carcinoma, *NSCLC* non-small cell lung cancer

treatment, alters cellular structures such as cell membrane, causes DNA double-strand breaks and leads to an increase in apoptosis [158]. In addition, the induction of apoptosis may vary between photons and particle irradiation in a dose-dependent manner [159]. Zhang and co-workers showed that proton irradiation of paclitaxel-resistant NSCLC cell lines kills more CSC-like cells than photons at the same radiation dose [160], whereas conventional photon therapy leads to the induction of migration and invasion of CSCs and eventually metastatic disease [161].

Modern radiotherapy has also to respect changes in the distribution of CSCs and may have to adapt radiotherapy based on their localization. Radioresistant cells such as CSCs often reside close to necrotic areas [162–164], which has to be considered in radiation therapy planning, e.g. CSC niches may be treated more extensively by higher irradiation doses. Furthermore, radiotherapy combined with specific (direct) or unspecific (indirect) targeting of CSCs (Table 3.1) would lead to a more homogeneous tumour with equal radiosensitivity and might improve radioresponse. Due to the CSC dynamics, clonal selection and treatment-related changes, it may be essential to target CSCs via different routes in order to achieve permanent tumour control and to prevent tumour recurrences and metastatic disease (see Fig. 3.2 for CSC-targeting strategies). Pre-clinical studies are needed to gain a deeper understanding of the biological CTV and imaging of CSC dynamics [111].

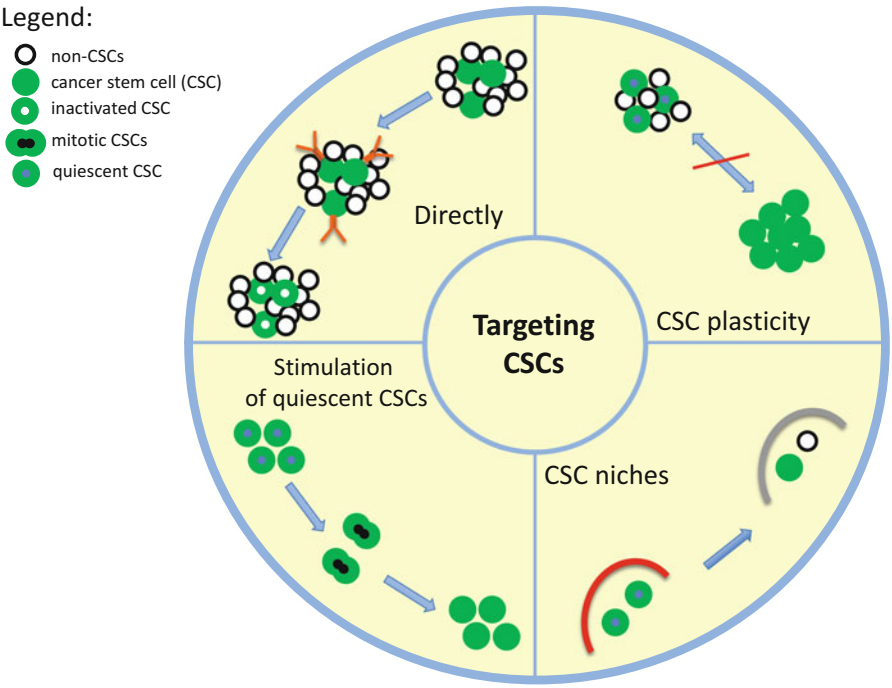

Fig. 3.2 Strategies for targeting CSCs

3.9 CSCs as Prognostic and Predictive Biomarkers in Radiation Oncology

In clinical routine, radiotherapy treatment planning is based on the anatomy of the patient, the individual tumour localisation, tumour size, stage and histology. However, patients with tumours of the same entity show different response to radiotherapy, which is being attributed to the inter- and intratumoural biological heterogeneity. Recent pre-clinical and clinical data show that specific biological tumour characteristics are excellent biomarkers that can be included and analysed in clinical studies and may help to further individualise radiotherapy in future. Putative CSC markers have been evaluated in many tumour entities and are currently considered as very promising specific targets for treatment individualisation [23]. In this chapter, the focus is on the role of CSCs as prognostic and/or predictive biomarkers in HNSCC.

A ground-breaking retrospective study on the impact of CSCs on loco-regional control after primary radiotherapy of early laryngeal carcinoma implemented gene expression profiling on tumour biopsies in order to identify potential biomarkers that could predict tumour recurrence following radiotherapy [165]. Patients who developed a local tumour recurrence were matched for T-stage, subsite, treatment,

gender and patients' age with patients, who did not develop tumour recurrences. Gene expression data were generated on pre-treatment biopsies of 52 patients who were diagnosed with laryngeal carcinoma. Promising candidate genes such as the putative CSC marker CD44 were then evaluated in an independent patient cohort of 76 patients. Both CD44 mRNA and protein levels, assessed by immunohistochemistry, were found to significantly correlate with the response to primary radiotherapy. The transmembrane glycoprotein receptor CD44 is interacting with a number of ligands of the extracellular matrix such as hyaluronan, collagen, laminin, fibronectin and osteopontin [166]. In vitro studies demonstrated that CD44 interacts with EGFR and activates c-Met, focal adhesion kinase (FAK) and AKT signalling pathways. It further contributes to ROS defense by up-regulation of reduced glutathione [167, 168] and thus, leading to increased cellular radioresistance [169–171]. Current data support that CD44 is a suitable marker to measure the CSC density, but the putative intrinsic radioresistance of CD44 positive HNSCC cells warrants investigations in further clinical studies [22]. Both parameters, CSC density and their intrinsic radioresistance, correlate with local tumour control probability after irradiation [22]. Experimental datasets also demonstrated that in tumours with the same histology both parameters can be attributed to intratumoural heterogeneity after clinically relevant fractionated radiotherapy [8]. This is further supported by multicentre studies of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). The pre-treatment expression of putative CSC markers was significantly associated with loco-regional tumour control after curative intended radiochemotherapy both in the primary (Linge et al., in preparation) and also the postoperative setting. The expression of CD44, solute carrier family 3A2 (*SLC3A2*) and hepatocyte growth factor (*HGFR*; *MET*) in the surgical specimen prior to radiochemotherapy significantly correlated with loco-regional tumour control and, in the case of *SLC3A2* and *MET*, also with distant metastasis in HPV-negative HNSCC. This is of special interest, since postoperative treatment is being applied in order to inactivate potentially remaining tumour cells. For interpretation of these data, it has to be considered that in some cases all tumour cells might have been removed by surgery already, i.e. the potential prognostic and/or predictive value of these biomarkers measuring the sensitivity of tumour cells to the respective treatment would be weakened. The significant association between CSC expression and its impact on clinical outcome after postoperative radiochemotherapy indicates that in tumours with high CSC density, more CSCs are remaining after surgery compared to tumours with low CSC density. If these results are merely attributed to the CSC density or if tumours of high number of CSCs are also associated with increased infiltrative or metastatic potential has to be elucidated in further studies [23].

3.10 Patient Stratification: Are CSCs Enough?

The overall aim of biology-based treatment stratification is to identify patients, who have a nearly 100 % chance of total tumour cure after current standard treatment and patients, who have a very poor chance of tumour control. Those patients can then be

recruited into respective anti-cancer treatment de-escalation and escalation trials. However, for patients with intermediate risk for tumour recurrence it is currently difficult to allocate them into appropriate studies without decreasing their chance of tumour control. Therefore, the latter group of patients should be recruited into clinical trials, which are based on standard treatment and combined with translational biomarker studies [23].

The tumour size is a well established biomarker for tumours treated by primary radio(chemo)therapy and has to be included as a parameter in all clinical studies which are aiming for patient stratification for individualised radio(chemo)therapy. From experimental and clinical data on different tumour entities, it has been shown that the relationship between tumour volume and tumour response to radiotherapy can be described as a sigmoid negative curve [4, 21, 172, 173] (Linge et al., in preparation). The predicted curve between tumour volume and local tumour control from radiobiological data was shown to be steeper than the curve created from clinical data, the latter assuming that the number of CSCs shows a linear increase with tumour volume and that all other biological parameters of differentially sized tumours are comparable. From clinical data, a shallower curve was obtained, implying that in addition to the number or density of CSCs, other biological parameters such as intrinsic radioresistance or the tumour micromilieu are impacting CSC radiosensitivity [23, 174]. An example of potential patient stratification based on CSCs and tumour volume for patients with locally advanced HNSCC undergoing primary radiochemotherapy is shown in Fig. 3.3 (Linge et al., unpublished data). There is also some evidence that the efficacy of different radiotherapy fractionation schedules depends on tumour size before radiotherapy [175]. Considering all this, it is important to include clinical parameters such as the tumour volume of primary irradiatiated tumours in biomarker analysis, which are aimed on patient stratification (Fig. 3.3).

For HNSCC, a number of clinical studies have shown that the human papilloma virus (HPV)-infection status is a strong prognosticator for local tumour control and overall survival after primary radio(chemo)therapy [176, 177] (Linge et al., in preparation). HPV-positive HNSCC have been shown to be more radiosensitive, which may be due to an impaired DNA repair capacity [178]. Higher immunogenic effects of HPV positive tumours may also play a role [179]. The HPV status is currently being included in a number of prospective clinical trials for patient stratification.

Tumour hypoxia is another important parameter, which has been shown to play a major role in tumour radioresistance [180]. Experimental [181] and clinical data [61, 153, 182, 183] showed a correlation of the extent of hypoxia or respective surrogate markers. Clinical trials demonstrated that modification of tumour hypoxia significantly improves loco-regional tumour control and overall survival in HNSCC [184]. In addition, tumour hypoxia also leads to a more malignant phenotype and indicates a higher intrinsic radioresistance of hypoxic tumours [8] (Linge et al., in preparation). Nowadays, tumour hypoxia can be measured by non-invasive approaches such as by the analysis of hypoxia-induced gene expression in tumour biopsies or surgical specimen [153, 185], or by positron emission tomography using hypoxia-specific

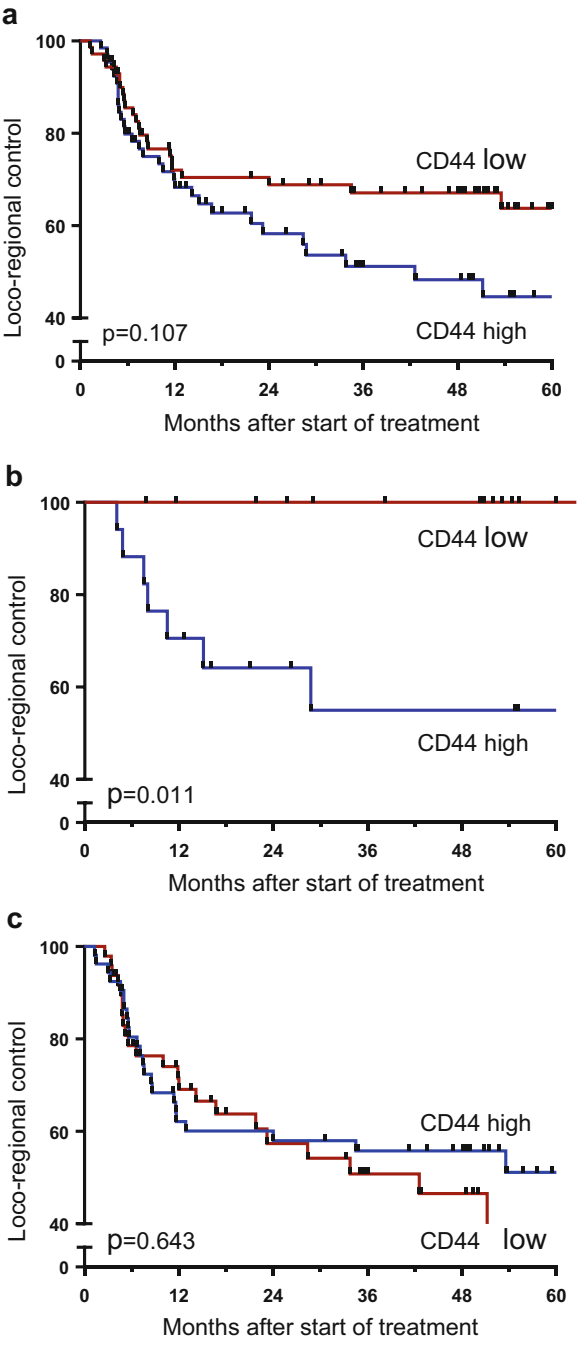

Fig. 3.3 Patient stratification based on tumour volume and CSCs. Patients with locally advanced HNSCC were treated by primary radiochemotherapy, CD44 expression was analysed in pre-treatment biopsies (**a**–**c**). CD44 expression in (**a**) all tumours, (**b**) small tumours and (**c**) large tumours

tracers such as ^{18}F-misonidazole, and showed a correlation with loco-regional control after primary radiochemotherapy [183, 186–188].

The additional clinical value of multi-biomarker assays to established clinical parameters has been demonstrated by a retrospective multicentre biomarker study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). In patients with loco-regionally advanced HNSCC, HPV16 DNA positivity was significantly associated with improved loco-regional tumour control (around 97 %) over 5 years after postoperative radiochemotherapy. Patients with HPV16 DNA negative tumours showed a loco-regional tumour control rate of about 80 % [189]. In the latter group of patients with HPV-negative HNSCC, tumour hypoxia-associated gene expression and high CSC expression within the surgical specimen were associated with poor loco-regional tumour control. In contrast, patients with CD44 protein negative tumours showed a nearly 100 % loco-regional tumour control rate [18]. This study awaits prospective validation.

CSC markers might be used not only for prediction of loco-regional control, but also for distant metastases. Within the above mentioned study of the DKTK-ROG, the gene expression of the putative CSC markers *SLC3A2* and *MET* have been shown to be associated with an increased risk of distant metastases [18]. This is in contrast to preclinical data, showing that CSC expression does not necessarily correlate with increased metastatic potential [190]. More functional studies are needed to further explore these findings.

Taken together, the inclusion of biomarker panels in addition to established clinical parameters is a promising approach for individualised radiotherapy. The analyses of CSC marker expression levels and/or other biological parameters, which are associated with tumour radioresistance, may lead to more patient subgroups, each consisting of less heterogeneous tumours and thereby a steeper radiation dose–response curve per treatment-subgroup and a higher effect of treatment modifications [23].

3.11 Outlook

Current pre-clinical and clinical developments in the field of CSC research suggest that the number of CSCs as well as their density and radioresistance are important determinants for local tumour control after radiotherapy. However, there is increasing evidence that not only CSCs as such but also other factors such as parameters of the tumour microenvironment determine radiosensitivity of CSC. Treatment stratification approaches require the use of well established parameters such as tumour volume as basic parameters along with markers determining radioresistance. Clinical trials for biology-based treatment individualisation will base on increasingly higher stratification and thus require large patient cohorts and multi-institutional co-operation. An under-researched area is the role of treatment-associated changes including selection of different CSC subclones for loco-regional tumour control in order to estimate the optimal time-points for CSC assessment.

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Chapter 4
Novel Strategies to Prevent, Mitigate or Reverse Radiation Injury and Fibrosis

Pierre Montay-Gruel, Gael Boivin, and Marie-Catherine Vozenin

Abstract Despite recent advances in Radiation Oncology with treatment planning and delivery of image-guided radiation therapy, acute tissue toxicity is still a dose-limiting factor for optimal local tumor control. Additionally, as the number of long-term cancer survivors is increasing, unacceptable complications emerge and dramatically impair the patients' quality of life. This means patients and clinicians expect therapeutic management of radiation-induced complications. Over the past four decades, research has enhanced our understanding of the pathophysiological, cellular and molecular processes governing normal tissue toxicity. This knowledge has provided us with tools to improve the therapeutic ratio of radiation therapy by enhancing its tumoricidal effect and protecting normal tissue. In this chapter, we review biology-driven efforts to develop translatable therapeutic approaches to prevent, mitigate or reverse radiation injury based upon cellular and signalling pathways targeting. We also highlight innovative approaches based upon manipulating external contributors such as the microbiota and applying novel radiotherapy delivery procedures.

Keywords Normal tissue complication • Fibrosis • Therapeutic strategies • Stem cells • Stroma • Inflammation • Immune response • Microbiome • Novel radiotherapy procedure

4.1 Introduction

The incidence of cancer is increasing worldwide with more than 14 million new cases per year. About 50 % of cancer patients are treated with radiation therapy (RT), making it, after surgery, the most important contributor to cancer cure. In the era of targeted therapies, RT is one of the best examples of a precise and

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powerful targeted treatment. Thanks to major advancements in physics, imaging and ballistics, high-precision dose delivery has succeeded in safely reducing the volume of irradiated normal tissues. New and very appealing RT approaches using high or very high dose per fraction (hypofractionation) such as stereotactic body radiotherapy (SBRT) also called stereotactic ablative radiotherapy (SABR) are increasingly used, both in early stages cancers and in some oligo-metastatic patients. In parallel, the particular dose distribution of protons and heavy ions has been therapeutically exploited with the aim to efficiently spare sensitive organs and enhance tumor cure.

At the biological level, the molecular response of cells and normal tissues to ionizing radiation involves a complex series of events that leads to the loss of tissue homeostasis caused by a direct killing of cells and an indirect stimulation of inflammatory mediators, as well as vascular alteration and release of thrombotic factors, recruitment of immune cells, remodeling of the extracellular matrix and stromal compartment associated with fibrosis initiation and maintenance. These phenomena may lead to genomic instability, persistent modulation of gene expression and alteration of the cellular phenotype leading to organ dysfunctions. This kind of modification of normal tissue homeostasis is the origin of disabling radiation-induced side effects which can have a huge impact on the patient's quality of life.

Therefore, increasing tumor sensitivity to radiation or increasing normal tissue tolerance to radiation are the two major paths toward improving the therapeutic index of radiotherapy. In this chapter, we will discuss the management of normal tissue complications, a research topic initiated decades ago by pioneer researchers in the field (including [1–5]). We will review the current status and future opportunities for clinical implementation of novel strategies to prevent, mitigate, and cure radiation injuries based upon the molecular understanding of cell and tissue responses to ionizing radiation.

4.2 Protection of Stem Cells

Recent studies have highlighted the importance of adult stem cells in restoring tissue homeostasis after radiation injury (reviewed in [6]). Because of their unique properties of self-renewal, pluripotency and the ability to differentiate into organ-specific functional cells, stem cells are fundamentally relevant in terms of maintaining life-long tissue homeostasis.

Radiation exposure may directly kill adult stem cells or induce degenerative-mutations leading to stem cell depletion. Therefore the protection, stimulation, recruitment or replacement of stem cells with intact functional properties facilitate tissue regeneration, and wound healing. Novel technological, pharmaceutical, or biological strategies to spare adult stem cells have been actively explored as well as replacement strategies based on stem therapy (Fig. 4.1).

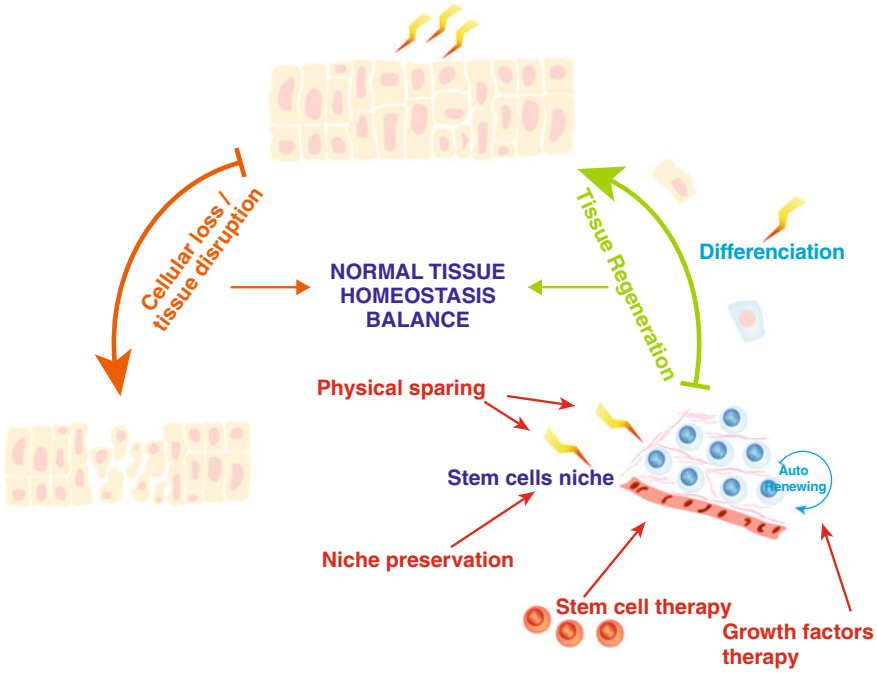

Fig. 4.1 A proper balance between tissue regeneration and disruption is key to normal tissue homeostasis through the stem cell pool. Radiation modifies this balance and different prophylactic, therapeutic agents, and technologies have been developed to preserve and restore it

4.2.1 Preventing the Depletion of Stem Cell Pools and Improvement of Tissue Regeneration

Recent studies have shown that adult stem cells are not evenly distributed in tissues but rather located in specific niches able to trigger regeneration and differentiation. The recognition of the stem cell niche and its relevance to stem cell function has prompted extensive research into the possibility of ballistic protection of stem cells. For instance, in the brain, Neural Stem Cells (NSCs) are mainly localized in the subventricular and subgranular zones (SVZ/SGZ). SGZ-NSCs are of major importance for cognitive skills as both retrospective and prospective trials have demonstrated radio-induced neurocognitive impairment upon hippocampal irradiation [7, 8]. Interestingly, recent technological advances (IMRT, Tomotherapy, proton) have shown it is possible to spare the hippocampus (or at least reduce the dose) with a good preservation of functional NSCs in the SGZ (preclinical model from [9]) as well as encouraging results in terms of verbal memory (phase II clinical trial from [8]). The major limitation of this strategy is tumor control or relapse in the radiation spared field.

Therapeutic agents have also been tested to protect stem cells from radiation injury especially by stimulating the stem cell pool. For instance, radiation-induced xerostomia can be counteracted by administrating Keratinocyte Growth Factor before and just after irradiation [10]. Along the same line, the trophic factor GLP-2 [11, 12] and the peptide TP508 prevent GI crypts ulceration. TP508 is able to up-regulate the expression of GI stem cell markers such as DCLK1 and LGR5 [13], increase the stemness potential of tissues and restore their integrity. A third method of stem cell preservation is via niche-mediated protection. One study, for instance, reported that the use of pharmacological inhibitors of Prolyl-hydroxylase Domains proteins (PHD) before and after abdominal irradiation of mice showed an HIF-mediated increase in crypt survival, enhancement of crypt regeneration, and increase in mice survival [14].

4.2.2 Stem Cell Therapy to Counteract Radio-Induced Toxicities

Restoration of the stem cell pool and function can also be achieved by stem cell transplantation from syngenic or xenogenic origin; impressive positive results have been reported in most organs (see Table 4.1). The transplanted stem cells repopulate the injured tissue leading to cellular differentiation and cell proliferation However, in most cases paracrine stimulation is also involved. Tissue restoration correlates with a decrease in local inflammation, apoptosis and microvasculature damage, altogether resolving the niche injury. Modifications in protein expression also drive the niche restoration such as TGF-β, CTGF, col1α2/col3α2 and MMP/TIMP balance in the case of skin fibrosis treatment.

4.3 Protection of Resident Cells

Besides the impact triggered by ionizing radiation on the fate and function of stem cells, irradiation also dramatically alters the immediate and long-term function of differentiated cells. The complex interplay and the various cross-communication that occur between the different cellular compartments, *i.e*. epithelial, endothelial, mesenchymal, and immune cells of a given organ after irradiation can initiate, amplify, and maintain tissue injury [34–40]. It is now clear that the complexity of these interactions induces a heterogeneous response, which can only be assessed *in vivo* or using sophisticated 3D-models.

Today, studies in stem cell biology (see Sect. 4.1) and microbiota (see Sect. 4.6) show how acute ulceration and epithelial apoptosis/anoikis function can initiate and amplify radiation injury. In addition, endothelial radiation sensitivity [41] and

Table 4.1 Preclinical and clinical trials using stem cell therapies for treatment of radiation induced normal tissue injury

Organ system	Endpoint	Toxicity	Preclinical studies (stem cell type)	Clinical trials (stem cell type)	References
Skin	Fibrosis, Radionecrosis	Stem cell depletion, Inflammation, Fibroblast death, Epidermis	MSC, ADSC, EPC	MSC	[15]
					[16]
					[17]
					[18]
					[19]
Brain	Cognitive dysfunction, Radionecrosis	NSCs depletion, niche destruction, inflammation	hESC, hNSC	–	[20]
Bone marrow	Aplasia	HSCs depletion, niche destruction	BMDC, HSC, MSC	BM	[21]
H&N	Xerostomia	Stem cell depletion	BMDC, MSC, SGSC	–	[22]
					[23]
					[24]
					[25]
					[26]
GI	Rectitis, Proctitis	Epithelial stem cells depletion, inflammation	MSC	MSC	[27]
					[28]
					[29]
					[30]
Bone	Bone growth	Niche destruction	BMDC, MSC	BM	[24]
	Radionecrosis				[31]
Liver	Liver disease	Hepatocyte cell death	Hepatocyte	Hepatocyte	[32]
			hMSCs		[33]

MSC mesenchymal stem cells, *BMDC* bone marrow-derived cells, *EPC* endothelial progenitor cells, *hESC* human embryonic stem cells, *hNSC* human neural stem cells, *NSC* neural stem cells, *HSC* hematopoietic stem cells, *SGSC* salivary gland stem cells

thrombogenic activation [42] has been extensively studied. The extravasation of blood fluids and leukocytes into the extracellular milieu generates a wounded area prone to long-term endothelium remodeling such as endothelial-mesenchymal transition (EMT). This chronic environment ultimately causes endothelium wall thickening, muscular media replacement by connective tissue, and the activation of myofibroblasts. Defined as the principal cellular effector of radiation-induced fibrosis [36, 43], myofibroblasts can arise from a variety of sources [44] such as trans-differentiated local fibroblasts or mesenchymal cells [45, 46], as well as from epithelial or endothelial cells *via* EMT. Tissue exposure to ionizing radiation induces phenotypic alteration of all resident cells orchestrated by TGF-β1 and a growing list of growth factors including CTGF/lysophosphatidic acid (LPA) and

Rho/ROCK axis, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) [47] as well proinflammatory mediators, cytokines, interleukin (IL)-6 [48] and reactive oxygen species (see Sects. 4.3, 4.4 and 4.5).

4.3.1 Cytoprotective Therapies

Several cytoprotective therapies [49] based upon administering trophic growth factors have been utilized to protect stem (see Sect. 4.1) as well as differentiated epithelial and endothelial cells [50]. Epithelial cells [11, 12] from the gut have been shown to be protected by the trophic factor GLP-2 which stimulates proliferation and integrity of the intestinal barrier [11, 12]. GLP-2 protects mice from radiation-induced mucosal ulceration prone to bacterial translocation and sepsis (see Sect. 4.6). Similarly, KGF has been shown to stimulate cell proliferation and promote epithelial cell survival and differentiation of oral mucosa both in pre-clinical and clinical trials [51, 52] as well as displayed an off-target effect by decreasing ROS levels and stimulating DNA repair [51, 52].

Endothelial cell apoptosis can be inhibited by the transient blockade of p53 and the exogenous administration of basic fibroblast growth factor (bFGF). Both approaches protect the gastrointestinal tract from radiation injury [53, 54]. Similarly, ceramide-targeting antibody [55], and the Flagellin-derivative, CBLB502 [56, 57], protect microvascular endothelial cells of the gut from radiation-induced apoptosis by activation of NF-κB. Finally, enhancing endothelial cell radiation resistance has also been achieved by blocking the TSP1/CD47 pathway [58] in addition to stimulating M1 macrophage infiltration known to be prone to wound resolution and restoration of tissue homeostasis (see Sect. 4.5).

Whether epithelial [59] or endothelial [60] cell death is the primary inducer of acute toxicity has been a long-term dispute within the radiobiology community; however, given the complexity of the pathogenic process it is today obvious that effective therapeutic strategies cannot target only one cell type or pathway but must rely upon coordinated stimulation of stem cell function, resident cell phenotype immunity, and reducing inflammation.

4.3.2 Phenotypical Modulators

Radiation exposure induces a persistent phenotypic activation of endothelial cells and fibroblasts. In endothelial cells this activated phenotype is composed of the expression of thrombogenic and adhesive markers. In fibroblasts, radiation results in the trans-differentiation of myofibroblasts that then synthetize fibrogenic molecules and oversecrete extracellular matrix. Antioxidant therapies including SOD [61–64], pentoxifylline-tocol combination [65–70], and anti-inflammatory agents such as statins [71–75] have been shown to reverse

these activated phenotypes by inhibiting specific signaling mediators such as ROS, thrombogenic factors i.e. Thrombin or fibrogenic pathways such as TGF-β, Protein C, and CTGF.

4.4 Modulation of Signaling Cascade That Regulates Resident Cell Fate Upon Radiation Injury

Of the many signaling cascades governing normal tissue response to radiation injury, we have selected some recently described pathways for their relevance and clinical implication. Most of these pathways are involved in multiple radiation response processes, such as vascular/microvascular damages as well as inflammatory and fibrogenic responses. This means that the drugs that target these pathways are effective in mitigating toxicity to normal tissues by their combined action on these multiple pathogenic processes.

4.4.1 Protein C Pathway

Microvascular injury is a prominent feature of normal tissue radiation injury and plays a critical role in both acute/inflammatory and chronic/fibrotic radiation responses. The dysfunction of the Thrombomodulin (TM)-protein C (PC) system is involved in the pathogenic process (Fig. 4.2). Acute radiation-induced ROS release inactivates the TM, its transcription and release into the circulation. TM alteration in endothelial cells causes loss of local vascular thrombo-resistance, excessive activation of protease-activated receptor-1 by thrombin, and insufficient activation of protein C. When they persist, these acute alterations are also involved in the fibrogenesis and maintenance of fibrogenic signals.

4.4.1.1 Inhibition of Coagulation

Direct inhibition of coagulation using anti-coagulant strategies such as Hirudin and Octreotide have demonstrated efficacy in experimental models when administered before irradiation [76–78]. Activated PC is one other potent anti-coagulant and cyto-protectant that inhibits blood clotting (through the proteolysis of factors V and VII), promotes fibrinolysis and exerts potent anti-inflammatory and cytoprotective effects on endothelial cells, neurons and innate immune cell populations [79]. It has shown considerable promise as a radiation mitigator as seen in a study in which the systemic administration of soluble TM or activated PC to lethally irradiated wild-type mice resulted in an accelerated recovery of hematopoietic progenitor activity in bone marrow [80].

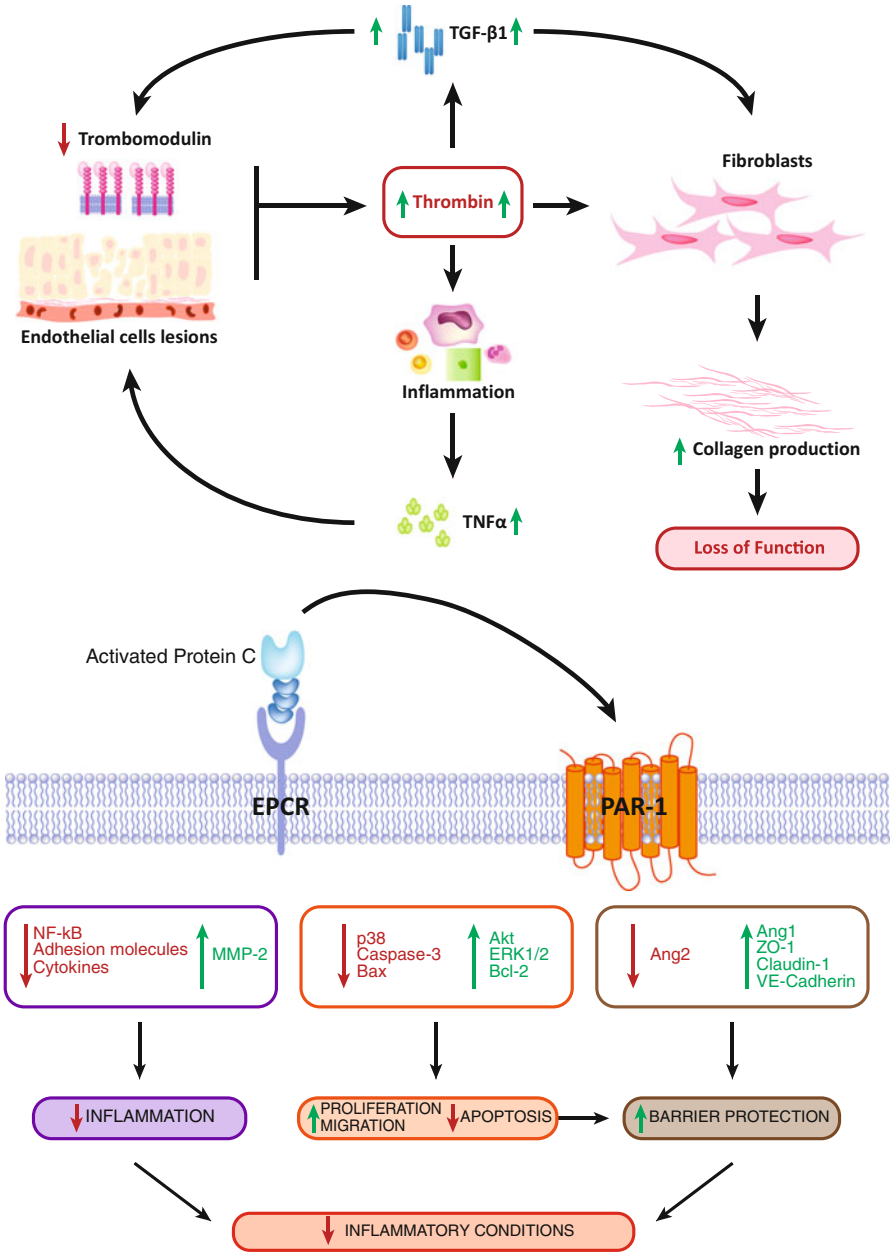

Fig. 4.2 *Radiation-induced alteration of the Thrombomodulin—protein C system.* Radiation exposure of microvessels induces a deficiency in thrombomodulin (TM) and leads to enhanced coagulation status via the accumulation of thrombin and deposition of fibrin. Thrombin also has powerful inflammatory, mitotic and pro-fibrogenic effects via TGFβ- activation. In addition TM deficiency prevents PC activation and anticoagulant activity of APC. The anti-inflammatory, anti-apoptotic activities of APC along with its protective effect on endothelial barrier function, require the cellular receptors EPCR and PAR-1

4.4.2 Transforming Growth Factor (TGF-β1)

The transforming growth factor-β (TGFβ) pathway contribution to radiation injury has been extensively studied and reviewed in many articles [36, 81–85], therefore we will shortly summarize its function and will focus on some of its less described properties.

Transforming growth factor is secreted as a large latent complex that must be released by proteolysis for full activity. Its signal transduction is mediated via two serine/threonine kinase receptors [86] that recruit and phosphorylate Smad proteins which are considered as the canonical TGFβ mediated signal transduction pathway [87] (Fig. 4.3). However, non-Smad mediated transduction also occurs via Erk, p38, and c-Jun N-terminal (JNK) MAP kinases, PI3K-Akt, and small GTPase pathways [86]. The TGFβ receptor can also—through as yet unknown intermediates—engage the Rho-ROCK1 signaling module [88] as well as the Cdc42/Rac1-PAK2 complex [89]. These molecular pathways are transactivating thrombogenic and fibrogenic genes: More recently, TGFβ has been shown to protect cells from radiation through activation of the NHEJ repair pathway [90].

A remarkable but less-explored feature of TGFβ-activated Smad2/3 is its ability to bind p68, a component of the microRNA (miRNA) processing complex DROSHA. First described to target the primary transcript of miR-21 (pre-miR-21) in vascular smooth muscle cells [91] where it regulates the contractile phenotype of the cells, this mechanism has been now extended to the regulation of cardiogenesis [92] and myocardial remodelling [93]. This new mechanism of selective microRNAs maturation mediated by TGFβ could be of great interest in the field of normal tissue injury since miRNA can be either biomarkers or mediators of normal tissue injury, as highlighted in recent publications that identified miR-21, -29 and 101 in fibrotic tissue [94, 95] and miR-210 as a possible anti-fibrotic target in radiation enteropathy [96].

4.4.2.1 Inhibition of TGF-β Using Antibodies and Pirfenidone

One of the earliest therapeutic studies targeting TGFβ was conducted with a neutralising antibody against TGFβ and was effective in a model of rat lung fractionated irradiation. A reduction in alveolar septal wall thickness, macrophage activation, TGFβ and its downstream signal transduction proteins was seen [97]. Subsequently, a small molecule inhibitor, SM16, targeting TGFβ type 1 receptor kinase was shown to be effective in a similar model [98]. Other studies used a human recombinant adenoviral vector carrying the gene for a TGFβ type II receptor, which acted as a plasmatic competitor trapping TGFβ and leading to an improvement of radiation pulmonary and intestinal toxicity [99, 100]. There are some limitations to interpretating these pre-clinical studies: the use of treatment schedules not fully representative of clinical settings and the fact that these interventional therapies have predominantly been tested during the early phase of the disease. However, some compounds, including Pirfenidone, described as a selective regulator of most fibrogenic molecules including TGFβ and PDGF, β-FGF,

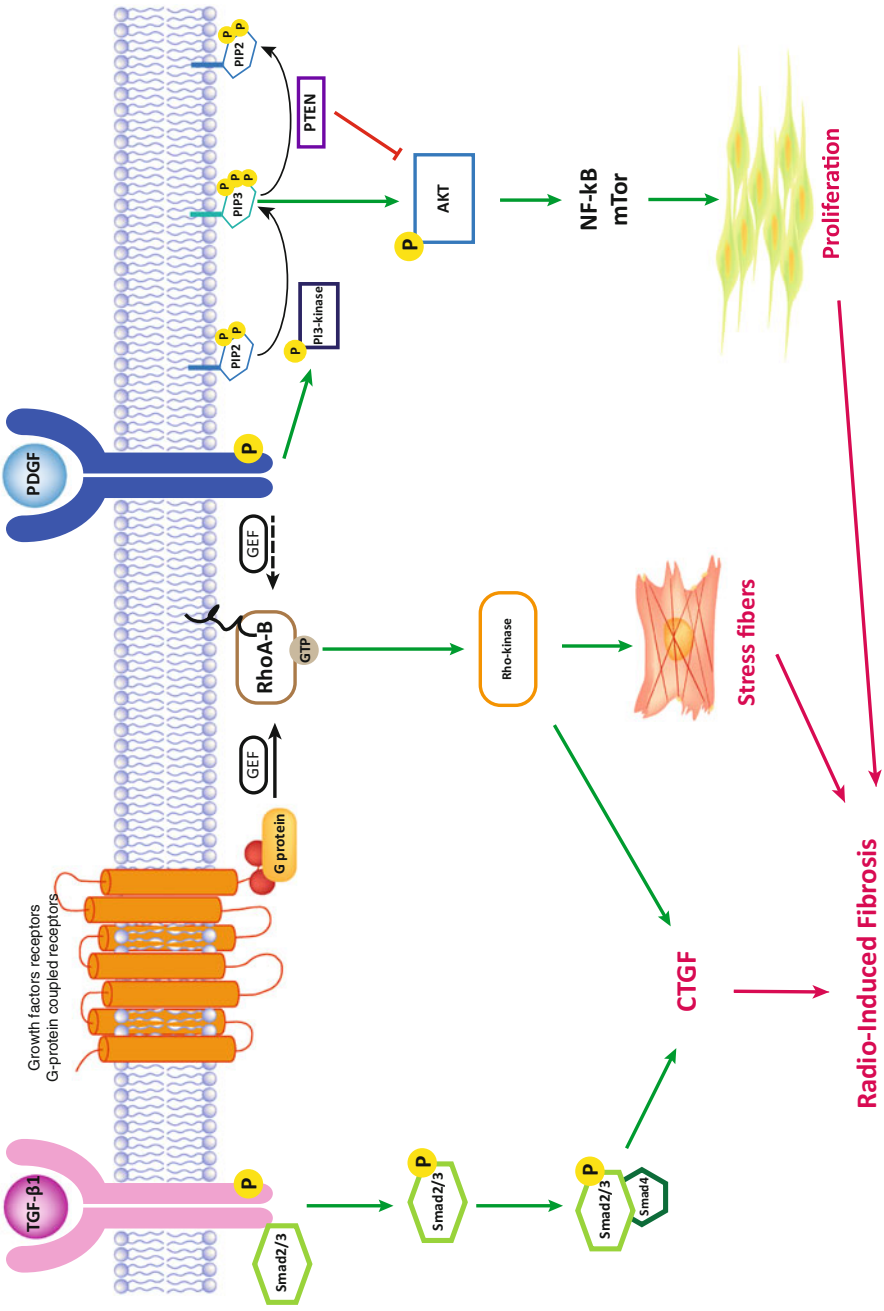

Fig. 4.3 *Signaling cascades that regulate radiation-induced fibrogenesis*. Fibrogenic growth factor including TGFß and Platelet-derived growth factor-BB (PDGF-BB) activate signaling cascade and lead to the development and maintenance of fibrosis mediated by the differentiation of fibroblasts and smooth muscle cells, enhanced CTGF expression and extracellular matrix synthesis and accumulation

EGF, TNF-α, have been used with a certain success in humans with IPF [101]. One pilot trial published in 2007 reported the stabilization of radiation-induced lung fibrosis [102] but a proper randomized trial is still missing and the efficacy of Pirfenidone disputed. In addition, clinical trials using TEW-7197, LY2157299, associated or not with anti-cancer treatment are ongoing, but to our knowledge none of them is associated with radiotherapy and the pleiotropic effects of TGFβ probably remain the main limitation for the clinical application of TGFβ inhibition.

4.4.3 RHO/ROCK Signaling Pathway

Guanosine triphosphatases (GTPases) from the Rho family (from "Ras homologous") are fundamental regulatory molecules in cells [103]. Post-translational modification by prenylation (geranylgeranylation) is required for Rho activation which is determined by the ratio of GTP/GDP-bound forms, and mediated by various activators: the guanosine nucleotide exchange factors (GEFs); and inactivators: the guanine dissociation inhibitors (GDIs). The biological effects of Rho are mediated by a number of downstream effector proteins, including the Rho-associated kinase (ROCK) (Fig. 4.3). Alterations in the expression of the genes coding for proteins of the Rho family have been reported both in human samples and mice models of delayed radiation injury affecting various organs including the gut, lung and heart [104–107] and can be modulated using pharmacological agents [108].

4.4.3.1 Modulation of Rho/ROCK Using Statins and ROCK Inhibitors

Regulation of Rho/ROCK pathway can be achieved using the approved drugs called statins which work inhibiting HMG-CoA reductase, the rate-limiting enzyme in mevalonate synthesis needed to produce isoprenoid intermediates. Several pre-clinical studies have shown that statins were able to modulate fibrogenic and thrombogenic differentiation of myofibroblasts and endothelial cells; they also reduce the expression of CTGF/CCN2, TGFβ, and Col Iα2 genes [71, 72, 74, 109–111] and help to restore the "gatekeeper" function of the endothelium after irradiation [112] without decreasing the tumor's radiation sensitivity [73]. These interesting pre-clinical findings were supported by retrospective trial conducted on statin users with rectal cancer [113] and further confirmed in a prospective trial that included 308 patients undergoing radiotherapy for the treatment of pelvic cancer [114]. In this study, the use of a statin in combination or not with ACEi medication reduced acute gastrointestinal symptom scores and also appears to have provided longer-term sustained protection. A second trial is currently ongoing to confirm this beneficial effect in Head&Neck cancer and make it available for patients.

4.4.4 The Connective Tissue Growth Factor (CTGF/CCN2)

CTGF/CCN2 is a matri-cellular protein with heparin-binding activity. Composed of four modules, it is susceptible to protease cleavage and can be found in its cleaved form in various biological fluids where they play distinct functions [115]. Its synthesis is stimulated by various fibrogenic mediators, such as endothelin-1 and TGFβ [116, 117], environmental changes such as hypoxia and biochemical stimuli such as stretch [118]. CTGF/CCN2 is overexpressed in radiation-induced fibrotic diseases [43, 105, 106, 119–123]. Despite many efforts, a specific CTGF/CCN2 receptor has yet to be identified; CTGF/CCN2 appears to perform many of its functions through integrins, heparin sulfate-containing proteoglycans, and the LPA axis [124, 125]. The effects of CTGF/CCN2 seem to mirror TGFβ's fibrogenic functions [126] but is a more attractive anti-fibrotic target as it does not display pleiotropic function but rather an almost selective action on mesenchymal cells.

4.4.4.1 Inhibition of CTGF/CCN2

In pulmonary fibrosis, the LPA–LPAR1/3 axis has been described as a potent modulator of CTGF/CCN2 expression. Its inhibition using VPC 12249 has demonstrated anti-CTGF/CCN2 action associated with decreased fibroblast proliferation, improvement of histological structures and pulmonary function [127]. More specific inhibition of CTGF/CCN2 using the monoclonal anti-CTGF antibody FG-3019 has been reported [128] to prevent and reverse lung radiation-induced lung fibrosis. This anti-CTGF/CCN2 antibody is being currently tested in the context of IPF, but to our knowledge no studies are ongoing in the context of radiation-induced fibrosis.

4.4.5 The Platelet Derived Growth Factor (PDGF)

Like TGFβ, the PDGF is released from platelets upon radiation exposure and binds to a tyrosine kinase receptor to transduce a mitogenic and fibrogenic signal that stimulates the transdifferentiation of fibroblasts into myofibroblasts (Fig. 4.3). PDGF is mainly synthesized by platelets and stored in their alpha granules; however numerous cells such as activated macrophages, endothelial cells and smooth muscle cells, have been shown to produce PDGF.

4.4.5.1 Inhibition of PDGF

Imatinib, desotinib and nilotinib are amongst the tyrosine kinase inhibitors that suppress the PDGF receptor signaling. Imatinib anti-fibrotic efficacy was proved more than 10 years ago in preclinical experiments [129] and is currently being assessed

in clinical trials. Nindetanib is another tyrosine kinase inhibitor that has shown encouraging results in the management of idiopathic pulmonary fibrosis and is being tested in lung cancer and neuroblastoma patients undergoing radiotherapy with assessment of normal tissue complications as secondary endpoints.

4.4.6 Blockade of Other Growth Factors (EGF, FGF2 and IGF) and Heparanase

Many other signaling cascades regulated by EGF, FGF and IGF are involved in the acute and delayed radiation response of normal tissue. Broad range molecules such as Suramin, a polysulfonated naphthylurea that acts as a potent competitive inhibitor of reverse transcriptase, have been described to block the activity of these growth factors. Their inhibitory action seems mediated via heparanase inhibition [130] and physical sequestration of the fibrogenic factors. Suramin has been combined with RT [131], but the outcome in terms of toxicities remains to be investigated.

4.4.7 Modulation of Redox Status

Exposure to ionizing radiation produces a burst of free radicals resulting from the ionization of water molecules. This is followed by a persistent and prolonged increase in both Reactive Oxygen and Nitrogen Species (ROS/RNS). Upon injury, if the initial increase in ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS scavenging capacity. However, when high and persistent ROS production occurs, following exposure to high radiotherapy doses for example, the antioxidant response is not sufficient to reset the system to the original level of redox homeostasis. This new steady state is called chronic oxidative stress. The radiation-induced vascular cell damage [53, 60] contributes to the redox imbalance with alternate sequences of hyper- and hypoperfusion-lead ROS burst and tissue hypoxia [132], leading to HIFs stabilisation, transativation of proangiogenic (VEGF) and pro-wounding (TGFβ) genes, all of which perpetuates the vicious circle.

4.4.7.1 Therapeutic Modulation of the Redox Status and Antioxidant Strategies

Treatment with hyperbaric oxygen (HBO) [133] and antioxidant therapy [64, 134, 135] were both successfully used despite their apparent antagonistic mechanism of action. HBO induces transient tissue hyperoxia (typically ~2 h/day) that should not overcome natural antioxidant defenses [136] but may help to remobilize tissue remodelling by activating signaling molecules in transduction cascades

(see the review [137]). Antioxydant therapies scavange ROS. Initial studies with Amifostine [138–140] and bovine liposomal Cu/Zn superoxide dismutase showed anti-fibrotic efficacy associated with TGFβ inhibition [61]. More recent trials investigated the benefits of tocol isoforms (Vitamin E analogs) such as high-dose alpha-tocopherol combined with pentoxifylline and Clodronate [66, 141], and γ-tocotrienol (GT3) [142]. In addition to their antioxidant action, both strategies have displayed off-target benefits with protective endothelial activity [69, 143] and miRNA regulation [96]. Interestingly, the efficacy of GT3 is enhanced when combined with pentoxifylline [68]. Lastly, hypoxia-regulating molecules such as 2-methoxyestradiol (2-ME) have been shown to downregulate HIF1α-mediated Smad activation and inhibit radiation-induced lung fibrosis in mice [144].

4.5 Modulation of Inflammation

Acute normal tissue response to radiation exposure is characterized by the orchestrated release of numerous pro-inflammatory mediators such as tumor-necrosis factor (TNF)-α, cytokines and chemokines. This early inflammatory phase is characterized by the rapid resolution of the vascular changes, oedema and neutrophil infiltration and can be followed either by a regenerative phase or by a chronic inflammation that persists over weeks and months. This chronic inflammation is today recognized as the main contribution to fibrosis, in which persistent immune responses occur alongside tissue remodeling and repair processes [145, 146] and the results obtained using anti-inflammatory interventions suggest that both processes do feed off each other (Fig. 4.4).

4.5.1 Corticosteroid to Reduce Inflammation

Corticosteroids have a long-standing history of use in patients with severe radiation complications after radiotherapy to inhibit inflammation; however their anti-fibrotic properties remain uncertain. Hirota et al. [147] noted that patients in their series who received corticosteroids as part of chemotherapy regimens had significantly lower incidences of severe fibrosis. However, well-controlled randomized clinical trials are lacking and similarly, experimental results are inconsistent [148, 149] which further supports their use as anti-inflammatory agents to be administered initially but not for their anti-fibrotic effects.

4.5.2 Blockade of TNF-α

TNF-α deficient mice have been described to be radioresistant [150] and TNFα inhibition with chitosan/DsiRNA nanoparticles [151] and ambroxol [152] has been shown to protect mice from acute inflammation. TNFα overexpression has also been reported in radiation fibrosis but its inhibition does not trigger an anti-fibrotic

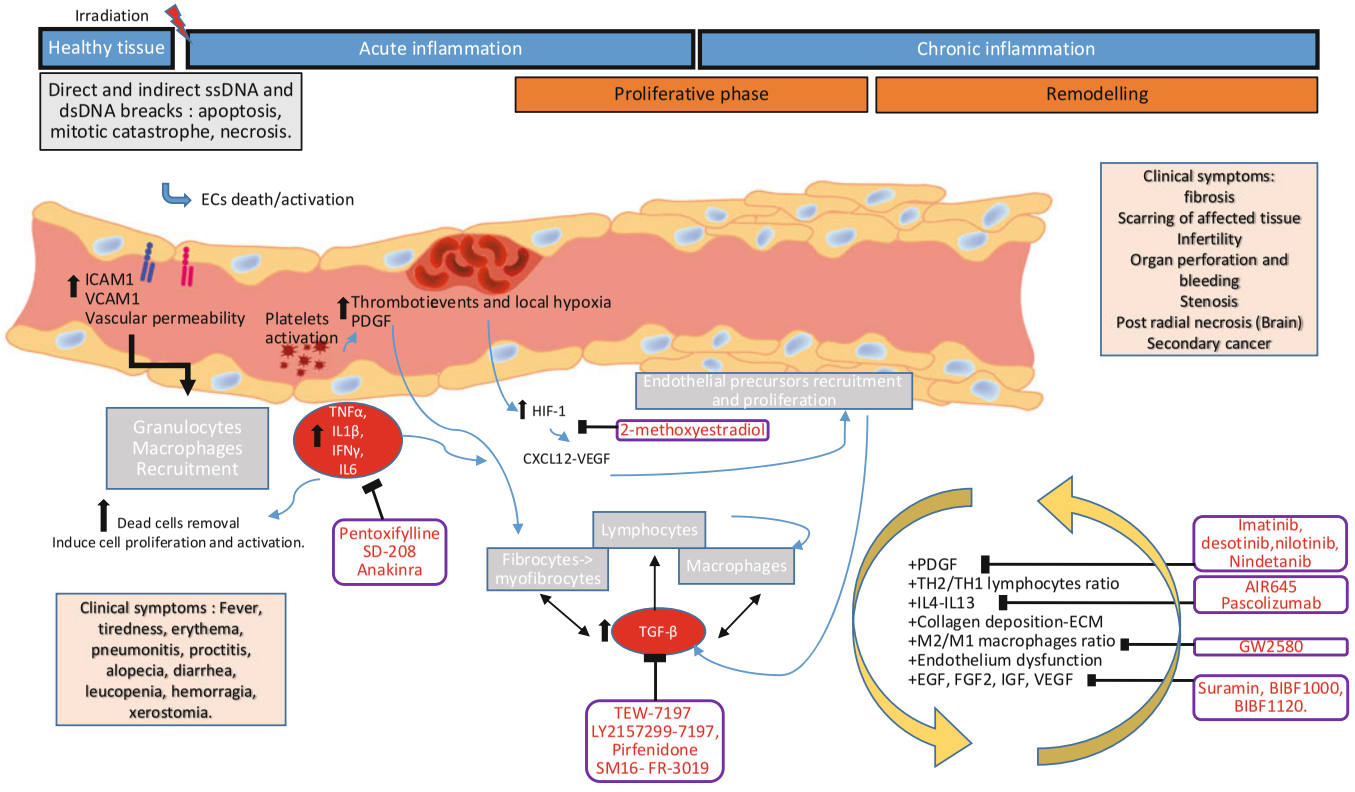

Fig. 4.4 Inflammatory and immune contribution to acute and chronic radiation response. *ICAM* intercellular adhesion molecule 1, *VCAM1* vascular cell adhesion protein, *ssDNA* single strand DNA, *dsDNA* double strand DNA, *Ecs* endothelial cells, *PDGF* platelet derived growth factor, *TNF α* tumor necrosis factor alpha, *IL1* interleukine 1, *INF γ* interferon gamma, *HIF-1* hypoxia inducible factor 1, *TH1/TH2* helper T cell 1 &2, *ECM* extra cellular matrix, *EGF* endothelial growth factor, *FGF* fibroblast growth factor, *IGF* insulin like growth factor, *VEGF* vascular endothelial growth factor, *TGFβ*

effect but can potentiate the anti-fibrotic action when combined with other molecules—one such example is the combination of pentoxifylline (PTX), a well-known anti-TNFα drug, with an antioxidant, the alpha-tocopherol (vitamin E) and an anti-macrophage, Clodronate [65, 153].

4.5.3 Blockade of Pro-inflammatory Cytokines

Strategies to inhibit pro-inflammatory cytokines have been developed to treat inflammatory diseases. Recent molecules such as the IL-1R antagonist (Anakinra-Kineret®) are currently used to treat rheumatoid polyarthritis [154] and might be of interest to treat radiation-induced fibrosis as the inhibition of IL-1β attenuates fibrosis [155]. IL-4 and IL-13 are other potential targets with common functional activities and a common receptor (IL-4Rα) which activates STAT6-dependant signalling pathway [156]. In vivo, blocking studies were successfully conducted and confirm the fibrogenic role of IL-4 and IL-13 in various fibrosis models including skin [157], liver [158], and lung [159, 160]. IL-4 inhibitors have been consistently used for managing airway inflammatory disease (AIR645, pascolizumab). This new compound may be of great interest for avoiding or reducing radiation-induced toxicities, but it has not yet been validated for radiation injuries and the sequence of administration needs to be accurately assessed to avoid protecting the tumor and impairing the start of wound healing.

4.5.4 Blockade of Chemokines

Tissue homeostasis is tightly controlled by chemokine balance. CCL3 (also known as macrophage inflammatory protein 1α, MIP1α) and CCL2 (also known as monocyte chemoattractant protein 1, MCP1) have been identified as key chemotactic molecules for recruiting mononuclear phagocytes. Anti-CCL3 and CCL2 antibodies prevent the development of bleomycin-induced fibrosis [161–163]. On the other hand, CXCL10 and CXCL11 are natural inhibitors of fibroblast recruitment and neoangiogenesis via the production of the antifibrotic cytokine IFN-γ, [164, 165]. Thus, modulating specific chemokine signaling pathways in order to restore the natural balance between profibrotic and anti-fibrotic signals is theoretically achievable, but the fine tuning required seems hardly compatible with a clinical application.

4.6 Modulation of the Immune System

The composition of the immune cell compartment is organ-specific and organized in a fragile balance to react against stress and restore homeostasis. However, in certain conditions of non-self-resolutive immune activation like wounds (ex: cheloids),

infections (ex: tuberculosis) or inflammatory diseases (ex: familial Mediterranean fever) involving severe tissue injury can occur. In the same way, tissue exposure to radiotherapy induces a dramatic remodeling of the tissue microenvironment. This means that elucidating the impact of radiotherapy on the immune compartment and subsequent immunomodulation is currently one of the most promising strategies for enhancing the differential effect of radiotherapy.

4.6.1 Modulation of the Pool of Adaptive Immune Cells

Studies have suggested that regulating the adaptive immune cell balance would reduce both acute and chronic injury of normal tissue. Radiation is known to modulate the polarization of CD4+ cells within normal tissues and prime tissue response towards fibrogenesis when TH2 polarization occurs [157, 159], whereas TH1 polarization would be anti-fibrotic *via* INF-γ secretion. Similarly, the role of FoxP3+ Tregs could be either anti-inflammatory/anti-fibrotic [166] or pro-fibrotic via secretion of the fibrogenic growth factor TGFβ [167, 168]. CD4+ Th17 seems pro-fibrotic via the secretion of IL-1β, IL-23 and TGFβ [169, 170] and the recruitment of neutrophil and MMP-1 [171].

Pharmacological interventions have been performed to eradicate or reprogram adaptive immune cells [172] but with limited success. Recent studies, however, performed in blood samples of whole body irradiated mice have shown differential radiosensitivity of subtypes of immune cells. Persistent changes in immune phenotype [173] with a permanent TH1 drop associated with an increase in the percentage of blood TH17+ or FoxP3+ T cells have been observed. This recent observation suggests that circulating cells may trigger a fibrogenic effect, something which has never been investigated and is worth future attention.

4.6.2 Modulation of the Pool of Innate Immune Cells

Researchers have recently given a lot of attention to the role of macrophage reprogramming occurring during radiotherapy. Their relevance has been demonstrated in both tumor and normal tissue response to radiotherapy with potential therapeutic implications [174, 175]. Macrophage phenotype is highly dependent upon the micro-environment and at least two functionally distinct populations—"classical, M1 macrophages" and "alternative, M2 macrophages"—have been described upon exposure to Th1 or Th2 cytokines, respectively [176]. A hybrid phenotype has also been reported [177], illustrating the high plasticity of these cells and corroborating their function as a sensor and rheostat of tissue homeostasis (Table 4.2). In fact, macrophage polarization seems to drive the balance between the exacerbation of tissue damage (M1 polarization) and tissue recovery and fibrosis (M2 polarization) [178–180]. M2 polarized macrophages are especially relevant to fibrosis as they display immunosuppressive properties, secrete large amounts of the fibrogenic mediator TGFβ [181], go on to activate the Smad pathway and stimulate fibrogenic

Table 4.2 Functional impact of macrophage phenotypes

M1 macrophages	M2 macrophages	Hybrid macrophages
Induced by Th1 cytokines including IFN-γ	Induced by Th2 cytokines including IL-13 and IL-4	
Produce TNF-α, IL-12 and IL-6 and increase inducible nitric oxide synthase (iNOS), superoxide anions ($O_2^{-\bullet}$) and oxygen radical	Produce PDGF, TGF-β1, arginase type 1 (arg-1)	
Arginase and iNOS		Arginase and iNOS to limit T cell function
CD40, ICAM-1, MHC class II, CD80, CD25	CD206, Dectin1, CD71, CD163 and chemokine receptors including CXCR1, CXCR2 and CCR2	
MCR1 low	MCR1 high	
CD11c high	CD11c low	

genes such as CTGF and PAI-1 [182]. The macrophages isolated from bronchoalveolar fluid from patients undergoing thoracic irradiation spontaneously released PDGF, another important fibrogenic growth factor [129] (Table 4.2).

Recent studies have suggested that depending on the dose administered, radiotherapy could induce Th1/M1 or Th2 /M2 polarization [183]. High doses of ionizing radiation induce immunogenic cell death and normalize tumor vasculature, thereby improving the recruitment of tumor-specific cytotoxic T cells [184, 185]. However, the balance is tight and in B16F10 melanoma, high doses of radiation promote M2 polarization and inhibit TNF-α expression, supporting tumor-induced anergy [186]. In a recent study, Klug et al. used a lower range of radiation doses (down to 2 Gy) in combination with immunotherapy to induce the reprogramming of M2 macrophages into M1 macrophages and subsequent elimination of the tumor [187]. Interestingly, tumor-associated macrophages and fibrotic tissue-infiltrating macrophages display similar M2-oriented phenotypes, suggesting that the modulation of macrophage polarization could improve radiotherapy outcomes by enhancing anti-tumor efficacy and preventing radiation-induced fibrosis.

4.6.2.1 Macrophage Reprogramming

Clodronate liposomes were used to deplete macrophages in several studies [188]. The reduction of the number of macrophages by clodronate in wounded tissue indeed reduced excessive scar formation and delayed cutaneous wound healing [189]. Froom and colleagues [190] showed that oral administration of clodronate (bisphosphonate) significantly reduced bone marrow fibrosis. Delanian and Lefaix proposed clodronate administration in combination with the pentoxifylline-vitamin E (PE) treatment, and showed improved efficacy in the treatment of radiation-induced fibronecrosis [153,

191]. The very targeted depletion of M2 macrophages by inhibiting CSF1/CSF1R signalling [192] seems to be even more promising. CSF1R inhibition using a neutralizing mAb (AFS98) showed a decrease in macrophage accumulation in atherosclerotic lesions of ApoE-deficient mice [193], in renal allografts [194] and damaged skeletal muscle [195]. Its effect on fibrosis is more disputed, as it may increase renal fibrosis [196–198] but may be beneficial in other fibrosis. Interestingly, a combination of CSF-1R inhibition using GW2580 with radiotherapy suppressed tumor growth more effectively than irradiation alone in a mouse prostate cancer model by TAM blockade, suggesting that CSF-1R inhibitor should enhance radiotherapy's differential effect [199].

4.6.2.2 Targeting Neutrophils, DCs and Other Immune cells

Neutrophils and DCs are also relevant to radiation injury. The recruitment of neutrophils at the injury site is important for removing tissue debris and killing invading pathogens. They also, however, secrete ROS/NOS that may exacerbate tissue damage and induce scarring [166]. Because of this, neutrophils have been described as either pro-fibrotic (bleomycin, hypersensitivity pneumonitis–induced fibrosis) [200] or anti-fibrotic via extracellular matrix clearance [201].

DCs are professional antigen-presenting cells (APCs) able to migrate into secondary lymphoid organs to activate T helper cells for pathogen control and clearance, but in pathological inflammation and autoimmune disease, DCs can contribute to local tissue injury [166]. Like neutrophils, the role of DCs in fibrosis is dual with high infiltration described in Hepatic and lung fibrosis [202, 203], but not in cardiac fibrosis [204].

Other innate immune cells, such as mast cells, eosinophils and basophils have also been implicated in the pathogenesis of fibrosis in multiple organ systems and are viewed as potential therapeutic targets. Indeed, mast cells have been described to promote fibrosis by recruiting inflammatory leukocytes and by producing pro-fibrotic mediators [205]. Eosinophils are important sources of TGF-β1 and IL-13 [206] and have been found to be associated with the development of pulmonary fibrosis [207], skin, liver and idiopathic retroperitoneal fibrosis [206, 208]. The role of basophils has not been explored in the context of radiation injury but they are an important source of type 2 cytokines such as IL-4– and/or IL-13.

4.7 Contribution of the Microbiome: An Emerging Contributor and a Possible Target?

There has not been much exploration, until recently, of the possible role of the microbiome in regulating susceptibility/resistance to radiotherapy. Yet, bacterial translocation induced by the disruption of the epithelial/mucosal barrier is one of the main consequences of radiotherapy. The gastrointestinal tract from oral

mucosa to rectum is an ideal model to study the contribution of the flora to normal tissue damage induced by radiation therapy and define possible innovative prevention and/or mitigating strategies [209]. The recent interest in flora is partly driven by technological advances, particularly metagenomic sequencing and marker gene-based phylotyping. These novel approaches have helped to understand that the microbiota is far more diverse than previously thought [210, 211]. The complex interactions that occur in between epithelial cells and microbiota is the guarantee for their respective homeostasis and constitute the hormesis concept. Host factors are known to influence the microbiota composition [212] and anticancer treatment, including radiotherapy, may alter this makeup. The alteration in the microbiota composition is named dysbiosis.

Recent studies have investigated the impact of radiation-induced damaging signals coming from host cells that can modulate microbiota composition. One of the primary effects of radiation therapy is ROS-mediated. The strong oxidative milieu generated upon irradiation interferes with many cellular functions, such as cell cycle progression and pro-apoptotic pathways. This causes ulceration that can be modulated using anti-oxidants including SOD, Amifostine and Vitamin E. The long-term breakage of the epithelial barrier is the first point of entry for bacteria and is mainly caused by the loss of adult stem cells. In the gut, the stem cell response is mediated by p53 activation, which in turn induces PUMA as a signal triggering progenitor and stem cell death via intrinsic apoptosis [213, 214]. But the mechanism is not as simplistic, because, at the same time, p53 induces p21, thereby facilitating cell-cycle arrest and DNA repair in progenitor cells, consequently increasing cell survival and tissue regeneration [215].

A direct role for the microbiota in regulating epithelial homeostasis has been described. In the gut, the regulation has been shown to be mediated through activation of Toll-like receptors [216]. The immuno-modulatory activity of the gut microbiome has been investigated by Zitvogel et al. who showed that gut flora elicited innate and adaptive immune responses [217]. Long-lasting dysbiosis has indeed been associated with cancer [218]; it may promote low-grade inflammation [219], and increase cell transformation [220]. Recent studies also suggest that the presence of crypt-associated flora bacteria could act as “gate keepers” and help in the protection against colonization by pathogenic bacteria, thus maintaining the homeostasis of the regenerative apparatus [221].

4.7.1 Therapeutic Modulation of the Microbiome

Studies focusing on the relevance of the microbiota to the pathogenesis of radiation-induced normal tissue complications are just emerging. Some bacteria, such as *Roseburia* or *Eubacterium,* seem to have beneficial effects by producing molecules such as butyrate. Whether it is the absolute composition or the relative changes in the microbiota that is relevant to understand and modulate the pathogenic process is another question. Some clinical studies profiled the intestinal [222–226] and the

oral [227–229] microbiome after radiotherapy. Andreyev et al. described gram-negative bacterial overgrowth in patients with radiation enteropathy, both in the acute and late settings [230, 231]. In a further study they assessed faecal microbial populations and reported an overall increase in *Bacilli* and *Actinobacteria*, and a decrease in *Clostridia* [227]. De Rick et al. measured shifts in the oral microbial community during radiotherapy with a decrease in the richness and presence of a small fraction of species. These shifts correlated with a poor functional outcome including pain and nutrition problems. However, these studies included only a small number of patients and were only associative, making it difficult to discern cause and effect. The use of probiotics has also been developed and preclinical studies with *Lactobacillus spp.* were able to partially treat proctitis in rats while preserving intestinal morphology [232, 233]. Similar clinical trials have been developed using *Lactobacillus spp.* as a probiotic treatment to mitigate gastrointestinal injury after radiation therapy. Prophylactic treatments also seem to be efficient [234, 235]. Nevertheless, no unique microorganism strain or product has been described in clinical trials and further studies are required to address this promising question.

4.8 Conclusion

The aim of modern targeted radiotherapy is to kill a maximum of cancer cells while reducing normal tissue injury and decreasing morbidity. To achieve that aim, selective protection of normal tissue function is a powerful approach to improve cure rates and simultaneously improve the quality of life of long-term cancer survivors. The development of complex models of radiation injury based upon the use of transgenic animals and targeted irradiation procedures with Image Guided Radiotherapy devices dedicated to small animals has lead to a better understanding of the normal tissue response to radiation injury. The complexity of the phenomena has been dissected and an interconnected series of processes has been deciphered. These series include inflammation, alteration of the vascularisation which leads to alternative sequences of perfusion and hypoxia within tissues, alteration of the immune cell composition and infiltration, remodeling of the extracellular matrix and tissue fibrosis that may ultimately lead to irreversible organ failure.

The therapeutic challenge is now driven by the complexity of radiation-induced processes. Combination strategies that target distinct pathogenic pathways with several "old" or existing molecules—such as the combination of anti-inflammatory agents, vascular protectors, antioxidants and immunomodulators—have given good pre-clinical and clinical results. However, dosage and administration sequences require a personalized and fine-tuned follow-up for each patient. More recent targeted therapies using specific pathway inhibitors or biological agents such as antibodies can now be foreseen as the next approach in modulating radiation injury. Lastly, fascinating clinical questions are being raised, aiming to study organ ecosystem and how it might be exploited in the future with direct and "natural" therapeutic agents to treat radiotherapy complications and restore the fine equilibrium altered by anti-cancer therapies.

Interestingly, the changes described at the normal tissue level also occur in the tumor's microenvironment. Numerous factors activated in response to irradiation in normal tissue such as TGF-β, CTGF and PDGF, cytokines, TNF-α and Interleukins, are similarly altering cellular phenotype in tumors *i.e.* CAFs display myofibroblastic differentiation; TAM display M2 polarization. Consequently, the next challenge will be to develop rational radiotherapy-drug combinations to target tumors and avoid normal tissue toxicity.

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Chapter 5
Technology Based Strategies to Enhance the Therapeutic Ratio

David V. Fried and Shiva K. Das

Abstract The general purpose of technology is to enable the accomplishment of new objectives or make current objectives easier and more attainable. In radiation oncology, the ultimate objective is to maximize tumor control while minimizing normal tissue complication (i.e., optimizing the therapeutic ratio). Advances in modern technology have continued to push the envelope in terms of maximizing the therapeutic ratio for radiation oncology patients through a variety of means.

Keywords Technology • Radiation • Therapeutic ratio

5.1 Introduction

The general purpose of technology is to enable the accomplishment of new objectives or make current objectives easier and more attainable. In radiation oncology, the ultimate objective is to maximize tumor control while minimizing normal tissue complication (i.e., optimizing the therapeutic ratio). Advances in modern technology have continued to push the envelope in terms of maximizing the therapeutic ratio for radiation oncology patients through a variety of means.

Treating cancer with radiation is a complex process and its effectiveness is dependent on an assortment of factors. Accurate ***localization/characterization*** of disease and patient anatomy is paramount if we are to deliver the best possible treatment. Underestimation of disease burden can enhance the probability of disease recurrence while overestimation can lead to excessive radiation dose to normal tissue and subsequent increases in probability and severity of toxicity. Once the location of disease is determined, **mitigation** of uncertainty involved in localization such as variations in patient setup, respiratory motion, and changes in anatomy are necessary to maintain treatment quality. Failure to account for or minimize uncertainty will also result in increased probability of recurrence and/or excessive radiation dose to normal tis-

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sue. Finally, **optimization** of dosimetry is required to ensure fulfillment of the dose prescription to the target(s) while minimizing the dose to the surrounding normal tissues. This chapter briefly reviews advances in routinely used and cutting edge technologies that are utilized in radiation oncology to improve patient treatment. Technologic improvements have directly increased our ability to maximize the therapeutic ratio by allowing for accurate localization of disease, mitigation of uncertainties, and optimization of treatment.

5.2 Localization/Characterization Technologies

The evolution of radiation oncology has always been inextricably linked to technological developments in imaging. The mainstay of radiation oncology imaging has traditionally been X-ray based modalities such as planar radiography and computed tomography (CT). The invention and eventual clinical implementation of magnetic resonance imaging (MRI) and dual-modality positron emission tomography (PET/CT and PET/MRI) systems have greatly improved radiation oncologists' ability to accurately localize and potentially further characterize patient disease in a variety of settings. These imaging modalities are increasingly being used in combination with treatment simulation CT scans for tumor delineation. The addition of these modalities allow for better tumor contrast and facilitates more accurate and reproducible contours for radiotherapy treatment alongside more accurate initial staging at time of diagnosis which dictates treatment [1–11]. In addition, functional imaging modalities such as PET and MRI are the subject of ongoing research to identify tumor sub volumes that may be candidates for dose escalation or enhancement of tumor characterization to enable more individualized treatments [12–16].

5.2.1 PET/CT

The basic process behind PET is that an intravenously injected radiopharmaceutical distributes within patient tissue according to the properties of the radiolabeled carrier molecule. The radiopharmaceutical emits positrons which interact with free electrons resulting in the production of two 511-KeV annihilation photons at approximately 180° with respect to one another. Due to this known geometry of annihilation emission, spatial and temporal detection of the photons emitted from the body can be used to localize the source of the annihilation event and subsequently be used in image reconstruction (see Fig. 5.1).

PET imaging most commonly utilizes ^{18}F-labeled fluoro-2-deoxyglucose (FDG) as the injected radiopharmaceutical. This glucose analog is formed by replacing the 2′ hydroxyl group in glucose with a flourine-18 atom. Whereas normal glucose enters glycolysis, FDG cannot proceed through glycolysis due to the previously

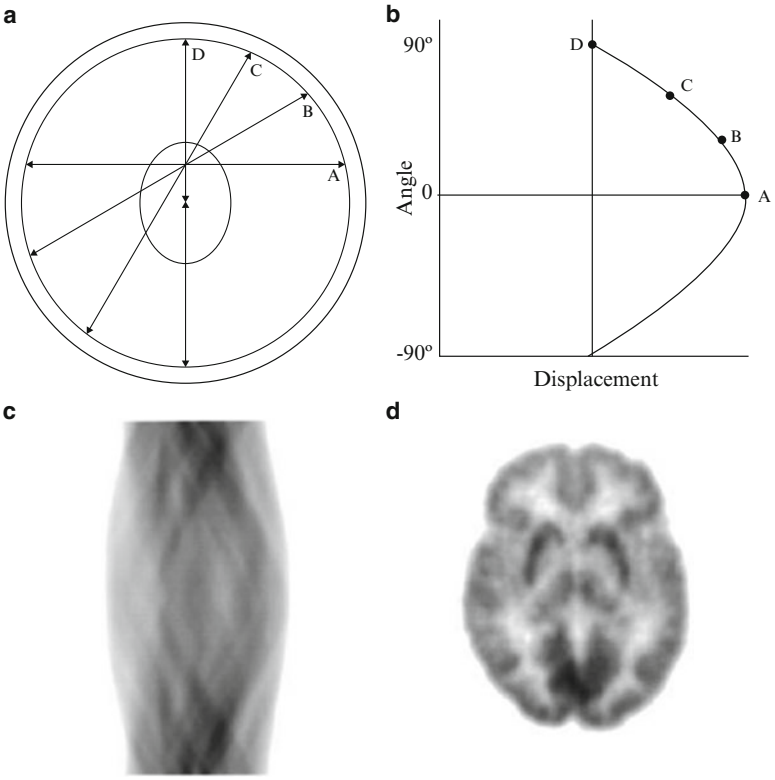

Fig. 5.1 (**a**) Lines of response due to the known geometry of positron annihilation. (**b**) Generation of sonogram using angle of detection and displacement from the center of the gantry. (**c**) Example sinogram. (**d**) Generated image [17]

mentioned substitution and remains temporarily trapped intracellularly as FDG-6-phosphate. Tumor cells are highly metabolically active and have increased numbers of GLUT-1 and GLUT-3 transporters that lead to substantial glucose demand. Therefore, tumors cells uptake and retain high levels of FDG compared to normal tissues making FDG an ideal multipurpose tracer for oncologic applications. However, FDG is not a cancer specific molecule and therefore will be retained in normal tissues with high glucose demand (e.g. brain, left ventricle, etc.) as well as areas of infection, inflammation, or high metabolic activity (see Fig. 5.2). This can lead to false positive diagnoses if the images are not read properly with adequate knowledge and experience in these issues.

The PET and CT portions of the scanner are serially connected such that a patient can have a CT obtained followed by a PET scan without having to change positions. The inherent fusion of these two images provides both anatomic and metabolic information simultaneously. Technological advances in PET/CT imaging have improved the quality of the resulting images and hence made them more capable of improving target localization and detection.

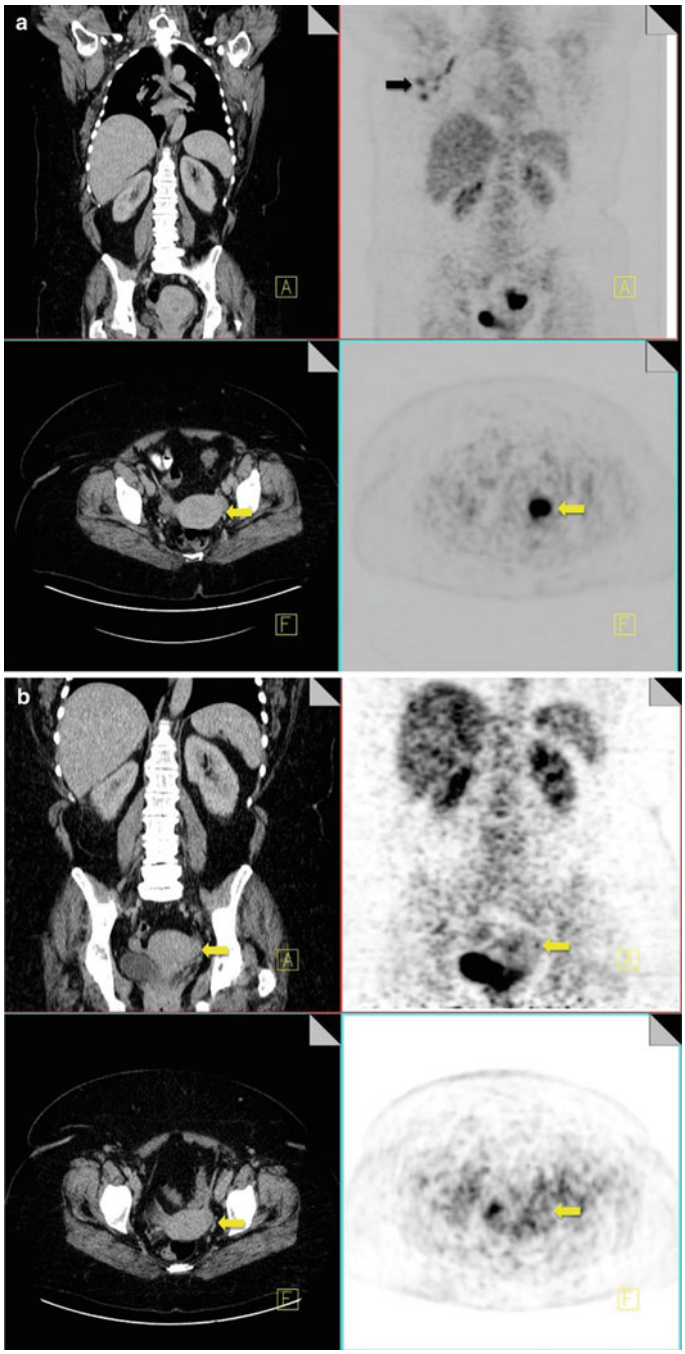

Fig. 5.2 FDG PET/CT scan of patient with inflammatory breast cancer. Increased uptake observed within endometrial canal due to menstruation (*top* four images). Same patient when not menstruating (*bottom* four images) does not demonstrate any increase in uptake [18]

Advances in both PET and CT hardware have increased the quality and speed/efficiency of imaging. Incorporation of spiral CT technology has progressed from single slice to current multi-slice models along with decreases in CT rotation times. PET detectors have incorporated new fast scintillators such as gadolinium oxyorthosilicate (GSO) and lutetium oxyorthosilicate (LSO) along with faster electronic acquisition hardware and higher resolution detectors [19]. Newer scanners are also beginning to incorporate extended field of view in their systems which allow more efficient detection of annihilation photons [20, 21]. Increases in detection efficiency allow for the development of protocols to optimize the tradeoff between scan time and coincident counts (i.e., signal to noise). Higher efficiency scanners allow a patient to be scanned for a shorter period of time with the same number of detected counts or over the same time with an increase in detected counts. Major software advances have also played an important role. The transition from 2D to 3D reconstruction, time-of-flight capability (software and hardware), and the use of point spread function in reconstruction have significantly impacted image quality and signal to noise ratio [22, 23]. Time-of-flight utilizes fast responding detectors in order to determine the region along the line of response the annihilation event occurred. This allows for a higher degree of confidence regarding where the annihilation event (and thus the FDG activity) is located within the body and therefore improves reconstruction. Point spread functions quantitatively relate the distribution of tracer to the measured counts and can yield improvements in image quality, accuracy, and contrast. These technological improvements have led to standardized uptake measurements (SUV) from PET to be more quantitative and therefore more useful for diagnosis and monitoring of disease.

The use of PET enables true metabolic imaging on a molecular level and has been used routinely in oncology. PET has been shown to be complimentary to CT in terms of patient staging and disease localization and has led to the combination of the two technologies into a single scanner (PET/CT). PET/CT has demonstrates significant improvements in staging/localization in a variety of cancer types (e.g. lung, head and neck, cervix, melanoma, etc.) [1, 3–10, 24, 25]. A prime example of how this technology can improve the therapeutic ratio for patients is in lung cancer. Lung tumors are frequently accompanied by normal lung collapse as seen in Fig. 5.3 making it difficult to discern the location of the primary disease based solely on CT. FDG-PET allows for better distinction between tumor and normal tissues that facilitates more effective localization and subsequent treatment dosimetry.

FDG-PET/CT nodal surveillance (i.e., at follow-ups post treatment) in patients with head and neck cancer has been shown to yield similar overall survival rates compared to patients undergoing planned neck dissection [10]. Accurate localization of potential disease during follow-up using FDG-PET/CT could therefore be used to reduce the rates of unnecessary surgery and its associated complications.

The use of radiotracers aside from FDG is also an area of technological advancement. PET imaging is not limited to quantification of tumor metabolism. There have been developments of tracers capable of measuring hypoxia ([^{18}F]-FMISO, [^{18}F]-HX4, [^{64}Cu]-ATSM) and cell proliferation ([^{18}F]-FLT) [27, 28]. Trials are

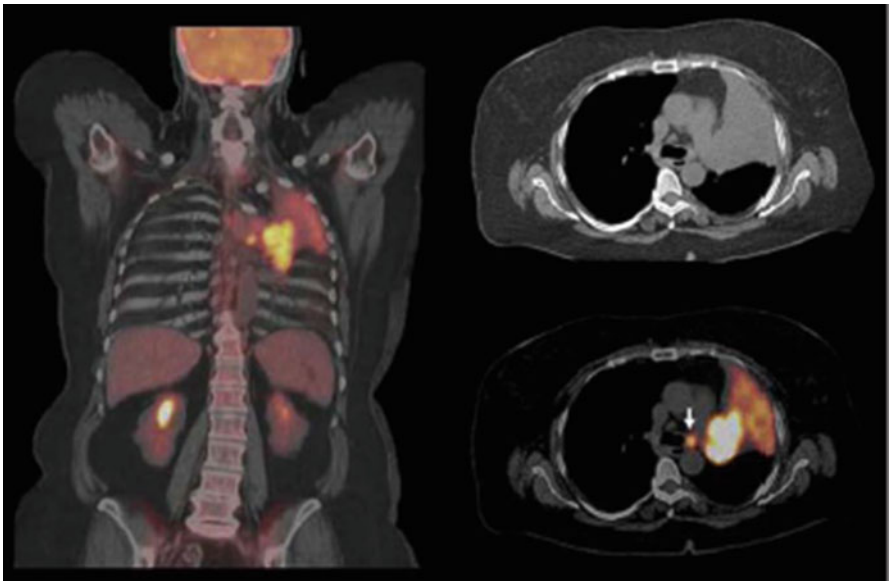

Fig. 5.3 Lung tumor with associated atelectasis [26]

ongoing that incorporate these tracers in patient diagnosis, phenotyping, and response. Recently, the advent of ^{89}Zr-based agents made it possible for the "immunoPET" or antibody-based PET imaging [29]. Analysis of these tracers are ongoing and very much developmental.

PET/CT has provided tremendous value in oncology for localizing patient disease for diagnosis, treatment planning, and surveillance. Clinically implemented scanners have shown in a large study of cancer patients across disease sites that PET leads physicians to change their intended management in 36.5 % of patients [30]. Advances in PET/CT hardware, software, and novel radiotracers will continue to improve the therapeutic ratio for cancer patients during diagnosis, treatment, and follow-up.

5.2.2 PET/MRI

Similar to PET/CT, PET/MRI is the combination of both PET and MRI systems in a single device. This technology is new with only around 70 systems installed worldwide [31]. There are several theoretical advantages to PET/MRI over PET/CT such as high soft tissue contrast, multi-modality functional imaging (both PET and MRI), and lower patient dose due to MRI being non-ionizing. However, with any new technology there are several drawbacks such as cost, lack of data regarding improvement over PET/CT, potential geometric distortions, and difficulty providing accurate data

for attenuation correction. It is difficult to assess how PET/MRI can improve patient care given its novelty and currently prohibitive cost (PET/MRI costs approximately 2–3 times more than PET/CT system). The capabilities of PET/MRI are similar to PET/CT in terms of its ability to combine quantitative functional imaging alongside anatomic imaging. However, an important distinction is that PET/MRI can perform multi-modality functional imaging. A majority of studies involving PET/MRI have focused on using MRI as a CT surrogate (i.e. providing anatomic information alone) and not investigating potential synergy of function modalities between PET and MRI images. Preliminary data do not suggest that PET/MRI (anatomic) is superior to PET/CT in terms of general disease localization [31]. It has been hypothesized that PET/MRI may be advantageous in malignancies of the head and neck. MRI and PET have been clinically used for sinonasal and skull base lesions and now these images could be generated simultaneously in one imaging session which would be more convenient for patients and obviate the need for registration between the two images. Further, PET/MRI may have benefits over PET/CT in lesions of the head and neck due to higher soft tissue contrast and metal artifact reduction (see Fig. 5.4) which may lead to more accurate tumor staging and contouring at the primary site of disease. It should be noted that it would be difficult for any technology to improve on

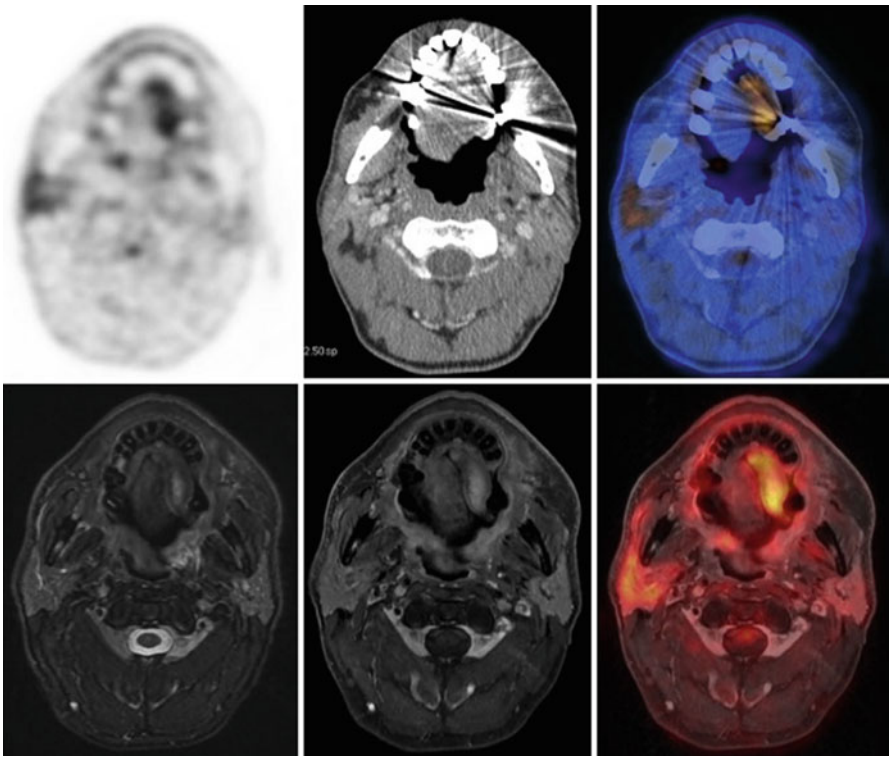

Fig. 5.4 Base of tongue cancer on PET/CT (*top*) versus PET/MRI (*bottom*) [17]

the high rates of sensitivity and specificity provided by PET/CT systems. Therefore, it is not overly surprising that PET/MRI has not demonstrated a dramatic improvement in this area as there is not a tremendous amount of room for improvement.

The future of this promising technology rests on its ability to improve the diagnosis and phenotyping of cancer. For instance, simultaneous acquisition of FDG-PET and DCE (dynamic contrast enhancement) and/or DWI (diffusion weighted imaging) images may be more prognostic in predicting tumor aggressiveness, radioresistance, and patient outcome. The use of PET/MRI in developing personalization of therapy for cancer patients needs to be investigating in the future. Currently, there has not been sufficient evaluation of PET/MRI as a distinct technology providing unsurpassed functional information rather than a PET/CT replacement.

5.2.3 Magnetic Resonance Imaging (MRI)

MRI is a non-ionizing imaging technique that uses high field strength magnets to generate images of patient anatomy with exquisite soft tissue contrast. MRI uses radiofrequency (RF) excitation pulses alongside multiple magnetic gradients in order to measure emitted RF energy from the patient. These signals can be processed to determine anatomic location and, depending on the pulse sequence, influence the contras of the resulting image. T1 and T2 MRI images are the primary contrast mechanisms used in diagnostic imaging and radiation oncology applications. T1-weighted images are used for general anatomic imaging while T2-weighted images are more indicative of biological characteristics of tissue and hence are used for extracting pathologic information. These standard sequences are used routinely for localization/delineation of lesions in the central nervous system (CNS). Diagnostic MRIs (with and/or without IV contrast) are frequently fused to simulation CT scans in order to assist in target delineation for radiotherapy. Figure 5.5 demonstrates images that can be obtained using CT and different MRI sequences.

This is particularly true for stereotactic radiosurgery (SRS) treatments where the use of high dose gradients necessitate extremely accurate target localization. The use of MRI is being incorporated more in radiation treatment planning for head and neck, prostate, gynecologic, and liver malignancies [33]. These sites in particular utilize MRI imaging in treatment planning as they rely heavily on contrast between the tumor(s) and surrounding normal tissue to determine appropriate planning target volumes. MRI also enhances the ability to determine tumor extension in comparison to CT scans which can alter patient tumor staging and subsequent treatment. Figure 5.6 demonstrates invasion from a base of skull lesion that can be seen using MRI but is not visible on CT.

Recent advances in functional MRI (fMRI) techniques have been evaluated in a variety of tumor sites. Dynamic Contrast Enhanced (DCE) MRI captures sequential images of contrast uptake (predominantly gadolinium-based) in order to characterize tumor tissue perfusion and blood volume. Contrast wash-in and wash-out can be analyzed using regions of interest or on a voxel-voxel basis using a parameters called Ktrans. Modeling can also be performed to identify parameters of physio-

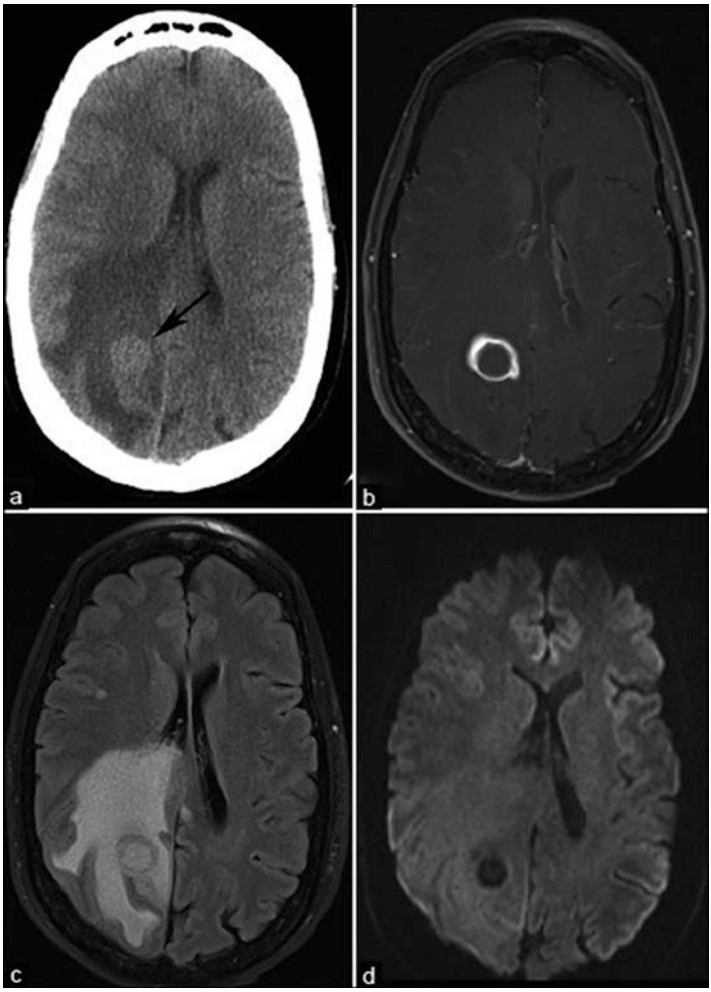

Fig. 5.5 (**a**) CT of brain lesions. (**b**) Post-contrast T1 MRI. (**c**). FLAIR MRI. (**d**) DWI MRI [32]

logic function. This technique has shown to have diagnostic and prognostic value in various tumors and has demonstrated usefulness in assessing response [35].

Diffusion weighted imaging (DWI) is another fMRI technique that is capable of generating images of water molecule mobility. Two phase shift pulses are used to measure phase differences that are dependent on the mobility of the water molecules. The apparent diffusion coefficient (ADC) is measured in mm^2/s and is the quantitative measure used in DW-MRI. Figure 5.7 demonstrates how ADC and Ktrans maps can be useful in assessing response to treatment. This imaging technique takes advantage of the fact that water mobility and tissue cellularity are inversely related. Therefore, in highly cellular tissue (i.e. tumors) the movement of water is quite restricted whereas in necrotic tissue or tissue undergoing apoptosis water is less restricted yielding increasing ADC values. Clinically, DW-MRI has

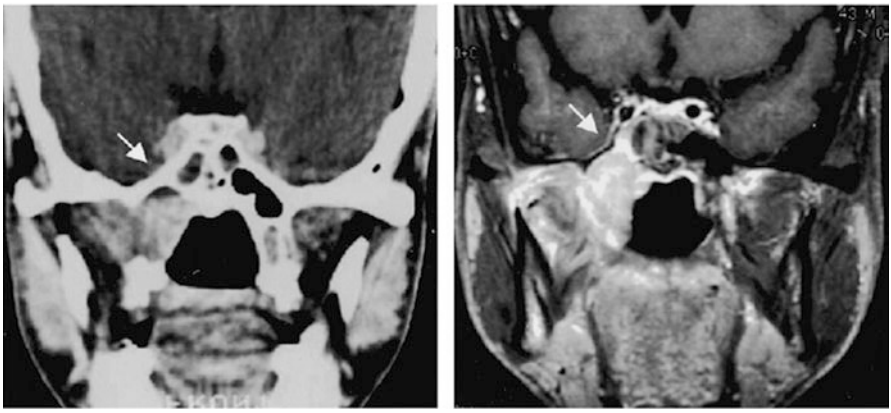

Fig. 5.6 Extension into right cavernous sinus is shown by T1 contrast enhanced MRI (*right*) but not appreciated on CT (*left*) [34]

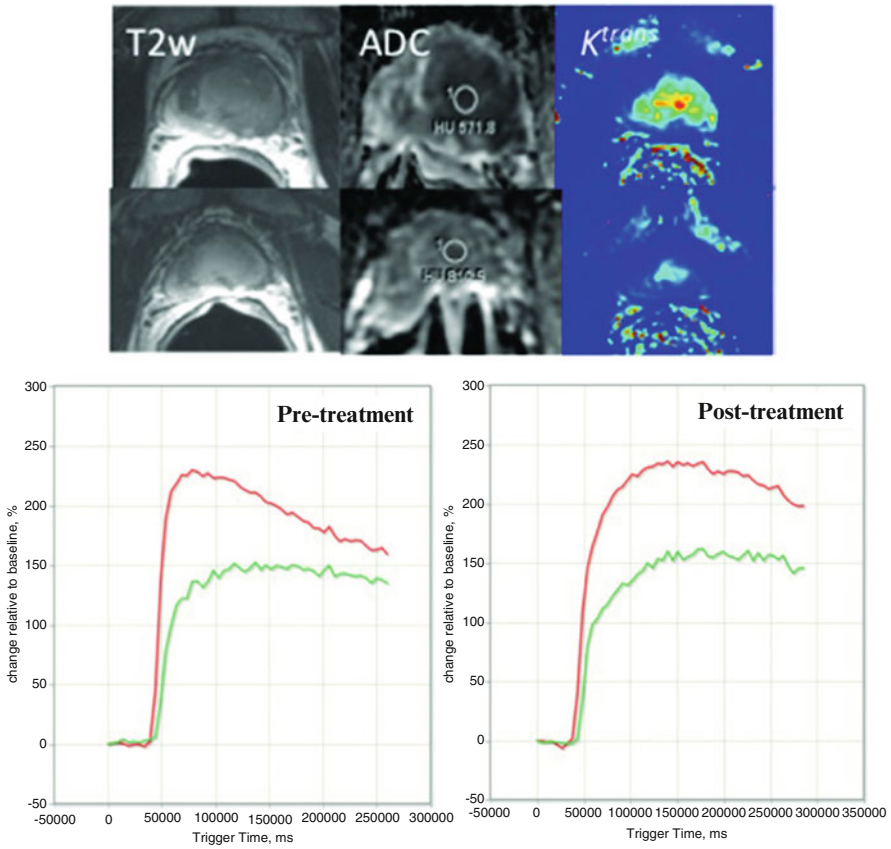

Fig. 5.7 T2, ADC, and Ktrans images of prostate pre and post treatment [39]

been investigated predominantly in assessing tumor response to treatment [36–38]. Additional characterization of tumors or early detection of recurrence may allow for escalation of dose to sub-volumes or more successful salvage treatments.

Other advancements in MRI such as magnetic resonance spectroscopy and hyper polarization are continuing to evolve through academic research but have not yet been able to demonstrate the feasibility and efficacy for widespread clinical adaptation. These imaging methods may continue to add to the ability of MRI to localize and characterize malignancy in the future. However, localization is not only important for tumor tissue but also normal tissue. Recent use of hyperpolarized helium-3 gas has been shown to provide 3D imaging of normal lung function [40]. This may pave the way for more personalized treatment in lung cancers where radiation dose to areas of highly functional lung can be reduced. In addition, MRI has been found to be able to quantitatively assess damage to irradiated parotid glands in patient undergoing treatment for head and neck malignancies (see Fig. 5.8).

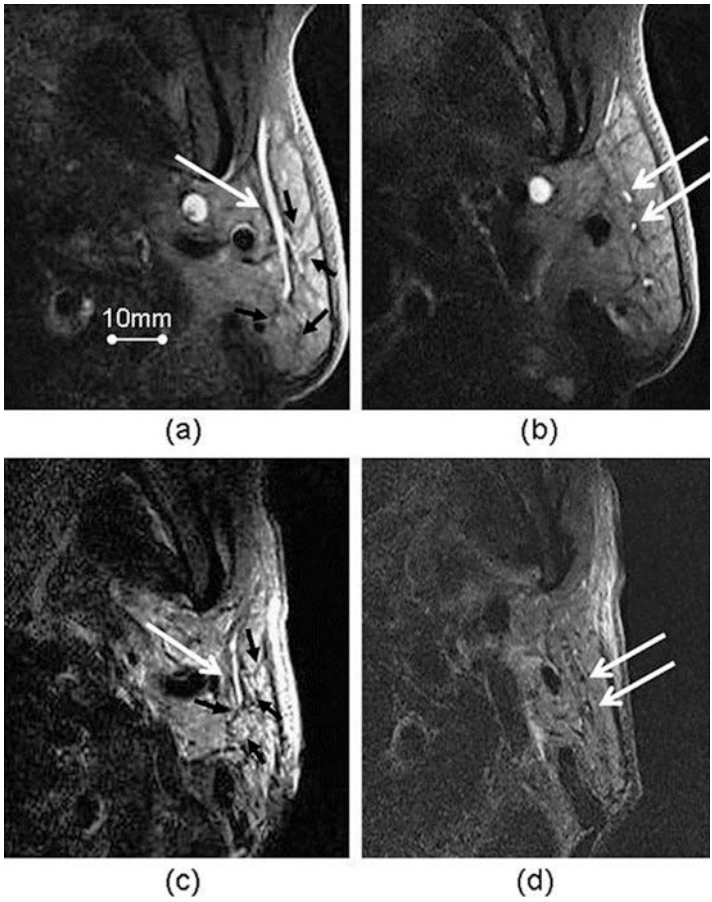

Fig. 5.8 MRI evaluation of parotid gland prior to RT (*top*) and post RT (*bottom*), showing reduction in cross-sectional volume and duct width [41]

MRI has demonstrated the ability to improve the therapeutic ratio by providing exquisite soft tissue contrast in the CNS alongside other sites. The strength of MRI outside of soft tissue contrast is its versatility in image generation using different contrast and functional techniques in a manner that is non-ionizing [33]. Future research will likely focus on using MRI for improved characterization of patient disease in addition to its ability to produce impressive anatomical images.

5.3 Mitigation Technologies

Accurate localization of gross disease is the foundation of any effective radiotherapy treatment. As previously discussed there are ever advancing imaging systems such as MRI, PET/CT, and PET/MRI that provide valuable information in addition to diagnostic and simulation CT scans to aid clinicians in target delineation. However, these technologies only go so far as to tell you where the disease is located in a single instance or averaging of instances during the imaging session. For actual patient variations over the course of treatment (inter-fraction) or during any single treatment (intra-fraction), the location of the target may remain constant, shifted due to variations in normal tissue anatomy, shifted due to alterations in patient positioning/setup, or constantly moving due to respiratory and/or cardiac motion compared to the location seen during the diagnostic imaging procedure or simulation CT. Mitigation of location uncertainty can be divided into corrections for anatomic/setup variation (predominantly inter-fractional changes) and motion management (predominantly intra-fractional changes). These two sources of uncertainty are particularly important because of the frequency with which modern treatment delivery utilizes high dose gradients to maximally deliver dose to the target(s) while minimizing dose to normal tissues. In the presence of high dose gradients, small errors in localization can lead to large differences in planned versus delivered dosimetry. Therefore, mitigation of localization uncertainty is a key component of maximizing the therapeutic ratio and has led to an emphasis on providing image guided radiation therapy (IGRT).

5.3.1 Mitigation of Anatomic/Setup Variation (Inter-Fraction Changes)

Accurate and reproducible patient setup is a hallmark of delivering optimal radiotherapy treatment. Traditionally, comparison of physician delineated field openings and blocks on planar X-rays were compared to megavoltage (MV) portal films from the linear accelerator to ensure adequate setup. As technology has improved, setup can be verified in 3-dimensions using CT-on-rails or cone beam CT (CBCT) with much higher soft tissue contrast compared to portal films. With modern treatment utilizing increasingly more beams, new imaging that is complementary to tradition portal films are needed to ensure appropriate radiation delivery.

5.3.1.1 CT-on-Rails

The need for mitigation of anatomic/setup variation is that the patient's tumor and/or normal tissue may shift between the CT simulation used for treatment planning and the actual treatment. An intuitive solution would be to perform a simulation scan prior to treatment delivery in order to determine if the geometry from the simulation scan still holds. However, the patient would have to be setup on the CT scanners, scanned, moved back to the linear accelerator (LINAC) for treatment, and be re-setup. This whole process would not be ideal as significant changes in tumor position and patient anatomy could occur during the patient transfer. Traditional megavoltage portal fields using the treatment beam are important in patient setup but are limited in that they are planar and lack significant soft tissue contrast due to being generated from megavoltage energy beams that make it difficult to visualize both the tumor and normal tissue. Therefore, the idea of putting a diagnostic quality CT scanner in the LINAC vault such that the patient could be imaged and treated on the same couch (i.e. CT-on-rails) was developed. Figure 5.9 illustrates a CT on rails system.

CT-on-rails can utilize a horizontal isocenter plane where the isocenter of the LINAC and CT imaging isocenter are in-line (common isocenter method). Alternatively, external markers can be placed that will be visible on the patient's CT and then these markers are used to re-align the patient to the LINAC isocenter after imaging (daily isocenter method). These two techniques allow for the patient to be setup for treatment on the couch and remain stationary while the CT traverses the patient. Contrary to diagnostic CT scans where the patient is translated through the CT bore using the table, the CT-on-rails system translates the entire CT scanner using rails embedded within the treatment floor. Accurate and consistent positioning and movement of the CT scanner is required in order to generate correct reconstructions without artifacts.

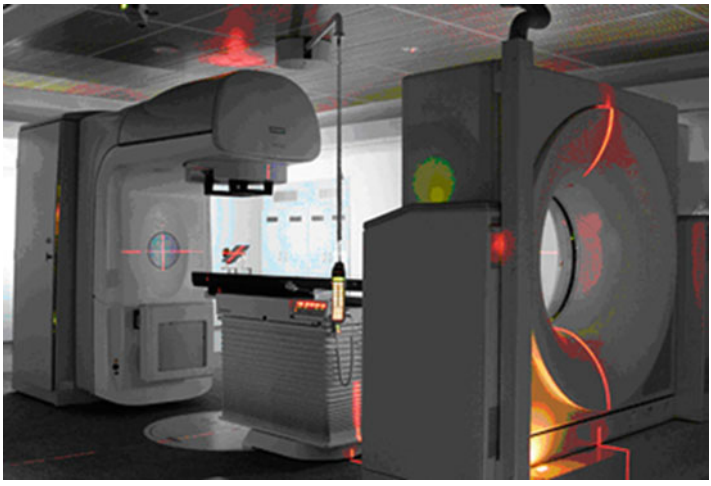

Fig. 5.9 CT on rails combined with a LINAC [42]

The functionality of the CT-on-rails system requires the couch to have two axes of rotation; one axis to rotate the table between the LINAC and CT and another for treatment isocenter. The mechanical precision of all aspects of the system are extremely important as instability will introduce errors in image reconstruction, positioning of the patient on the couch, and corrections applied for patient treatment. This is particularly true for the common isocenter method since this relies on the imaging and treatment isocenters to be perfectly in-line.

There are many demonstrated benefits to having diagnostic quality CT scans at time of treatment with the patient in the treatment position. Most notably, this technology enables truly adaptive planning. Conventionally, the CT simulation scan provides the framework for treatment planning that is assumed to hold for the duration of treatment. CT-on-rails can provide daily CT scans which makes it possible to re-plan at each patient treatment or at fixed intervals during a patient's treatment.

This has been shown extensively in prostate cancer where re-planning from CT-on-rails images has proven the ability to reduce the dose to rectum and bladder and quantify target shifts for establishing appropriate treatment margins [43–48]. CT-on-rails has also shown to be useful in adaptive therapy for head and neck malignancies in terms of re-planning as well as generating predictive models for tumor shrinkage and optimal tumor margins [49–51].

CT-on-rails is a fusion of technologies in order to lessen the challenge of dealing with anatomic/setup variation. There are advantages of having diagnostic quality imaging in the treatment vault, however there are also considerable drawbacks. There are high initial upfront costs in purchasing a unit and building a vault that is capable of accommodating the system. Additionally, the CT delivers additional dose to the patient and adds extra time to the treatment. As with most technology, CT-on-rails has proven benefits but also some drawbacks impeding widespread use.

5.3.1.2 kV Cone Beam CT

The goal of having 3D daily patient imaging at each treatment was not fully solved with the advent of CT-on-rails system due to the complexity and cost of installation and use. The incorporation of CT has not only been done using a separate scanner but using a cone-beam X-ray tube and imaging panel attached to the LINAC gantry (i.e. cone beam CT [CBCT]). This setup can also be used to generate kilovoltage portal films as well as fluoroscopic images. While these technologies are clinically available and have been implemented, they will not be the focus of this section. The kV source and imaging panel are affixed to the gantry in a 90° orientation to the treatment beam. Figure 5.10 illustrates the configurations as used by Varian and Elekta LINACs. By using the LINAC gantry, the X-ray tube and imager can rotate entirely around the patient in a similar manner to a diagnostic CT scanner. The placement of both the kV source and imager are paramount for CBCT as accurate geometry of the system is required for optimal reconstruction. In addition, it must be routinely checked that the imaging isocenter and treatment isocenter are coincident. However, the X-ray beam, detector(s), and reconstruction differ significantly from diagnostic scanners. As the name implies, CBCT scans are generated using an

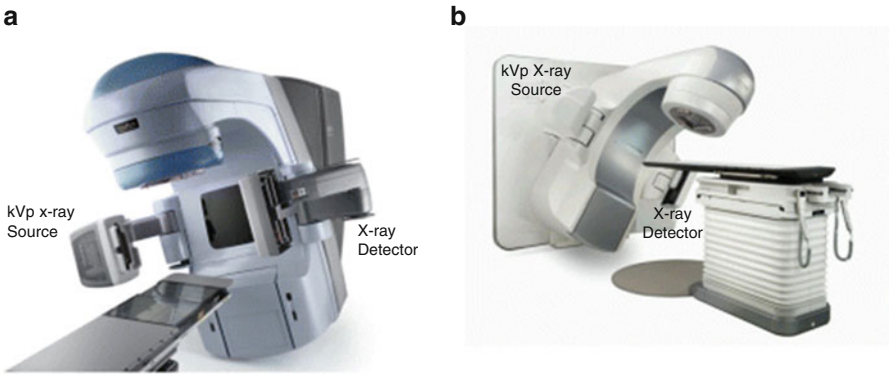

Fig. 5.10 kV X-ray source and detector on (**a**) Varian and (**b**) Elekta systems [52]

X-ray tube emitting a "cone" of X-rays through the patient, whereas diagnostic scans emit a fan shaped beam collimated to a particular axial width. The X-rays are detected by a 2D panel of detectors for cone beam and an arc shaped array of detector rows for diagnostic CT. Reconstructions using back projection are available for both modalities. However, there are differences due to the different geometry of X-ray emission and detection. Iterative reconstruction methods are currently being evaluated for both CBCT and diagnostic CT applications.

The real power of CBCT is its integration into the routine treatment workflow. Unlike CT-on-rails, the CBCT can be generated without any major alterations to the treatment vault except for having a LINAC that is CBCT capable. The patient does not have to be rotated from the LINAC to the CT scanner since the LINAC/CBCT are an integrated system. For CBCT, the patient is setup as they normally would for treatment. The gantry rotates around the patient with the kV source emitting the cone of X-rays that pass through the patient and are detected by the kV image panel on the opposite side. The image is reconstructed and the CBCT is aligned in all three dimension with the reference simulation CT (see Fig. 5.11). This alignment is performed using a variety of tools such as: automated software registrations, bony anatomy, regions-of-interest, and color overlays.

Once shifts required to align the images are computed, the patient table is then shifted by the corresponding amount and the CBCT arms retracted (if need be) in order to deliver the treatment. Using CBCT to ensure accurate setup is performed routinely for treatment of the thorax, liver, brain, head and neck, stereotactic body, and radiosurgery cases. Geometric accuracy of CBCT is less than 1 mm and therefore facilitates highly accurate evaluations of positioning.

CBCT can also be useful when using a traditional 2D simulator that is CBCT capable for brachytherapy treatment planning. This enables 3D treatment planning without having to move the patient between imaging and treatment procedures which minimizes applicator motion. While CBCT has multiple advantages over CT-on-rails, it does not deliver diagnostic quality images due to the nature of the system and reconstruction. CBCT suffers from more noise and potentially more artifacts being present in the images compared with diagnostic CT. Larger patients can be particularly problematic due to increased patient scatter leading to lower quality images.

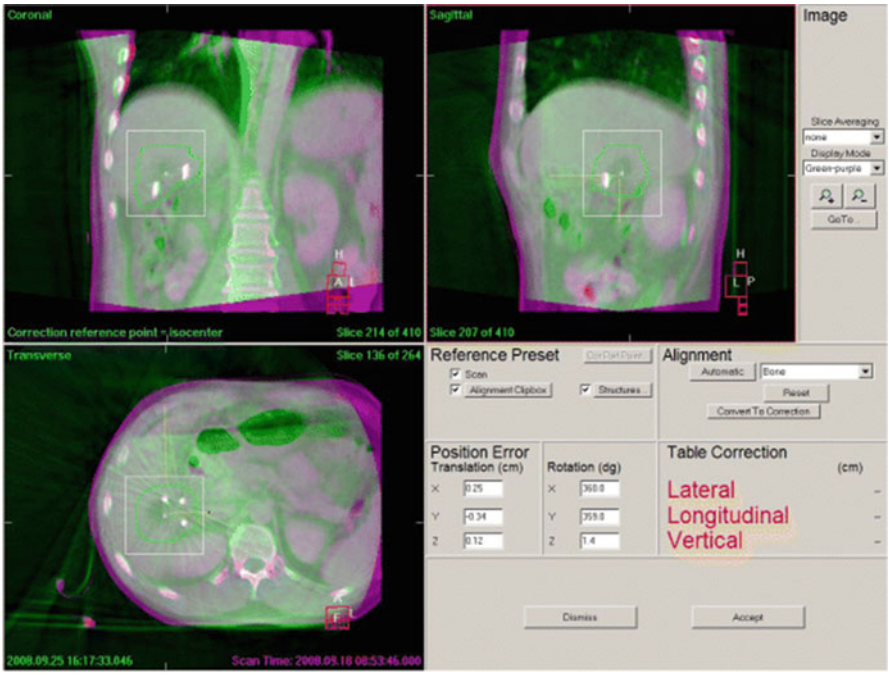

Fig. 5.11 Registration of daily cone beam CT to simulation CT [53]

CBCT also does not have the quantitative accuracy of diagnostic CT in terms of calculating Hounsfield units. In brachytherapy applications, the current TG-43 dose calculation method assumes constant tissue density and therefore the Hounsfield inaccuracies of CBCT are of no consequence.

CBCT has become a staple of modern radiation therapy practice that heavily relies on IGRT. New investigations in treatment delivery such as upright treatment have also led to the investigation/feasibility of upright CBCT [54]. Inter-fraction monitoring of patient setup and anatomic changes using CT-on-rails or CBCT are necessary when it is desired to deliver highly conformal radiation therapy appropriately. The ability of both technologies to ascertain information regarding reproducibility of patient setup alongside changes in internal anatomy allows for optimal delivery of treatment to ensure adequate coverage of targets alongside optimal sparing of normal tissue.

5.3.1.3 Vision-RT

Both CT-on-rails and CBCT can be used to mitigate changes in patient anatomy along with changes in patient setup. VisionRT's AlignRT platform is another technology used to monitor patient setup and does so without the use of ionizing

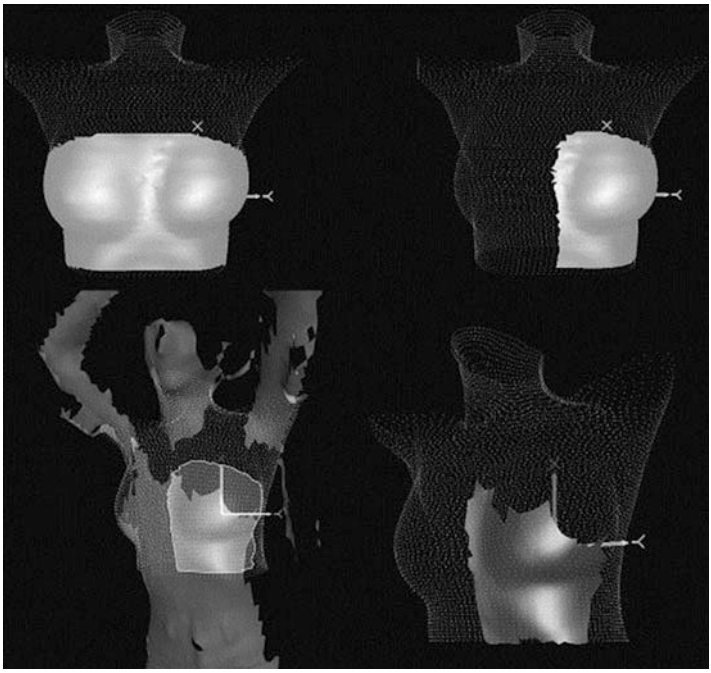

Fig. 5.12 Surface rendering for left sided breast cancer patient [55]

radiation or markers on the patient's skin. The AlignRT system uses 3 in-room cameras to create a surface rendering of the patient similar to what is shown in Fig. 5.12. Clinicians can define which region(s) of the patient's surface to monitor prior to delivery. This surface map is then continually checked against the simulation scan over the course of the treatment. Real-time positional errors in the 6 degrees of freedom (3 translational and 3 rotational) are displayed during setup and treatment on monitors on the inside and outside of the treatment vault. Continuous monitoring and error display allow treatment therapist to ensure that the patient not only has a quality initial setup but maintains proper positioning and posture during the course of the radiation delivery.

AlignRT has the versatility to improve patient setup in a variety of treatment sites. A study by Krengli et al. found high agreement (correlation range = 0.77–0.92) between AlignRT measurements versus electronic portal imaging device (EPID) measurements for prostate cancer patients [56]. For extremity sarcomas, Gierga et al. found that AlignRT may reduce setup errors and may complement daily imaging in patient setup to ensure motion does not exceed typical PTV margins [57]. AlignRT has even been investigated for enabling frameless and maskless stereotactic radiosurgery treatment. Cervino et al. found that setup using AlignRT resulted in average shifts from CBCT of 1.85 mm anterior-posterior and less than 1.0 mm in the lateral and superior-inferior directions [58]. However, the authors did state that an additional

degree of semi-rigid immobilization would be helpful for patient that feel asleep or had involuntary movement. AlignRT has most often been investigated for aiding in deep inspiration breath hold (DIBH) for breast cancer patients. This is discussed further in the subsequent gating/breath-hold section.

The complementary nature of AlignRT alongside clinically routine imaging can improve patient setup and subsequently the quality and consistency of radiation delivery.

5.3.2 Mitigation of Motion (Intra-Fraction Changes)

As described previously, patient setup is crucial for optimal radiotherapy delivery. However, it has been recognized that even perfect setup and monitoring of anatomy with each treatment is not necessarily sufficient in some situations. New LINACs are capable of delivering millimeter and even sub-millimeter accuracy according to phantom studies. However, this accuracy is usually measured on a static phantom and is inconsequential for moving targets. Patients are not static objects. Issues such as conscious movement can be mitigated by technology dealing with setup uncertainty and involuntary movement (e.g. movement associated with digestion) can be mitigated by daily imaging. However, movement such as respiration and cardiac motion are entirely separate issues and must be mitigated using different methods.

5.3.2.1 4D-CT

CT simulation scans are the basis of 3D radiation treatment planning. CT scans provide localization of patient disease and normal tissue organs in three dimensional space along with the Hounsfield unit information (and subsequently electron density) needed to calculate radiation attenuation. For a majority of CT simulations, patients are breathing normally when they undergo their scan. The resulting images are reflective of a random phase of breathing (i.e., could be at any point during inspiration or expiration) or an averaging of breathing motion phases depending on the scan duration and speed of motion. While this may not be problematic for lesions located in areas that are not significantly influenced by respiratory motion, it is potentially problematic for lesions in the thorax/abdomen (e.g. lung, esophagus, liver, chestwall, etc.). These lesions can potentially move multiple centimeters during respiration due to the expansion/contraction of the lungs and diaphragm. This motion may not be appreciable on a routine (non-4D) CT scans as the tumor may appear blurred or appear shorter/longer along the axis of motion. Additionally, the tumor may appear static if the motion period is slow compared to the scan time. For instance if a patient with a very slow respiration rate is being scanned using a modern multi-slice CT scanner, the resulting images may not demonstrate any evidence of motion such as blurring and/or distortion. The need to visualize tumor motion led

to the advent of 4D-CT which is now routinely used for simulation of any tumor suspected to have substantial motion or when precise targeting is required.

4D-CT in principle is exactly the same as routine CT. The major difference involved in 4D-CT is that the patient is over-sampled along the axis of motion (long axis). Repeat images are taken over the same region of the patient as to capture the entirety of the respiratory and/or cardiac cycle. These images are tagged using a surrogate signal for respiration. This signal can be generated using a monitor such as a real-time position management system (RPM), pressure belt, variation of the patient's anterior surface, etc. Once the image acquisition is complete, the images are binned according to their tagged respiratory signal (usually into 10 phases). These phases correspond to particular phases of the breathing cycle. Binning images with the same or similar breathing phases reduces tumor distortion. From these different phases, an alternative image set can be created called the maximum image projection (MIP) where the maximal Hounsfield unit value of each pixel is used from across all phase images to determine the maximal extent of tumor motion.

There are a few variations regarding 4D CT reconstruction and acquisition. In terms of respiratory signaling one can use phase or amplitude based binning. Phase based binning uses time intervals between peak inspirations to divide the signal into different bins while amplitude based binning uses differences in signal amplitude. There are also different acquisition methods of the CT itself [59, 60]. Helical acquisition uses a very small pitch in order to oversample during the respiratory cycle while cine mode repeats acquisition at the same couch position over the respiratory cycle. Both techniques have advantages and disadvantages compared to one another. For instance, cine acquisition may be more effective at removing motion artifacts while helical acquisition can be acquired in a shorter time and is more similar to standard helical imaging performed during routine CT scans.

The phase images and MIP image can be used to reduce the motion artifacts that would be present in a routine CT acquisition and estimate the amplitude of tumor motion. Figure 5.13 demonstrates how 4D CT can acquire images representative of different phases of respiration that reduce motion artifacts. Figure 5.13 illustrates both the max inhale and max exhale tumor positions and allows for the dosimetry to take this into account by treating to a volume that encompasses the extent of the motion. The quantification of tumor motion is then used to determine the internal target volume (ITV) which is the planning volume that encompasses the entirety of the tumor plus its motion. ITV margins allow the tumor to move within the patient and while still delivering adequate coverage of the prescribed dose due to the added ITV margins. 4D CT enables better characterization of tumor motion and therefore allows for enhanced radiation therapy that takes this motion into account. With 4D CT generated ITVs, planners are able to ensure adequate tumor coverage even in the presence of motion which reduces the probability of tumor recurrence and improves the therapeutic ratio. However, ITVs are not the only technique to address tumor motion. Techniques such as gating/breath-hold and real-time tracking are alternatives that have been introduced to improve radiation treatment.

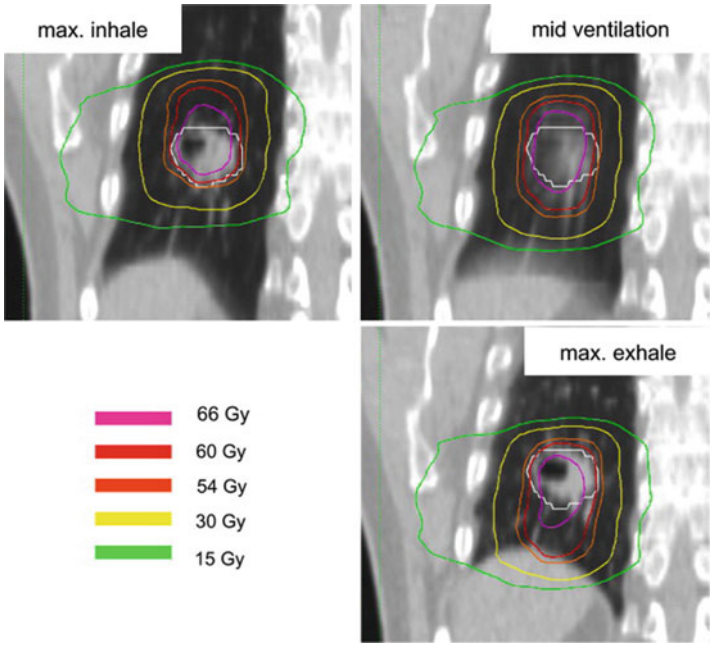

Fig. 5.13 Dose distributions planned for different breathing phases. The use of an ITV allows for the dose distribution to provide adequate coverage from maximum inhale to maximum exhale [61]

5.3.2.2 Breath-Hold/Gating

Tumors that move due to respiration and/or cardiac motion are problematic as their location may or may not be the same as observed during routine simulation scans. However, a current practice in radiation oncology is to employ a "breath-hold" during simulation and treatment. This technique has the patient hold his or her breath during the simulation scan and then this same process is repeated during treatment. Similar to 4D-CT, a signal indicating the patient amplitude/phase of breathing is required. This is used in order to ensure that the signal is consistently within a particular interval for both the simulation and treatment. The idea is that by maintaining a constant lung volume, the tumor and normal tissues will remain in the same location.

This technology for conducting breath hold simulation and treatment has been most extensively used and investigated in patients with breast cancer; particularly left sided-breast cancer. Since the heart is located in the left side of the chest, patients with breast cancer on the left side are at an increased risk to receive increased radiation doses to the heart. Multiple studies have demonstrated that the risk of cardiac related mortality and morbidity are associated with irradiation of left sided breast cancers due to higher cardiac doses [62–64]. Studies have shown that upon deep inspiration the separation of the heart and chest wall increases and the heart moves

inferiorly away from the upper chest. This has led to patients performing a deep inspiration breath hold (DIBH) during left sided breast cancer radiation treatment.

One technology to facilitate breath hold simulation and treatment is the Vision-RT system. As described previously, this system compares the patient's real-time surface rendering to their reference surface during simulation. In the case of breath-hold, the comparison is used to ensure adequacy of the patient's breath hold during the treatment. Other systems are available such as the spirometry-based active breathing coordinator system (Elekta, Stockholm, Sweden) and the video-based real-time position management (RPM) system (Varian Medical Systems, Palo Alto, CA). The spirometry-based and RPM systems exclusively deal with respiratory motion management whereas the Vision-RT system can be used to manage respiration alongside setup (AlignRT).

In conjunction with breath-hold treatments is the ability to gate the radiation beam. All respiratory monitoring systems have a particular range of movement that is deemed acceptable within tolerance limits. Linking respiratory signal technology with radiation delivery allows for the beam to be terminated when the signal falls outside the acceptable range (i.e. beam gating). Gating enables the beam only when the tumor is believed to be within a pre-specified position. The use of gating leads to smaller margins due to not needing an ITV and subsequently reduces the dose to surrounding tissue along with decreasing the probability of tumor under dosing.

5.3.2.3 Tracking

4D-CT and breath-hold technologies allow clinicians to take motion into account during planning or stabilize motion during treatment, respectively. However, neither of these systems are ideal since the generation of ITVs from 4D-CT lead to higher doses delivered to normal tissues and not all patients are capable of performing breath-holds due to poor lung function or length of treatment. A more recent advance in mitigating issues related to tumor motion is real-time tracking. Tracking can be divided into two serial categories: real-time localization and real-time adaptation. Real time localization involves determining target position at a specific point in time during treatment. This is mostly useful for gating more precisely by determining when to enable the treatment beam using direct tumor location rather than anatomic surrogates (patient surface, RPM, or spirometry readings). Real-time adaptation takes this concept one step further. In real-time adaptation the position of the tumor is known during treatment and methods are in place to adapt delivery according to the position (as opposed to only treating when the target is in an acceptable location).

Real-time localization currently involves a combination of fiducials, optical markers, and orthogonal X-ray units mounted in the treatment vault (real-time MRI imaging will be addressed briefly in subsequent sections). Two systems implementing real-time tracking are the Brainlab Exactrac System and the Accuray Cyberknife System. Both technologies use orthogonal in-room imagers to triangulate patient and/or fiducial positioning. These systems are capable of using bony anatomy (e.g. spine, skull, etc.) to quantify patient movement and use this as a surrogate for tumor

movement. The addition of fiducials implanted within or near a lesion allows for repeated imaging to be performed during treatment to identify tumor location in a more direct manner than surface based markers.

The Cyberknife system is capable of real-time adaptation of treatment. Since Cyberknife uses a LINAC mounted on a robotic arm, modification in beam delivery can be made as motion is occurring during the treatment. This allows the treatment beam to be on continuously with no potential beam interruptions, which is the case for gated treatments. The Cyberknife system uses the implanted fiducials (3–5, ideally) seen on the orthogonal X-rays along with surface optical markers to generate a model of tumor motion using optical marker position to generate tumor (i.e. fiducial) position at the beginning of treatment. This model is periodically updated during the course of treatment. There is a system delay of approximately 115 ms but this is taken into account within the model predictions so that the system can follow the motion in real time [65]. A gimbal-based system known as Vero (BrainLAB AG, Germany; Mitsubishi Heavy Industries Ltd., Tokyo, Japan) also performs in a similar fashion [66]. Rather than using a robotic arm, Vero is held by two gimbals that allow for small angle pan and tilt. Similar to Cyberknife, it uses both optical and orthogonal kV images for motion modeling. This system is reported to have smaller latency than Cyberknife but does not routinely update the model during treatment automatically [66].

New technologies are being investigated to enable more routine widespread use of real-time tracking on standard LINAC machines. Both MLC (multi-leaf collimator) and couch tracking may play a role in mitigating tumor motion during treatment in the near future. Tumor tracking can improve the therapeutic ratio for patients by unveiling exactly what is happening during treatment. Conventionally, assumptions are made that tumors behave a certain way and remain in certain location(s), however without real time tracking it is generally unknown if our assumptions hold on a patient by patient basis. By localizing the tumor during treatment, a higher degree of certainty is conveyed regarding both adequacy of coverage and normal tissue dose minimization.

5.4 Optimization Technologies

After efforts are made to localize each patient's disease and ensure consistency between simulation and treatment, the process of actually treating the patient begins. Classic radiotherapy utilized large open fields from a limited number of directions. Radiotherapy has evolved with advent of the multi-leaf collimator (MLC) to delivering highly modulated beams using multiple angles/arcs in a process known as intensity modulated radiation therapy (IMRT). The basic concept of IMRT is to deliver beams with varying intensity profiles that sum together at the target(s) in a manner than delivers uniform dose to the target(s) while maximally sparing surrounding normal tissue. To accomplish this, many degrees of freedom are needed not only for beam modulation but also the angles of beam delivery. This had led to the widespread adoption of arc based treatments and higher dimensional (quasi four pi based) systems.

5.4.1 Arc Based Treatment

IMRT customarily was delivered using a fixed number of equally spaced beams using step-and-shoot (MLC segment shaping only at specific beam angles) or dynamic MLC delivery (MLC segment shaping at fixed angles and during movement between angles). Multiple implementations of arc based treatments exist such as VMAT (Elekta), RapidArc (Varian), SmartArc (Pinnacle), and Tomotherapy (Accuray), which is a helical delivery that is similar enough to fall under the general arc-based treatment paradigm. The hypothetical advantage of arc based treatments are that they provide higher potential degrees of freedom compared to fixed beam IMRT and are a more efficient delivery method leading to shorter treatment times. During arc based treatments, beam segments are constantly being delivered while the gantry is continually rotating around the patient. Multiple arcs are routinely used when additional modulation is needed. A number of dosimetric planning studies have been performed comparing arc based treatments versus fixed-beam IMRT.

The results across multiple sites are often conflicting due to the nature of dosimetric of planning studies. Ost et al. found that VMAT was able to improve rectal dose sparing compared to IMRT, but studies from Yoo et al. came to the opposite conclusion [67, 68]. Similar results were found in lung where studies are conflicting as to which methodology provides better normal lung sparing. One constant is that arc based deliveries appear to be faster than fixed beam IMRT treatments. Having the patient on the table for less time is advantageous in that the patient has less time to move during the treatment. The optimization and dose calculation engines behind arc based treatments are just as important as the mechanical aspects of the LINAC. Accurate optimization and calculation is crucial in making the treatments deliverable and able to pass quality assurance testing. Optimization engine improvements have also reduced planning time, delivery time, and increased normal tissue sparing.

5.4.2 Quasi 4π Treatment Delivery

Arc based treatment expanded on fixed-beam delivery by adding more potential degrees of freedom. This concept has been pushed further into systems that are "quasi 4pi" meaning they can more easily deliver non-coplanar beam from virtually anywhere in space. This methodology has been implemented particularly in systems designed to treat lesions of the brain, specifically Cyberknife and Gamma Knife. The differences in delivery between Cyberknife and Gamma Knife are vast (e.g. non-isocentric versus isocentric, 6MV versus cobalt, dynamic versus fixed beam position, whole body vs. brain only delivery, etc.). However, the geometric delivery of both systems were designed with the same goal; to deliver a high number of small beams from a wide range of possible positions to maximize conformity of dose and thereby limit the delivered dose to the surrounding normal tissue [69]. Both systems also are used primarily for ablative or radiosurgery procedures where treatments are

delivered in single or a small number of fractions at very high doses per fraction making conformity of high dose regions paramount. These technologies are being used more commonly in patients with oligometastatic brain metastases in combination or as an alternative to whole brain radiation.

5.4.3 Proton Treatment

Proton therapy is becoming more widely available as centers are currently under construction across the country. Proton therapy is fundamentally different from photon treatments due to the difference in depth dose distribution of charged particles.

The benefit of proton treatments is that protons deposit their energy predominantly at depth known as a Bragg peak whereas photons deposit a majority of their energy centimeters below the surface and continue depositing gradually decreasing amounts of energy with depth. Figure 5.14 demonstrates how proton beam treatment (14.e–14.f) delivers considerably less dose to the anterior structures in craniospinal treatments compared to photon based approaches (14.a–14.d). This allows

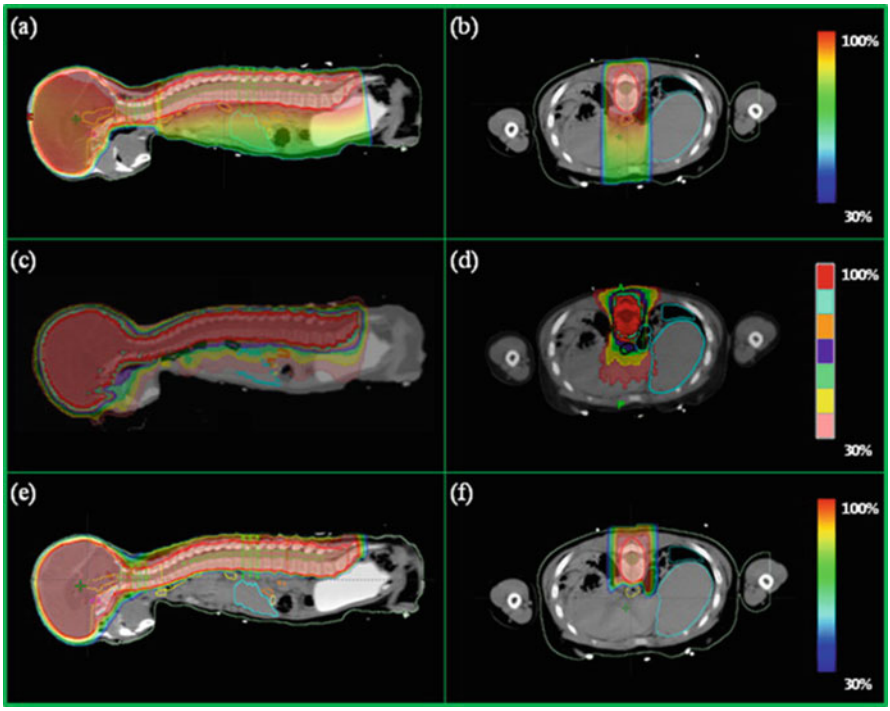

Fig. 5.14 Comparison of photon 3DCRT (**a**, **b**), photon IMRT (**c**, **d**), and proton beam treatment (**e**, **f**) for craniospinal irradiation [61]

proton therapy to theoretically spare normal tissue distal to the target(s) being irradiated. Clinically, proton treatments use what are known as spread out Bragg peaks but the advantage of sparing distal tissue remains the same. Protons also are believed to have higher radiobiological effectiveness (RBE) compared to photons. In treatment planning, protons are assumed to have an RBE of 1.1 compared to photons which have an RBE of 1.

Much of the benefits of proton therapy remain hypothetical at this point in time. There is not an abundance of evidence of proton therapy's superiority over photon therapy in terms of patient outcome or toxicity. It has become more widely recognized that certain aspects of proton therapy are still not well understood and/or able to be mitigated. For instance, range uncertainty of proton dose delivery due to changes in anatomy, position of the patient, and motion must be taken into account during planning. There is also the issue that while we assume a RBE of 1.1, in reality there is evidence that RBE may be variable, which could negatively impact treatments. These aspects, along with potentially suboptimal patient selection, may contribute to the as yet unclear clinical benefit of this technology compared to photon therapy. It is generally agreed upon that proton therapy does have a place in pediatric patients where radiation induced malignancies and long term side effects are of greater concern. However, it is unclear whether this technology will demonstrate benefits in alternate settings to offset its associate costs. Moving forward, more proton therapy trial results are needed in a variety of treatment sites alongside maturation of robust planning and delivery.

5.5 Integrated MRI Teletherapy

The concept of combining MRI and a LINAC is one that brings together imaging and therapy delivery in a way we have never seen before. An MRI/LINAC has the potential to provide real-time 3D imaging with superb soft tissue contrast during patient treatment. Since an MRI radiotherapy device is a meshing of technologies that can improve tumor localization, mitigation, and treatment using a single (albeit incredibly complex) device it is described briefly here in a separate section. Unlike CT-on-rails or CBCT there would potentially be minimal delay between imaging and the ability to beam-on. This is obviously advantageous as it is ideal to mitigate issues with tumor or normal tissue changes with the shortest time possible between imaging and potential correction or gating. Unlike current tracking technologies (orthogonal radiographs and fiducials) an MRI/LINAC could produce real time 3D images of the tumor itself (i.e. not 2D and not using any surrogate). The possibility of adaptive treatments is also a possibility and preliminary data exists from Washington University. Acharya et al. found that the average time for recontouring, reoptimization, and quality assurance was 26 min in 20 patients (170 fractions).

There are a currently a number of MRI/LINAC versions each with their own pros and cons and variations in regards to field strength and method of therapy beam generation. For instance, the ViewRay MRIdian system uses a 0.35 T magnetic field in combination with a three source cobalt unit while an Elekta/Philips system uses

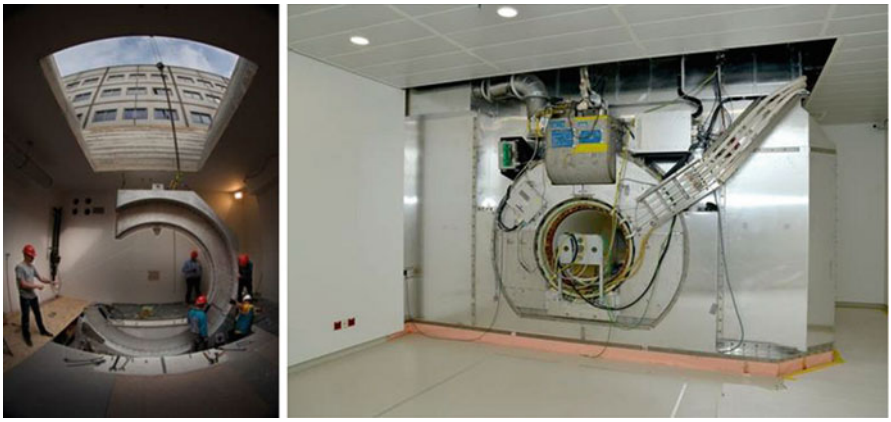

Fig. 5.15 UMC Utrecht installation of MRI/LINAC [70]

a LINAC alongside a 1.5 T magnetic field. Figure 5.15 shows the UMC Utrecht installation of the MRI/LINAC. The goal of all system versions is the same; combine state of the art therapy and imaging. Time will tell if the potential benefits of these systems will translate into clinical improvement and subsequent widespread implementation. It is also unknown if in the future a single platform will prove most efficacious or a variety of platforms will be in clinical use.

5.6 Conclusion

Technology has facilitated, and will continue to facilitate, the therapeutic ratio for patients receiving radiation treatment. Overall, technology is gradually reducing the physical/dosimetric unknowns of radiation therapy. Imaging is more accurately targeting disease location. Integration of imaging and tracking methods are being used to better mitigate the effects of motion, and enhanced delivery techniques are more efficiently sparing normal tissues while ensuring adequate target coverage. Moving forward, technology will allow practitioners to monitor the details of treatment in real-time, allowing for computation of actual delivered dose as opposed to planned dose, in turn facilitating treatment personalization/adaptation to compensate for suboptimal target dosage and tumor/normal tissue anatomic/physiologic changes.

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Chapter 6
Nitric Oxide Synthase Uncoupling in Tumor Progression and Cancer Therapy

Ross B. Mikkelsen, Vasily A. Yakovlev, Christopher S. Rabender, and Asim Alam

Abstract High levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are hallmarks of solid tumors, promoting genomic instability as well as uncontrolled proliferation. In inflammatory diseases such as diabetes, hypertension, atherosclerosis and cancer, loss of nitric oxide (NO) production is a common feature. Recent experiments demonstrated that under these conditions the relative levels of the nitric oxide synthase (NOS) cofactor, tetrahydrobiopterin (BH4), are relatively low resulting in an "uncoupled NOS" and reduced NO bioavailability and increased ROS/RNS. Similar evidence suggest that NOS uncoupling is also a critical "switching mechanism" essential for tumor progression. Furthermore, uncoupling can be exploited therapeutically as both in vitro and in vivo treatment with the BH4 precursor, Sepiapterin (SP), restores NOS coupling and shifts the balance of signaling from ROS/RNS and pro-proliferative to NO dependent and anti-proliferative pathways.

Keywords Nitric oxide synthase • Uncoupling • Tetrahydrobiopterin • Sepiapterin • NADPH oxidase • Reactive nitrogen species • Reactive oxygen species • Chromosomal instability

Rudolf Virchow 150 years ago noting leucocytes in neoplastic sites described solid tumors in terms of sites of chronic inflammation [1]. Epidemiologic evidence accumulating over subsequent years has supported the positive correlation between cancer incidence and chronic inflammation, and it is now a well-recognized hallmark of cancer development [2–5]. This correlation is also seen in studies demonstrating the crucial role of redox signaling and free radicals in carcinogenesis [4, 6–8]. Normal cells and tissues of healthy mammals are characterized by a low steady-state level of 'oxidizers' (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) and relatively constant level of 'reducers' (endogenous redox pairs: NADH/NAD+, NADPH/

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NADP+, FADH2/FAD, reduced/oxidized glutathione, reduced/oxidized ascorbate, etc.). Increasing ROS/RNS above the critical level provokes genomic instability and triggers uncontrolled proliferation [4, 8–11].

The main endogenous initiators of the redox imbalance in cancer cells are defective mitochondria, uncoupled NO synthase (NOS), and elevated NADPH–oxidase activity. They are involved simultaneously in two processes affecting tissue redox status: (a) an excessive generation of ROS (in particular, superoxide radicals, $O_2^{\bullet-}$) and nitric oxide (•NO), and (b) an increased consumption of two of the main cellular reducers—NADH and NADPH [8, 12–14]. The increased $O_2^{\bullet-}$ production from uncoupled NOS synthase, mitochondria and NADPH oxidase contributes to reduction in •NO availability by reacting with •NO to form peroxynitrite ($ONOO^-$) [15–21]. Through its decomposition products of nitrogen dioxide ($\bullet NO_2$), carbonate radical ($CO_3^{\bullet-}$), and hydroxyl radical (•OH), $ONOO^-$ stimulates Tyr nitration of multiple proteins [6, 22–24]. Protein Tyr nitration is well-accepted marker of tissue inflammation and is gaining attention because of its impact on carcinogenesis and tumor growth. This is seen in studies demonstrating that NOS inhibitors attenuate tumor xenograft growth and enhance the effects of radiation on tumor control by selectively reducing tumor blood volume and inducing tumor cell killing [25–28]. With mouse mammary tumors, endothelial NOS (eNOS) expression characterizes metastatic breast tumor cells in the lung and NOS inhibition blocks tumor cell migration and invasiveness [27, 29]. Importantly, recent investigations described in detail below show that a general characteristic of all solid tumors is an uncoupled NOS activity that generates $O_2^{\bullet-}$ and $ONOO^-$ instead of •NO as occurs in normal non-inflammatory tissues. As we will see the coupling state of NOS has important implications for both tumor progression and the effects of radiation on normal tissue toxicity.

Elevated levels ROS or RNS not only directly damage DNA, but at physiological relevant doses, regulate expression of genes necessary for high fidelity homologous recombination repair (HRR) of DNA double-strand breaks (DSB). This potentially leads to mutagenesis by two mechanisms: (i) directly damaging DNA by oxidative and nitrosative stress, and (ii) decreasing high fidelity DNA repair thereby stimulating the accumulation of DNA mutations. Our studies demonstrate that elevated RNS levels of the tumor microenvironment stimulate tyrosine nitration and activity of PP2A with one consequence being downregulation of BRCA1 expression [30]. The inhibition of BRCA1 expression significantly reduces the ability of cells to fix DSB and the reduction in high fidelity HRR is compensated with a subsequent increase of error-prone non-homologous end joining DSB repair. This provides a mechanism for chromosomal instability essential for tumor progression.

The extent of Tyr nitration in biological systems is responsive to increases in either the •NO or $O_2^{\bullet-}$ production [31, 32]. For this reason Tyr nitration and activation of PP2A depends on both the RNS and ROS levels. In recent studies we show that inhibition of ROS production by targeting ROS generation using mitochondrial electron transport deficient cells (ρ^o cells) or inhibiting NADPH oxidase activity with a selective peptide inhibitor (gp91 ds-tat) significantly reduces PP2A Tyr nitration and its activity in different cancer cell lines. As a result of the decreased PP2A activity BRCA1 expression is restored along with a significantly enhanced level of DNA HRR (Yakovlev, in preparation).

ROS/RNS are also mechanistically involved in autocrine growth regulation of tumors. This is elegantly shown for oncogenic Ras-driven tumor growth [33]. Akt phosphorylation and activation of eNOS stimulates S-nitrosylation (or RNS-dependent oxidation) of Cys118 of Ras resulting in cytoprotective signaling through Akt. We demonstrated an alternative RNS-dependent mechanism for tumor cell autocrine regulation and cytoprotective response to mild oxidative events, e.g. radiation [34, 35]. This mechanism requires Ca^{2+}-dependent NOS and involves S-nitrosylation (or RNS-mediated oxidation) of the active site Cys of protein Tyr phosphatases (e.g. SHP-1 and SHP-2). SHP2 oxidation and inhibition correlates with enhanced Tyr992/Tyr1173 phosphorylation of ERBB1 [34–38]. Phosphorylated Tyr992 and Tyr1173 are targets of SHP2 and initiate phospholipase C and ERK1/2 cytoprotective signaling following a radiation exposure [35, 39, 40]. Akt is also negatively regulated by the phosphatase PTEN. PTEN like other protein Tyr phosphatases has an active site Cys-containing domain sensitive to oxidation [41, 42]. Peroxynitrite inhibits PTEN, which in turn activates Akt signaling [43]. Thus, in autocrine regulated cells, inhibition of protein Tyr phosphatases by ROS/RNS is critical in sustaining growth promoting and anti-apoptotic receptor tyrosine kinase activities [44, 45]. This also appears to be true in supporting stromal cells. In tumor endothelial cells, for example, Akt-dependent phosphorylation and activation of eNOS is necessary for endothelial cell migration and formation of capillary-like structures [28, 46].

A characteristic of most tumor cells is an elevated NF-kB stress response pathway [47–50]. Mild oxidative stress such as radiation doses <10 Gy activates NF-kB in diverse cell types including breast cancer cells by a mechanism requiring constitutive eNOS or neuronal NOS (nNOS) and nitration of Tyr181 of the inhibitor protein IkBα. Tyr181 nitration results in dissociation of IkBα from the active transcription factor complex, p50/p65 [51]. Low basal levels of Tyr-nitrated IkBα are also observed, probably as a consequence of the elevated levels of ROS/RNS generated by tumor cell metabolism and the inflammatory microenvironment. This activation mechanism differs from the classical model observed with cells treated with tumor necrosis factor or very high radiation doses (>10 Gy) and involving the IKKβ-dependent phosphorylation and proteolysis of IkBα. S-nitrosylation of p65 Cys38 can also inhibit NF-kB transcriptional activity [52]. Recoupling NOS with oral sepiapterin, a tetrahydrobiopterin (BH4) salvage pathway precursor, inhibits NF-kB promoter activity in the spontaneous mammary breast carcinoma MMTVneu by a mechanism involving both reduced IkBα nitration and increased S-nitrosylation of p65 Cys38 [21].

Wink and associates established a concentration scale for •NO effects on cell proliferation [53]. High doses of exogenous •NO-donors induce DNA-damage and promote p53 transcriptional activity by a classical DNA-damage-response ATM/ATR- dependent mechanism, e.g. [54, 55]. These early studies also reported that lower •NO donor concentrations still stimulate unequivocal nuclear retention of p53 but by mechanism(s) not requiring ATM/ATR-dependent p53 Ser15 phosphorylation. An alternative mechanism for low •NO donor concentrations involves nitration of Tyr327 in the p53 tetramerization domain promoting oligomerization, nuclear accumulation and increased DNA-binding [56]. A difference in the pattern

of p53-target gene expression at low- and high-doses of •NO is also observed. Regulation of Bax, Cyclin B, GADD45, MDM2, and MSH2 expression levels by high doses of •NO mimics the radiation-dependent regulation pattern. This expression pattern is significantly different quantitatively and qualitatively from that following treatment at low doses of •NO. These results demonstrate a new post-translational mechanism for modulating p53 transcriptional activity responsive to conditions characteristic of the inflammatory tumor microenvironment.

Although well described in the vasculature literature, studies of NOS in cancer cells generally ignore the fact that NOS can have two activities: "coupled" that generates •NO or "uncoupled" $O_2^{\bullet-}$/$ONOO^-$ synthase activities The NOS catalytic cycle involves transfer of electrons coupled to the oxidation of Arg with the cofactor BH4 donating electrons to the NOS Fe^{2+}-O_2 complex initiating oxidation. The ratio of [BH4] to its oxidation product [BH2] (BH4:BH2) is critical since both bind to the active site with equal affinity. With low BH4:BH2 as found in inflammatory conditions, uncoupling is observed and more $O_2^{\bullet-}$/$ONOO^-$ and less •NO are generated [17, 57]. Moreover, since $ONOO^-$ oxidizes BH4 to BH2, a futile feed forward destruction mechanism of BH4 is established.

We recently showed that diverse tumor cell types *in vitro* and *in vivo* have low BH4:BH2 (≤1) compared to normal tissues (>4) [21]. Furthermore the BH4:BH2 can be increased by doping culture medium or mouse diet with the BH4 salvage pathway precursor, sepiapterin, or by over-expressing GTP cyclohydrolase-1 (GCH1), the rate limiting enzyme for BH4 synthesis. Increasing BH4:BH2 with sepiapterin reduced L-NNA-sensitive $O_2^{\bullet-}$ generation while simultaneously increasing cGMP. The functional consequences of increasing the BH4:BH2 include a shift from RNS-dependent pathways, e.g., decreased NFkB activity due to increased S-nitrosylation of p65 and decreased IkBα Tyr nitration coupled to increased •NO-dependent soluble guanylate cyclase (SGC) cGMP-dependent protein kinase G (PKG) signaling. Suppressed sGC/PKG signaling is a general characteristic of breast and other epithelial tumor cells expressing high levels of cGMP phosphodiesterases, PDE5 and PDE9. Inhibition of these PDEs, resulting in a corresponding increase in cGMP/PKG activity, blocks growth promoting pathways (e.g., β-catenin/TCF signaling) and increases tumor cell apoptosis [58–61]. Sepiapterin by increasing cGMP and PKG activity also inhibited β-catenin expression and TCF-4 promoter activity in MCF-7 and MDA-MB-231 breast cancer cells. *In vitro* clonogenic and *ex vivo* clonogenic assays for breast tumor MCF-7 and MDA-231 xenografts demonstrate that sepiapterin inhibits tumor growth [21]. Oral sepiapterin also inhibits spontaneous MMTVneu tumor growth as shown by ^{18}F-fluorodeoxyglucose (FDG) PET/CT imaging and Ki67 staining [21]. Not all MMTVneu tumors respond identically to 5 days of oral sepiapterin even with tumors in the same mouse. However, when the normalized % decrease in FDG uptake of each tumor is plotted versus tumor BH4:BH2 there is a linear negative correlation ($r = -0.9576$) indicating that a low BH4:BH2 and, as a consequence, uncoupled NOS is critical for tumor growth and that increasing the BH4:BH2 represents a possible therapeutic approach.

In a model for inflammatory bowel disease, prophylactic oral sepiapterin blocks dextran sodium sulfate (DSS)-induced colonic inflammation as shown by blocked

IL-1β, IL-6 and IL-17A expression and reduced infiltration of the colon by inflammatory cells. Furthermore prophylactic sepiapterin significantly reduced colorectal cancer in azoxymethane (AOM)/DSS treated mice [19]. Oral sepiapterin provided continuously by oral gavage on a daily basis for weeks post AOM and during three cycles of DSS treatment reduced the numbers of tumors in the AOM/DSS-treated mice from approximately 7 ± 2.5/per unit length of colon to 1.5 ± 1.5/per unit length ($p < 0.05$). However, the tumor volumes of those tumors that persisted through the sepiapterin treatment were no different from those of animals without sepiapaterin. This suggests an undefined mechanism that defeats the sepiapterin driven recoupling. In unpublished work the BH4:BH2 of colorectal cancer cells isolated from the colons of AOM/DSS mice was 1.4 ± 0.3 compared to 7.1 ± 0.6 in isolated normal colon epithelial cells (Alam *et al.* in preparation). In isolated colorectal cancer cells from animals treated with sepiapterin BH4:BH2 increased to 4.6 ± 0.9 with 5 days of oral sepiapterin. As with the MMTVneu mammary cancer model, increasing BH4:BH2 correlated with reduced colorectal tumor growth as assessed by FDG PET/CT. A low BH4:BH2 is also observed in human colorectal cancer biopsies compared to paired "normal" adjacent colon tissue (2.3 vs. 4.5, matched 2- tailed t-test, $n = 4$, $p < 0.001$) [21]. That the paired "normal" biopsy sample has a relatively low BH4:BH2 from what is expected for normal tissue is probably a consequence of residual tumor tissue and the chronic inflammatory state of tissue adjacent to tumor.

NOS uncoupling also occurs under conditions of low [Arg] as with elevated expression of arginase or S-glutathiolation of eNOS [62, 63]. Regardless of the mechanism, NOS uncoupling is a critical switching mechanism for tumor cell growth. When coupled, the primary product of all NOS isoforms is •NO and downstream signaling is dominated by •NO-dependent pathways (eg, sGC/PKG). Uncoupled NOS, on the other hand, produces potent oxidants, e.g., $ONOO^-$ and $O_2^{\bullet -}$ initiating different downstream signaling that is pro-proliferative and anti-apoptotic, e.g., NF-kB [51]. Recoupling NOS by increasing BH4:BH2 with an orally provided BH4 metabolic precursor inhibits the growth of both a spontaneous mammary carcinoma and a colorectal tumor.

The above studies focused on the tumor cell. However, the inflammatory microenvironment of the tumor also affects •NO signaling of the endothelium leading to poor vascular function as found in other chronic inflammatory diseases (e.g. diabetes and atherosclerosis). Previous efforts have attempted to therapeutically target the tumor vasculature either by vascular disruption or vascular normalization. The former, exemplified by the use of combretastatin compounds to disrupt the existing tumor vasculature, causes ischemia and tumor cell death [64]. The NOS inhibitor, L-N^G-Nitroarginine (L-NNA), also appears to disrupt tumor vasculature. By selectively inhibiting tumor blood flow relative to normal tissues, L-NNA increases tumor cell death and more than additively radiosensitizers squamous carcinoma xenografts [25]. In a Phase 1 clinical trial relatively low concentrations of L-NNA selectively decrease tumor blood measured by dynamic contrast enhanced CT in diverse tumors by 40 % within 1 h of iv infusion [65]. This reduction in tumor blood volume persists for at least 24 h with minimal and self-limiting cardiovascular toxicity. This decrease in blood volume is associated with significant increases

in the number of non-perfused pixels from 7.3 % at baseline to 25.1 % at 1 h and 18.2 % at 24 h indicative of tumor antivascular activity.

In contrast to this approach, vascular normalization by agents that inhibit vascular endothelial growth factor (VEGF)-dependent pathways target the immature vasculature of tumors (pruning) and angiogenesis to improve blood flow, reducing hypoxia, and enhancing drug delivery [66, 67]. Vascular normalization using anti-VEGF strategies is, however, transient. Targeting VEGF activity is complicated clinically by effects on normal endothelium and is associated with hypertension, proteinuria, and impaired wound healing [68–71]. Vascular disrupting agents also have cardiovascular toxicities and dose limiting profiles similar to their anti-angiogenesis inhibitor counterparts [72]. Another approach using a PI3K inhibitor causes a more sustained normalization indicated by reduced hypoxia, elevated tumor blood flow, increased tumor vascular density, and increased sensitivity to doxorubicin [73].

Uncoupling of eNOS in tumor endothelial cells may partly explain the poor vasculature structure found within the solid tumors and thus recoupling may provide an alternative approach to normalizing tumor vascular function. Earlier work demonstrated that sepiapterin-induced NOS recoupling stimulates angiogenesis and functional recovery of dysfunctional endothelium in diverse cardiovascular diseases [74–77]. In a preliminary investigation measuring $\%HbO_2$ in spontaneous MMTVneu tumors by multispectral optoacoustic tomographic imaging results demonstrate that sepiapterin normalizes tumor vasculature. Mice fed 10 mg/kg sepiapterin daily by oral gavage for 6 days were imaged starting on day 0 just prior to the first sepiapterin treatment. The time course of increased $\%HbO_2$ with sepiapterin is similar to that found with other vasculature normalization methods, e.g. avastin, with a maximal 20 % increase in $\%HbO_2$ at 8 days relative to tumors in non-treated animals. These results suggest that sepiapterin not only has direct anti-tumor cell activity but potentially chemo- or radiosensitizes hypoxic tumors through enhanced anti-tumor drug delivery and re-oxygenation [78–80].

Key players in the angiogenic process include the endothelial angiopoietin receptor Tie2, its ligands, angiopoietin 1 and 2 (Ang 1 and Ang 2), and PI3K/Akt/eNOS signaling. A recent study with an orthotopic xenograft ovarian tumor model shows by multispectral optoacoustic tomography that tumor HbO_2 was increased by a neutralizing antibody against both Ang1 and Ang2 [81]. The investigators argue that elevated Ang1 found in the blood circulation of vehicle-treated mice is consistent with previous work showing that excess Ang1 promotes vascular remodeling and plasticity in tumors and that this is normalized in response to anti-Ang1/Ang2 therapy. Reduced Ang1 signaling would lead to more functional, less tortuous tumor vessels, which is confirmed *ex vivo* by a significant increase in smooth muscle cell number and coverage of endothelial cells. One complication in interpreting these results is the use of xenograft tumors in immune compromised mice. Other studies demonstrate that •NO/eNOS-dependent expression of Ang1 in mature vasculature is essential for normal vasculature maturation including the recruitment of smooth muscle cells [82–85]. In contrast, Ang2 is expressed in growing vessels and destabilizes blood vessels during angiogenesis by interfering with Ang1 function [82, 86, 87].

Ang2 through α3β1 integrin signaling may also induce smooth muscle cell apoptosis [88]. Ang2 expression is increased by hypoxia and VEGF and this may enhance neo-vascularization of tumors [89]. Furthermore, targeting of Ang2 signaling restores vascular stability in multiple mouse breast cancer model systems and decreases tumor growth and metastasis [90]. Enhanced Ang2 expression correlates poorly with outcome in patients with breast cancer [90]. The relationship between •NO/eNOS and Ang2 has become more apparent in recent studies on PI3K/Akt regulation of angiogenesis. Angiogenesis defects in Akt1 null mice can be rescued by expression of a constitutively active eNOS, a major target of Akt1 [91]. Furthermore, endothelial cell secretion of Ang2 packaged in exosomes is inhibited by the PI3K/Akt/eNOS pathway and inhibition of this Ang2 secretion rescues vascular defects in Akt1 null mice [92]. Thus the "normalization" observed with the anti-Ang1/Ang2 may actually be due to blocking Ang2 activity. Future studies evaluating a mechanism of sepiapterin stimulated tumor vascular normalization will need to test whether sepiapterin-recoupling of tumor NOS increases Ang1 expression but suppresses Ang2 secretion and enhances recruitment of smooth muscle cells to the tumor vasculature.

Radiotherapy and chemotherapy cause normal tissue injury and mitigating these toxicities can potentially enhance the therapeutic ratio. Radiation-induced cardiomyopathy, for example, is characterized by dose-dependent progressive decreases in contractile reserve and left ventricle systolic function accompanied by increased myocardial and pericardial fibrosis and ultimately premature death [93–96]. The underlying mechanisms remain to be defined but several studies show that radiation-induced injury to the heart and other normal tissues is a consequence of a chronic inflammatory response and endothelial dysfunction that stimulates unbalanced overexpression of pro-fibrotic cytokines, irreversibly increased collagen, extracellular matric expression and fibrosis [97–102]. The mechanisms underling endothelial dysfunction in normal tissues following radiation injury are not well understood. However, radiation induced-oxidative stress has been shown to decrease BH4 levels inducing NOS uncoupling and subsequent endothelial malfunction [103–105]. Loss of endothelial function has been reported to anticipate myocardial degeneration and symptoms of heart diseases after single radiation to the rat heart [97, 106]. Doxorubicin-induced cardiotoxicity is similarly described as the result of chronic inflammation due to infiltrating inflammatory cells and elevated levels of ROS/RNS and pro-inflammatory cytokines stimulating fibrosis [107–109]. ROS/RNS generation mediates endothelial cell and cardiomyocyte apoptosis that characterized doxorubicin-induced cardiotoxicity [110]. Doxorubicin also induces iNOS expression and enhanced $ONOO^-$ and $O_2^{\bullet -}$ in the heart [111]. However, whether doxorubicin-mediated cardiotoxicity is in part mediated by the uncoupled NOS through the reduction of BH4:BH2 is unknown. Investigations are underway to test whether radiation or doxorubicin by uncoupling NOS promote cardiac injury and whether this injury can be minimized by recoupling NOS with SP. Support for this hypothesis comes from work showing that •NO reduces plasma membrane expression of TFGβ-R1 in vascular smooth muscle cells by enhancing dynamin-dependent endocytic internalization of the receptor [112]. Furthermore in

cardiac endothelial cells •NO through a PKG-dependent mechanism inhibits transforming growth factor-beta1 (TGFβ) expression and enhances proteasomal degradation of activated Smad2 [113]. By interfering with TGFβ/Smad2 activity, •NO serves as a molecular restraint to the excessive actions of TGFβ. When NOS activity is uncoupled as is found in dysfunctional endothelium, this restraint on TGFβ signaling is lost promoting abnormal fibrosis.

An accepted model for the progressive nature of late tissue toxicity involves persistent oxidative stress and endothelial dysfunction leading to an abnormal wound healing [99]. Normal wound repair involves three overlapping phases: (1) a transient inflammatory phase of increased expression of pro-inflammatory cytokines and recruitment of pro-inflammatory cells; (2) a subsequent phase of neovascularization and re-epithelization or re-endothelialization, including the expression of TGFβ, recruitment of fibroblasts and deposition of collagen; and (3) extracellular matrix (EM) formation for wound closure. When fully recovered, remodeling occurs with apoptotic removal of fibroblasts forming an acellular scar. Late normal tissue RT toxicity on the other hand is seen as a chronic inflammatory response stimulating unbalanced overexpression of pro-fibrotic cytokines and irreversibly increased collagen, EM expression and fibrosis. In the case of the radiation-induced lung injury the resulting fibrosis is seen as a chronic inflammatory response leading to unbalanced overexpression of pro-fibrotic cytokines such as TGFβ and as a consequence irreversible increased collagen and the extracellular matrix expression [99]. The appearance and severity of radiation-induced lung injury is both dose and time dependent with the response categorized into phases. An early acute phase is defined as a period immediately following RT and consists of a large number of molecular, cellular and physiological changes including increases in ROS/RNS, cytokine expression and hypoxia, and decreases in lung perfusion [6, 114–117]. Many early responses are reversible with increases in most cytokines returning to lower levels within 2 weeks post-radiation exposure [118]. After a subsequent latent period, an acute phase presents characterized clinically by the onset of pneumonitis and also by a second increase in ROS/RNS, hypoxia, decreased lung perfusion and the expression of TGFβ and pro-inflammatory cytokines [116–118]. Unlike the early acute response, the appearance of the acute phase is dose-dependent and for some events, irreversible. In addition, many of the changes that occur during pneumonitis, such as increased TGFβ expression do not return to basal levels but remain elevated until the occurrence of the late phase which is often characterized by the appearance of fibrosis [100, 116, 117, 119]. Our proteomic study of the rat lung after a 28 Gy hemithoracic radiation exposure [120] demonstrated changes in protein expression that correlated with these functional and structural events including transient increases (days 1–3) in expression of an anti-inflammatory and anti-fibrotic enzyme HO-1, and an increase in IkBα Tyr nitration (an indicator of RNS-dependent NF-kB activation [51], followed at 6–8 weeks with irreversible decreases in a number of structural proteins (e.g. talin and filamin), anti-oxidant proteins (biliverdin reductase and peroxidoxin 1). We have not repeated these experiments in detail with a mouse model, but this pattern of events appears too hold true with mice as well. Thus radiation induces a transient expression of anti-inflammatory and anti-fibrotic HO-1 in mouse

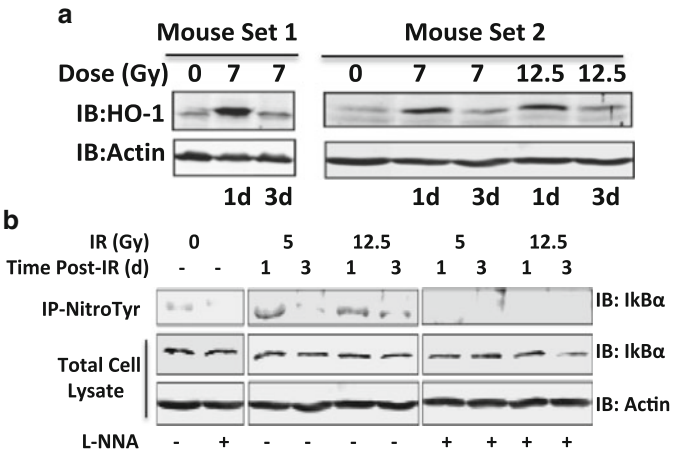

Fig 6.1 (**a**) IR induces HO-1 expression in mouse lungs. (**b**) IR stimulates transient Tyr-nitration of IkBα, indicating increased RNS and potential activation of NFkB

lungs (Fig. 6.1a). Previous studies [121] demonstrated that nitrosative stress activated HO-1 expression. We have shown in unpublished experiments that a NOS inhibitor, L-NNA, blocked radiation-induced HO-1 expression in mouse and rat lungs. HO-1 transcription is regulated by two RNS/ROS sensitive transcription factors, NF-kB and Nrf-2. Radiation stimulates nitration of Tyr181 of IkBα, dissociating the IkBα/NF-kB complex stimulating NF-kB p65 DNA binding and transcriptional activity in CHO and MCF-7 cells [51]. IkBα of irradiated lung is also nitrated after a radiation exposure (Fig 6.1b). Only a short period post-radiation was followed in these experiments. However, radiotherapy-induced NF-kB activation in human arteries is sustained for years post-radiotherapy [122]. Since NF-kB is a key pro-inflammatory transcription factor, it is not surprising that further analysis revealed enhanced mRNA levels for inflammatory cytokines and their receptors (e.g. IL-1β, IL-6), HO-1 and tissue remodeling proteins (e.g. MMP-1, TIMP1). A persistent elevated NF-kB activity is also found with porcine arteries weeks post-radiotherapy [123].

The enzymatic products of the HO-1 catalytic cycle are CO, a •NO mimetic, and biliverdin/bilirubin, anti-oxidants, thus representing a critical anti-inflammatory mechanism [124]. CO, like •NO, binds to the heme of sGC stimulating activity and cGMP synthesis. Furthermore CO binds to the heme of NOS but inhibits NOS activity [125]. This suggests that activation of HO-1 may circumvent uncoupled NOS activity and reduce ROS/RNS generation while at the same time performing the main function of •NO stimulating PKG signaling and regulating vascular tone and preventing platelet aggregation. CO also dilates blood vessels by directly activating Ca^{2+} dependent K^+ channels. We tested whether CO is able to block radiation-induced lung Tyr nitration. As shown in Fig 6.2a, the CO donor, Tricarbonyldichlororuthenium (II) dimer (CORM2), when introduced intraperitoneally (ip) at 3.5 mg/kg, like the NOS inhibitor L-NNA in drinking water, inhibits protein Tyr nitration in the mouse

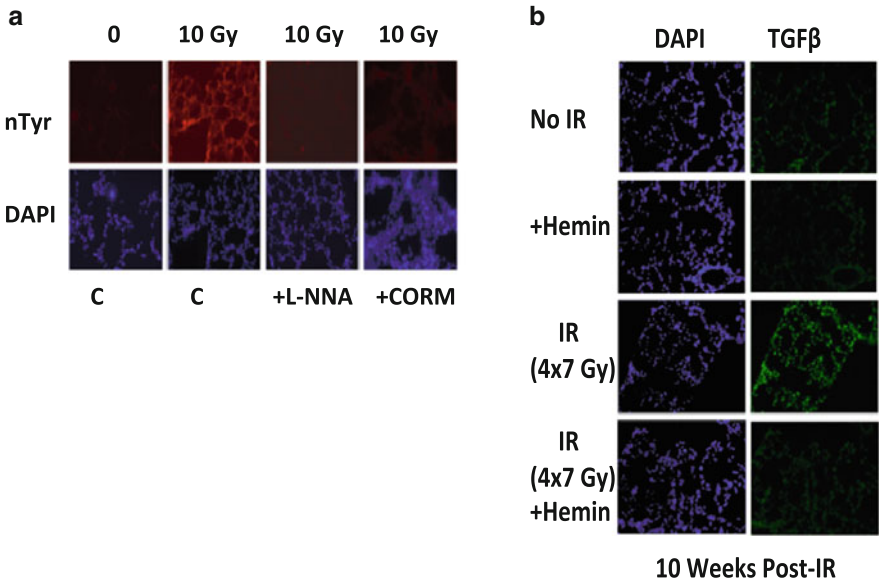

Fig 6.2 (**a**) L-NNA and the CO donor CORM-2 block radiation induced protein Tyr nitration in the mouse lung. Animals were sacrificed 24 h after a 10 Gy radiation dose to the thorax and the lungs after perfusion with PBS were frozen in optimal cutting temperature medium. (**b**) Hemin (4 mg/kg) ip for 3 consecutive days during fractionated radiotherapy to the thorax (4 × 7 Gy) inhibits TGFβ expression in the mouse lung

lung after a 10 Gy radiation exposure. Treatment of mice ip with hemin, an inducer of HO-1, during fractionated radiotherapy also blocked expression of TGFβ1 at 10 weeks (Fig 6.2b). Studies with different rodent models of cardiac injury have demonstrated the cardioprotective effects of HO-1 expression. For example in a rat model of heart failure with permanent ligation of the left coronary artery, administration of hemin (4 mg/kg body weight) every other day for 4 weeks induced a robust and sustained increase in HO-1 expression and activity, as shown by increased levels of bilirubin and CO [126]. These effects were associated with a significant improvement in survival (out to 28 days when the animals were sacrificed) and reduced the extension of myocardial damage. The ischemic hearts of the hemin-treated animals displayed reduced oxidative stress, as shown by reduced lipid peroxidation, free-radical-induced DNA damage, caspase-3 activity and Bax expression. Chronic HO-1 activation also suppressed neutrophil infiltration, and production of IL-1β but increased the plasma level of the anti-inflammatory cytokine IL-10.

A mouse model for radiation-induced cardiomyopathy has been established testing the role of the pro-inflammatory cytokine receptor, IL-1R [95, 127]. Female IL-1R1 KO mice or treatment with recombinant human IL-1R1 antagonist, anakinra, 10 mg/kg twice daily for 7 days, are used as independent approaches to determine the role of IL-1 in response to single 14 and 20 Gy radiation doses to the heart using a CT-based treatment plan and a Gulmay small animal radiation research platform.

Echocardiography (before and after isoproterenol challenge) and left ventricle (LV) catheterization is performed to evaluate changes in LV dimensions and function. The contractile reserve, a measure of exercise capacity, is impaired in wild type mice at day 3 post-radiation, and the LV ejection fraction (LVEF) is reduced after 4 months when compared with sham-radiation. IL-1R1 knock out mice had preserved contractile reserve at 3 day and 4 months and LVEF at 4 months after radiation. Anakinra prevented the impairment in contractile reserve. A significant increase in LV end-diastolic pressure, associated with increased myocardial interstitial fibrosis and pericardial thickening, is observed in both wild type and IL-1R1 knockout–or anakinra-treated mice. Thus, induction of IL-1 by radiation mediates the development of some but not all aspects of the radiation-induced cardiomyopathy. Clinical trials with cardiotoxic chemotherapy agents show that an absolute decrease in LVEF of 5–10 % is associated with a > fourfold increased risk of symptomatic heart failure and cardiac death [128]. Impaired exercise capacity with reduced peak oxygen consumption has been observed in long-term survivors of Hodgkin's disease treated with chest radiotherapy [15]. Decreased peak O_2 consumption (measure of contractile reserve in patients) is a hallmark of heart failure and its severity is exacerbated by inflammation [16]. In our mouse model the reduction in contractile reserve is observed within 3 days of radiation and persisted until the animals died.

The contractile reserve depends on the energy metabolism of the heart. Several studies have demonstrated an association of decreased creatine kinase (CK) activity with decreased contractile reserve [128]. Transgenic mice overexpressing the myofibrillar form of CK show attenuated cardiotoxic effects of chronic doxorubicin administration including a reduced decline in contractile reserve and enhanced animal survival [129]. Reduced ATP synthesis via CK also appears to be a predictor of heart failure and death even after correction for a number of other predictors including LVEF [130]. The mechanisms leading to impaired CK activity are not completely understood. Chronic inflammation leading increased ROS/RNS, and associated with endothelial dysfunction, has been mechanistically linked to reduced CK flux and reduced contractile reserve [131, 132]. For example, elevated levels of ROS/RNS can cause oxidation of a critical Cys in CK inhibiting its enzymatic activity and blocking stress-induced contractile function [132].

Endothelial dysfunction is a hallmark of chronic inflammatory diseases such as diabetes and atherosclerosis, e.g. [133], and parallels have been drawn between atherosclerosis and the late effects of radiotherapy, e.g. [134]. There is evidence that relative radiosensitivity of endothelial cells is the critical lesion in initiating radiation-induced normal tissue toxicity [135]. In diabetes and atherosclerosis endothelial dysfunction is explained in part by an "uncoupled" NOS activity that generates $O_2^{\bullet-}$ and $ONOO^-$ rather than •NO. The one possible mechanism for uncoupling in inflammatory conditions is a reduced cofactor BH4:BH2 [136]. Previous studies [103–105] have shown that the lungs after total body irradiation have a decreased BH4:BH2, and exhibit increased vascular oxidative stress, increased nitrated Tyr, and reduced white blood cell counts compared with non-irradiated controls. Oral BH4 reduced the amount of nitrated Tyr and hematopoietic toxicity. Tetrahydroneopterin, a biopterin

derivative that is ineffective as a NOS cofactor, neither reduced the amount of nitrated Tyr or radiation induced toxicity. These results provide evidence for the role of the NOS coupling in radiation-induced normal tissue toxicity.

Blood samples have been collected from consenting patients in our Department in an IRB approved study involving both retrospective and prospective arms. The retrospective arm consists of patients showing late effects at 6 months or later post treatment at all disease sites except brain. The prospective arm consists of patients providing blood samples before radiotherapy commenced, the day after, every 2 weeks, at the end of treatment, at 6 months post-treatment, and annually thereafter. An initial analysis of the retrospective data looking at selected gene polymorphisms in the promoters of genes encoding HO-1, TGFβ, Nrf-2, and eNOS has been published [137]. An increased frequency of the long GT repeat in the HMOX1 promoter was associated with late normal tissue toxicity in both African American and white populations With increasing length of the GT repeat there is a corresponding decrease in transcriptional activity that has been associated in clinical trials with increased cardiovascular disease [138, 139]. Expression of the minor allele at the single nucleotide polymorphism (SNP) rs1800469 in the TGFβ1 promoter is associated with increased promoter activity and fibrosis [102]. A number of studies on rs1800469 and radiation effects in humans have produced mixed results in terms of predicting late effects [140, 141]. One possibility is seen in our results showing that the association of this minor allele with fibrosis was only significant with African Americans and not Caucasians. However, enhanced severity of the late effects was predictable in Caucasians but not African-Americans by the presence of the minor allele. This same racial disparity is found with rs6721961 SNP in the promoter of the gene encoding Nrf-2. Increased minor allele frequency at this position is associated with reduced promoter activity and acute oxidant lung injury in mice and humans [142]. These results combined with the extensive studies on NOS uncoupling in chronic inflammatory diseases and the anti-inflammatory and anti-fibrotic role of HO-1 argues for investigation as to their potential role in RT-induced late normal toxicity.

Another potential area of study is whether specific GCH1 gene polymorphisms are also potential predictors of normal tissue toxicity. A particular GCH1 haplotype has been found to be a major determinant of BH4 bioavailability both in plasma and in the vascular wall in patients with coronary heart disease [143]. These GCH1 haplotypes are defined by 3 polymorphisms, rs8007267G < A, rs3783641A < T, and rs10483639C < G where the X haplotype is A,T,G; and the O haplotype is any other combination. In the studied patient population haplotype frequencies are OO 70.6 %, XO 27.4 %, and XX 2.0 %. The X haplotype, either XX or OX, is associated with significantly lower vascular GCH1 mRNA expression and significantly reduced plasma and vascular BH4 and total biopterin levels. NOS inhibitor sensitive vascular $O_2^{\bullet-}$ is significantly increased whereas acetylcholine induced vasorelaxation is reduced in the X haplotype carriers. These findings are indicative of endothelial dysfunction in the X haplotype patients. More recent studies showed that these haplotypes are associated with endothelial dysfunction and oxidative stress in patients with type two diabetes [144].

Uncoupling of NOS is an essential feature of tumor progression. Uncoupled NOS by generating RNS/ROS enhances chromosomal instability by down-regulating BRCA1 expression, stimulates anti-apoptotic mechanisms such as NF-kB and Akt signaling while promoting autocrine growth factor-dependent pathways. Uncoupled NOS also may be a component of the chronic inflammatory response of normal tissues to radiation that contributes to radiation toxicity. Currently a BH4 derivative, Kuvan, is used clinically in treating certain forms of phenylketonuria and is in clinical trials to improve endothelial function, in treatment of hypertension and other cardiovascular diseases (clinicaltrials.gov). In addition induction of HO-1 with hemin is in clinical trials to alleviate not only porphyria but also improve reperfusion injury after ischemia. Thus, both approaches are readily testable clinically and may prove to be of potential use in enhancing the therapeutic ratio by both enhancing tumor cell kill and by mitigating normal tissue injury.

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Chapter 7
Aiming the Immune System to Improve the Antitumor Efficacy of Radiation Therapy

Chunqing Guo, Timothy Harris, and Xiang-Yang Wang

Abstract Radiation therapy (RT) has historically been the most common approach used to achieve local tumor control in cancer patients. However, emerging evidences over the last decades suggest an important role for RT in modulating or amplifying the antitumor immune response upon induction of cancer cell death through its direct cytocidal effect. RT alters multiple components of the tumor microenvironment which affect both the immune cell phenotype and function as well as the interactions between tumor and the immune system. Despite the documented immunostimulatory effects, RT alone rarely induces effective antitumor immunity resulting in systemic tumor rejection. RT can also reinforce immunosuppressive mechanisms within the tumor microenvironment, which negatively impacts on the tumor response to RT. Preclinical and clinical data show that combination RT and immunotherapy can elicit powerful antitumor efficacy through either strengthening the immune activation or counteracting immune suppression. In this review, we summarize the immunological changes in the tumor microenvironment upon exposure to radiation. We also highlight radiation triggered molecular and cellular pathways that may contribute to immune evasion and tumor recurrence. Rational and optimized combination of RT and immunotherapy to achieve synergistic antitumor activities for systemic eradication of cancer cells and development of durable antitumor immunity will also be discussed.

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7.1 Introduction

Radiation therapy (RT) is a well-established conventional cancer treatment modality that is administrated up to 50 % of the cancer patients [1]. Tumorical effect of RT lies in its ability to cause DNA damage in irradiated cancer cells. RT is frequently used to achieve local or regional control of cancers either alone or in combination with other treatments, e.g., surgery or chemotherapy. Although RT has been well recognized for its direct cytotoxic and cytostatic effect on neoplastic cells, it is increasingly clear that the immune system also has a major role in the long-term control of tumor growth by RT [2, 3]. This may provide an immunological basis for the abscopal effect of RT [4]. The mechanisms underlying RT-induced antitumor immune responses are complex and involve an active interaction between irradiated cancer cells and the tumor stroma. Historically the tumor cell itself has been the focus to improve the outcomes of RT, while the interplay between the tumor cells and tumor microenvironment (TME) were largely ignored.

The immune compartment within the tumor stroma is primarily constituted of resident or recruited leukocytes with both lymphoid and myeloid origins. Depending on their phenotype and activation state, these immune cells can either promote or suppress tumor progression as well as modulate the therapeutic response to anticancer treatments, including RT [5–7]. Although RT transiently depletes resident leukocytes via direct cytocidal activity, the rebound effects of immune cells following RT are known to impact on tumor response. In this review, we describe immunological changes in the TME following RT and discuss how this immune profile alteration may promote radio-resistance and tumor recurrence. We will reveal the capability of RT to provoke or modulate an immune response and the immune system's role in regulating the local or regional effects of RT implicate the potential rationale of combination RT with immunotherapy. Lastly, we will discuss how RT may exploited to counteract tumor-mediated immune evasion and how immunotherapy can be integrated into RT regimen to achieve improved treatment outcome by promoting immune activation and/or overcoming tumor-associated immune suppression.

7.2 Immune Stimulatory Effects of RT

7.2.1 Induction of Immune Stimulatory Factors by RT

RT can directly stimulate production of immunostimulatory cytokines and chemokines from both tumor cells and tumor stroma. Tumor necrosis factor-α (TNF-α), which can enhance the radiation lethality of tumor cells upon treatment with X-rays

in human sarcoma cells [8]. Irradiation of B16 mouse melanoma tumors induces production of interferon-γ (IFN-γ), which can act directly on cancer cells to induce upregulation of surface the major histocompatibility complex class I (MHC-I), an antigen-presenting molecule critical for T cell mediated recognition and elimination of cancer cells [9, 10]. Clinically, serum IFN-γ levels were found to increase in a dose-dependent fashion in the 56 of 63 patients with esophageal squamous cell carcinoma who were treated with RT alone. The remaining 7 patients whose IFN-γ levels remained unchanged in response to RT developed local recurrence despite radiation [11]. Type I interferons (IFN-α and IFN-β) not only play important roles in the immune responses to viral infection, but are also actively involved in antitumor immunity [12]. RT can induce production of Type I interferons through activation of intracellular DNA sensors, such as the STING-dependent pathway [13, 14]. Induction of type I interferon within the TME is required for generation of type I interferon-dependent innate and adaptive antitumor immunity by potentiating the cross-priming capacity of tumor-infiltrating dendritic cells (DCs) as well as recruitment and effector function of $CD8^+$ T effector cells [15, 16]. It is demonstrated that RT substantially enhances the secretion of the chemokine (C-X-C motif) ligand 16 (CXCL16) by mouse and human breast cancer cells. Upon binding to its receptor CXCR6 on T helper cells (Th1 cells) or activated $CD8^+$ effector T cells, CXCL16 plays an important role in recruiting antitumor immune effector cells [17]. Therefore, it is suggested that in addition to the direct cytotoxic effects, RT can exert its immune stimulating effects through triggering the production of immune activating cytokines or chemokines, which might be additive to radiation lethality through autocrine and paracrine mechanisms.

7.2.2 RT-Increased Antigen Presentation Within the TME

Activation of naïve T cells requires both the recognition of antigen-MHC complexes by the T cell antigen receptor and additional costimulatory signals, including B7 molecules (CD80 and CD86) on the antigen-presenting cells (APCs) [18, 19]. Killing of cancer cells and the subsequent inflammatory responses can make the tumors visible to the immune system if released tumor antigens are taken up by DCs and presented to T cells along with effective co-stimulation signals [20]. RT can induce extensive immunogenic alterations of dying and surviving tumor cells within the TME. The resulting stress and death of tumor cells could activate tumor-specific immune responses through the liberation of damage-associated molecular patterns (DAMPs) upon binding to their corresponding pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) on APCs [21]. It was recently reported that RT triggered immunogenic cell death (ICD), which is defined by translocation of stress protein calreticulin from the endoplasmic reticulum (ER) to the tumor cell-surface or extracellular release of the nuclear high-mobility group protein-1 (HMGB1) and adenosine triphosphate (ATP) [22–25]. Although TLRs in the mammalian immune system was first described as innate receptors recognizing pathogen-associated

molecules, there is growing evidence that the TLRs also sense and respond to DAMPs, endogenous molecules or signals associated with cellular stress and tissue injury [26]. In breast cancer patients with loss-of-function alleles in TLR4, which mediates a signaling response to the HMGB1 stimulation, relapse occurs more quickly after chemoradiation compared with patients with wild-type alleles, indicating that the mode of host response to cancer cell death can affect clinical outcomes of cancer therapy [27].

As opposed to normal self-antigens, immune responses can be biased against the tumor-specific antigens that the immune system has been tolerated without resulting in side effects associated with standard cytotoxic therapies [24, 27]. Studies in mouse models revealed that the antigenic repertoire of tumor cells was substantially altered following RT. Radiation-induced exposure of antigenic peptides have been identified as a mechanism underlying RT-elicited antitumor immune response [28]. The 'danger' signals, including immunostimulatory cytokines, generated by radiation within the TME can activate DCs phenotypically and functionally for effective cross-presentation of tumor antigens [29]. Intratumoral injection of DCs alone does not show evident antitumor effects in mice with squamous cell carcinoma; however, significant tumor regression were observed when combined with chemoradiation, suggesting that the immune environment conditioned by the RT favors DC activation and fosters generation of antitumor immunity [30]. In both mice and humans, activation of tumor antigen-specific T cell immunity following RT requires TLR4 on DCs. Efficient processing and cross-presentation of antigens from dying tumor cells by DCs during RT are dependent on signaling through TLR4 and its adaptor MyD88 [27]. Similarly, local high-dose irradiation of B16 tumors results in activation of tumor-associated DCs as well as the consequent mobilization of tumor-reactive $CD8^+$ T cells [31]. Ablative RT can dramatically improve the cross-priming capacity of tumor-infiltrating DCs. The autocrine effect of type I IFNs is required for the enhanced cross-priming ability of DCs after their infiltration into the irradiated tumor tissues [15]. A recent study demonstrated that adaptor protein STING in DCs and downstream type I IFN signaling are essential for RT-induced adaptive immune responses [13]. In addition, the cytokine secretory profile and its relevance to DC function upon direct radiation exposure have been noted. DCs show enhanced expression of IL-2, IL-12 and IFN-γ after exposure to low dose irradiation, which is positively correlated with their enhanced capability to prime T cells compared with non-irradiated DCs [32].

7.2.3 Activation and Recruitment of T Cells by RT

In addition to direct damage to the tumor cell DNA, accumulating data supports the notion that T cell recruitment and activation represent important mechanisms mediating the antitumor effect of RT. Stone et al. provided the first evidence supporting T cell repertoire dependent tumor response to RT by comparing the efficacy of RT in immunocompetent and T cell deficient mice [33]. RT can also

remodel the abnormal tumor vessels and facilitate efficient tumor infiltration of anti-tumor T cells in a transgenic mouse model of insulinoma with multiple carcinogenesis. The remodeling of the tumor vasculature directly affects lymphocyte extravasation and effector function [34]. Up-regulation of vascular cell adhesion molecule (VCAM)-1 after RT promotes T cell infiltration into mouse B16 melanomas [10, 35]. Recruitment of $CD8^+$ cytotoxic T lymphocytes (CTLs) into 4 T1 mammary tumors was found to depend on RT-induced CXCL16 release from tumor cells [17]. Indeed, the chemokine CXCL16 has been identified as a prognostic factor that correlates with improved survival and increased numbers of tumor-infiltrating lymphocytes in colorectal cancer and renal cell carcinoma [36, 37]. Prostate cancer patients developed detectable tumor-specific $CD4^+$ and $CD8^+$ T cells responses following RT that were undetectable prior to the treatment [38]. RT has been reported to dramatically increase the T-cell priming in lymphoid tissues or tumor tissue. The efficacy of RT can be abolished upon depletion of $CD8^+$ T cells through administration of anti-CD8 monoclonal antibodies [39, 40]. Combination of RT with Th1 cell therapy augments the generation of tumor-infiltrating CTLs, resulting in complete regression of mouse EG7 lymphomas, which suggests that $CD4^+$ T cells are also critically involved in RT-induced CTL response and tumor eradication [40].

7.2.4 Other Immune Cells Activated by RT

Natural killer (NK) cells are considered to be innate lineage cells based on their characteristic that there are no specific antigen receptors on their surface unlike T and B cells. NK cells play an important role in antitumor immunity by directly targeting tumor cells through cytolysis or the secretion of soluble immune mediators [41]. Ionizing radiation can increase the expression of natural-killer group 2 member D (NKG2D) ligands in human cancer cell lines, including KM12, NCI-H23, HeLa and A375. This makes the irradiated cancer cells more susceptible to NK cell-mediated cytotoxicity via the activating receptors NKG2D [42, 43].

7.3 Immune Suppressive Mechanisms Engaged by RT

7.3.1 Immunosuppressive Factors Induced by RT

Although RT can induce immune responses to tumor antigens, it is not sufficient to prime T cells specific for endogenous antigens that can efficiently reject the poorly immunogenic tumors. This may be attributed to the preexisting immune suppression in the TME and the immunosuppressive factors induced by RT. Activation of TGF-β is an early as well as a persistent event in tumors exposed to RT [44, 45]. Serum levels of TGF-β during the course of RT was evaluated in patients with

non-small-cell lung cancer to adaptively deliver higher doses of radiation [46]. TGF-β plays a dual role both limiting tumor growth and stimulating tumor cells progression. Although TGF-β seems to be an antitumor factor at the early stages of cancer progression, it eventually becomes protumorigenic [47, 48]. In the mouse mammary tumor virus-polyoma virus middle T antigen (MMTV-PyVmT) transgenic model of metastatic breast cancer, RT significantly increases the circulating TGF-β and lung metastases, which can be suppressed by the deficiency of type II TGF-β receptor. This implicates RT induced TGF-β as a pro-metastatic signal for tumor cells [49]. TGF-β neutralization in mice bearing 4 T1 mammary tumors enhances radiation sensitivity and significantly delays tumor growth [50]. A recent study reported that TGF-β activity is a major obstacle that hinders the ability of RT to induce antigen-specific anti-tumor immunity. Neutralization of TGF-β by antibody injection during RT effectively rescues a $CD8^+$ T-cell response against poorly immunogenic mouse carcinomas [51].

Hypoxia-inducible factors (HIFs) are the main molecular transcriptional factors in the hypoxia response [52, 53]. HIF-1 is highly induced in the irradiated tumors and high HIF-1 activity is often used as an independent predictor of poor prognosis after RT [54–56]. Expression of HIF-1 and HIF-2 is strongly associated with RT failure in patients with head and neck squamous cell carcinoma [57]. HIF-1 can stimulate the production of stromal-derived factor-1 (SDF-1), a chemokine that recruit tumor-promoting and immunosuppressive myeloid cells through the chemokine receptor CXCR4 [58, 59].

7.3.2 Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs), derived from circulating monocytes, make up a critical component of immune cells in solid tumors [60, 61]. Although a few studies showed that RT enhances the anti-tumor properties of TAMs, including enhanced cytolytic activity and increased secretion of IL-12 and IL-18 [62, 63], there exists extensive literature indicating that TAMs enhance resistance to RT. $CD11b^+$ myeloid cells, including TAMs, are believed to be the major source of pro-tumor growth factors that support angiogenic programs during tumor progression, e.g., vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) [58]. Murine tumors are more sensitive to RT when are transplanted in CD18 hypomorphic or CD11b knockout mice. Resistance of tumors to RT is partially restored by rescue of CD18 hypomorphism with the reconstitution of wild-type bone marrow [64].

Depletion of TAMs by injection of liposomal clodronate prior to RT enhances tumor control, emphasizing an important role of TAM for modulating tumor response to RT. Radiation exposure upregulates VEGF expression in macrophages and VEGF-neutralization subcutaneously improves the antitumor potency of RT

[65]. Recently, it was revealed that $CD11b^+$ monocytes/macrophages restored the damaged vasculature by promoting vasculogenesis and growth of surviving cancer cells following RT in a human glioblastoma xenograft model. Blocking the influx of $CD11b^+$ monocytes/macrophages by pharmacologic inhibition of HIF-1 or SDF-1-CXCR4 pathway can prevent tumor recurrence [66]. Similar observation was made using human breast and lung carcinoma xenografts, further supporting a critical role of myeloid cells, primarily macrophages, in promoting tumor regrowth after RT. It is proposed that TAMs facilitate tumor recurrence by promoting the survival of endothelial cells (ECs) and tumor revascularization [67]. Studies of three murine tumors (TRAMP-C1 prostate adenocarcinoma, ALTS1C1 astrocytoma, and GL261 glioma) demonstrate that $CD11b^{low}/F4/80^+$ macrophages locate at the junctions between central necrotic and surrounding hypoxic regions in the irradiated tumors. Hypoxia-aggregated TAMs are more polarized toward an immunosuppressive and pro-angiogenic M2 phenotype, indicated by the higher expression of arginase I [68]. Thus, despite a potential stimulatory effect of radiation on cytolytic activity of macrophages, the recruitment and alternative activation of macrophages in the TME shifts the balance toward immunosuppression and pro-angiogenesis that benefits tumor recurrence.

7.3.3 Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells, comprised of myeloid progenitor cells and immature myeloid cells [69, 70]. MDSCs are often expanded in tumor-bearing hosts and have been well documented to act as a suppressor of antitumor immunity [71–73]. MDSCs are believed to be one of the mechanisms by which cancers escape from immune surveillance or resist immunotherapy [71–73]. MDSCs are characterized as $CD11b^+Gr\text{-}1^+$ cells in mice [69, 70] and $CD11b^+CD14^-CD33^+$ in human [74]. Two distinct subsets of MDSCs have been identified in mice, i.e., monocytic MDSCs (M-MDSCs; $CD11b^+Ly6G^-Ly6C^{high}$) and granulocytic MDSCs (G-MDSCs; $CD11b^+Ly6G^+Ly6C^{int}$), both characterized by the expression of Gr-1 on the cell surface [75]. CSF signaling has been documented to expand and recruit myeloid cells or MDSCs to the tumor sites [76, 77]. Use of selective inhibitor of colony-stimulating factor 1 receptor (CSF1R) can suppress tumor growth more effectively when combined with RT, highlighting the significance of CSF1/CSF1R signaling in the recruitment of myeloid cells (e.g., MDSCs) that limit the efficacy of RT [78]. The role of MDSCs in limiting the efficacy of RT has also been demonstrated in RT in combination with Sunitinib, an angiogenesis inhibitor [79, 80]. A recent study found that Sunitinib treatment decreased M-MDSC levels and enhanced T-cell proliferative activity in cancer patients with oligometastases [81]. Moreover, the synergetic effect of Sunitinib and stereotactic body radiotherapy (SBRT) was only seen in the responders whose $CD11b^+CD33^+$ myeloid cell populations were reduced by Sunitinib [81].

7.3.4 Regulatory T Cells

FoxP3$^+$ regulatory T (Treg) cells are suppressive immune cells that promote tumor progression through suppressing anti-tumor immune responses [82–84]. Treg cell ablation in a polyoma middle-T antigen-driven tumor model significantly reduces tumor burden and improves overall survival following RT. Combining Treg cell ablation with RT could provide beneficial effects for the poorly immunogenic malignancies [85]. Epidermal mononuclear phagocytes Langerhans cells (LCs) are resistant to the depletion by high dose irradiation. Upon exposure to RT, LCs upregulates MHCII molecule and induces the expansion of Treg cells that can dampen anti-tumor immunity [86, 87].

7.4 Combining RT with Immunotherapy to Improve Therapeutic Index

RT alone is often insufficient to achieve a permanent cure in many clinical scenarios. This is primarily a result of insufficient radiation doses to control tumor without resulting in unacceptable toxicity related to normal tissues. This also suggests that despite the numerous pro-immunogenic or immunostimulating effects, RT as a sole modality fails in shifting the immunosuppressive TME. Systemic antitumor responses following local RT or abscopal responses are also extremely rare in clinical practice. However, RT-induced systemic abscopal response through development of effective and durable antitumor immunity can be promoted by additional immune manipulation. Therefore, it provides a scientific rationale for integrating RT with immunotherapy to amplify the systemic antitumor immunity and to improve overall therapeutic outcomes.

Irradiated tumor cells have been shown to be a source of tumor-associated antigens which can elicit anti-tumor T cell responses after capture and presentation by DCs [88]. Combination of RT and concurrent administration of DCs may result in *in situ* vaccination against tumors. Injection of unpulsed autologous DCs directly into irradiated D5 melanoma or MCA 205 sarcoma tumors was shown to activate tumor-specific reactive T cells and generate a potent systemic antitumor response causing regression of established tumors [89]. In a recent phase I clinical trial of combining external beam RT and intraprostatic DC injection, patients with high-risk prostate cancer showed increased tumor-infiltrating CD8$^+$ T-cells as well as prostate specific CD8$^+$ T-cells in the peripheral blood [90]. In patients with high-risk soft tissue sarcoma that received this combinational therapy, 9 of 17 patients developed tumor-specific immune responses and 12 patients remained free of progression 1 year after treatment [91]. Another recent trial was conducted in 40 patients with recurrent, metastatic, or locally advanced tumors [92]. Patients were treated with conformal RT and autologous DCs pulsed with autologous tumor cell lysates or tumor-specific peptides. Of 9 patients with evaluable tumor response outside the

RT target site, 22 % had a partial response and 33 % had stable disease, indicating that the combination of RT and DC-based vaccination induces measurable clinical responses [92].

An alternative approach that combined local RT and concomitant expansion of DCs *in vivo* through systemic administration of fms-like tyrosine kinase-3 ligand (Flt3L) was also shown to improve survival of animals bearing Lewis lung carcinoma by generating a long-term tumor-specific immune response [93]. The use of Flt3L was also shown to facilitate abscopal effect of RT, indicated by inhibition of both the irradiated breast tumor and the contralateral untreated tumor [94].

Manipulation of the TLR signaling can increase the functional activation of APCs and provide co-stimulation signals to T cells, thereby facilitating an effective adaptive antitumor immunity after RT [95, 96]. A phase II trial conducted in patients with recurrent anaplastic glioma showed that combined RT and intramuscular injection of poly-ICLC, a TLR3 agonist, improved 1-year overall survival compared to RT alone [97]. The TLR7 agonist imiquimod has been approved for the treatment of basal cell skin carcinomas and melanomas. Topical imiquimod can synergize with RT to inhibit tumor growth in a mouse model of skin-involving breast cancer, which is associated with increased number of tumor-infiltrating $CD11c^+$, $CD4^+$ and $CD8^+$ cells [98]. Based on a recent clinical study demonstrating imiquimod-induced immune rejection of skin metastases in breast cancer patients [99], a trial is ongoing to test combination of imiquimod and RT for improving therapeutic outcomes in brain cancer (ClinicalTrials.gov: #NCT01400672).

Our studies have recently identified scavenger receptor A (SRA) or CD204, a pattern recognition innate receptor, as an immunosuppressive molecule expressed on DCs that dampens DC function and T cell activation against several cancers by suppressing DC-intrinsic TLR signaling [100–102]. Our subsequent work revealed that absence of SRA/CD204 significantly increased the immunogenicity of ionizing radiation–treated mouse prostate cancer cells [103], which provides a scientific rationale for combining RT with *in situ* vaccination using SRA/CD204-downregulated DCs. We showed that intratumoral administration of SRA/CD204-silenced DCs, not DC counterparts without genetic modification, profoundly enhanced the control of RT-treated mouse prostate tumor as well as its metastases, which was mainly mediated by IFN-γ-producing CTLs [104]. These preclinical evidence supports the further development of TLR or SRA-targeting strategies for combinational use with RT to convert the tumor into an effective individualized vaccine.

Adoptive cell therapy (ACT) is a passive cancer immunotherapy by transferring of tumor-specific T cells that have been expanded *ex vivo* to cancer patients [105, 106]. Local irradiation of mouse MC38 colon tumors causes up-regulation of Fas on tumor cells and potentiates tumor eradication by adoptively transferred antigen-specific CTLs [107]. Local tumor irradiation combined with intratumoral DC vaccination regimens significantly enhances the therapeutic efficacy of ACT in a mouse liver cancer model, evidenced by reduced local tumor size, decreased metastasis, and prolonged survival. The enhanced antitumor activity is correlated

with the activation of endogenous $CD4^+$ T cells [108]. Therapeutic vaccination represents an active immunotherapy that aims to stimulate T cell response against specific tumor antigens. The synergistic antitumor effect of RT and therapeutic immunization is supported by a preclinical study in colon tumor-bearing mice that received RT plus recombinant vaccinia encoding carcinoembryonic antigen [109]. In a randomized phase II trial patients received RT alone or RT plus a viral vaccine targeting tumor antigen PSA and co-stimulatory B7.1. The results showed an elevated PSA-specific T cell response in the combination group compared to the RT alone arm [110].

Immune modulators that target immunosuppressive signaling in T cells to overcome immune suppression and restore and/or sustain antitumor function of T cells for tumor eradication have shown promise in cancer patients [111]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are two primary immune checkpoint molecules that inhibits T cell activation upon binding to their ligands, B-7 molecules and PD-L1, respectively [20, 111, 112]. As T cell activation relies on the engagement of both antigen receptor and the costimulatory molecule CD28 [113], it is conceivable that using RT to increases antigen availability and APC activation in conjunction with anti-CTLA-4 or anti-PD-L1 therapy will further augment antitumor immune responses.

Anti-CTLA-4 antibody, ipilimumab, has been approved by FDA in the treatment of patients with metastatic melanoma [114]. Combination of local RT of 4T1 mouse mammary carcinoma with the anti-CTLA-4 monoclonal antibodies 9H10 significantly elicited an anti-tumor immunity that inhibited metastases [115]. In another preclinical study administration of 9H10 was shown to optimize tumor response to fractionated RT by inducing an abscopal effect involving activation of $CD8^+$ T cells [116]. The abscopal effect was also reported in a patient with metastatic melanoma following treatment with RT plus ipilimumab. Clinical observation obtained several months after last dose of RT revealed that tumor masses in the spleen and hilar lymph nodes eventually reached the point of stable minimal disease [4]. Complete response in both the primary tumor and the metastatic lesions was also achieved in another patient with asymptomatic melanoma treated with ipilimumab and concurrent RT [117]. A recent case report described that a patient with non-small-cell lung carcinoma also showed abscopal response upon the combination therapy [118]. A phase III trial that evaluated RT combined with ipilimumab therapy in metastatic castration-resistant prostate cancer (mCRPC) was recently completed [119]. The trial did not meet its primary endpoint, however, there was an improvement in overall survival of patients treated with RT plus and ipilimumab compared to RT plus placebo arm (11.2 months vs. 10 months; $p = 0.053$) [119]. Currently, more than ten phase I/II clinical trials that are testing the combination of RT and ipilimumab for treatment of multiple cancer types including melanoma, head and neck cancer, and cervical cancer are ongoing.

PD-1 receptor is another important immune checkpoint molecule that downregulates T cell-mediated immune responses. Expression of PD-1 on T cells in

the TME is an indicator of their exhaustion that is often associated with an impaired T cell response. Overexpression of the PD-1 ligand (PD-L1), also known as B7 homolog ligand 1 (B7-HL1), in a variety of malignant cancers such as renal, lung, ovarian, breast, head and neck cancers, represents one of the mechanisms responsible for tumor immune evasion [20, 120]. Low doses of fractionated RT increases the tumor expression of PD-L1 in a number of syngeneic mouse cancer models, which is attributed to IFN-γ produced by $CD8^+$ T cells [121]. Upregulation of the PD-L1 on tumor cells were shown to contribute to radio-resistance of cancer and suppress the antitumor function of tumor-infiltrating T cells [121–123]. Recently, antibodies targeting PD-L1 (BMS-936559, MEDI4736, MPDL3280A) and its receptor PD-1 (Nivolumab, Pidilizumab, Lambrolizumab) have been developed to overcome PD-1/PD-L1 signaling-mediated immune suppression. Clinical studies showed that anti-PD-1/PD-L1 antibodies have achieved significantly increased objective response (~20–30 %) in the treatment of several types of cancers including non-small cell lung cancer, melanoma, and renal-cell cancer [124–126]. Studies using pre-clinical models demonstrated the synergistic effects of RT combined with PD-1 checkpoint inhibitors. Adding anti-PD-1 antibody to combination therapy of RT and anti-CD137 therapy resulted in cure of the primary mammary tumors [127]. Treatment with RT in conjunction with anti-PD-1 antibody resulted in synergistic inhibition of mouse glioma, TUBO mammary carcinoma, and MC38 colon adenocarcinoma, probably through increasing the infiltration of IFN-γ- or TNF-α-expressing CTLs while decreasing the accumulation of Treg and MDSCs within the TME that normally suppress T cell function [122, 128]. Despite the impressive clinical responses resulted from immune checkpoint inhibitors, optimization is required to overcome multiple non-redundant mechanism of immune resistance. A recent phase I trial reported that melanoma patients with high expression of PD-L1 did not respond to RT plus anti-CTLA4 therapy [129]. Mouse studies found that this resistance was due to upregulation of PD-L1 on melanoma cells during RT and consequent T-cell exhaustion, which allows tumors to escape anti-CTLA4 therapy. Thus, triple combination of RT, anti-CTLA4 and anti-PD-L1 treatments, which enhances the diversity of the T-cell receptor repertoire of intratumoral T cells, inhibits Treg cells, thereby increasing the ratio of CTL to Treg, and reverses T-cell exhaustion, can achieve maximum antitumor response by engaging distinct mechanisms [129].

In addition to targeting immune checkpoint molecules to rescue and sustain T cell functions in the TME, other approaches directed to promote co-stimulation can also enhance T cell priming and effector function. OX40 signaling is one of the co-stimulatory mechanisms involved in T cell activation [130–132]. Administration of OX40 agonistic antibodies in combination with RT significantly extends the mouse survival in a model of primary sarcoma by augmenting the activity of tumor antigen-specific CTLs following RT [133]. Clinical trials of combining RT and OX40 agonist for treatment of metastatic prostate cancer (ClinicalTrials.gov: #NCT01303705) and breast cancer (ClinicalTrials.gov: #NCT01642290) are ongoing.

7.5 Challenges and Opportunities

Encouraging preclinical results of combining RT and immunotherapy for cancer treatment have stimulated clinical translation of this combinatorial therapeutic modality. However, clinical data from trials that combined RT with other immune modulating agents, e.g., vaccines, immune checkpoint blockade, have only shown a modest promise. While these outcomes provide the rationale for current clinical trials of both treatments, further investigation is required to realize the full potential of the combination.

The immunogenic alterations in the TME induced RT at molecular and cellular levels are just beginning to be elucidated. The immunoregulatory effects of dose and fractionation schedules as well as delivery during RT remain to be further defined. The timing of RT relative to immune manipulation should also be carefully determined in preclinical and clinical studies. Extrapolation of data derived from mouse models may have its own limitation because currently most animal studies involve radiation to tumor xenografts. Use of spontaneous transgenic mouse model instead of transplantation mouse model to address these questions may better guide the optimal design of clinical RT in combination with immunotherapy. Mechanistic understanding of immunological and biological changes in the TME has an important impact on capitalization of tumor destruction capacity of radiation and immune augmentation.

The immunosuppressive effect of the TME remains a major hurdle for clinical success of combined RT and immunotherapy. The promising results observed in clinical trials with immune checkpoint blockade therapy highlights the therapeutic potential by modification of the TME to overcome immunosuppressive pathways. Exploring and identifying additional and non-redundant immunosuppressive molecules or mechanisms that operate in the TME will provide new therapeutic targets to synergize with RT to mount an effective and durable antitumor immunity.

Personalized medicine has been an important part of therapeutic endeavors in the field of cancer research and treatment. Emerging data suggests that each patient's own immune system have the potential to develop a tailored immune response to the unique clonal populations within the tumors. Thus, immunotherapy is at the forefront of personalize anticancer therapy and precision oncology. With the advantage of its very focused and localized nature, RT is ideal for combination with proper modulations of the immune system and has a great potential to convert the tumor into an individualized vaccine. Ongoing and future randomized trials with RT and immune-based combinations will help determine if such regimens can change the RT paradigm and, more importantly, revolutionize cancer treatment (Table 7.1).

Table 7.1 Ongoing clinical trials of combined radiation therapy with immunotherapy

Description	Cancer type	ClinicalTrials.gov Identifier
Radiation therapy plus dendritic cells vaccination	Metastatic melanoma	NCT01973322
	Pancreatic cancer	NCT00868114
	Glioblastoma	NCT02010606
	Glioblastoma	NCT02366728
Radiation therapy plus toll-like receptor signaling-targeting agents	Soft tissue sarcoma	NCT02180698
	Breast cancer	NCT01421017
	T cell lymphoma	NCT02061449
	B cell lymphoma	NCT02254772
Radiation therapy plus immune checkpoint blockade	Pancreatic cancer	NCT02311361
	Metastatic melanoma	NCT02406183
	Non-small cell lung cancer	NCT02599454
	Non-small cell lung cancer	NCT02434081
Radiation therapy plus adoptive T cell transfer	Merkel cell carcinoma	NCT02584829
	Merkel cell carcinoma	NCT01758458
	Nasopharyngeal carcinoma	NCT01462903
	Hepatocellular carcinoma	NCT01462903
	Breast carcinoma	NCT01462903

7.6 Concluding Remarks

The effort in improving the therapeutic efficacy of RT have focused on the capability of ionizing radiation to kill neoplastic cells while sparing normal healthy tissues. However, accumulating evidence supports the immune modulating effects of RT (Fig. 7.1a). RT-mediated destruction of cancer cells releases tumor antigens along with 'danger' signals or PAMPs that defines the immunogenicity of tumor or ICD. These immunostimulatory factors result in recruitment and activation of APCs (e.g., DCs), which facilitates subsequent T cell priming and antitumor immune response. However, it is recognized that multiple mechanisms in the TME, which involve induction of immunosuppressive factors (TGF-β, CTLA-4 and PD-1) and recruitment of immunosuppressive cells (TAM, MDSC, Treg cell), dampen or impair immune effector function and promote immune tolerance. The substantial expansion and recruitment of myeloid cells following RT is known to facilitate tumor revascularization and possibly immune suppression as well. Therefore, it is unlikely RT alone is capable of generating an effective immune response that can eradicate tumors locally and abscopally. Nevertheless, interaction of RT and the immune system offers new opportunity to strengthen and improve antitumor

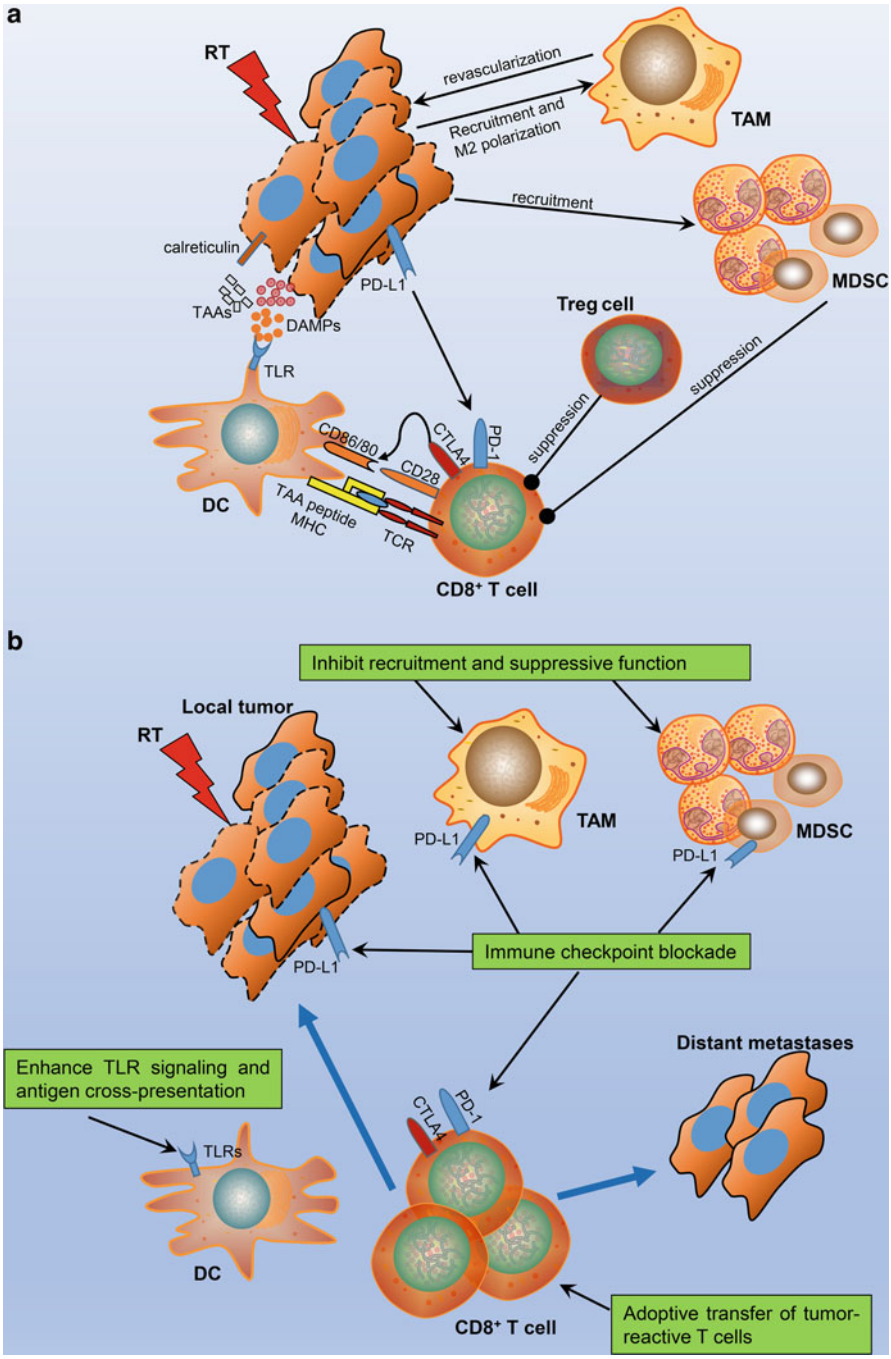

Fig. 7.1 Exploiting the interplay between radiation therapy (RT) and the immune system to improve cancer therapeutic index. (**a**) RT can induce immunogenic cell death associated with release of tumor-associated antigens (TAA) with damage-associated molecular patterns (DAMPs), which recruit and stimulate dendritic cells (DCs) via toll-like receptor (TLR) for antigen cross-presentation.

response by strategically combining RT and immune interventions or immunotherapy, e.g., in situ DC vaccination, TLR activation, immune checkpoint blockade, immune co-stimulation (Fig. 7.1b). Cancer immunotherapy has emerged as a viable therapeutic option and with multiple agents in clinical development the immunotherapeutic portfolio is expected to expand significantly in the near future. While more research is needed to precisely understand and rationally optimize the protocol of this combinatorial treatment, clinical studies have started to show the promise in improved treatment outcome, which we believe may lead to ultimate elimination of cancers and metastases by amplifying immune-mediated abscopal effects after standard RT.

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Fig. 7.1 (continued) Subsequent priming of $CD8^+$ T cells though engaging surface T cell receptor (TCR) contributes to cancer cell killing during RT. However, concurrent increase in the number of pro-angiogenic and immunosuppressive cell types, such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg), as well as induction of immunosuppressive factors, such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1), compromise immune effector function within the tumor microenvironment (TME) and promote cancer escape and recurrence. (**b**) While RT-induced immune response is not adequate to achieve systemic eradication of tumors, integrating RT with immunotherapy could amplify the systemic antitumor immunity and improve overall treatment outcomes. Selectively stimulation of TLRs or strategic administration of ex vivo expanded DCs can enhance DC function and antigen cross-presentation by providing additional co-stimulatory signals. Adoptive transfer of tumor-reactive T cells, either ex vivo expanded T cells or genetically modified T cells, can further strengthen the effector arm of antitumor mechanisms since radiation exposure sensitize cancer cells to the killing of T cells. Additionally, immune checkpoint inhibitors can be used to rescue the effector function of T cells by counteracting the immunosuppressive signaling mediated by CTLA-4 or PD-1/PD-L1. Other approaches directed to block the recruitment and/or to overcome the immunosuppressive activity of myeloid cells (e.g., TAMs, MDSCs) can also boost antitumor immune response. The increased magnitude and duration of systemic activation tumor-specific T cells combined with direct tumoricidal effect of RT could lead to synergetic and optimized destruction of local/regional tumors as well as metastases

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Chapter 8
The Role of MicroRNAs in Modulating Tissue Response to Radiation

Rebecca J. Boohaker and Bo Xu

Abstract MicroRNAs are a critical class of regulators for cells to deal with DNA damage. Abnormal miRNA function is associated with tumor initiation and progression, and altered miRNA expression found in tumor tissues are frequently associated with heterogeneity of tumor responses to therapeutic agents, including radiotherapy. In this chapter, we review recent advances of the functional role of microRNAs in the context of the DNA damage response, tissue specific tumor initiation and progression. We further discuss clinical implications of using miRNA signatures as biomarkers for radiosensitivity and targeting specific miRNAs as therapeutic approaches.

Keywords miRNA • DNA damage response • Radiotherapy • ATM • Biomarker

8.1 Introduction

Radiotherapy is used to treat more than half of patients diagnosed with cancer, either as the primary mode of treatment or in combination with chemotherapy or surgical resection. The success of radiotherapy relies on the inability of cancerous cells to efficiently repair DNA damage relative to their normal tissue counterparts, pushing the cancerous cells into death pathways. Specifically, ionizing radiation (IR) is intended to induce DNA damage, engage the DNA double-stranded break repair machinery, and to push the cell into mitotic catastrophe, apoptosis, or stress-induced senescence. Some tumors, however, exhibit an insensitivity to an otherwise

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curative dose and are deemed radioresistant. Tumor radioresistance is a common problem linked to tumor heterogeneity and underlying biochemical factors such as abnormal DNA damage response pathways, microenvironment alterations, deregulated survival pathways, and altered expression of oncogenes and tumor suppressors. An increasing number of studies have shown that regulation of these pathways is modulated in part by microRNAs.

MicroRNAs (miR) are highly conserved, small, non-coding RNAs that are involved post-transcriptional regulation of target mRNAs, and also regulate roughly 30 % of human genes at the DNA level [1]. Biogenesis of these miRs involves a series of enzymatic cleavages that begins with primary microRNA transcripts (pri-miRNAs). These primiRNAs are converted to hairpin pre-miRs via activity of the Drosha/DGCR8 complex for export out of the nucleus and into the cytoplasm. Finally, the pre-miRs are cleaved by a Dicer, a RNase, and the cleaved mature miR is assembled into the RISC complex. The RISC then targets a specific mRNA for repression and degradation [2]. Regulation of protein expression subsequently affects the pathways in which the proteins function, resulting in a measurable change in intracellular processes. MiR biogenesis can be triggered by a number of external and internal cellular signals. Here the focus will be on the miR expression patterns, and the affected pathways in the context of ionizing radiation (IR), the DNA damage response (DDR), and tumor progression.

Exogenous genotoxic agents, in the form of IR and oxidative stress inducers, have been shown to influence the biogenesis of a certain subset of the identified ~3700 miRs [3], and within that subset there are tumor-type specific miR expression profiles [4]. While there are type-specific differences in miR expression, there also exists a significant overlap [Fig. 8.1]. So, it is within the differential expression, coupled

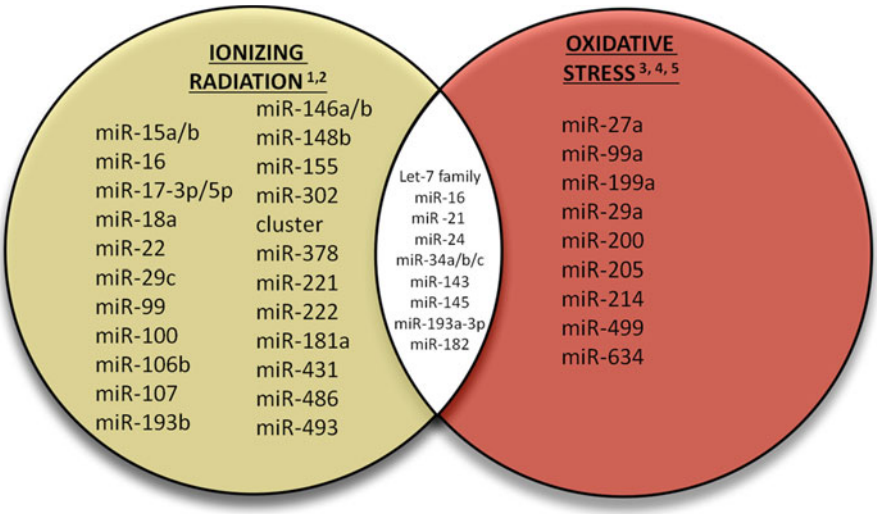

Fig. 8.1 miR expression in response to genotoxic stress. MiR expression is unique to the nature of the DNA damaging agent. However there is some overlap in a small subset of miRs. [1][41], [2][42], [3][43], [3][44], [4][45], [5][46]

with the common alterations, that diagnostic and therapeutic approaches can be taken to determine on a patient-by-patient basis whether radioresistance is likely. The use of miRs as prognosticators of tumor sensitivity to ionizing radiation has clinical significance in determining the best course of action for treatment, sparing the patient from therapies that are not viable options based on tissue micro-environment.

8.2 The Role of miRs in the DDR

A properly functioning DDR is essential for the maintenance of genomic integrity. Double-stranded breaks (DSBs) trigger activation of the Ataxia-Telangiectasia Mutated (ATM) kinase which results in the phosphorylation of H2AX at lesion sites and recruitment of repair proteins. This process stalls cell cycle progression until the lesions are repaired, or, if the damage is too catastrophic, transitions the cell into senescence and apoptosis. The DDR pathway is extensively regulated by miRs and components of the miR biogenesis pathway. Specifically, the enzymatic functions of Dicer and Drosha regulate the expression of miRs that target pATM and its substrates that subsequently sense and form foci at DNA damage sites [5]. ATM is also directly modulated by miR-18a, or indirectly by miR-421 and miR-106a, underscoring the importance of regulation of the DDR pathway via ATM activity [6–8]. Additionally, DNA damage directly regulates biogenesis of a small subset of miRs linked to Drosha/DGCR8 by complexing with p68 and p72 to facilitate processing of pri-miRs to pre-miRs. ATM activates KH-type splicing regulatory protein (KSRP) which also complexes with Drosha/DCGR8 to allow for pri-miR processing [9] [Fig. 8.2.].

During the course of the DDR, nearly all major players involved in the process of clearing the damage are subject to direct regulation by miRs. From the damage sensor H2AX, to ATM as a signal transducer, to downstream effector pathways miRs target and directly regulate key components of the process. Indirectly, miRs modulate the expression of upstream regulators of the process in order to provide a fine-tuning of the pathway [10]. The extent to which the DDR, gatekeeper of genomic stability, is regulated by miRs underscores the importance of proper expression and function of these RNAs in this pathway [Fig. 8.3].

8.3 MiRs Expression in Carcinogenesis

MiRs, by their nature, regulate both tumor suppressor genes and oncogenes, and are divided into oncomircroRNAs which regulate tumor suppressors or anti-oncomicroRNAs which regulate oncogenes [11]. This regulation depends on the tissue in which the miR is expressed. The first indication that miRs could be implicated in cancer pathology was from the early descriptive studies in *C. elegans* and *drosophila* model systems where mutations in let-7 resulted in

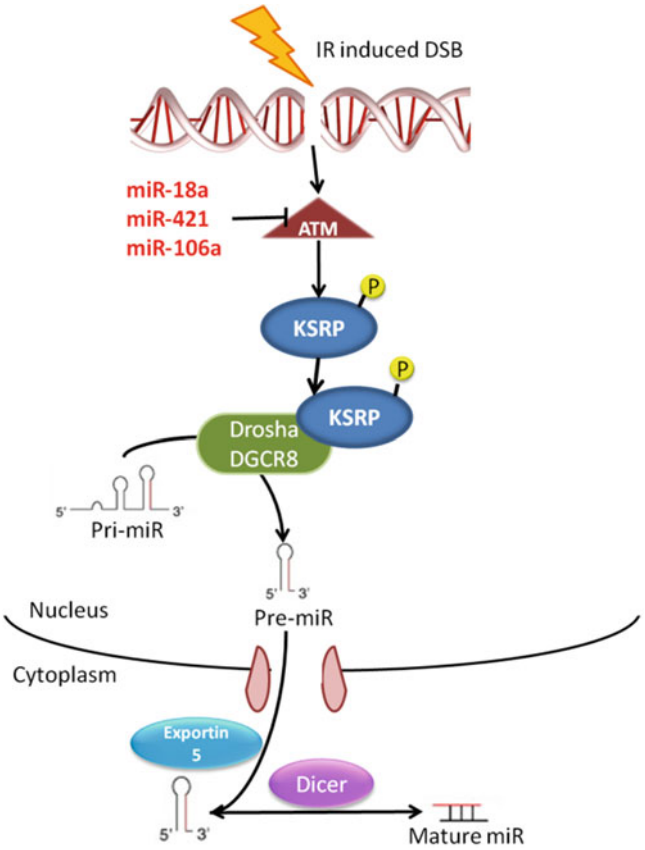

Fig. 8.2 ATM Regulates and is Regulated by miRs During DDR. DNA damage induced ATM activation triggers miR biogenesis via phosphorylation of KSRP. ATM itself is regulated by miRs 18a, -421, and -106a. Regulation of ATM fine-tunes the response to DNA damage

loss-of-function phenotypes as seen in loss of proliferation regulation [12, 13]. The association of miR expression with carcinogenesis was shown initially in chronic lymphocytic leukemia (CLL) which linked loss of expression of miRs 15 and 16 to disease progression [14]. This was the first in a series of studies that critically examined the miR expression patterns, mutation rates, and physiological consequences in neoplastic tissue arising from every tissue type [4]. Determination of the gene locus of these miRs found that a majority of these coding sites can be found in fragile sites, susceptible to alteration, and also in genomic regions frequently associated with carcinogenesis [15]. The distribution of these genes is not random as most of these coding regions are positioned to be flanking oncogenes and common translocation sites that result in altered expression or even deletion of these miRs [16]. As such, a stressed system is likely to expose these fragile sites to damage [17].

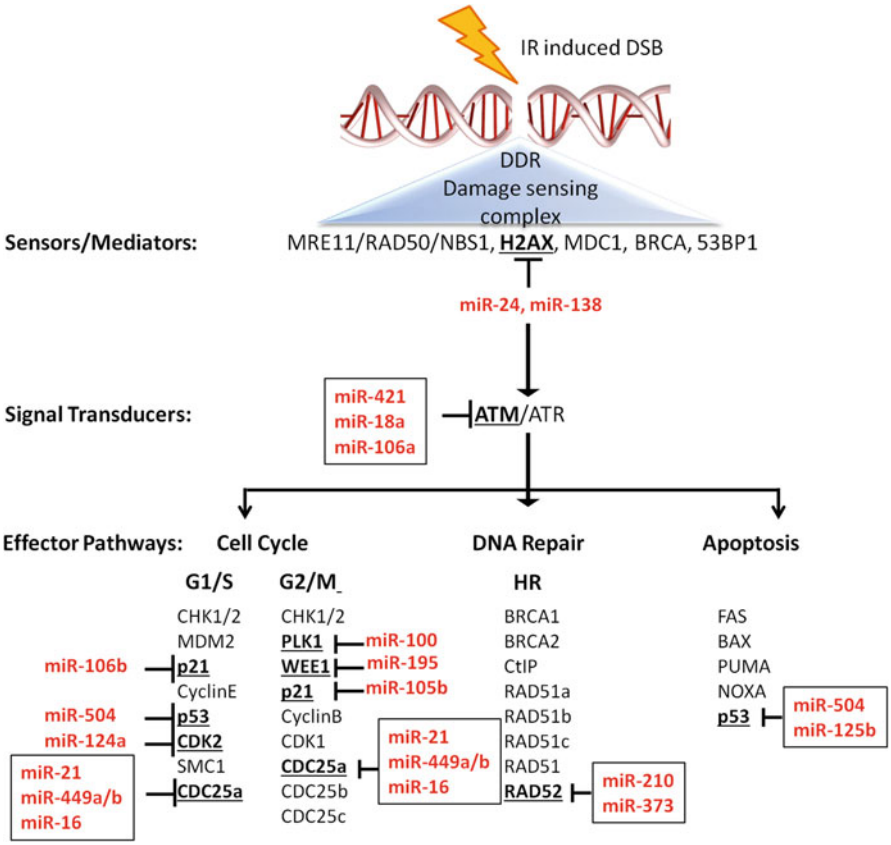

Fig. 8.3 The DDR is Regulated at all Levels by miRNAs. MiR expression as the result of ionizing radiation exerts a regulator effect on all phases of the DNA damage response. This regulation determines whether damage is able to be detected and whether the appropriate effector pathways can be signaled

The effect of genomic changes to miR sequences or transcription rates is compounded because of the mechanisms by which miRs are processed into maturity. MiR clusters encompass precursors to mature miRNA products; as such alterations in any given cluster can have wide-ranging, deleterious effects. The localization of these miR coding genes at fragile sites, combined with the documented high mutation rate is further affected by alterations in protein-coding genes crucial in miR biogenesis, specifically Dicer and Argonaute coding genes [16]. Such alterations fall into the following catagories: (1) loss of miR expression due to deletion, transcription error, or mutation, (2) over-expression due to gene translocation, and (3) altered expression due to changes in the biogenesis pathway and machinery. The physiological consequences manifest themselves in the form of hyper-proliferation, evasion of apoptosis, and invasiveness due to the inability to properly regulate target mRNAs.

8.4 MiRs as Biomarkers for Radiation Sensitivity

An ideal biomarker meets the following characteristics: it must be specific to the pathology in question, is rapidly detectable upon onset of the pathology, is proportional, or inversely proportional, to the severity of the pathology, is a preclinical predictor of a clinical outcome, and is readily accessible [18]. The quest for biomarkers as prognosticators for positive therapeutic outcomes has focused attention on circulating miRs. Since miRs are critical regulatory elements of key pathways, changes in their expression levels could be reliably linked to the mRNA targets and subsequent pathways they regulate. The discovery of detectable miRs in serum and other bodily fluids points to use of these extracellular miRs as potential biomarkers. These miRs are packaged in such a way as to avoid RNase digestion and likely act as cell-to-cell communicators, and because of their stability and specificity, can indicate tissue specific pathology.

Normal, non-cancerous tissues exhibit a predictable change in miR expression in response to radiation. Notably, the biogenesis of miRs that regulate cell cycle progression and DDR are regulated in a dose-dependent manner to IR [19, 20]. Acting as anti-oncomiRs, the let-7 cluster of miRs is linked to cell cycle progression and apoptosis regulation through regulation of KRAS, while the miR-34 family is targeted by p53 to halt cell cycle progression and modulate apoptosis proteins [21]. Additionally, miR-21 is reliably up-regulated in both normal and cancerous tissue and is reported to target key components in the apoptotic pathway such as the transcript for programmed cell death 4 (hPDCD4) [22]. At the front end of the DDR, initiated by ionizing radiation-induced double-stranded DNA breaks, ATM expression is regulated by miR-421 [8] and miR-101 [23] while at the back end, H2AX, a histone variant phosphorylated by ATM, is targeted by miRs-24 and -138 [24, 25]. Over-expression of any of these miRs results in down-regulation of their protein targets and subsequently leads to accumulation of chromosomal damage and sensitivity to IR.

The pathway-specificity and ubiquitous expression profiles of these few miRs provide promising biomarkers for predicting radiosensitivity. Nearly all have direct targets at key non-redundant junctures in the DDR, cell-cycle, proliferation and apoptotic pathways. The differential expression in normal tissues upon treatment with ionizing radiation gives a baseline with which to measure efficient pathway execution when compared to cancerous tissue. The ability to detect and quantify these miRs prior to and in the course of treatment has the potential to increase therapeutic efficacy and improve patient outcome. Use of patient databases such as The Cancer Genome Atlas (http://tcga-data.nci.nih.gov) and cBioPortal (http://www.cbioportal.org) provide standardized sets of data gathered from patient biopsies. Meta-analysis of these datasets links expression patterns in tumor types to survivability and treatment efficacy, identifying clinically relevant miRs that may be key regulators in the response to radiation. These studies are currently being done for most tissue specific cancers from head and neck cancers to glioblastomas [26–28].

Table 8.1 Tissue specific miR expression alterations after IR exposure

Tissue type	Increased expression	Decreased expression
Blood and lymphocytes	Let-7f, miR-16, miR-17-3p/5p, miR-19a, miR-20a/b, miR-24, miR-27a, miR-29a/c, miR-34a/b, miR-106a, miR-126, miR-142-5p, miR-145, miR-155, miR-221, miR-222, miR-601	Let-7e, miR 10a, miR-17, miR-19b, miR-99a, miR-100, miR-143, miR-152, miR-181a, miR-196a
Breast	–	miR-302a/b/c/d/e
Central nervous system	Let-7 family, miR-15a, miR-16, miR-17-3p/5p, miR-19a/b, miR-22, miR-21, miR-142, miR-143, miR-155,	miR-107, miR-181a, miR-521
Colorectal	miR-125a-3p, miR-137, miR-188-5p, miR-483-5p, miR-630, miR-765, miR-1183, miR-1909	miR-1274b, miR-720
Lung	Let-7a, miR-15, miR-16, miR-17-5p, miR-19a/b, miR-20, miR-24, miR-27a/b, miR-30a-5p, miR-99a, miR-106a, miR-126, miR-128b, miR-148a, miR-221, miR-365, miR-451, miR-495	Let-7 family, miR-15b, miR-17-5p, miR-19b, miR-21, miR-26b, miR-125a, miR-130a, miR-155
Urogenital	miR-9-1, miR-22, miR-24, miR-29b, miR-141, miR-191, miR-200c, miR-379	miR-100, miR-106b, miR-107, miR-133b, miR-143, miR-145, miR-196a, miR-199a
Head and neck	Let-7c/d/g, miR-17-3p/5p, miR-27b, miR-34a/b, miR-188-5p, miR-365	Let-7f/g, miR-10a, miR-106a, miR-152

Adapted from [47]

Can circulating miRs be used to predict radiosensitivity? Recent studies have indicated that, yes, tumor specific circulating miRs can be detected in blood serum. Examples of this can be found in breast cancer [29–31], and in a recent study that showed xenograft-specific miR detection in the blood serum of mice correlated to clinical samples from patients with pancreatic, lung and colorectal cancers [32]. The real key to the feasibility of miRs as biomarkers in a clinical setting is that the detectable, circulating miRs at least partially overlap with the tumor-specific ones. To date, radiosensitivity miR biomarkers have been identified in every major tissue specific cancer [Table 8.1]. The development of clinical assays to rapidly and accurately evaluate the radiosensitivity of cancers depends in part on the ability to isolate and detect miRs in a minimally invasive manner.

8.5 MiRs as Therapeutic Targets to Enhance Radiation Efficacy

Given the baseline expression of key miRs involved in the radiation response, coupled with changes in expression when exposed to radiation, a list of therapeutic targets begins to emerge in disregulated systems. The therapeutic avenues for rescuing normal phenotypes are to either inhibit the activity of over-expressed miRs or

exogenously express deficient miRs. The major obstacle to this is the ability to package and deliver the therapeutic agent in a way that will ensure both stability and tissue specificity. Current strategies in miR-based cancer therapy have used modified, or locked, nucleic acids to competitively bind and inhibit mature miRs [33]. To combat under-expression or deletion of key miRs, synthetic naked miRs can be loaded into tagged lipid vesicles or nanoparticles for delivery [34]. Viral delivery of deficient miRs has shown promise in laboratory settings and even *in vivo*, but the concern of chromosomal integration and other off target effects has limited success in pre-clinical and clinical trials [35, 36].

A less direct method to affect miRNA expression is through the use of drug compounds to induce miR biogenesis pathways subsequently resulting in a synthetic lethality in cancerous cells when coupled with radiation treatment. A prime example of this is the identification of MiRNA-145 as an indicator of tumor sensitivity to radiation in prostate cancer [37]. This miR also plays a role in ovarian cancer where its expression can be induced by the flavonoid quecetin subsequently inducing apoptosis [38]. Also within the class of flavonoids, Rhamnetin and cirsiliol have been shown to induce miR-34a expression. MiR-34a inhibits Notch-1 expression and renders tumors more susceptible to IR treatment [39]. Another example is cantharidin, a terpenoid, affecting the expression of miR-214 regulating p53-mediated apoptotic pathway to swing the Bax/Bcl-2 balance towards cell death [40]. The use of these compounds and others like them depends heavily on identification and targeting of the tissue specific miR responsible for pathway dysregulation.

8.6 Clinical Significance

The extent to which miRs regulate critical survival and repair pathways in a cell makes these small oligonucleotides ideal as predictors and therapeutic targets in the treatment of a wide array of cancers. MiR biogenesis pathways are incredibly sensitive to internal and external changes, allowing for the characterization of expression patterns in the face of DNA damage resulting from ionizing radiation. It is important to establish and validate cancer-specific biomarkers using meta-data analysis and bench-top verification of radiosensitive miR signatures for relevant carryover into the clinical setting. Properly vetted biomarkers can be used both as prognosticators as to whether the cancerous tissue will be responsive to therapeutic doses of radiation, and if readily accessible, as with circulating miRs, can be assessed during the course of treatment to assure that the cells are responding to treatment. This is beneficial from the perspective of patient wellbeing because radiotherapy can be discounted out right, fine-tuned in dosage, or coupled with other therapies with greater confidence in the sensitivity of the cells to treatment.

As clinically relevant therapeutic targets, miRs can either be inhibited or induced. The balance in this strategy is in the delivery mechanism and ensuring that drug delivery is tumor specific with limited off target effects. Because of the nature of miR regulation, in that one mature miR has multiple mRNA targets, it is critical to target miRs

with direct effects on pathways involved in DNA damage. Over-expressed miRs are an easier target to address because the drug or oligomimetic cargo can be packaged in such a way to direct the therapy to specific cells with specific receptors. Drug-induced specific miR biogenesis with the intent of weakening a survival or repair pathway could prove more challenging. A more focused approach to manipulating miR expression is needed to effectively produce the synthetically lethal radiosensitivity. As a class of nucleotides though, miRs are emerging as critical components.

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Chapter 9
Adapting Therapy Based on Tumor Response

Christina Hunter Chapman, Yue Cao, Martha Matuszak, Matt Schipper, and Theodore S. Lawrence

Abstract Though radiotherapy techniques have improved significantly over the past few decades, high rates of toxicity, recurrence and mortality persist for many cancer types. This chapter describes how traditional radiation therapy, which involves application of common dose prescriptions and normal tissue constraints across heterogeneous populations, limits personalization of treatment. It presents an explanatory model of the potential benefits of individualizing and adapting radiotherapy and places these benefits in the appropriate clinical context. It highlights the importance of utilizing novel imaging sequences and biomarkers, and illuminates the advantages of incorporating intra-treatment data into adaptation algorithms. It outlines a framework for identifying opportunities to investigate and develop individualized radiotherapy protocols. Using various tumor types as examples, it discusses the rationale for radiotherapy, current standards of care, and emerging evidence for the utility of adaptation strategies. Finally, it suggests areas for future adaptive radiotherapy research, with the goal of improving cancer outcomes through precision radiation medicine.

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9.1 Introduction to Adaptive Radiotherapy

9.1.1 Rationale for Adaptive Radiotherapy

Traditional radiation therapy has involved applying common dose prescriptions and normal tissue constraints across heterogeneous populations. Normal dose limits are based on the most sensitive individuals in the population, thus potentially undertreating the tumors of the vast majority of patients. Furthermore, the dose limits and prescriptions that were historically defined at the beginning of treatment were rarely altered mid-treatment to account for individual variation in toxicity and tumor response. Individual adaptation often focuses on correcting for daily systematic and random setup variations, and is therefore still aimed at the goal of delivering the population-based standard prescription. Significant toxicity and failure rates, coupled with the limited success of recent dose escalation trials, highlight the weaknesses of these population-based approaches to radiotherapy delivery.

New imaging modalities and other dynamic assays have made it clear that the original prescription and plan may no longer be optimal as treatment progresses. Valuable information can become available during treatment that should factor into the decision of how to alter the radiation plan. The suboptimal specificity and sensitivity of temporally rigid, population-based radiotherapy algorithms has therefore generated interest in identifying techniques to individualize and adapt radiotherapy. Attempts have been made to explore intratreatment adaptation using anatomic imaging (including CT [1] and MRI), but they have not led to major changes in radiotherapy delivery for most cancers. Anatomical adaptation has largely been unsuccessful for a number of reasons, including the lack of correlation between outcomes and early radiographic changes on anatomical sequences [2, 3] and concerns about underdosing of occult microscopic disease.

Another strategy for adapting radiotherapy is the incorporation of radiogenomics—the study of the relationship between germ line genotypic variation and variation in response to radiotherapy [4]. While genomics has led to successful advances in systemic therapy development and utilization, it has not yet proven to be a practical technique for improving radiation delivery. Ongoing research will clarify whether genomics can improve radiation delivery in the future [5]. Though radiogenomics is not ready for clinical application, novel imaging modalities and assays used before and during radiotherapy have shown promise in improving individual level outcomes. Incorporation of these new technologies has ushered in the era of "precision radiation medicine."

9.1.2 *Theoretical Benefits of Radiotherapy Adaptation*

Though modern radiotherapy techniques have reduced normal tissue toxicity, dose limits continue to be based on the most sensitive 5–15 % of the population, thus potentially undertreating the other 85–95 % of patients. Furthermore, we assume that a tumor is homogeneous in its response to treatment, when we know that it is not. Therefore, there is great potential to improve treatment outcomes by assessing the tumor and normal tissue response during the course of treatment, and using this information as a bioassay of each patient's normal tissue sensitivity and tumor response, which may be spatially and temporally heterogeneous. A hypothetical illustration of intended innovative approaches will help clarify the potential benefits of precision radiation medicine.

For a given severe treatment related complication, we can assume each patient has an individual threshold "equivalent uniform dose" to that organ (the absorbed dose that, when homogeneously given to a tumor, yields the same mean surviving clonogen number as the given non-homogeneous irradiation, EUDt), such that EUD values in excess of that threshold would lead to the complication and EUD values less than that threshold would not. Then, if we assume that across the population of patients, EUDt is normally distributed with some mean EUD_{50} and standard deviation, we get Lyman's normal tissue complication (NTCP) model [6]. Thus, an allowed complication probability level would essentially translate to an upper bound on EUD for the population. This is the situation illustrated by the "prior" population curve in Fig. 9.1**.** We hypothesize that there are subpopulations of patients with distinct distributions of EUDt, and that we can obtain, during treatment, information on the probability distribution that the current patients belong to a particular subpopulation. These probability distributions would follow, for example, a Bayesian Network analysis. Using Bayesian inference or maximum likelihood methods, we might expect to be able to determine if an individual patient resides in the most tolerant portion of the population (the "posterior" distribution in Fig. 9.1). With this new information, we could then establish a new upper bound on the EUDt for the most tolerant patients at the same level of expected complication rate; and (if the threshold changed "significantly") we could reoptimize the radiation therapy treatment plan, escalating the tumor dose up to this new EUDt level. That is, during the course of treatment, new data leads us to believe that the patient is likely (with some likelihood distribution) to be in the most tolerant part of the distribution. This type of pretreatment stratification for normal tissues and tumors has already been performed in the setting of liver radiotherapy (using Child-Pugh score) [7] and can be applied using intratreatment imaging and biomarker changes in additional tumor types.

The next step is to ensure that escalating the tumor doses for patients most tolerant to radiation complications while lowering the doses to the very sensitive members of the population (in order to maintain the same overall rate of complications as the original population) produces higher overall tumor control rates. Figure 9.2 uses a hypothetical scenario to graphically illustrate that it is possible to use better prediction models of NTCP to select dose for patients in such a way that the overall

tumor control probability (TCP) is increased while the overall NTCP is maintained at a constant level. In this figure, we assume that there is a group of patients with the same mean tumor volume and location. This assumption, which implies that there is a 1:1 relationship between mean lung dose (or mean dose delivered to the entire lung excluding the tumor, MLD, which drives NTCP) and prescription dose (which drives TCP), is not necessary for the result to hold, but allows the point to be made graphically. The solid black line gives the overall "total population" probability of toxicity. The solid red line gives the overall total population probability of tumor control. For example, if we were to treat all patients at 70 Gy, this would result in an NTCP of about 20 % and a TCP of 37 % for the entire population. The dotted black lines represent a hypothetical increase in prediction ability in terms of NTCP. They give the probability of toxicity for the least sensitive and most sensitive patients (50 % of each.) With this additional information, we could continue to utilize the same treatment strategy of treating all patients at a fixed NTCP level, but with two corresponding TCP levels which could then be averaged to give a new predicted combined, two-subpopulation, TCP. The dotted red line represents the associated hypothetical increase in TCP associated with the ability to segregate the

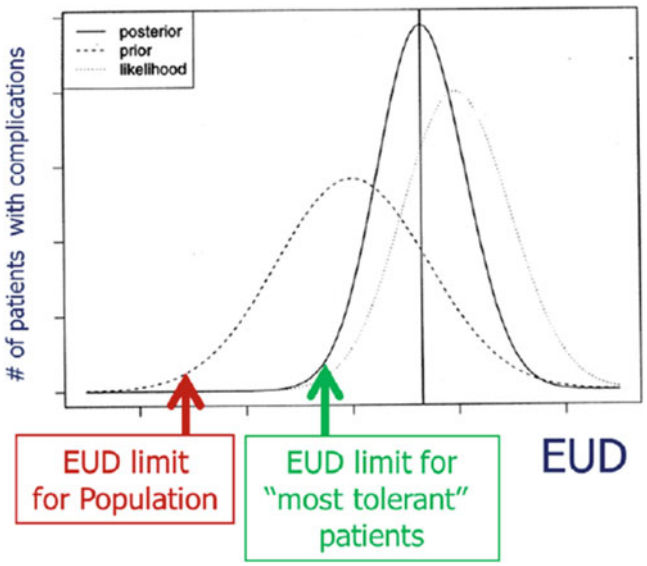

Fig. 9.1 The prior curve shows the distribution of EUD values that would result in toxicity for an overall population of patients. This data could come from a 'dose only' model for toxicity and the dose value that corresponds to some desired overall limit of toxicity (e.g. 10 %) is denoted by the red arrow. If biomarkers are identified that allow more precise identification of which patients are likely to experience toxicity, we could estimate separate curves for more or less sensitive patients. In this figure, we show these curves for the less sensitive patients estimated via maximum likelihood (likelihood curve) or in a Bayesian analysis (posterior curve). The dose corresponding to the same overall rate of toxicity for this group of patients is higher than from the dose-only model and is denoted in this figure by the *green arrow*

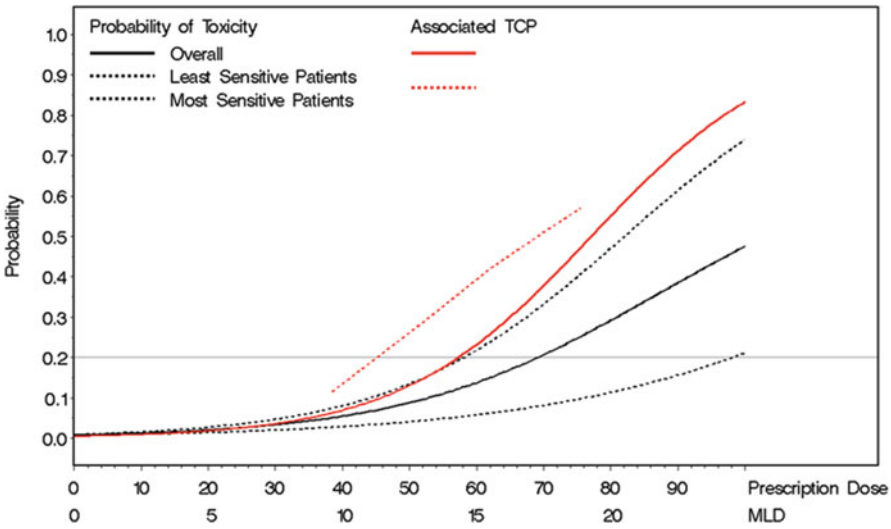

Fig. 9.2 Hypothetical overall gain in tumor control probability (TCP)-dotted red line for two sub-populations treated to the same NTCP level as that of the total population-solid red curve. (see text)

original total population into those two populations and treating each population at its own equivalent sub-population NTCP. For example, at the same 20 % NTCP level as above, this would correspond to a prescription dose level of 58 Gy for the most sensitive patients and 98 Gy for the least sensitive patients, increasing the overall TCP rate to 51 %. Further gains are possible if prediction models for TCP are improved.

9.1.3 Incorporation of Intratreatment Parameters into Adaptive Radiotherapy Algorithms

There are two types of individual patient level data that can be incorporated into the model described above: pretreatment data and information that is revealed only during a course of treatment. The great emphasis in oncology has been on the former, and there have been some important advances using this approach. Prominent examples include EGFR and K-ras mutation status is lung and colorectal cancer, respectively [8, 9]. Though knowledge of mutation status has utility in guiding selection of systemic therapy, these assays are unlikely to predict response to radiotherapy. The response to radiation, which produces single and double strand breaks as well as base damage, is determined by multiple genes controlling multiple DNA repair pathways. Although there are some well-known syndromes that demonstrate dramatic sensitivity to radiation, such as ataxia-telangiectasia, these are exceedingly rare. Furthermore, the radiation sensitivity of, for instance, the lungs of genetically

identical twins can be dramatically different as a function of the part of their lung that is being irradiated (for example, well versus poorly perfused regions near to the tumor) or their smoking history. These environmental differences act as noise in any genetic analysis. These and other factors have stymied attempts to discover a set of genes that can predict normal tissue sensitivity. Indeed, a recent analysis of SNPs that have been proposed to predict radiation toxicity revealed that not a single SNP set was validated as a predictor of radiation sensitivity [10]. Predictive assays for tumor sensitivity have faced many of the same problems, highlighting the need for utilization of novel imaging modalities and biomarker assays that can improve radiotherapy delivery.

9.1.4 Clinical Significance of Maximization of Tumor Control

One key potential advantage of radiation precision medicine is maximization of tumor control. This is critically important, given that local failure remains prevalent in many cancer subtypes. For some cancer types, such as glioblastoma, metastatic disease is largely unheard of, and local control is the primary determinant of survival. Even in cancer types with high rates of metastatic dissemination, such as lung and pancreatic cancer, a significant subset of patients die from locally recurrent or persistent disease [11]. This serves as the rationale for exploring further dose escalation as a means of improving local control. Modern studies of dose escalation have shown mixed results, with improved non-survival endpoints in prostate cancer [12–14], and worse survival in lung cancer [15] . While a lack of survival or significant local control benefit may suggest that the dose–response curve levels off in the higher dose range, a separate possibility is that increasing dose would improve local control (and potentially survival) if much higher doses can safely be delivered (e.g. in the range of 20–50 Gy as opposed to 8–16 Gy as is typically investigated in trials). The excellent local control observed with stereotactic radiotherapy supports this hypothesis [16, 17]. Though data from stereotactic radiotherapy trials have been practice changing in many ways, eligibility is limited to a small subset of patients given that many require treatment of areas that are either large or are in close proximity to critical normal structures. It is therefore critically important to identify scenarios in which dose escalation could be beneficial (perhaps using tumor types in which SBRT has already been shown to be effective), and then explore individualized normal toxicity prediction algorithms that could allow for safe dose escalation in many patients who are at high risk of experiencing local failure. Applying dose escalation in an individualized fashion may prove to be the most effective from both risk-benefit ratio and resource utilization standpoints. Because dose escalation is often associated with increased toxicity, it should be avoided in situations where adequate local control is obtained with lower doses. Furthermore, dose escalation may require more complex and costly radiotherapy planning or imaging guidance. Radiation precision medicine will ideally incorporate individualized

adaptation to identify the subset of patients who are likely to benefit from dose escalation with low risks of toxicity.

To date, the vast majority of dose escalation studies have involved delivering an increased dose to the entire tumor without regard for intratumoral heterogeneity. Though tumor heterogeneity was histologically described many decades ago, radiotherapy approaches were limited by the inability of standard imaging to sufficiently capture this heterogeneity. Within the past few decades, new imaging modalities have been developed that provide better spatial resolution and allow for assessment of tumor function. Emerging data show that these modalities can identify small regions with tumors, referred to as "subvolumes", that are less likely to respond to standard radiotherapy doses. These advances in functional and spatial imaging, coupled with the improved precision and accuracy of modern radiotherapy, can allow for selective dose escalation of high-risk tumor subvolumes. Because dose escalation is restricted to smaller volumes, higher absolute doses can safely be delivered with selective boosting that with uniform dose escalation.

Tumor control could also be maximized if normal tissue constraints (e.g. mean liver dose) could be safely relaxed in select patients. Improved individualization and adaptation strategies could minimize or eliminate tumor underdosing in the region of normal structures and lead to improved local control.

9.1.5 Clinical Significance of Minimization of Radiotherapy Toxicity

Another fundamental component of radiation precision medicine is the reduction of acute and late normal tissue toxicity. The process of minimizing radiation toxicity is complicated by the fact side effects are often delayed. Even acute toxicities generally present in the latter half of treatment. Late toxicities can present years to decades after radiotherapy has completed, when there is no longer an option of de-escalation or treatment adaptation. In addition to the later time course, there is significant heterogeneity across the population in the development of acute and late toxicity.

While acute toxicities are typically temporary, they can compromise care through a variety of mechanisms. Toxicities may interfere with the receipt of the full course of definitive therapy. Development of acute radiation toxicities such as severe dermatitis or mucositis may necessitate radiotherapy breaks that decrease local control and survival [18]. In addition to interrupting radiation, acute toxicities may also delay or prevent receipt of other treatment modalities including chemotherapy or surgery. Even when full courses of therapy are completed, acute toxicities worsen performance status and increase the risk of secondary complications (e.g. pulmonary emboli, wound infections).

Late radiotherapy toxicities such as secondary malignancies and cardiopulmonary damage can lead to significant morbidity and mortality for patients. This is especially relevant for malignancies in which many patients experience long term survival.

Individual level prediction of both acute and late toxicity remains a challenge. Individualized radiotherapy adaptation that can reduce toxicity while maintaining tumor control has great potential to improve quality and quantity of life in cancer patients.

9.1.6 Technical Requirements for Adaptive Radiotherapy

Over the past two decades, there have been significant advances in technology that allow for individualization and adaptation of liver radiotherapy. Intensity modulated radiation therapy has allowed for delivery of more conformal plans with improved normal tissue sparing. Motion assessment and management are critical for patients with tumors in the thorax or abdomen. Technologies such as active breathing control and SDX have allowed for reduction in margins that account for motion. For patients who cannot tolerate motion management, 4D CT has allowed for more accuracy in assessing the maximum extent of tumor motion, eliminating the use of unnecessarily large margins. Image registration algorithms have improved target delineation when non-CT modalities (MRI, PET, etc.) are used. On board imaging with cone beam CT has decreased setup uncertainty and allowed for further reduction field sizes. These advances have improved delivery of IMRT treatments and allowed for uptake of stereotactic body radiotherapy, which involves delivering high doses in a conformal fashion with a small number of fractions (typically less than five.)

9.1.7 Cautions with Radiotherapy Adaptation

Though adaptive radiotherapy is promising, a number of cautions must be heeded. When considering strategies to minimize toxicity, a lack of specificity of normal tissue toxicity algorithms may lead to overly conservative dosing, and thus missed opportunities to dose escalate. Additionally, a lack of sensitivity in tumor response algorithms may lead to increased failures. For example, imaging may not be sensitive enough to identify microscopic disease that persists after gross tumor shrinkage, which could lead to failures in the setting of field size reduction.

9.1.8 Deciding When to Consider Adaptive Radiotherapy

How does one then decide when to consider adaptive radiotherapy? Adaptive radiotherapy can be useful under a number of different circumstances. Individualization and adaptation should be considered for cancers with poor local control rates (e.g. pancreatic, glioblastoma, locally advanced lung and head and neck cancers) with the goal of improving outcomes. Adaptive techniques can also be considered in

tumor types with good local control but significant rates of acute or late toxicity, given the cost, morbidity, and mortality association with toxicity. Before adaptation protocols are initiated, it will likely be beneficial to first establish that the imaging sequences or biomarkers being utilized are predictive of treatment response or toxicity at a phase of treatment when adaptation is still feasible. When new imaging sequences or biomarkers are used, detailed protocols should be outlined to ensure that findings are reproducible. Adaptation protocols should ideally be investigated in the setting of a randomized trial before they are widely adopted.

9.2 Adaptive Liver Radiotherapy

9.2.1 Rationale for Using Radiotherapy for Liver Tumors

Given the increasing burden of primary and metastatic cancer in the liver, there is a dire need to develop individualized and adaptive hepatic radiotherapy techniques. Hepatocellular carcinoma, the most prevalent primary hepatic malignancy, contributes significantly to cancer mortality in the United States [19, 20]. While liver transplant is the most effective therapy for hepatocellular carcinoma, the vast majority of patients present with unresectable disease, which underscores the importance of identifying alternative treatment strategies. Any alternative strategies must consider safety in the setting of suboptimal liver function, given the high prevalence of concomitant cirrhosis. Common non-surgical therapies for managing primary liver tumors include chemoembolization and radiofrequency ablation. Though chemoembolization improves survival in patients with Child's A (good liver function) patients without portal vein thrombus, it is associated with post-embolization syndrome (nausea, pain, and fatigue, sometimes requiring hospitalization) and rarely provides durable tumor control. Radiofrequency ablation can control some small tumors, but cannot be applied to tumors larger than 4 cm in diameter, those that abut important vessels or bile ducts, or those that cannot be seen on ultrasound. Most of the limitations of the previously described modalities do not apply to modern hepatic radiotherapy, making it an attractive treatment option.

The liver is also a common site of metastasis across many cancer types. Though some patients with hepatic metastases have short survival times that might not justify liver-directed therapies, advancements in systemic therapy have extended survival times in some cancers, resulting in a population of patients who might benefit from aggressive management of hepatic oligometastatic disease. The use of liver-directed therapies might be particularly beneficial in patients with colorectal primaries, given the extended median survival observed in metastatic colorectal cancer (relative to other cancers) coupled with the high incidence of hepatic metastases. Patients with metastatic disease who are being considered for liver-directed therapies typically have better liver function than patients with HCC, suggesting that they might safely tolerate higher doses. Though better baseline liver function might

allow for more flexibility in radiotherapy planning, timing of radiotherapy might be a stronger consideration than in patient s with primary liver disease if the goal is to minimize breaks from systemic therapy. Precision radiation medicine for the liver must therefore account for differences within and across subsets of patients with primary and metastatic disease.

9.2.2 Current Standard of Care for Liver Radiotherapy

9.2.2.1 Radiotherapy Dose and Technique

Historically, liver radiotherapy involved treating large volumes with a large degree of technical uncertainty. Given its radiosensitivity, the whole liver dose was limited to approximately 30 Gy. Adjacent structures such as the duodenum and stomach were also dose limiting. Respiratory motion restricted the ability to reduce field size to conform to the tumor. In many cases, the doses that could be safely delivered under these conditions were palliative in nature, as they were unable to provide durable local control. Technological advances including motion management, SBRT and on board imaging have allowed for treatment of liver tumors to higher doses with less toxicity.

Despite the advances in radiotherapy technique, challenges remain in treatment patients with hepatic malignancies. While data show that patients with cancer metastatic to the liver can be safely treated with up to 60 Gy in three fractions of 20 Gy each with excellent results [21, 22], SBRT for HCC has produced more toxicity than with colorectal cancer. In one small phase I trial (17 patients) patients with small tumors were able to receive three fractions of 16 Gy if they had Child's A disease, but suffered unacceptable toxicity with Child's B disease [23]. In a second prospective phase I/II trial, patients received 6 fractions based on a normal tissue complication probability model (to be discussed in further detail below) to a median dose of 36 Gy [24]. Twenty-three percent (7/41) patients experienced progression from Child's A to B within 3 months of treatment. The 1-year in-field control was 65 %. Therefore, although radiation can clearly control HCC, there is a critical need to increase control rates while preserving adequate liver function.

9.2.2.2 Normal Tissue Complication Probability Models

While the introduction of three dimensional radiotherapy planning allowed for delivery of more complex radiotherapy plans, it also necessitated a more nuanced understanding of the relationship between dose and toxicity. Mean doses or parameters that reflect a single point on the dose-volume histogram (e.g. volume of an organ receiving greater than or equal to a given dose) can be the same across two plans that otherwise have very different dosimetric properties. Though these individual parameters may have a reasonable degree of sensitivity and specificity for

predicting acute or late effects, toxicity prediction can be improved when information from the entire dose-volume histogram (DVH) is used. NTCP models were developed as a strategy to incorporate data from the entire DVH to generate a probability of developing a specific level of organ toxicity. The use of NTCP models for liver radiotherapy has allowed for delivery of significantly higher doses than was permissible on protocols that used dose limits based on broad-volume groupings or specific dose-volume limitations (e.g. <33 % vs. 33–66 % of liver included in high dose region, and $V_{50\%}$, respectively) [25]. Separate models were subsequently developed for patients with primary hepatic malignancies and liver metastases to account for differences in baseline liver function, largely attributable to the prevalence of cirrhosis in patients with hepatocellular carcinoma [7].

9.2.3 Novel Strategies for Adaptive Liver Radiotherapy

9.2.3.1 Serum Biomarkers

Serum biomarkers provide opportunities for quantitative assessment of global liver function and toxicity prediction. Over the past decade, there has been increasing interest in exploring how these biomarkers can be used to optimize delivery of hepatic radiotherapy.

Indocyanine Green as a Marker of Global Liver Function

Indocyanine (IC) green is a green dye that, after intravenous injection, is rapidly bound to plasma proteins, taken up almost exclusively by hepatic parenchymal cells, and secreted entirely into the bile. Therefore, the serum clearance rate (determined from serial serum concentration measurements at various times after intravenous injection) is a quantitative measure of liver function. It has extensively been used to predict 30-day mortality following hepatic resection, short-term prognosis for patients with cirrhosis and survival for critically ill patients [26–28]. Therefore, IC green would seem to be an ideal technique for assessing liver function and permitting adaptive therapy based on changes in liver function during treatment.

Preliminary data support the approach of using IC green for adaptive liver radiotherapy. Overall liver function via the change in IC green retention at 15 min ($ICGR_{15}$) between pre-radiotherapy and 1 month post predicts for eventual development radiation induced liver disease (RILD) [29]. Though prediction of toxicity is useful in some settings, there is limited utility in predicting RILD once the full radiotherapy course has been delivered because it is difficult to treat and is fatal in up to 15 % of patients. Data show that it takes a minimum of 2 weeks and more typically 4–6 weeks for early evidence of RILD to develop.

The delayed presentation of RILD serves as the rationale for the introduction of a break in radiotherapy that permits mid treatment assessment of liver function. This technique, pioneered at the University of Michigan, involves delivery of 60 % of the

radiotherapy course, followed by a 1 month treatment break. At the end of the 1 month break, a repeat $ICGR_{15}$ assessment is performed. A protocol has been developed that takes into account both the change in $ICGR_{15}$ between pretreatment and 1 month after 60 % of the treatment has been delivered and the absolute $ICGR_{15}$ predicted if the additional 40 % of the treatment were delivered unaltered. The dose for the last two fractions is decreased if it is predicted that $ICGR_{15}$ will exceed 0.44. This adaptive and individualized protocol has led to excellent local control rates without development of RILD [30].

Other Serum Biomarkers

TGF-β_1 is a cytokine that plays a pivotal role in the development of normal tissue injury after radiotherapy in many organs, prior to, during, and following completion of radiotherapy for liver tumors. Preliminary data demonstrates that TGF-β_1 levels at the 1-month break after delivery of the first 60 % of treatment correlates with $ICGR_{15}$ after completion of all treatment. Thus, TGF-β_1 levels have the potential to be an early predictor of radiation-induced liver damage.

Other potentially useful biomarkers for predicting liver damage include hepatocyte growth factor and CD40 ligand (CD40L). Preliminary data demonstrate that high HGF and low CD40L levels 1 month after delivery of 60 % of the radiation dose correlated with eventual development of liver toxicity (as measured by change in Child-Turcott-Pugh score) [31].

9.2.3.2 MRI: Dynamic Contrast Enhanced and Perfusion Sequences

MRI is likely to become of vital component of precision radiation medicine by providing useful information on both normal liver function and tumor response. Using perfusion sequences, a significant correlation has been demonstrated between ICG clearance rate and mean portal venous perfusion at pre-, intra-, and post-treatment time points [32]. Given previously described data showing a correlation between ICG clearance and prediction of RILD, this suggests that portal venous perfusion can be used to assess liver function at baseline, during treatment, or after completion of radiotherapy.

Though global measures of radiotherapy can be useful in determining the total tolerable radiation dose, spatial information may allow for higher level individualization of radiotherapy planning. Indeed, a spatial correlation has been observed between delivered dose and the amount of portal venous perfusion on MRI [32]. Understanding individual function in spatial dimensions will allow for investigation of radiotherapy plans that differentially deposit dose in areas that already have poor, unsalvageable function and selectively spare regions with preserved function to maximize total organ function at the completion of treatment.

The benefits of spatial correlation can be capitalized on further if changes in function can be predicted at a time when the radiation plan is still modifiable. Data

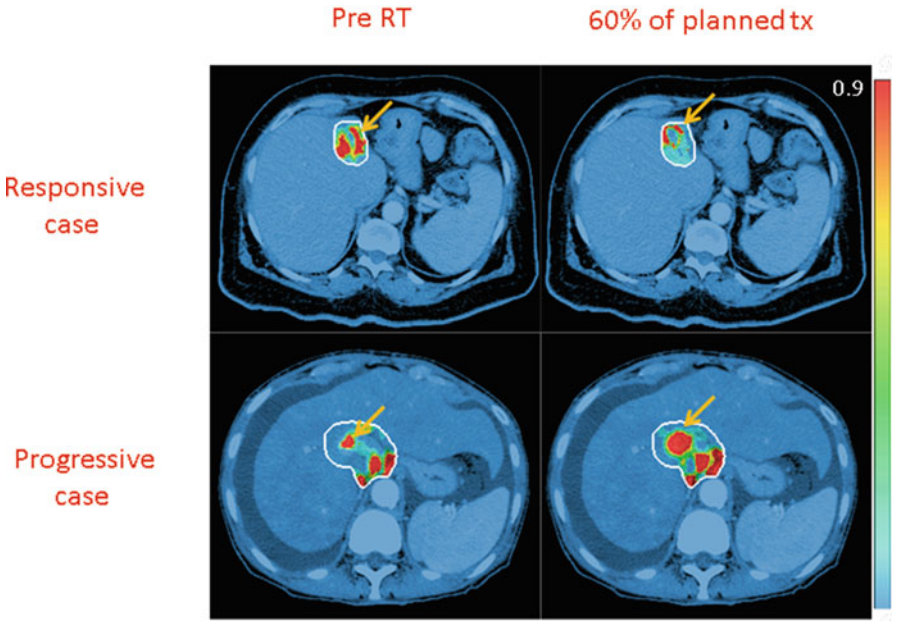

Fig. 9.3 Elevated arterial perfusion probabilities of the tumors pre-RT (*left*) and after receiving 60 % of planned treatment (*right*). *Top*: a responsive case; *bottom*: a progressive case

have shown that regional portal venous perfusion measured during treatment is correlated to perfusion 1 month after radiotherapy [32]. Mid-treatment data that predict late toxicity might be actionable if intra-treatment plan modifications can decrease or eliminate the chance of further damage.

In addition to providing meaningful data on normal liver toxicity, MRI has the potential to provide novel information about the primary tumor via dynamic contrast enhanced sequences. Liver tumors are typically associated with an abnormal increase in arterial perfusion, which allows them to be distinguished from the normal hepatic parenchyma that derives the majority of its blood supply from the portal venous system. While using arterial perfusion to distinguish tumor from normal tissue is critically important, it would also be beneficial to better understand tumor heterogeneity by determining whether the most aggressive subvolumes within tumors are also the most perfused. Data using dynamic contrast MRI (DCE-MRI) suggest that increases in the volume of tumor with elevated arterial perfusion can predict for tumor progression after radiotherapy [33] Fig. 9.3. Baseline features such as tumor volume and pre-RT tumor perfusion were not predictive of treatment outcome, suggesting that this temporal and spatial information is key to predicting tumor response. These important data provide the rationale for exploring the individualized adaptation strategy of selectively boosting tumor subvolumes that have elevated arterial perfusion on mid-treatment imaging.

9.3 Adaptive Lung Radiotherapy

9.3.1 Rationale for the Use of Lung Radiotherapy

Lung cancer is the leading cause of cancer mortality in the world. The standard of care for management of early stage non-small cell lung cancer (NSCLC) is surgical resection. In contrast, most patients are treated with concurrent chemoradiotherapy. Radiotherapy is preferred in most cases of locally advanced lung cancer for a variety of reasons. For patients with technically resectable disease, but with involvement of multiple mediastinal lymph nodes, there is no survival benefit to the addition of surgery. Other patients have extensive central disease that would require pneumonectomy, but have insufficient pulmonary reserve to tolerate such an extensive surgery. Another subset of patients exist who have features such as mediastinal invasion render the disease unresectable altogether. The prevalence of locally advanced lung cancer combined with the poor survival underscores the importance of improving radiotherapy techniques for this disease.

9.3.2 Current Standard of Care for Lung Radiotherapy

Standard radiation doses for NSCLC are in the range of 60–66 Gy, which produces a median survival of 16–18 months with 5-year overall survival rates of only approximately 20 % [34, 35]. The benefit of increasing radiation doses on tumor control has been confirmed for stereotactic body radiotherapy [16], and data demonstrated that dose is more important in patients with larger tumors [36]. It was estimated that a dose of at least 80 Gy (BED 96 Gy) was required for local tumor control in lung cancer, and 100–180 Gy BED was associated with over 90 % long-term tumor control. However, delivery of these doses also requires increased treatment of normal structures and thus increases radiation toxicities.

As discussed in the context of liver tumors, NTCP models have been utilized in lung radiotherapy. These models have higher sensitivity and specificity for predicting toxicity than parameters such as mean lung dose and V20. Nonetheless, NTCP remains a population based strategy.

RTOG 0617 sought to determine whether dose escalation could improve outcomes for patients with locally advanced lung cancer [15]. In this randomized phase III trial, the standard 60 Gy dose was compared to 74 Gy delivered to the primary tumor and involved lymph nodes. Though it was hypothesized that dose escalation would improve outcomes, excess mortality was observed in the 74 Gy. This mortality decrement has been attributed to increased toxicity, particularly cardiac and pulmonary, in the high dose arm. It is therefore clear that high dose uniform dose prescription to the entire tumor has not been successful, and is likely limited by radiation toxicities.

9.3.3 Recent Advances in Adaptive Radiotherapy for Lung Cancer

9.3.3.1 PET/CT

PET/CT has multiple potential applications in adaptive radiotherapy for lung cancer. Data from the University of Michigan show that when performed at appropriate times, midtreatment FDG-PET is only negligibly confounded by treatment related inflammation, and can potentially be used for prediction of treatment outcome, evaluation of response during treatment, and adaptation or alteration of the remaining treatment. These findings have been independently confirmed. A series of prospective trials showed that FDG uptake and tumor volume were reduced significantly after 40–50 Gy of fractionated radiotherapy [37], that adapting the planned target volume to this decreased tumor volume with a fixed composite NTCP allowed escalation of the total dose by 30–102 Gy (mean 58 Gy) or a reduction in NTCP if the dose remained unchanged, that reduction in the PET-MTV was greater than the reduction of the CT-GTV (gross tumor volume) during treatment, and that using the MTV during treatment, tumor dose can be escalated about 74 Gy while keeping lung NTCP unchanged in a majority of patients with stage III NSCLC Fig. 9.4. These results led to the design of a prospective clinical trial of adaptive radiotherapy using mid-treatment FDG-PET. Preliminary results show significantly better local control and overall survival at 1 and 2 years than standard radiation in patients with stage III NSCLC treatment with concurrent and adjuvant carboplatin and paclitaxel. The results from this trial led to the development of RTOG 1106, which is an ongoing randomized phase II clinical trial investigating FDG-PET based adaptive radiotherapy in patients with locally advanced NSCLC.

In addition to providing information about the primary tumor, PET/CT can potentially be useful for evaluating radiosensitivity of normal tissues, including the esophagus. Esophagitis is a common side effect of thoracic radiotherapy and a source of considerable morbidity. Late in the course of fractionated radiotherapy, patients often complain of dysphagia and/or odynophagia, which can lead to complications such as dehydration, weight loss, treatment interruption, esophageal perforation or obstruction, or even death. Clinical and dosimetric studies have shown that the dose and volume of esophagus irradiated, as well as the use of concurrent chemotherapy correlate with the severity of esophagitis, but the predictive value of these correlations is only modest [38]. Preliminary data show that the increase in FDG uptake in the esophagus at 40–45 Gy identifies the sensitive esophagus and improves the ability to estimate esophagitis over the predictions made by using maximum esophageal radiation dose alone. Thus, another adaptation strategy is to obtain mid-treatment FDG PET and decrease the esophageal dose to keep esophagitis levels at no greater than that produced by 60 Gy of standard radiotherapy.

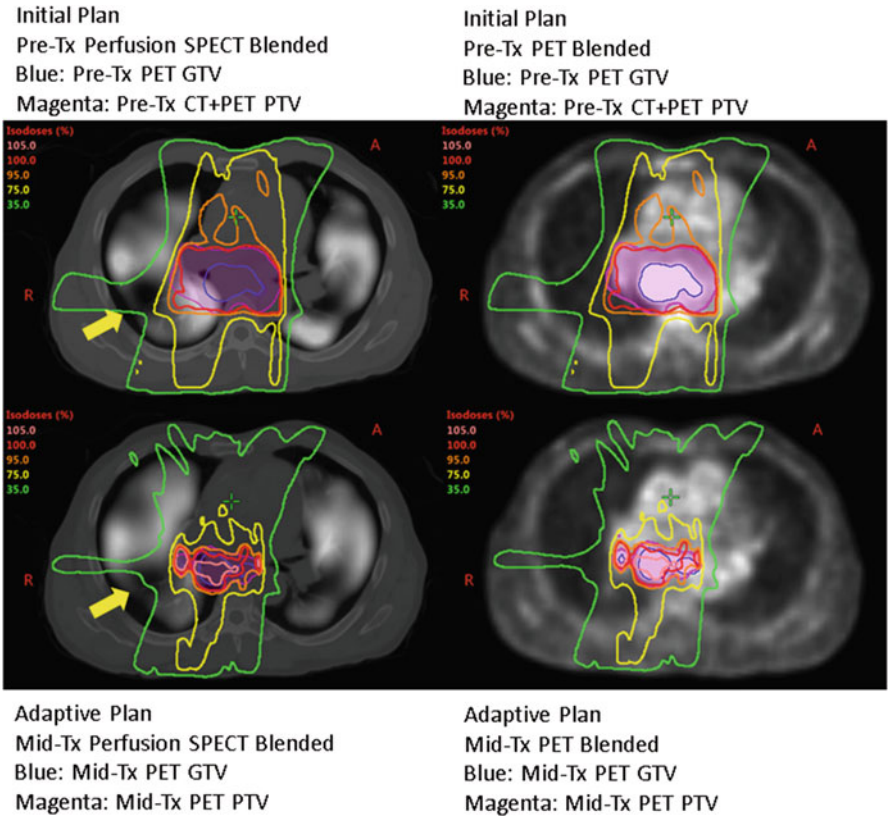

Fig. 9.4 *Top panels*: Initial plan treating CT + PET defined PTV (*magenta*) and avoiding highly perfused lung (*top left*: pre-treatment perfusion SPECT shown blended with planning CT, *top right*: CT + PET). The *yellow arrow* indicates a low perfusion lung area that is being preferentially dosed to avoid highly perfused areas. *Bottom panels*: Adaptive plan treating only a PET defined PTV (*red*). The PET is shown blended with the planning CT (*bottom right*). The during-treatment SPECT scan is also used for functional avoidance (*bottom left*)

9.3.3.2 V/Q SPECT-CT

While adapting radiotherapy based on PET/CT has the potential to improve tumor control, toxicity remains a concern. In fact, in the previously described institutional protocol of adaptive radiotherapy, toxicity appeared to be greater than that produced by standard therapy. A key dose-limiting toxicity of thoracic radiotherapy is radiation induced lung toxicity. Though NTCP models were an important advance in predicting the risk of pneumonitis, current models treat the whole lung uniformly, despite data that show that patients with NSCLC frequently have heterogeneous lung function due to comorbidities such as chronic obstructive pulmonary disease. Furthermore, the presence of the tumor itself

often affects local vascular supply and ventilation, and changes function level. V/Q SPECT-CT is a commonly available technique in most hospitals that images the perfusion (Q) and ventilation (V) of the lung. Q-SPECT images can be used to guide treatment planning so that radiation is directed to the non-functional lung regions [39] Fig. 9.4. Q-SPECT guided plans produce more favorable dose functional volume histograms compared to non-SPECT guided plans, with the fV20 and fV30 values reduced by an average of 13.6 % ± 5.2 % and 10.5 % ± 5.8 % respectively. Additional data show that NSCLC often presents with defect regions on (1) V/Q SPECT-CT, some of which are more resistant to post-treatment function reduction, (2) SPECT-CT defection regions are more resistant to post-treatment function reduction and (3) V/Q SPECT-CT guided treatment can reduce dose to the functional lung without increasing doses to the total physical lung. These data suggest that avoiding V/Q SPECT-CT functional regions in based on pre- and intratreatment scans can minimize damage to the functional lung. V/Q-SPECT-CT adds lung ventilation mapping on top of the Q-SPECT, providing more information (including the mechanism for lung function defects and their potential for recovery.) Midtreatment V/Q SPECT-CT allows adaptive because lung function changes globally and locally during treatment, largely due to treatment-induced tumor volume reduction and improvement of the vascular supply and ventilation. The combination of pre and intratreatment V/Q SPECT-CT can classify the lung into different functional regions and strategize to differentially prioritize certain regions, a technique that can be used to minimize lung damage.

9.3.3.3 Biomarkers for Toxicity Prediction

Patients receiving the same doses of radiation therapy often have very different levels of toxicity or toxicity patterns, largely due to their biologically different intrinsic sensitivity to radiation damage. Many studies have been conducted to understand the correlation between pro-inflammatory and pro-fibrogenic cytokines, including TGF-B1, IL1B, IL-6, IL-8 and TNF-alpha and radiation-induced normal tissue injury. TGF-B1, a fibrogenic and radiation-inducible cytokine, has been known to play a key role in this process. Data show that TGF-B1 elevation in the middle of treatment (2–4 weeks) relative to pre-treatment is highly correlated with late-onset grade >= 2 RILT in NSCLC patients [40] and a model of combining mean lung dose, pre-treatment IL-8 and TFG-B1 ratio provided a more accurate prediction Figs. 9.5 and 9.6. Thus, an additional adaptation strategy is use TFG-B1 to decrease lung dose to keep RILT levels at no greater than that produced by 60 Gy standard therapy.

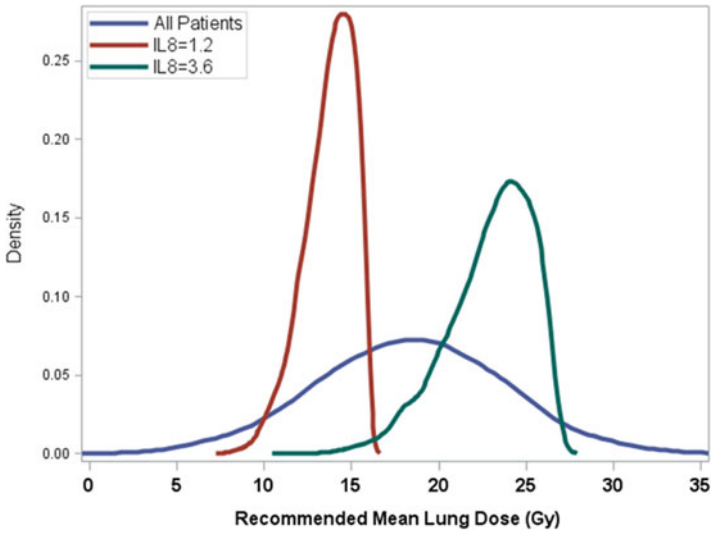

Fig. 9.5 This figure shows the distribution (histogram) of the mean lung dose (MLD) value that corresponds to a 15 % risk of grade 2+ pneumonitis for a population of about 100 patients treated for NSCLC. In the curve for all patients (*blue curve*), there is a lot of variation in the 'recommended' MLD values because patients varied in their IL-8 and TGFbeta1 values. To further illustrate how the model might work, we 'fixed' the IL-8 value to one of 2 specific values and then calculated the recommended MLD values corresponding to all the observed week 2 TGFbeta values (*red curve*: IL-8 = 1.2, *green curve*: IL-8 = 3.6). Once the baseline IL-8 value is known, the range of 'recommended' MLD values becomes much narrower. Having a higher IL-8 value permits delivery of a higher MLD for the same risk of pneumonitis

9.4 Adaptive Head and Neck Radiotherapy

9.4.1 Rationale for the Use of Radiotherapy for Head and Neck Cancer

Chemoradiotherapy is the cornerstone of treatment for many patients with locally advanced head and neck cancer. Many of these patients present with unresectable disease, leaving chemoradiotherapy as the only definitive treatment option. In other cases of locally advanced disease, the primary tumor and nodes are technically resectable, but surgical resection may results in the need for permanent tracheostomy or gastrostomy tube placement, which may contribute to a substantial decrement in quality of life. Furthermore, many patients who undergo surgery for locally advanced head and neck cancer will have high risk features such as extracapsular extension or positive margins, which necessitate the need for adjuvant chemoradiotherapy. Though the additional of surgery to chemotherapy improves treatment outcomes for some patients with locally advanced disease, for many others, trimodality therapy produces more toxicity than chemoradiotherapy alone without an added survival benefit. In order to maximize the therapeutic ratio, a substantial percentage of patients with locally advanced head and neck cancer receive definitive chemoradiotherapy alone.

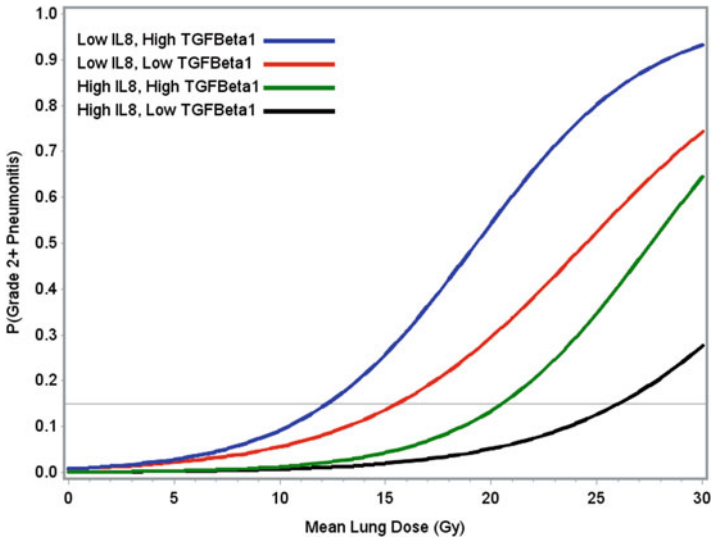

Fig. 9.6 Estimated risk of toxicity for 4 possible combinations of dichotomized values of IL-8 and TGFbeta (there are included continuously in the model so there are essentially an infinite number of possibilities). The curves demonstrate how dose can be maximized using biomarker stratification without increasing the risk of toxicity. A 15 % risk of pneumonitis (*horizontal grey line*) corresponds to 25 Gy for those with high IL-8 and low TGFbeta versus only 12 Gy for those with low IL-8 and high TGFbeta

There are a number of unique features of head and neck cancer that emphasize the need for improved radiotherapy delivery. One important feature is that a substantial portion of deaths from locally advanced head and neck cancer are caused by locoregional, as opposed to metastatic progression. Locoregional failure can lead to aspiration, severe malnutrition and electrolyte imbalances, and hemorrhage, from either the tumor or the neck vasculature. Radiotherapy delivery is also complicated by the proximity of head and neck cancers to important normal structures, such as the brain, spinal cord, optic structures, constrictors, and salivary glands. This is in contrast to radiotherapy of the lung and liver, for example, where tumors might be surrounded by normal tissue in all directions, which can permit delivery of high dose radiotherapy without clinical organ compromise. The high frequency of locoregional failure, combined with challenge of minimizing normal tissue toxicity highlights the need for advanced adaptation strategies in head and neck cancer.

9.4.2 Current Standard of Care for Head and Neck Radiotherapy

The current standard of care for locally advanced head and neck cancer typically involves IMRT with concurrent platinum-based chemotherapy. Randomized trials have shown that the additional of concurrent chemotherapy leads to improved

survival [41]. IMRT has allowed for reduction of dysphagia [42–44] and xerostomia [45] with maintenance of local control. These toxicity-reducing strategies have been combined with simultaneous integrated boost techniques that deliver the highest doses (typically in the range of 70 Gy) to areas of gross disease and progressively lower doses (50–60 Gy) to areas at risk of harboring microscopic disease [46]. The introduction of HPV testing has identified a more favorable-risk cohort of patients with locally advanced oropharyngeal squamous cell carcinoma [47]. Ongoing studies are evaluating whether chemoradiotherapy can be de-escalated in HPV-positive patients, without compromising survival [48].

Despite these recent technological advances, once patients are risk stratified, primarily based on histology and stage, most will be subject to the same normal tissue limits and receive common doses without intratreatment adaptation. Though many of the dose limits and prescriptions are based off of high quality evidence, the significant rates of both toxicity and locoregional failure after head and neck radiotherapy suggest that these population based metrics may be inadequate, indicating that strategies for individualized adaptation should be investigated.

9.4.3 New Strategies for Adaptation in Head and Neck Radiotherapy

9.4.3.1 Dynamic Contrast-Enhanced (DCE) MRI

DCE MRI is being investigated as a strategy for identifying poorly perfused and thus potentially hypoxic areas of tumor that are at higher risk for recurrence. Previous research using CT has demonstrated that the presence of low tumor perfusion on pre-treatment imaging is correlated with poor treatment response and prognosis [49–51]. Though perfusion can be assessed with CT, more granular information may be obtained with MRI. DCE-MRI allows for assessment of blood flow and volume, and its excellent soft tissue resolution allows for quantification of perfusion in individual tumor subvolumes and improved discrimination of tumor from adjacent normal structures. In a prospective longitudinal cohort of patients undergoing chemoradiotherapy for locally advanced head and neck cancer, changes in blood flow and volume were evaluated using DCE MRI. When blood volume changes were analyzed within the GTV before treatment and at 2 weeks after initiation of treatment, a significant increase in blood volume was observed in patients with local control, while minimal change was observed in patients who experienced local failure [2]. While changes in blood volume were predictive of local control, changes in GTV volume at 2 weeks were not. This suggests that anatomical adaptation that relies only on changes on CT might not be a useful adaptation strategy in locally advanced head and neck cancer. While the predictive ability of global changes in blood volume indicated potential utility in adaptation algorithms, additional information could be obtained when the heterogeneous distribution of blood volume is taken into account. In

order to better assess blood volume changes given the potential for heterogeneity, a further analysis of this cohort was performed using a method called global-initiated regularized local fuzzy clustering [52]. ROC analysis showed that regardless of sensitivity, the subvolume of tumor with low blood volume at week 2 had greater specificity for predicting local control than other metrics, including pretreatment tumor volume, change in tumor volume, or change in average blood volume values of the entire tumor.

A number of other small studies have explored the relationship between DCE-MRI characteristics and outcomes, with most showing correlations between baseline, intra, or post-treatment DCE-MRI parameters and outcomes [53–57]. However, the directionality of the correlation between parameters and outcome varied across studies depending on which parameters were measured and when imaging was performed relative to treatment initiation. Important themes that appear to be consistent are that better outcomes are associated with higher baseline perfusion and with intratreatment increase in perfusion (compared to baseline). In order to better understand these trends, an ongoing randomized phase II trial at the University of Michigan is using DCE MRI to investigate whether outcomes can be improved when hypoperfused head and neck tumor subvolumes are selectively boosted.

9.4.3.2 PET/CT

Studies are investigating whether PET might have utility as an early predictor of treatment response. FDG-PET is commonly used in target delineation, but data regarding its predictive and prognostic abilities are mixed. While some studies have found correlations between high baseline standardized uptake value (SUV) or metabolic tumor volume and worse outcomes [58–60], others have not confirmed this hypothesis [61]. An ongoing prospective study in the UK will help clarify the potential utility of FDG-PET in radiotherapy adaptation. It will explore correlations between clinical outcomes, serum biomarkers, and functional imaging (MRI and FDG-PET) parameters measured at various time points in patients undergoing head and neck radiotherapy with or without induction chemotherapy [62].

While FDG has been the most commonly used PET tracer, there is increasing interest in using tracers that identify hypoxic, radioresistant regions of tumor, such as ^{18}F-fluoromisonidazole (F-MISO). A study on which F-MISO PET scans were obtained at baseline and various intratreatment time points showed that F-MISO image parameters at weeks 1 and 2 were the strongest predictors for local-progression-free survival [3]. Of note, neither F-MISO parameters at baseline nor other factors (clinical, FDG- or CT-delineated volumes) were predictive of LPFS on multivariate analysis, which highlights the importance of utilizing sufficiently predictive functional tracers and performing intratreatment assessments.

9.5 Future Directions

9.5.1 Adaptive Brain Radiotherapy

9.5.1.1 Rationale for the Use of Brain Radiotherapy

Radiotherapy is an important modality for both benign and malignant primary lesions of the brain. While benign lesions are often slow growing, they may present in unresectable locations and eventually lead to development of severe neurological symptoms, necessitating the use of radiotherapy in some cases. There is even more urgency to improve treatment techniques for malignant brain tumors, with an emphasis on glioblastoma. Even after gross total section, almost all patients with glioblastoma recur, which has been attributed to its infiltrative nature. Studies with radiographic and pathological correlation show that malignant cells extend beyond the gadolinium enhancing region into the T2/FLAIR component. Despite the infiltrative nature, studies have not demonstrated a benefit to whole brain radiotherapy compared to radiotherapy with smaller fields. After survival resection, limited field adjuvant radiotherapy has been shown to improve survival [63]. An additional improvement in survival was achieved with the introduction of temozolomide-based chemoradiotherapy after maximal survival resection, but 5 year overall survival remains dismal at 9.8 % [64]. While prognosis is poor for patients who undergo maximally resection and adjuvant chemoradiotherapy, outcomes are even worse for patients who present with unresectable disease, highlighting the need for improved treatment strategies.

Radiotherapy also plays an important role in the management of brain metastases. The majority of patients with brain metastases will die of their intracranial disease, even if extracranial disease is present. Whole brain radiotherapy can provide effective palliation, but intracranial disease often progresses in patients who live more than a few months. Stereotactic radiosurgery has been increasingly used, especially in patients with limited extracranial and intracranial disease and good performance status. While particularly SRS is effective for small lesions, there is an increased risk of toxicity and suboptimal local control in patients with larger tumors, stressing the need for improved radiotherapy techniques for these patients. Although advances in systemic therapy are leading to prolonged stability of extracranial disease in some patients, many of these agents are unable to cross the blood brain barrier. The opportunity for extended survival due to controlled extracranial disease further increases the importance of improving strategies to controlling brain metastases.

9.5.1.2 Current Standard of Care in Brain Radiotherapy

Dose escalation has been investigated as a means of improving local control in glioblastoma. SRS-based boosting was investigated in a randomized fashion with no improvement in survival [65]. Proton boosts have also been investigated, though

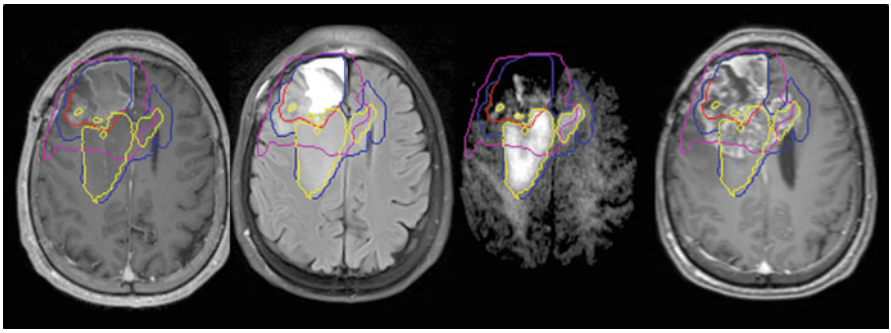

Fig. 9.7 Post-Gd T1-weighted image pre-RT (*left*), T2 FLAIR image pre-RT (*second left*), diffusion weighted image with b-value of 3000 s/mm2 pre-RT (*second right*), and post-Gd T1 weighted image 4-month post-RT at progression (*right*) of a patient with glioblastoma. Contours: Gross tumor volume based upon contrast enhancement (*red*), FLAIR abnormality volume (*yellow*), hypercellularity volume (*yellow*) and 95 % of the prescribed dose volume (*pink*). *Gd* gadolinium, *RT* radiotherapy

there are no data from randomized trials to support this approach [66, 67]. Phase I data indicate that dose escalation to 72–75 Gy is safe and results in favorable median overall survival (20.1 months), support that investigating of this fractionating in a randomized trial [68]. The current standard of care remains 60 Gy with concurrent and adjuvant temozolomide [64].

The management of brain metastases varies based on performance status, extent of intra and extracranial disease and other factors. Whole brain radiotherapy with or without resection was recently considered the standard of care, but SRS alone is increasingly being used for patients with a good prognosis who are non-operative candidates.

9.5.1.3 Emerging Strategies in Adaptive Brain Radiotherapy

Diffusion MRI

Emerging evidence supports the utility of diffusion MRI for prediction of radiotherapy response in the treatment of brain metastases and glioblastoma. Data show that change in the diffusion abnormality index (2 weeks post-radiotherapy initiation versus pre-treatment) was superior to other metrics (change in gross tumor volume and conventional diffusion metrics) at predicting the response of brain metastases to whole brain radiotherapy with or without bortezomib [69]. Assessment of diffusion abnormality index may therefore allow for treatment adaptation, regardless of whether patients are treated with radiotherapy alone or concurrently with a radiosensitizer such as bortezomib.

For patients with glioblastoma, functional diffusion maps (fDM) have been analyzed as a means of predicting treatment response. One study investigated metrics measured at three time points, including changes in tumor volume, mean apparent diffusion coefficient, and three submetrics of fDM, and showed that the volume of tumor with increased diffusion by functional diffusion mapping at 3 weeks was the best predictor of patient survival [70]. Radiologic response (RR)

and fDM differed in 25 % of cases and the combination of fDM and RR provided more accurate survival prediction than either metric alone. These data highlight the potential for earlier and improved survival prediction with fDM, suggesting an opportunity to incorporate this modality into intratreatment adaptation strategies.

High b-value diffusion-weighted sequences are also being investigated for use in adaptive radiotherapy algorithms. High b-value diffusion (defined as 3000 s/mm^2) might be superior to FLAIR and conventional (b ≤ 1000 s/mm^2) DWI at differentiating hypercellular GBM components from high vascular components, edema, and normal tissue [71]. Preliminary data show that when high b-value DWI is use to define hypercellularity volumes, poor coverage of these volumes predicts for worse progression free survival Fig. 9.7. Future research will help determine whether targeting high b-value DWI subvolumes can improve outcomes in glioblastoma.

MET-PET

Another promising approach for integrating into adaptive radiotherapy is amino acid position emission tomography using ^{11}C-MET-PET. Data show that MET-PET is useful after resection of glioblastoma, given that it identifies regions of concern beyond the gadolinium-enhancing area [72]. Data from a phase I GBM dose-escalation trial showed that MET-PET volumes were typically smaller than contrast enhancing volumes in patients who underwent resection, but there were many cases were MET-PET uptake extended beyond the contrast enhancing region (though typically within the FLAIR region) [68]. Follow up data from this study showed that suboptimal coverage of the MET-PET tumor volume resulted in a higher risk of non-central failure Fig. 9.8. Given the natural history of GBM, central failures of often considered inevitable, but the presence of margin failures suggests an

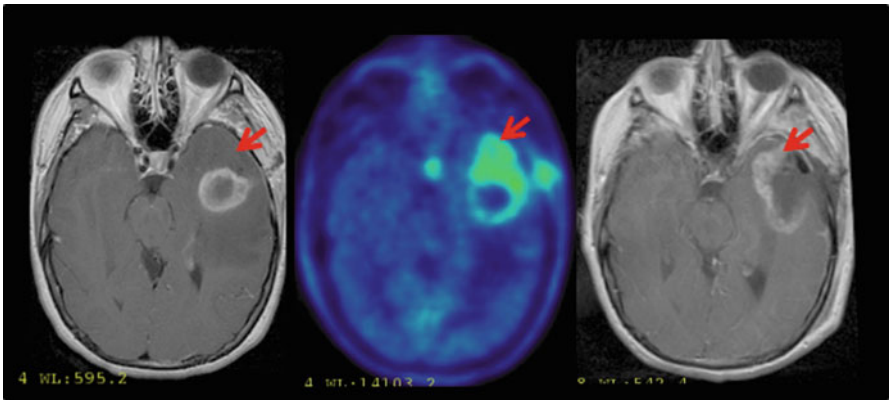

Fig. 9.8 Post-Gd T1 weighted image pre-RT (left), 11C-MET PET pre-RT (*middle*) and post Gd T1 weighted image at recurrence (*right*) of a patient with glioblastoma. The *arrow* points to the MET uptake but non-enhanced tumor, which was coincident with the contrast enhanced lesion at recurrence. *Gd* gadolinium

opportunity to improve local control with enhanced coverage. This provides the rationale for investigating MET-PET-based target coverage into glioblastoma trials. Data also support further investigation of the use of MET-PET in patients with low-grade glioma [73]. One limitation of MET-PET is that it may not identify low-proliferating components [71], suggesting that in should be used in conjunction with as opposed to serving as a replacement for MRI.

9.5.2 Additional Cancer Types

While much of the adaptive radiotherapy research has focused on glioblastoma, head and neck, liver and lung cancers, there are other tumor types for which adaptive radiotherapy could be beneficial. Data suggest that diffusion weighted MRI could be useful in predicting response to chemoradiotherapy in pancreatic cancer. Further research should identify adaptive strategies for improving outcomes and reducing toxicity in other cancers.

9.5.3 Circulating Tumor Cells

Over the past decade, there has been increasing interest in identifying tumor cells in locations that are distinct from the primary site, most commonly in the form of circulating tumor cells (CTCs) in the peripheral blood or disseminated tumor cells (DTCs) in the bone marrow. These cells are thought to originate from either the primary tumor or metastatic sites.

Data have shown that higher level circulating tumor cells are associated with poorer prognosis in patients with non-metastatic [74] and metastatic disease [75, 76]. Studies are now being undertaken in which systemic therapy regimens are modified based on the results of CTC or DTC analysis [77]. While most of the studies of CTCs have been performed in patients with metastatic disease undergoing systemic therapy, there is potential applicability in patients undergoing definitive radiotherapy. Emerging evidence shows that CTCs can be identified in patients with no evidence of metastatic disease who are undergoing definitive [78] or adjuvant [79] radiotherapy. Preliminary data suggest a correlation between CTC counts appear to correlate with outcome. Furthermore, CTC counts appear to change before therapy is completed [78], suggesting that there assays can be incorporated into adaptive radiotherapy algorithms that modify total dose or intensify systemic therapy. Though imaging has the advantage of providing spatial information to allow selective boosting, CTCs have the potential to provide complementary information that is a combination of primary tumor viability (whether it continues to shed CTCs) and the existence of otherwise occult metastatic disease (evidenced by rising CTC levels.) Future research is needed to determine whether CTC quantification has utility in adaptive radiotherapy and to identify which assays as the most robust and cost effective.

9.6 Conclusion

Though radiotherapy techniques have improved significantly over the past few decades, high rates of toxicity, recurrence and mortality persist for many types of cancers. Through incorporation of novel imaging modalities and biomarkers, adaptive radiotherapy has the potential to improve outcomes and reduce toxicities that occur when population-based dose limits and prescriptions are used. Emerging evidence reveals promising adaptation strategies in liver, lung, head and neck, and brain tumors, among others. Carefully designed and executed trials will help establish optimal adaptive radiotherapy protocols.

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Chapter 10
NQO1 Bioactivatable Drugs Enhance Radiation Responses

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Abstract Inhibitors of cancer-specific pathways can selectively kill off tumor cells. However, heterogeneity of neoplastic tissue often allows other cancer cells to repopulate the tissue area, leading to regeneration of resistant disease. β-Lapachone is a novel bioactivatable drug that relies specifically on tumor-directed upregulated levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) to kill most solid cancers, such as 90 % of pancreatic and non-small cell lung, 60 % of breast, colon, and prostate, as well as 50 % of head and neck cancers. Once β-lapachone is bioactivated by the NQO1 enzyme, massive levels of hydrogen peroxide are produced that, in turn, damage the DNA of cancer cells, while associated normal tissues, which lack NQO1, are protected by high levels of catalase. If tumors are irradiated prior to applying β-lapachone, the drug (clinical form, ARQ761) can work in combination with the vast spectrum of DNA lesions created by ionizing radiation, particularly DNA base lesions, single and double strand breaks (SSBs and DSBs), in addition to the mas-

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sive hydrogen peroxide-based lesions created by β-lapachone, to cause tumor-dependent poly(ADP-ribose) polymerase 1 (PARP1) hyperactivation. Once tumor-selective PARP hyperactivation is induced in cancer cells, they die due to low concomitant catalase levels. In contrast, associated normal tissue, as well as other normal tissue, lack elevated levels of NQO1 and have high catalase levels. Cancer cell death ultimately occurs by NAD^+-depletion, where resistance to NQO1 bioactivatable drugs has not been noted to date. Current studies are focused on pancreatic and non-small cell lung cancers, as NQO1 is elevated in nearly all of these cancers.

Keywords Oxidative stress • NQO1 expression • PARP hyperactivation • Tumor-selectivity • NAD^+ loss • NAD^+-Keresis

10.1 Introduction

Developing effective agents that can selectively increase the sensitivity of cancer cells to ionizing radiation (IR), so called 'radiosensitizers,' while not affecting normal cells or tissues, has been difficult. IR therapy is effective due to the spectrum of DNA lesions produced and its ability to generate complex DNA double strand breaks (DSBs), where only one non-repaired lesion is required for cancer cell lethality. Nevertheless, IR therapy is subject to the classical four Rs of radiobiology [1, 2]: (**i**) **Re-oxygenation** and resistance by hypoxia-efficient sensitization of cells (see Chap. 2); (**ii**) **Repair** where tumor cells often have heightened DNA repair mechanisms; (**iii**) **Redistribution** of tumor cells into more radioresistant phases of the cell cycle; (**iv**) **Repopulation** in which resistant tumor cells (e.g. cancer stem cells [CSCs]) rapidly expand and often have increased capacity for metastasis. In recent years, another R has been added, (**v**) **Radioresistance**, to indicate 'inherent radiation resistance' mechanisms that develop during carcinogenesis. Strategies for developing radiosensitizers (briefly summarized below and specifically addressed

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in this review), have attempted to overcome one of these five Rs. Unfortunately, many of these strategies have focused on differential cell division and not on underlying non-dividing tumor cells (that may include CSCs) that remain unperturbed by nearly all radiosensitizing agents. In this review, we will present data on NQO1 bioactivatable drugs from the past 20 years, and suggest that these agents should be explored as radiosensitizing agents which, when used properly, can kill independent of cell cycle status, or presence of tumor suppressor (e.g., p53, pRb) or oncogene (e.g., KRAS, MYC) status. Additionally, NQO1 bioactivatable agents can efficiently alter tumor-specific metabolism and inhibit a spectrum of DNA repair pathways by a unique poly(ADP-ribose) polymerase (PARP) hyperactivation mechanism. This occurs via the X-ray-inducible transcript leading to protein (xip3) gene [3], now known as NAD(P)H:quinone oxidoreductase 1 (NQO1) which is overexpressed 5- to 100-fold in most solid cancers, including extremely recalcitrant cancers, such as non-small cell lung, pancreas, as well as breast, prostate, colon, bladder, and head and neck cancers [4–8]. We briefly review current and past radiosensitizer strategies, and then describe the mechanism of action of NQO1 bioactivatable drugs alone [7, 9–11], and how these agents exploit a novel NQO1-dependent, tumor-selective PARP hyperactivation mechanism when combined with IR therapy.

10.2 Prior and Current Strategies for Radiosensitizing Cancers (Fig. 10.1)

1. Halogenated Pyrimidines. Halogenated pyrimidines (HPs), such as chloro-, bromo- and iodo-deoxyuridine (CldU, BrdU, IdU), are well-known radiosensitizers and have been reviewed in detail elsewhere [12–17]. In an effort to develop additional tumor-selectivity and to exploit elevated deoxycytidine deaminase (dCD)-deoxycytidine kinase (dCK) levels, halogenated deoxycytidine derivatives (particularly CldC and FdC) were developed by Dr. Sheldon Greer [9, 18–20].

Since the 1960s, HPs were investigated for their potential to replace thymidine and incorporate into the DNA of replicating tumor cells to increase cell sensitivity to IR [21–28]. Bromine, chlorine and iodine have van der Waals radii that are larger than hydrogen and similar to the 5-methyl group of thymine. They mimic thymidine and become better substrates for thymidine kinase [29]. Several mechanisms are simultaneously at work to explain how HPs cause radiosensitization. First, IR exposure of cells that have unifilar or bifilar incorporation of HPs causes de-halogenation and subsequent formation of $HP^{\bullet}$ radicals that cause complex DSBs due to the formation of multiple damaged sites, making these lesions difficult to repair [30]. Accordingly, bifilar incorporation is more effective than unifilar incorporation [31]. Multiple clinical trials and applications have been developed for HPs over the years with varying degrees of success, with the main challenge being the incorporation of sufficient HPs into DNA to achieve a therapeutic result [32–38]. Clinical trials have not been definitive about the use of HPs in cancer treatments, but there may be a subset of patients that benefit [39, 40]. Toxicities, including increased sensitization of the eyes to light and blindness, have greatly impeded the use of HPs as radiosensitizers [41].

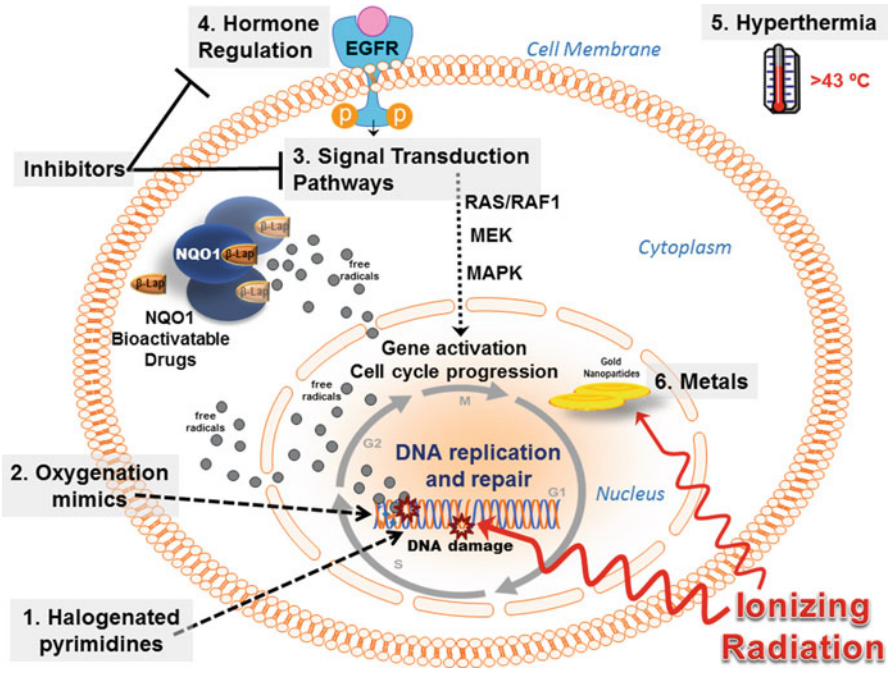

Fig. 10.1 Cellular map of radiosensitization targets

2. Oxygenation mimics: The use of oxygen to increase IR effects. The aggressive nature of tumors relies on their ability to adapt to environmental stress factors. Regions of the tumor contain necrotic areas where oxygenation levels remain low (i.e., are hypoxic, with oxygen (O_2) levels less than 10 mmHg). This hypoxic environment in tumors induces the activation of certain compensatory pathways to protect the cell, including the hypoxia-inducible factor (HIF) pathway [42, 43]. Activation of these pathways in response to low O_2 levels allows for a selective advantage against apoptosis and can lead to resistance to chemo- and radiotherapies [44, 45]. When the level of O_2 is enhanced in the tumors, radiosensitization is increased up to threefold [46]. This increased radiobiological effect (RBE) led to the development of oxygenation methods to enhance the response to IR therapy.

One technique to sensitize tumors to radiotherapy is the use of oxygenation mimetics. These mimics utilize the chemical properties of oxygen without being rapidly metabolized by cells undergoing cellular respiration, which allows for increased distribution into hypoxic areas of tumors [44]. The most common class of oxygenation mimetics is the nitroimidazoles. These agents are able to "fix" and prolong DNA radical lesions produced by IR. Nitroimidazoles take advantage of nitroreductase enzymes that are upregulated in hypoxic conditions found in tumors to generate anion radicals [47, 48]. The radical anions created are highly reactive and undergo irreversible fragmentation that promote cross-linking in DNA, rendering irradiated cells unable to divide, eventually leading to apoptosis [49].

3. Signal transduction inhibition strategies. Aberrant activation of various signal transduction pathways is frequent in neoplastic growth. In response to IR, cancer cells critically depend on a cascade of multiple signal transduction responses, such as the stimulation of (1) plasma membrane receptors [50, 51]; (2) cytoplasmic protein kinases [52, 53]; (3) specific transcriptional activation [54–56]; and (4) altered cell cycle regulation [53, 57] to ultimately evade the toxic effects of IR-induced damage. In cancer, for example, overexpression or mutational activation of RAS and PTEN can regulate the PI3K pathway needed for the eventual repair of IR-induced DSBs [58]. This inherent "addiction", however, opens new avenues for innovative therapeutic strategies toward the development of novel anticancer drugs that inhibit key signal transduction cascade steps to potentiate the effects of low-dose IR [59–63]. The PI3K/mTOR dual inhibitor, NVP-BEZ235, is an excellent example of a radiosensitizer [61–63]. This inhibitor targets both ATM and DNA-PKcs that are apical kinases involved in repair of IR-induced DSB [62]. Simultaneous inhibition of these two kinases blocks both homologous recombination (HR) and non-homologous end joining (NHEJ) pathways that are critical for repair of IR-induced DNA damage [62]. In combination with IR, this inhibitor potently induces apoptosis both *in vitro* and *in vivo* using models of mutant KRAS-driven non-small cell lung cancer (NSCLC) and glioblastoma [61–63]. This combination therapy could be broadly applicable to other cancers that exhibit aberrant activation of PI3K. Pharmacological inhibition of specific signal transduction pathways is often quite effective initially, but over time, cancer cells upregulate compensatory pathways to overcome their dependence on the targeted pathways. In such scenarios, combination therapies simultaneously targeting multiple pathways become a necessity to eliminate cancer.

4. Hormone Regulation. The most well-studied growth factor pathways modulating radiosensitivity have been targetable EGFR and insulin receptors. The mechanism of radiosensitization by inhibiting EGFR are under intense investigation, but are likely related to both regulation of the cell cycle [64–71] and modulation of DSB repair [72, 73]. Kriegs et al., demonstrated that in NSCLC, radiosensitivity increases by promoting G_1/S-arrest in tumor cells [64]. In clinical trials, the most substantial benefit of EGFR inhibitors appears to be in a subset of head and neck cancers, with significant enhanced survival in patients receiving combination therapy with IR plus Cetuximab [74]. The main difficulty in improving clinical outcomes has been identifying the cohort of patients who would best respond to therapy. Less well investigated are other hormonal pathways, such as insulin and insulin-like growth factor 1 (IGF-1) regulation. Wang et al. demonstrated radiosensitization in pancreatic cancer via metformin's effect on the G_2-checkpoint and increased rates of mitotic catastrophe [75] and Fasih showed similar results via the AMPK pathway [76]. Another strategy being developed is to alter the TGFβ1-IGF-1 extracellular expression axis, which leads to the pro-survival protein and extracellular protein chaperone, secretory clusterin (sCLU) [77–79]. Suppressing the TGFβ1-IGF-1-sCLU expression pathway is likely to decrease resistance and suppress glycolytic and TCA metabolic reprogramming that can occur post-IR by suppression of fatty acid synthase (FASN) and lipogenesis [79].

5. Hyperthermia. Modifying the tumor microenvironment has long been a therapeutic target in cancer therapy. Multiple studies *in vitro* demonstrated the dramatic radiosensitization effects of hyperthermia [12, 80]. Heating tissue over 43 °C impairs the cell's ability to effectively perform DNA repair, which is potentiated by its combination with IR [81–83]. Among a host of other responses, hyperthermia can cause enhanced ATM kinase activity, inactivation of HSP70, and increased telomerase activity [82]. Heat also diminishes chromatin condensation and leads to nucleolar disintegration as a marker for impaired DNA repair [84]. Though many of these effects were demonstrated in various cell lines, clinical application of hyperthermia continues to be problematic. Strategies are now being employed to target heat shock proteins instead. As technology has improved, there is now considerable interest in the use of magnetic resonance high intensity focused ultrasound (MR-HIFU) for delivery of chemotherapy and palliative pain control [85–91].

6. Metals. Various metals, with a predominant emphasis on gold nanoparticles, have been investigated as radiosensitizers [92–94]. Gold particles absorb energy from IR, thereby potentiating SSBs and DSBs *in vitro*, as well as by generation of toxic free radicals [95–98]. Studies *in vivo* have also demonstrated antitumor effects in mice [99–103]. The main difficulty has been enhancing the delivery of gold nanoparticles to tumor sites. Other metals with radiosensitization properties such as copper, iron, nickel, and gadolinium are in early phases of exploration for potential clinical use [104–110]. Most metals, even gold, however, have major toxicity issues that must be dealt with for future therapies [111].

7. Dietary supplements, vitamins, and complementary and alternative medicine. Over the years, a large effort has been devoted to developing complementary and alternative medicines for the treatment of cancers, and many of these agents have been investigated as potential radiosensitizers. Caffeine has been described in several papers to induce radiation sensitivity via elimination of the radiation-induced G_2 checkpoint and inhibition of both ATM and ATR [112–114]. Indeed, examination of the literature on caffeine [115] led to a search for DNA repair inhibitors, and ultimately, the identification of β-lapachone, the first NQO1 bioactivatable drug [10]. Neem leaf extract was also implied in radiosensitization by modulation of apoptotic pathways in neuroblastoma, and via NF-kB in pancreatic cancer cell lines, where curcuma and black raspberry extract were also indicated to be effective [116–120]. Likewise, caffeic acid phenethyl ester (CAPE), the active ingredient in honeybee resin, demonstrated growth inhibition in human lung cancer cell lines via decreased glutathione and elevated H_2O_2 levels by unknown mechanisms. [121, 122] Soy products, likely via antioxidant effects, demonstrate synergistic anticancer effects in combination with radiation therapy in prostate cancer [123]. Finally, vitamins, such as riboflavin, were suggested as a radiosensitizer in mouse thymocyte models and human hepatoma cells [124]. Though none of these compounds have become adjuncts to radiation therapy, the widespread use of complementary and alternative medicines warrants clinical awareness of the effects of these particular xenobiotics, and specifically inquiring about their use.

10.3 Targeting NQO1 Expression for Cancer Therapy

1. NQO1: A Phase II detoxification enzyme. The cellular detoxification of foreign chemicals occurs in a step-wise manner facilitated by enzymes that carry out specific metabolic processes, including biotransformations (Phase I), conjugations (Phase II) and transportation (Phase III) [125]. Phase II detoxification involves glucuronidation, acetylation, and sulfonation conjugation reactions. These reactions add polar moieties to very reactive and toxic xenobiotic molecules, rendering them more water-soluble. Following conjugation reactions, the newly created quinone-metabolites are less reactive, and thus, are less harmful to cells. For most quinones, NQO1 is a phase II detoxification enzyme, and as such, interacts with various xenobiotic quinone substrates. NQO1 converts these reactive quinones to more stable hydroquinones, allowing more efficient phase II conjugation reactions, and subsequent rapid excretion [126]. The classic example of NQO1 metabolism of a quinone resulting in its detoxification is the interaction between NQO1 and menadione. In the early 1980s, studies involving menadione metabolism in hepatocytes discovered that in the presence of the NQO1 inhibitor dicoumarol, increased free radical formation and elevated oxygen consumption [127]. The relevance of NQO1's detoxification of menadione was further confirmed in the late 1990s by Jaiswal's characterization of NQO1-deficient mice who were hypersensitive to quinoid compounds, such as menadione [128].

2. NQO1 expression in cancers. In normal tissue, particularly normal lung, expression of NQO1 has been shown to be inducible, and the induction of endogenous NQO1 levels in tissues is primarily a response to increased levels of oxidative stress [129]. NQO1's inducible expression is tightly regulated by the transcription factor, Nrf2. Nrf2 is held in abeyance in the cytosol by Keap1, an E3 ubiquitin ligase. In the presence of Keap1, Nrf2 undergoes rapid proteasomal degradation. The Keap1-Nrf2 pathway controls expression of NQO1 as well as many other oxidative stress regulatory genes, resulting in their expression only when Nrf2 is released from Keap1. The dissociation of Nrf2 from Keap1 occurs when cells are confronted with oxidative stress. The severance of Nrf2 from Keap1 permits Nrf2 to translocate to the nucleus where it interacts with the chaperone, small Maf-1 protein. Together, the Nrf2/Maf-1 complex regulates the transcription of various antioxidant genes whose common thread is the presence of an antioxidant response element (ARE) within specific promoters of certain genes. These Nrf2-activated genes, including NQO1, are activated and tightly controlled in normal cells to protect the genome from various deleterious forms of oxidant stress. In contrast to the tightly regulated low levels of NQO1 in normal cells, expression of NQO1 in cancer cells was constitutively elevated well above those observed in normal tissues [130, 131]. In retrospective analyses, NQO1 expression in most solid cancerous tissue is elevated 5- to 100-fold more than levels noted in associated normal tissue for the same patient [130, 131]. Increased NQO1 expression in cancerous tissues is typically a disruption in the Keap1-Nrf2 association [132]. In fact, studies have reported finding mutations in Keap1 in various cancers, where NQO1 levels were elevated [133, 134]. These

studies showed that when Keap1 was mutated, expression levels of Nrf2 were increased in the nucleus and an elevated expression of NQO1 and other Nrf2-regulated genes were observed. Thus, elevated NQO1 expression in many cancers has led to an increase in studies investigating the plausibility of developing quinones whose bioactivation could potentially be harnessed for development of NQO1-directed antitumor chemotherapeutics.

3. Previous studies on initial NQO1 bioactivated drugs: Mitomycin C, Geldanamycin, EO9 and Streptonigrin. NQO1 plays a significant role in 'bioactivating' a select few quinone substrates. These NQO1 activatable drugs fall into two classes: (***i***) compounds, such as mitomycin C (MMC) and geldanamycin that are converted to their hydroquinone forms in one-step reactions, and become either DNA alkylating agents (MMC) or inhibitors of specific pathways, such as HSP90 or HSP70; or (***ii***) compounds that undergo futile redox cycling (e.g., EO9 and streptonigrin), potentially generating tremendous oxidative stress. Streptonigrin is unique in that it causes both elevated reactive oxygen species (ROS) levels and induces DNA alkylation.

Mitomycin C is a naturally occurring compound originally derived from the Gram-positive bacterium, *Streptomyces*. MMC is used clinically to treat a variety of tumor types, including stomach, pancreas, breast, and lung [135]. However, its use as an NQO1-directed killing compound has been limited by its dependence on a narrow pH range [136]. Outside this pH range, its antitumor activity is not NQO1-specific. In fact, MMC, in most cases, acts as an inhibitor of NQO1 activity. Thus, this compound, although used to treat a wide assortment of cancers with limited success, does not truly define the potential of selective NQO1-directed chemotherapy.

Geldanamycin is a 1,4-benzoquinone originally isolated from *Streptomyces* in 1970 [137]. Its antitumor activity has been correlated with three main factors, including free radical formation, tyrosine kinase inhibition, and binding and interfering with heat shock proteins. 17-AAG is a geldanamycin derivative produced to avoid dose-limiting toxicities that included hemolytic anemia and hepatotoxicity [138]. In clinical studies with patients with NQO1 homozygous *2 polymorphisms, and thus no NQO1 expression, no correlation was observed between responses to 17-AAG therapy and NQO1 status, suggesting that although NQO1 bioactivates 17-AAG [139], the activity of the drug was not related to NQO1 expression, but to its off-target effects.

EO9 (3-hydroxy-5-aziridinyl-1-methyl-2(1*H*-indole-4,7-dione)prop-β-en-α-ol), also known as apazoquinone, is another example of an NQO1-bioactivated compound that has undergone clinical trials [140]. Studies *in vitro* showed that EO9 bioreduction by NQO1 caused DNA damage in the form of SSBs, which was suppressed by catalase, implicating hydrogen peroxide formation during EO9 reduction by NQO1 [141]. In clinical studies with EO9, its low water solubility led to extremely poor systemic pharmacokinetics, actually due to metabolism by peripheral red blood cells themselves [142]. Thus, poor responses in patients with solid cancers were noted [143, 144]. However, when used in trials to treat local regional bladder tumors, the compound faired much better. Although EO9 had limited success in clinical trials, an interest still exists in its utilization in treating local regional tumors [140].

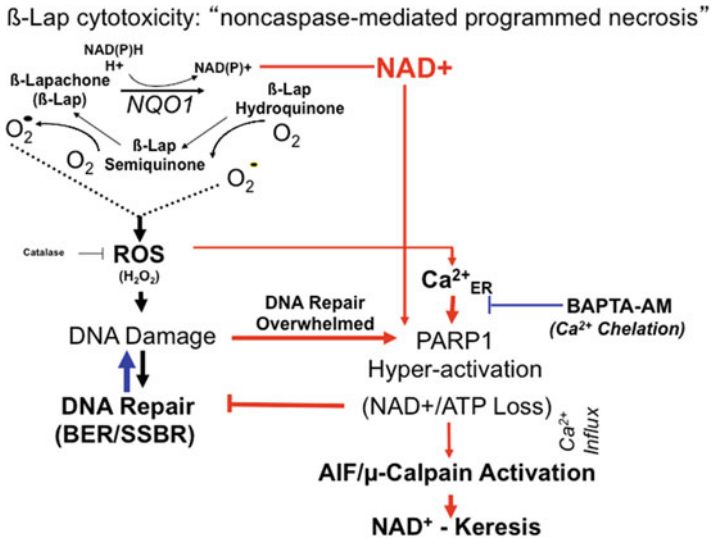

Fig. 10.2 Tumor-selective, NQO1-mediated futile redox cycle of β-lapachone (β-lap) triggers a novel PARP hyperactivation-dependent pathway of programmed necrosis, referred to as NAD^+-Keresis. Using β-lapachone, this pathway is being exploited for cancer therapy, but also as a general treatment against metabolic syndromes to correct NAD(P)H:$NAD(P)^+$ ratios

Streptonigrin is another quinone antibiotic discovered in the early 1960s to have antitumor activity. However, the drug was extremely difficult to synthesize and subsequently failed in initial clinical trials due to poor water solubility [145]. As a result, limited studies are available to prove its entire mechanism of action. In more recent studies, streptonigrin was found to be active in an NQO1-dependent manner in human colon cancer cells [146]. As noted above, streptonigrin represents a potent potentially NQO1-dependent agent that undergoes NQO1-dependent redox cycling, but also alkylates DNA [147]. Further studies on streptonigrin, including improved and more efficient synthetic procedures, may be warranted.

4. NQO1 bioactivatable drug mechanisms of action and cellular consequences. β-Lapachone (β-lap) is a unique NQO1 bioactivatable drug that exploits the NQO1:catalase therapeutic window due to its elevated expression in many solid tumors [5]. β-Lapachone undergoes a futile redox cycle, which attempts to detoxify the drug, thereby forming its unstable hydroquinone (Fig. 10.2). The hydroquinone form of β-lapachone undergoes a spontaneous back-reaction, creating two superoxides. The reaction is robust, using 60 mol of NAD(P)H and creating 120 mol of superoxide in just 2 min in NQO1 positive cancer cells. Reactions are prevented by dicoumarol (DIC), a specific inhibitor of NQO1 and do not occur in NQO1 negative cells. Impressively, a 1 h exposure of NQO1+ pancreatic ductal adenocarcinoma (PDA) cells to 4 µM β-lapachone is equivalent to 300–500 µM H_2O_2 [8, 148]. A minimum of 35–120 min of exposure to NQO1 bioactivatable drugs is required to kill all NQO1+ cancer cells in vitro, strongly suggesting that key lethal responses

occur in this time frame [149, 150]. Loss of NAD(P)H reducing equivalents, accumulation of NAD^+ pools, DNA base damage, and Ca^{2+} release from the endoplasmic reticulum (ER) result in 'PARP1 hyperactivation' which degrades NAD^+ pools and causes tumor-selective NQO1-mediated cell death (Fig. 10.2). Major advantages of using β-lapachone include its unique NQO1-dependent mechanism of action and lack of major exposure-related resistance pathways. A small population of NQO1- polymorphic individuals (~5 %) are predicted not to respond. Prior work [4, 8, 131, 149–154] demonstrates that β-lapachone-induced cell death is: ***(i)*** not dependent on p53 status; ***(ii)*** not dependent on cell cycle; ***(iii)*** not affected by bak/bax loss; ***(iv)*** not affected by known oncogenic driver or carrier mutations [5]; and ***(v)*** not affected by caspase (e.g., bcl2 expression or caspase loss in MCF-7 cells) loss [7, 153, 155–157]. β-Lapachone also causes a potent bystander effect, wherein $NQO1^{low}$ (<1 Unit) cancer cells in a mixed NQO1+/NQO1− tumor are killed by apoptosis, while normal tissues are unaffected due to high catalase levels [5, 6, 8, 158]. Cell death is mediated by μ-calpain/AIF activation [131, 148, 151, 152]. β-Lapachone causes extensive DNA base damage and SSBs, even at sublethal doses (≤2.5 μM) for NQO1+ cancers [5].

5. Metabolic consequences of NQO1 bioactivatable drugs. The loss of NAD^+ can be seen in several examples of NQO1 bioactivatable drugs. The mechanism of NQO1 bioactivable drugs (Fig. 10.2) shows how NQO1 catalyzes the rapid conversion of NADH to NAD^+. The buildup of NAD^+ in the cell is quickly depleted upon PARP1 hyperactivation. A lethal dose of the drug deoxynyboquinone (DNQ) revealed NAD^+ is depleted within 30–60 min, as well as complete loss of ATP within the cell [154]. A similar phenomenon is observed with a lethal dose of β-lapachone [159]. β-Lap treatment also caused a persistent reduction in the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in glycolysis, noted by the accumulation of glyceraldehyde 3-phosphate (GA3P) in the cells. This observation was also complemented by a decrease in glucose utilization and subsequent lactate production [160].

The exploitation of metabolic pathways that utilize NAD^+/NADH and ATP can be advantageous in enhancing the effects of NQO1 bioactivatable drugs. In theory, the rapid depletion of these essential cofactors suggests downstream metabolic consequences might occur. One pathway to target would be the nicotinamide recycling pathway which is primarily responsible for NAD^+ synthesis. The cell naturally tries to recover from the dramatic NAD^+ loss caused by NQO1 bioactivatable drugs by regenerating this essential nucleotide. The rate-limiting step of the reaction is catalyzed by nicotinamide phosphoribosyltransferase (NAMPT) and the most well-known inhibitor is FK866 [161, 162]. Pre-treatment with FK866 reduced overall NAD^+/NADH pool sizes prior to β-lap treatment, which leads to an accelerated decrease in overall NAD^+/NADH and shifted lethality of β-lap to smaller doses of the drug [160]. Overall, treatment with FK866 attenuated the effects of β-lap, without causing excess PAR formation (due to lower NAD^+ levels) and renders the NAMPT inhibitor tumor-selective.

Alternate pathways to target for combination therapy are those that feed directly into glycolysis and/or the TCA cycle. One approach targets cancer cells

whose metabolism is driven by mutant KRAS. KRAS-driven metabolism utilizes glutamine as an anaplerotic carbon source. The first step in this pathway is catalyzed by mitochondrial glutaminase (GLS1) and is responsible for converting glutamine to glutamate within the mitochondria [163]. Current inhibitors against GLS1 include BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide) and CB-839 [164, 165]. Recent studies have shown that treatment with BPTES sensitized pancreatic cells to β-lap in a mutant KRAS-dependent manner and caused a significant decrease in NAD^+ at lower, sublethal β-lap doses. *In vivo* studies with CB-839 reveals an increase in overall PAR formation and an increase in a survival advantage when treated in combination with β-lapachone [6, 166].

A more recent NQO1 bioactivatable drug, KP-372, shows many downstream metabolic effects. A KP-372 treatment results in an increase in the cytosolic NAD^+/NADH redox state in a dose-dependent manner. The most significant observation is the increase (seven to eightfold) of several intermediate metabolites within the pentose phosphate pathway (PPP), which most likely occurs to regenerate depleted NAD(P)H. Additionally, the glycolytic and TCA cycle metabolites also showed a perturbation upon treatment. However, the lactate production after treatment showed no significant change, indicating KP-372 had no measurable effect on glycolysis [167].

6. Pathways of sensitizing cells to NQO1 bioactivatable drugs. Early on, in searching for a DNA repair inhibitor, β-lapachone was discovered for its abilities to inhibit recovery of irradiated cells, only if added during or immediately after exposure to IR, affording efficacy as a radiosensitizing agent *in vitro* [9–11]. In those early studies, $NQO1^{low}$ radioresistant malignant melanoma (U1Mel) cells were used in an effort to counter potentially lethal DNA damage repair (PLDR) processes [10]. Simultaneously, it was discovered that IR-treated U1Mel cells significantly induced NQO1 expression, then identified as X-ray-inducible transcript leading to protein 3 (xip3) [3]. Therefore, IR treatment predisposed U1Mel cells to β-lap, which were relatively resistant to this NQO1 bioactivatable drug in its basal state. Dose enhancement ratios (DERs) of 1.8- to 2.5-fold, with >3.5-fold when halogenated pyrimidines (HPs) were incorporated, were found [9]. Further analyses revealed that numerous cancers have constitutively elevated levels of NQO1 [131, 148], and that NQO1 expression appears to be a pro-survival gene for CSCs [168]. Additionally, cancer cells with constitutively elevated levels of NQO1 could still be radiosensitized to the same extent as $NQO1^{low}$ cells [4], dramatically broadening use of β-lap and other NQO1 bioactivatable drugs as radiosensitizers. Mechanistically, low doses of IR, which do not hyperactivate PARP, combine with sublethal doses of β-lap (not capable of hyperactivating PARP) synergistically create enough DNA base damage, SSBs, and DSBs to hyperactivate PARP. In this case, synergy is the culmination of a number of events in the following sequence: (**a**) treatment with IR, causing a spectrum of DNA lesions, including DNA base alkylation, as well as single- and double strand breaks, with DSBs being the most lethal; (**b**) simultaneous treatment with β-lapachone, resulting in significant H_2O_2 production

and specific DNA base damage and SSBs. Co-treatment reaches the threshold level of DNA base, SSBs and DSBs, resulting in PARP hyperactivation, where significant decreases in $NAD(P)^+$ result creating conditions where DNA repair is prevented (Fig. 10.2). Most significantly, DSB repair is prevented, causing a synergistic killing effect which triggers a form of programmed necrosis (PN), known as NAD^+-Keresis. Due to the spectrum of DNA lesions, specifically DSBs, and triggering of PARP hyperactivation-dependent NAD^+/ATP loss, the combination shows significant synergistic lethality against many NQO1+ human cancers, including neoplasms of the prostate, breast, non-small cell lung, and head and neck carcinomas.

7. Other synergistic combinations with β-lapachone. Elucidating the mechanism of action of β-lap allows the prediction of various synergistic combinations with other pathways, and the development of specific inhibitors of these pathways for improved efficacy of treating NQO1+ human cancers (Table 10.1). Note that IR exposure is clearly the most efficacious, but also the only combination therapy where DNA lesions are initiated by both agents. In all cases, tumor selectivity arises from β-lap, where exposures exploit the elevated NQO1 levels present in most solid cancers, with concomitant lower levels of catalase [5]. After IR + β-lapachone, the most efficacious combinations with β-lap are indicated in decreasing order of efficacy, and include combination with (Table 10.1):

(a) **Gemcitabine,** the DNA base analog incorporates into DNA and creates DNA lesions with or without DNA repair mechanisms. The lesions synergize with β-lapachone-induced DNA damage to decrease survival. The exact mechanism of synergy is believed to result from PARP hyperactivation-dependent NAD^+ loss. However, studies confirming this have yet to be performed.
(b) **Methoxyamine (MeOX),** the base excision repair (BER) inhibitor and abasic-modifying agent. MeOX prolongs abasic sites allowing enhanced PARP binding and hyperactivation. DNA base damage by β-lapachone is the essential DNA lesion forming mechanism.
(c) **CB-839,** glutamine transaminase 1 (GLS1) inhibitor. Pancreatic cancers with activated KRAS concomitantly elevate NQO1, as well as GLS1 and other glutamine anaplerotic pathways, in order to move electrons within the cell for the ultimate synthesis of NAD(P)H. Depleting cells at the first steps of this pathway with GLS1 inhibitors, BPTES or CB-839, depletes NAD(P)H making β-lapachone-induced PARP hyperactivation-dependent NAD^+ loss more efficient.
(d) **FK866,** NAMPT inhibitor. NAMPT is the sole *de novo* enzyme responsible for the major pool of NAD^+ in the cells. Since most cancer cells, particularly pancreatic cancer cells overexpress the enzyme, inhibiting this salvage NAD^+ synthetic pathway lowers the pool and makes β-lapachone-induced PARP hyperactivation more efficient, causing NQO1+-dependent efficacy. Studies *in vivo* are warranted.

A common mode of synergy between the agents listed in Table 10.1 and β-lapachone is the formation of threshold levels of DNA lesions leading to PARP

Table 10.1 Mechanism of action of synergy of antitumor agents in combination with β-lapachone, an NQO1 bioactivatable, tumor-selective agent

Agent (Refs)	Synergy Y/N (DER LD_{50})	Source of damage	Induced DNA lesions	Mechanism of action (death)	Basis of tumor-selectivity
[a]IR [4, 9, 10, 149, 169, 170]	Y (2.2–3.1)	IR, β-Lap	DSBs, SSBs, Base Damage	DSBs, PARP Hyperactivation, NAD^+/ ATP loss, DNA Repair Inhibition, (PN)	NQO1:Catalase Ratio Focused IR beam
[a]Gemcitabine [171]	Y (2.0)	Gem, β-Lap	SSBs, Base Damage	Base-SSBs, PARP Hyperactivation, NAD^+/ ATP loss, DNA Repair Inhibition	NQO1:Catalase Ratio
[a]MeOX [5]	Y (2.0)	β-Lap	Base Damage	NQO1-induced H_2O_2, AP-MeOX Sites, PARP Hyperactivation, NAD^+/ ATP loss, DNA Repair Inhibition, (PN)	NQO1:Catalase Ratio
[a]BPTES, CB-839 (GLS1 Inhibition) [6, 166]	Y (1.5)	β-Lap	ROS Stress, H_2O_2-induced DNA Damage	NQO1-induced H_2O_2, PARP Hyperactivation, NAD^+/ ATP loss, (PN)	NQO1:Catalase Ratio, KRAS Gln/Glu dependency
FK866 (NAMPT Inhibition) [160, 172]	Y (1.5)	β-Lap	ROS Stress, H_2O_2-induced DNA Damage	NQO1-induced H_2O_2, PARP Hyperactivation, NAD^+/ ATP loss, (PN)	NQO1:Catalase Ratio, NAMPT Overexpression
Cisplatin/Melphalan [9, 10]	N	Crosslinks, β-Lap	Crosslinks, Base Damage	Not synergistic	None
Ultraviolet Radiation [9, 10]	N	UV, β-Lap, 6-4 photo, DNA Base Damage	Thymine dimers, DNA Base Damage	Not synergistic	None

Listed are agents that synergize with β-lapachone. It is the NQO1 bioactivatable nature of β-lapachone that affords tumor-selectivity in each case of synergy (*PN* programmed necrosis)

[a]Confirmed *in vitro* and *in vivo*

hyperactivation, NAD^+/ATP loss, repair inhibition and programmed necrosis-medicated cell death. Accordingly, agents that do not induce DNA lesions that PARP (specifically PARP1) typically binds, such as ultraviolet radiation (UV), DNA alkylators or cross-linking agents, do not synergize with β-lapachone (Table 10.1).

10.4 NQO1 Bioactivatable Drugs as Tumor-Selective Radiosensitizers

1. Prostate Cancer. In general, therapy for advanced prostate cancer therapy, particularly androgen-independent castration-resistant prostate cancer, highlights well the five Rs of radiobiology and the difficulties in treating these diseases. Its slow growing, metastatic nature makes it difficult to treat with agents developed for therapy against actively replicating cancer cells. The ability of β-lap to kill cells, regardless of cell cycle position, is a major advantage and its increased efficacy in combination with very low doses of IR makes such therapy using NQO1 bioactivatable drugs very attractive [4]. β-Lap kills prostate cancer cells by NQO1 metabolic bioactivation, triggering a massive induction of ROS, irreversible SSB and DNA base damage that hyperactivate PARP1, resulting in NAD^+/ATP depletion and μ-calpain–induced programmed necrosis [151, 152]. In combination with IR, β-lap radiosensitizes NQO1+ prostate cancer cells under conditions where nontoxic doses of either agent alone achieved threshold levels of DNA base damage and SSBs required for hyperactivation of PARP. Combination therapy significantly elevates DNA base and SSB lesions, γH2AX foci formation, and poly(ADP-ribosylation) of PARP1, which are associated with NAD^+/ATP losses and induction of μ-calpain–induced programmed necrosis [4]. Radiosensitization by β-lap was blocked by the NQO1 inhibitor dicoumarol, or temporarily by PARP inhibitors. β-Lap synergized with IR to promote antitumor efficacy in a mouse xenograft model of prostate cancer. NQO1 levels were elevated in 60 % of human prostate tumors evaluated relative to adjacent normal tissue, where β-lap might be efficacious alone or in combination with ionizing radiation [4]. These data warrant a clinical trial to use β-lap as a radiosensitizer against prostate cancers that overexpress NQO1, offering a potentially synergistic targeting strategy to exploit PARP hyperactivation. Completion of the ongoing first-in-man clinical trial of ARQ761 against solid cancers should pave the way for future β-lap radiosensitization trials.

2. Head and neck cancer (HNC). This aggressive cancer accounts for ~3–5 % of all cancers in the United States with over 45,000 new cases and 8000 deaths estimated as of 2015 [173]. The majority of these cancers are squamous cell carcinomas and are highly curable with surgery in combination with radiotherapy and/or chemotherapy if detected early. Although there has been a progressive improvement in therapy, current treatment approaches still result in an overall survival (OS) rate of only ~50 % for locally advanced HNCs.

NQO1 was overexpressed in ~50 % HNC tissues compared to adjacent normal tissues [191]. Expression of catalase is also examined since NQO1-mediated β-lap lethality kills cancer cells through an NQO1-dependent futile redox cycling to generate massive amounts of H_2O_2, which is degraded by catalase [8, 148]. Interestingly, catalase was significantly elevated in adjacent normal tissues compared to HNC tissues. This inverse expression pattern of NQO1 and catalase suggests an ideal therapeutic strategy, since the NQO1-dependent anticancer mechanism of β-lapachone will selectively kill HNC cancer cells. In contrast, catalase will efficiently protect adjacent normal cells by detoxifying the H_2O_2 generated by NQO1-mediated β-lapachone futile redox cycle. In addition to NQO1/Catalase IHC staining in HNC tissue microarrays (TMA), Western blotting was used to examine NQO1 and catalase expression in 41 HNC cell lines, including several pairs of primary and lymph node metastasis-originated cell lines, as well as one normal human fibroblast cell line. A corresponding inverse relationship was found between NQO1 and catalase expression compared to normal human IMR90 fibroblasts.

Since many HNC cell lines overexpress NQO1, cell survival was determined after treating HNC lines with β-lapachone. A total of 41 HNC cell lines were selected and cells with elevated levels of NQO1 expression respond very well with β-lapachone exposure, while NQO1-deficient cell lines, which carry *2 or *3 single nucleotide polymorphisms (SNPs), did not. HNC cell death was dramatically increased with increasing concentrations of β-lapachone through a very sharp inflection point in dose–response studies, and completely abrogated by co-treatment with dicoumarol, and significantly decreased by an shRNA specific knockdown of NQO1 [191]. Furthermore, NQO1 activity (in terms of units of enzyme) were determined for a panel of 41 HNC cell lines and one primary normal fibroblast, where the data further confirmed that ~100 NQO1 enzymatic units were required for lethality due to NQO1 futile redox recycling of β-lapachone. In cells with higher NQO1 enzymatic activity, NAD(P)H (electron donor) most likely became rate-limiting, in which case higher NQO1 levels did not confer enhanced lethality or increased β-lapachone lethality (i.e., lowered LD_{50} values) for β-lapachone treatments in HNC cells. In addition, NQO1-mediated, β-lapachone-induced cell death was partially blocked in the presence of exogenous catalase in HNC cell lines, as previously noted [131, 148]. These data further confirm that β-lap selectively kills HNC cancer cells in an NQO1-dependent manner, regardless of clinic pathological status, while sparing adjacent normal tissue. Finally, β-lapachone killed NQO1+ HNC cells through massive formation of ROS/H_2O_2, dramatic increases in Ca^{2+} efflux, creation of SSBs and DSBs, PARP1 hyperactivation, NAD^+/ATP depletion, and programmed necrosis.

3. β-Lapachone radiosensitizes NQO1 overexpressing HNC cell lines. Given the massive amount of ROS generation and SSBs and DSBs, enhanced anticancer lethality with a combination of IR + β-lap could be used for the treatment of HNC. Since radiation therapy is used to treat a majority of HNCs, radiosensitization using sublethal doses of β-lapachone was explored using a combination of relative cell survival assays and colony formation assays. In agreement with previous data, HNC cell lines that express NQO1 were all radiosensitized by β-lap at low (other-

wise nontoxic) concentrations, while there was no radiosensitization of NQO1-deficient cells. To further assess the role of β-lap in radiosensitization, DNA damage status was assessed with γH2AX foci formation 2 h after a 2 Gy exposure in the absence or presence of sublethal doses of β-lapachone. Damage was assessed using immunofluorescence *in vitro* assays, as well as *in vivo* mouse models. Data clearly demonstrated that γH2AX formation was significantly increased in the presence of sublethal doses of β-lapachone (doses that cause significant stress, but no lethality) with 2 Gy compared to β-lapachone or IR alone [191]. As in prostate cancer, significant NAD^+/ATP losses was confirmed, and programmed necrosis played an essential role in NQO1-mediated radiosensitization of HNC cells with β-lapachone.

IR is a central therapeutic modality for the treatment of locally advanced and locally regional recurrent HNC [174]. Currently, chemotherapy is used as the standard of care to radiosensitize HNC tumors, but the use of chemotherapy also suffers from non-specific normal tissue cytotoxicity. Since the current standard of care, which includes radiation therapy with concomitant chemotherapy only cures ~50 % of patients with locally advanced disease, there is a need to identify better tumor radiosensitizers to increase the effectiveness of IR. The inverse expression of the NQO1:catalase ratio provides a favorable microenvironment to exploit the therapeutic window of NQO1 bioactivatable drugs, such as β-lapachone, for the treatment of HNCs, since catalase in normal tissue protects against NQO1-dependent β-lapachone lethality by neutralizing the bystander effect (H_2O_2-generated) of the β-lapachone futile redox cycle [8]. Since a similar inverse expression pattern was seen in HNC cell lines and patient samples, these data support further evaluation of radiation therapy + β-lapachone as a treatment strategy for HNC. This therapeutic strategy will leverage tumor-selective cytotoxicity, as well as the radiosensitizing capacity of β-lapachone [9, 10], while greatly reducing exposure to β-lapachone-HP-β-CD-induced side-effects, which are restricted to a non-NQO1-induced methemoglobinemia. These data warrant a clinical trial of IR + β-lapachone for the treatment of HNCs, where methemoglobinemia would not be an issue with far lower doses of β-lapachone needed for therapy.

10.5 Conclusions

Use of NQO1 bioactivatable drugs, for example β-lapachone (ARQ761), in combination with ionizing radiation (IR) is applicable to many of the most non-treatable forms of cancer (e.g., pancreatic and non-small cell lung cancers). Mechanistically, IR + β-lapachone synergistically kills cancer cells by combinational DNA lesion formation, with DNA base, SSB and DSB lesions leading to PARP hyperactivation, as PARPs bind all of these lesions with differential hypersensitivity. However, the dramatic down-regulation of glucose utilization via glycolysis (GAPDH) and TCA cycle suppression, along with tremendous NAD^+/ATP losses likely plays havoc on DSB repair. Studies on DSB repair activities in cells exposed to IR + β-lap, compared to control, low doses of β-lap and IR are ongoing. The IR + β-lap synergistic

responses in cells likely causes interesting DNA metabolic alterations in cells leading to atypical routes of attempted recovery. The massive production of H_2O_2 may also cause alterations in the tumor microvasculature that could have impacts beyond just what is happening within cancer tissue. Limited experiments demonstrate that hypoxia plays no role in cancer cell metabolism to β-lap alone, or contribute to IR + β-lap responses.

10.6 Future Directions

1. Improved delivery is key to enhancing therapy with NQO1 bioactivatable drugs. Although β-lapachone shows significant synergy with many drugs in NQO1-specific tumors, its poor water solubility (0.038 mg/mL) limits its systematic administration and clinical applications *in vivo* [175]. To improve solubility, Nasongkla et al., formulated β-lapachone with cyclodextrins (CD), finding that hydroxypropyl-β-cyclodextrin (HP-β-CD) can improve solubility to 16.0 mg/mL and the complex offers a major improvement in bioavailability [175].

However, non-specific distribution raises the risk of methemoglobinemia, and quick clearance significantly impedes drug effectiveness. To improve tumor specific distribution and exposure, biocompatible polymers that can adjust drug release and drug distribution become the first choice. Blanco et al. [176, 177] developed β-lapachone-containing poly(ethylene glycol)-co-poly(D,L-lactic acid) (PEG-PLA) polymer micelles for the treatment of NQO1-overexpressing tumors. Compared with β-lapachone-HP-β-CD, β-lapachone-PEG5k-PLA5k in mice bearing subcutaneous A549 lung tumors showed prolonged circulation ($t_{1/2}$, ~28 h) of the drug and increased accumulation in tumors. In addition, antitumor efficacy analyses in mice bearing subcutaneous A549 lung tumors and orthotopic Lewis lung carcinoma models showed significant tumor growth delay and increased survival relative to HP-β-CD [177]. Wang et al. [178] designed poly(D,L-lactide-co-glycolide) (PLGA) millirods through cyclodextrin complexation and Díaz-Rodríguez et al. [179] designed Pluronic F127® (PF127) gel that forms a complex of β-lap and cyclodextrin. Dong et al. [180] intratumorally delivered β-lap via polymer implants for prostate cancer therapy showing that inclusion complexes of β-lap-HP-β-CD in PLGA millirods released constant β-lap (~0.4 mg/kg/day) after a burst of 0.5 mg in 12 h and improved antitumor efficacy. Zhang et al. [181] encapsulated β-lap with paclitaxel into the PEG-PLA micelles with significantly (>10 fold) improved drug encapsulation efficiency, although only additive effects resulted. Ma et al. [182–184] developed an esterase-activatable prodrug of β-lap formulated into PEG-PLA micelles. They synthesized diester derivatives of β-lap and the resulting micelles yielded fairly high β-lap solubility (>7 mg/mL), physical stability, and an ability to reconstitute after lyophilization. Moreover, β-lapachone-dC_3 prodrug micelles significantly improved antitumor efficacy against orthotopic A549 mouse models versus β-lapachone-HP-β-CD and provide a promising strategy for NQO1-targeted therapy of lung cancer with improved safety [182].

Besides polymers, liposomes have also been used to formulate β-lapachone. Cavalcanti et al. [185, 186] encapsulated β-lapachone-HP-β-CD into liposomes and evaluated their antimicrobial activity. Release kinetics *in vitro* of β-lap from liposomes showed initial faster drug release (almost 40 % in the first 4 h). In sum, formulation of β-lap has significantly improved over previous studies. Nevertheless, to overcome blood toxicity seen in the clinic caused by non-specific distribution and fast release from capsules, tumor-specific accumulation, and sustainable effective release at the tumor site is an important future direction for β-lap delivery to improve the clinical efficacy and safety.

2. Metabolic consequences and anaplerotic recovery. The one phenotype that is consistent among all the NQO1 bioactivatable drugs is the drastic change in the metabolic state of the cell due to the depletion of NAD^+ and/or NADH. The cell is expected to rescue this phenotype through anaplerotic pathways that produce NAD^+/NADH in order to recover a metabolic steady state. There are multiple pathways in cancer cells that are upregulated, therefore targeting those highly expressed pathways, which also generate NAD^+/NADH, provide several targets that could synergize with β-lapachone treatment. Several of these pathways are highlighted in Fig. 10.3. These pathways can regenerate the energy necessary in both the cytosol and mitochondria for proper metabolism to occur while also providing substrates needed to feedback into glycolysis and/or TCA cycle (Fig. 10.3).

3. NQO1 in Cancer Stem Cells and reducing recurrence and metastatic spread with NQO1 directed therapies. Developing novel chemotherapeutics that target tumor associated genes that are overexpressed in tumor tissues versus associated normal tissues, such as the KRAS protein, EGFR, and hepatocyte growth factor receptor (cMet), has become a staple in the anticancer drug discovery field [187]. Over the past few years, there has been an increasing interest in genes that regulate oxidant stress, such as catalase, hemoxygenease-1, and glutathione transferase, as these genes have been found to be critical factors in tumor associated oxidative stress regulation [188]. As such, these genes may also be credible targets for the development of anticancer therapies [189]. Interestingly, NQO1 overexpression in tumors is partly due to its critical role in quelling oxidative stress associated with tumor development. Depleting NQO1 in lung cancer cells causes an increase in oxidative stress, inhibits anchorage independent growth, increases sensitization to anoikis, inhibits tumor invasion, blocks cell proliferation, and reduces the growth of tumors *in vivo* [168]. NQO1's role in tumorigenesis suggest that the regulation of oxidative stress plays a critical role in tumorigenesis and developing therapeutics targeting oxidative stress regulatory genes, such as NQO1, is critically needed as an anticancer strategy [189].

One common goal for the development of novel anticancer drugs is discovering therapeutics that can kill bulk tumors, as well as chemo-resistant tumor initiating/cancer stem cells that are purportedly the cause of tumor recurrence [190]. Interestingly, our findings on NQO1's role in tumorigenesis also revealed that depletion of tumor associated NQO1 levels decreased the population of $ALDH^{high}$ cancer cells, suggesting that the reason we observed far less tumor growth in our *in vivo* NQO1 knockdown studies was due to the depletion of the $ALDH^{high}$ cancer

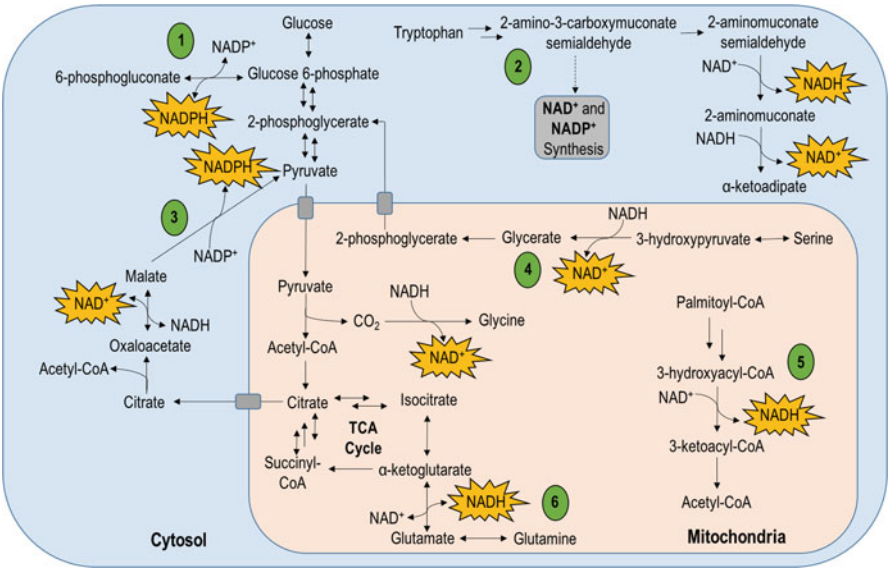

Fig. 10.3 Multiple pathways are upregulated in cancer cells, with some known for generating NAD^+/NADH. (*1*) **Pentose phosphate pathway (PPP)**: The enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase both produce NADPH from $NADP^+$; (*2*) **Tryptophan Biosynthesis**: The intermediate metabolite 2-amino-3-carboxymuconate semialdehyde is a precursor for de novo NAD^+ and $NADP^+$ biosynthesis while 2-aminomuconate semialdehyde dehydrogenase generates NADH from NAD^+ and 2-aminomuconate reductase produces NAD^+ from NADH; (*3*) **Pyruvate-malate cycling**: The cytosolic isozyme of malate dehydrogenase is responsible for producing NAD^+ from NADH while malic enzyme further generates NADPH from $NADP^+$; (*4*) **Serine/glycine metabolism**: The enzyme 3-hydroxypyruvate dehydrogenase can convert NADH to NAD^+ from mitochondrial serine; (*5*) **β-oxidation**: The breakdown of fatty acids within the mitochondria afford an NADH molecule from NAD^+ through 3-hydroxyacyl dehydrogenase; (*6*) **Glutamine/glutamate metabolism**: Uptake of glutamine into the mitochondria can convert to glutamate which will then produce NADH from NAD^+ through glutamate dehydrogenase

stem cell population within the bulk tumor [168]. Thus, further studies on NQO1's role in tumorigenesis may lead to the development of novel therapeutics targeting NQO1 expression levels directly in patient tumors.

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Chapter 11
DNA Repair Pathways as a Potential Target for Radiosensitization

Mahmoud Toulany

Abstract Nearly two-thirds of all cancer patients benefit from radiotherapy in their treatment regimen. Ionizing radiation induces a variety of lesions in DNA, of which double-strand breaks are considered the most lethal. Following irradiation, a multicomponent of signal transduction network referred to as DNA damage response (DDR) is activated to sense DNA damages, to initiate cell cycle checkpoints and to provoke repair in the nucleus. Many malignancies overexpress DDR proteins, which orchestrate cell cycle arrest followed by efficient DNA repair leading to radiotherapy resistance. DDR is also stimulated by cytoplasmic signaling pathways. Therefore, those components of DDR with enzymatic activity can be targeted to selectively inhibit repair of radiation-induced DNA damage in tumor cells. Such a strategy may provide therapeutic options to improve radiotherapy outcome. In this chapter, the current status of such components of classical DNA repair pathways will be discussed with regard to radiosensitization. Some of the mechanisms by which well-described cytoplasmic pro-survival receptor tyrosine kinase signals such as epidermal growth factor receptor cascades facilitate DNA repair after irradiation will also be summarized. The state of the art in terms of targeting these pathways will be discussed in the search for the potential approaches to improve radiotherapy outcome.

Keywords Ionizing radiation • Signal transduction • Molecular targeting • DNA repair • Radiosensitization

11.1 Introduction

Although concurrent chemoradiotherapy has improved treatment outcome compared with radiotherapy alone in multiple solid tumors, the progression-free survival rate in many cancers such as glioblastomas, lung cancers and pancreatic cancers is still very low. This indicates the necessity of combining novel approaches

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with conventional therapies to improve treatment outcome. DNA damage is the major cause of cell death induced by radiotherapy. Ionizing radiation-induced DNA damage can generally be classified into two groups, single-strand breaks (SSBs) and double-strand breaks (DSBs). Base damage and DSBs are the major types of ionizing radiation-induced DNA damage during radiotherapy [1]. DSBs are the main cause of radiotherapy-induced cell death.

Upon recognition of damage, a complex network of signal transduction referred to as DNA damage response (DDR) is activated to repair lesions and by that to protect cells against irradiation, induced cell death. A variety of post-translational modifications such as phosphorylation, methylation and acetylation is involved in the activation of DDR signaling [2]. Cells recognize damage by activating sensors, amplifying the signals through transducers and, finally, inducing the appropriate cellular response by effector molecules [3]. In order to process DNA repair following irradiation, cells are transiently arrested by checkpoint activity. Depending on the type of DNA damage and cell cycle in which damage occurs, cells engage specific repair mechanisms such as nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) pathways. Radiation-induced DSBs are repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ) [1].

Cancer cells are often defective in one of the DNA repair pathways [4]. Tumor-specific defects in the DDR provide new options for cancer-specific therapy. This is well evidenced by the defect in the HR repair pathway caused by mutations in BRCA1 (breast cancer 1) and BRCA2 (breast cancer 2) genes, contributors to hereditary breast cancer and ovarian cancer. BRCA1/2 defective tumor cells are sensitive to Poly(ADP-ribose) polymerase (PARP) inhibitors. Another reliable example is mutation in the TP53 gene. TP53 mutation is the most common mutation in human cancers and promotes tumor growth and metastasis [5]. Tumor cells with P53 mutation are defective in the G1/S checkpoint. Thus, targeting the G2/M checkpoint as a necessary step in DNA repair can be a very effective therapeutic approach for sensitizing P53 mutant cells to DNA damage. Since higher doses of radiotherapy are often limited by normal-tissue toxicity, tumor-specific targeting of DNA repair based on the principles of synthetic lethality [6] might be an effective therapeutic approach for killing tumor cells and minimizing the normal-tissue toxicity, leading to enhanced therapeutic ratio [7]. Besides mutations in classical DDR pathways, oncogenic mutations can result in addiction of cancer cells to the specific survival pathway through regulating DNA repair. For example, RAS is mutated in about 30 % of human cancers leading to the constitutive activation of the gene product and the activation of downstream pathways. Mutated RAS modulates Chk1 activity downstream of ATR and enhances DNA repair capacity through BER and NHEJ repair pathways in tumor cells, leading to radioresistance [8–10]. Thus, enhanced DNA repair capacity in RAS-mutated tumor cells [10, 11] may foster tumor-specific effectiveness of radiotherapy in combination with targeting strategies against checkpoints and the underlying repair pathways upregulated in RAS-mutated tumors.

It is well accepted that expression and post-translational modification of many of the components of classical DDR signaling pathways are regulated by cytoplasmic signaling cascades as well. Membrane-bound receptor tyrosine kinases (RTKs) are the major initiators of signaling amplification cascades from the plasma membrane

induced by ionizing radiation. These receptors consist of 58 members that are distributed in 20 subfamilies. More than half of these receptors have been frequently found to be mutated or overexpressed in human malignancies [12]. Epidermal growth factor receptor (EGFR) and its family members are well-accepted models for investigating the functions and regulations of other membrane-bound RTKs [13]. EGFR, in addition to its canonical role in regulating cytoplasmic signaling cascades [14], regulates histone modification, gene expression, DNA replication and DNA damage repair [13, 15]. Thus, based on the alterations of RTKs in human cancer and their function in DNA damage repair, many of these receptors have been proposed as potential targets in oncology. In this context, EGFR targeting has been approved in combination with radiotherapy for treatment of squamous cell carcinoma of the head and neck [16, 17]. Together, direct or indirect targeting of DNA repair seems to be a promising strategy to improve radiotherapy outcome. However, it is essential to develop functional assays for selecting those patients who might benefit from such treatment strategies. Below, the latest development in DNA repair targeting strategies in combination with radiotherapy will be discussed in the light of preclinical mechanistic studies and current clinical trials.

11.2 Ionizing Radiation-Induced Activation of DNA Damage Responses

Exposure to ionizing radiation induces a variety of DNA damage including SSBs and DSBs as well as DNA-DNA and DNA-protein cross-links. In the case of DSBs, after damage induction the lesions are recognized by sensor proteins including MRE11-Rad50-NBS1 (MRN) complex [18] and K70/80 as the regulatory subunit of DNA-dependent protein kinase (DNA-PK). This is followed by the activation of the major phosphoinositide 3-kinase (PI3K) related protein kinases, i.e. ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-PK catalytic subunit (DNA-PKcs) mediating cell cycle arrest, chromatin modification and DNA repair [19]. In response to DNA damage, ATM phosphorylates checkpoint kinase 2 (Chk2) at Thr68 leading to the activation and stabilization of p53. Cellular p53 regulates expression of cell cycle regulators such as p21 that through interaction with cyclin-dependent kinase (CDK) complex leads to G1 arrest. Furthermore, ATM regulates the G2 checkpoint by direct phosphorylation of Chk2 as well as ATR-mediated phosphorylation of Chk1. In coordination with cell cycle checkpoint activity, depending on the type of damage, cells undertake a specific repair pathway to remove and tolerate DNA damages. Interruptions of the DNA sugar phosphate backbone are referred as SSBs, which are localized in only one of the two strands of double helix and the intact strand is used as a template for repair. SSBs are repaired by mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER) [20]. DSBs are repaired by homologous recombination (HR) and non-homologous end-joining (NHEJ). Details on the repair pathways are found in review papers [21–24]. Because of the targetability of the components of BER and DSBs repair, these pathways are briefly described below.

Base damages are the most frequently occurring radiation-induced DNA damage. BER is responsible for repairing endogenous base damage as well as damages induced by environmental agents such as ionizing radiation through reactive oxygen species production. BER initiates base excision by cleaving the N-glycosyl bond between the base and the sugar using DNA glycosylases, which remove damaged bases without cleaving DNA backbone, resulting in apurinic/apyrimidinic (AP) sites [25]. After making a nick by AP endonuclease (APE) as well as polynucleotide kinase (PNK), DNA polymerase beta (polβ) inserts a single nucleotide in the repair gap in the short patch pathway of BER, simultaneously removing the 5′-deoxyribose phosphate left behind by the APE [26–28]. The X-ray cross-complementing 1 (XRCC1)-DNA ligase IIIα complex then seals the nick and complets the repair. An alternative long patch pathway, resulting in the incorporation of 2–10 nucleotides, involves polβ and/or polδ/ε in gap synthesis [27]. XRCC1 functions as a scaffold protein for binding to PARP and many other proteins involved in BER and SSB repair.

DSBs are considered as the most deleterious damages, causing chromosomal aberrations and cell death induced by radiation. Generally, DNA lesions other than DSBs are easily repaired. DSBs are either directly caused by DNA-damaging agents or are a consequence of non-repaired SSBs. Following cell cycle arrest, radiation-induced DSBs in cells of higher eukaryotes are repaired by the classical or canonical DNA-PKcs-dependent NHEJ (C-NHEJ) repair pathway. This pathway utilizes a molecular scaffold Ku70/80 heterodimer as a DSBs sensor, DNA-PKcs, Artemis endonuclease, XRCC4, XRCC4-like factor (XLF; also called Cernunnos or NHEJ1) and DNA ligase IV. Recently, PAXX as a paralog of XRCC4 and XLF was described to function with XRCC4 and XLF as an essential component of DSBs repair through C-NHEJ [29, 30]. Expression of the proteins regulating C-NHEJ is invariant throughout the cell cycle phases. During NHEJ repair, Ku70/Ku80 heterodimer as the regulatory subunit of DNA-PK binds to DSBs ends and recruits other necessary proteins. Ku70/80 through binding to serine/threonine kinase DNA-PKcs and other proteins such as X family DNA polymerases forms a multi-protein complex, which promotes synapsis of the broken DNA ends. Cells lacking the functional C-NHEJ pathway use a DNA-PKcs-independent repair pathway as an alternative backup NHEJ (B-NHEJ) pathway. Ligase III, PARP-1, XRCC1, MRE11, histone H1 and CtIP are the major components in the B-NHEJ repair pathway [21, 24].

HR is a critical pathway for high-fidelity repair of DSBs. Large variety of proteins including replication protein A (RPA), BRCA1/2, RAD51, RAD51 paralogs, and p53 are involved in this repair pathway [31]. HR repair proteins are expressed in the S and G2/M, but not in the G1 phase. Binding of MRN complex to DNA ends initiates HR. Resection of double-stranded ends is the key step of HR, which is initiated through recruitment of CtIP by the MRN complex. The resulting ssDNA is rapidly coated by RPA. The ssDNA-RPA recruits ATR interacting protein (ATRIP) leading to the activation of ATR. RPA is then replaced by Rad51 in a BRCA1/2-dependent manner to perform the recombinase reaction using the intact homologous DNA strand. Details of HR repair can be found in other review papers [32, 33].

The choice between NHEJ or HR depends on the cell cycle phase, complexity of DNA damage and chromatin structure [34]. NHEJ functions in all phases of the cell cycle with a dominant role in the G1 phase while HR is the predominant pathway for repairing DSBs in the S and G2 phases. Both pathways participate in the fast components of DSBs repair occurring within the first 2–3 h post-irradiation with half times of 15–30 min followed by the slow component that functions up to 24 h after irradiation [22]. NHEJ (C-NHEJ and B-NHEJ) are error-prone in terms of the simple rejoining of two ends of DNA while HR through copying the missing genetic information from the undamaged sister chromatid is a highly accurate repair process. HR is crucial for the repair of complex DSBs such as two-ended-DSBs, DSBs in proximity to additional DNA damage or DSBs within heterochromatin [35]. Cancer cells often present dysregulated DNA damage repair and signaling to cell cycle checkpoints, which may be compensated by another repair pathway. This cancer-specific phenomenon can be used as a synthetic lethal approach to selectively kill cancer cells without undesired effect in normal cells.

11.3 Targeting DNA Repair for Radiosensitization

Classical DNA damage repair pathways function to prevent genomic instability and attenuate the efficacy of radiotherapy after irradiation. Accumulated evidence supports the fact that a link exists between radiation-induced membrane signaling and DNA damage repair leading to post-irradiation cell survival. Thus, in addition to targeting components of classical DNA repair pathways, cytoplasmic signaling pathways can be targeted to interfere with DNA repair in tumor cells and increase radiosensitization. Altogether, the potential approaches for radiosensitization through interference with DDR will be discussed within the following four topics:

1. hampering cell-cycle arrest
2. targeting components of single strand break repair pathways
3. direct targeting of NHEJ and HR repair pathways
4. targeting cytoplasmic signaling pathways that indirectly regulate DSBs repair pathways

11.3.1 Hampering Cell Cycle Arrest

Checkpoint dysfunction is a common phenomenon acquired during tumorigenesis. DNA-damaging agents induce a variety of SSBs and DSBs. DSBs are detected by the MRN complex. The MRN complex bound to DNA ends initiates activation of Chk2 through phosphorylation at Thr-68 by ATM leading to G1 arrest as a consequence of p53-dependent regulating expression of CDK inhibitor p21 [36, 37]. Likewise, as shown in Fig. 11.1, Chk2 activity phosphorylates CDC25A

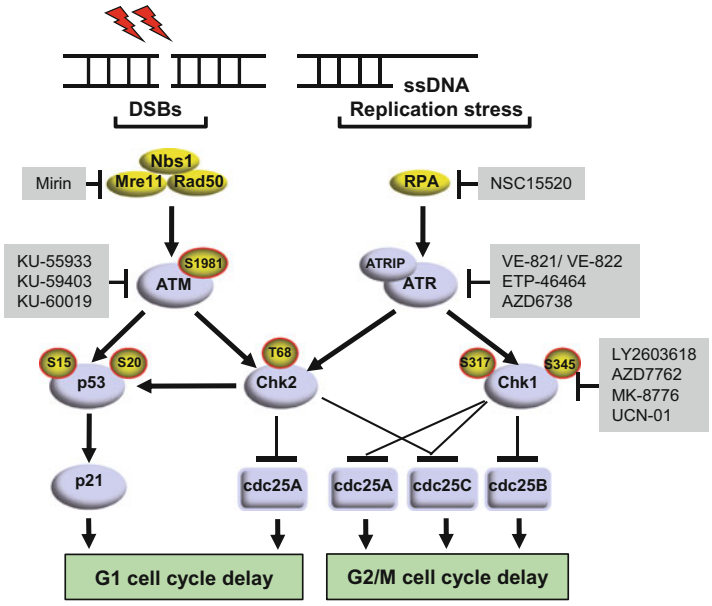

Fig. 11.1 Potential druggable target molecules and the related inhibitors for blocking DNA repair by hampering cell-cycle arrest after exposure to ionizing radiation. Please see text for detailed explanation of the pathways

phosphatase that prevents cyclin E(A)/CDK2, leading to G1 arrest [38]. Single strands of DNA that are exposed at stalled replication forks or after double-strand resection are sensed by RPA. RPA-coated SSBs stimulate localizing ATR-ATR interacting protein (ATRIP) complex to DNA. They also facilitate the recognition of ATR-ATRIP substrates for phosphorylation of checkpoint kinase 1 (Chk1) and subsequent steps initiated by phosphorylation of CDC25A, CDC25AB and CDC25c phosphatases leading to inhibited transition of cells to M-phase (G2/M arrest) [38, 39]. Cell cycle arrest is mediated by many proteins and enzymes. However, the current status of targeting MRN, ATM, ATR, Chk1 and RPA will be discussed in combination with radiotherapy.

Mutations of members of the MRN protein complex lead to hypersensitivity to DNA-damaging agents, indicating the MRN complex is a promising target for radiosensitization. Mirin as a small-molecule inhibitor has been alleged to interfere with the function of the MRN complex in DNA repair. Mirin prevents activation of ATM as downstream of MRN without affecting ATM kinase activity. Mirin also inhibits Mre11-associated exonuclease activity [40, 41]. Since MRE11 activity is required for the survival of BRCA2 mutant cells, targeting of MRE11 by mirin could be an efficient approch to enhance radiation-induced cell death through synthetic lethality. Additionally, degradation of the MRN complex with oncolytic adenovirus has been shown to be a promising strategy to enhance radiosensitivity [42].

Cells obtained from patients with ataxia-telangiectasia display DNA damage checkpoint defects with increased chromosomal instability and hypersensitivity to ionizing radiation. These observations highlight ATM as a potential target to enhance radiosensitivity of tumor cells. The role of ATM in checkpoint activity and repair of DNA damage was initially demonstrated with non-specific inhibitors (caffeine and wortmannin) and the effect was approved with genetic approaches. Currently, KU-55933, KU-60019 and KU-59403 are the most specific ATM inhibitors with promising data obtained from preclinical *in vitro* and *in vivo* studies [43]. In terms of inhibition of radiation-induced phosphorylation of ATM, KU60019 as an improved analog of KU-55933 is 10 times more effective [44]. KU60019 has potential radiosensitization *in vivo*, as shown in the orthotopic model of gliomas [45]. Details of ATM as a potential target for radiosensitization are provided in a separate chapter in this book.

In parallel with the initiation of HR following CtIP-mediated DSBs resection, exposed ssDNA is coated by RPA that is recognized by the activation of the ATR-ATRIP complex leading to the activation of Chk1 (Fig. 11.1). Because of its critical role in Chk1 signaling, ATR targeting has been proposed as a potential radiosensitizing approach, demonstrated with ATP competitive inhibitors VE-821, ETP-46464 and AZD6738 in solid tumor cells [46, 47] and in leukemic cells *in vitro* [48]. VE-822, a close analog of VE-821 with a marked increase in potency against ATR and improved pharmacokinetic properties, inhibits Chk1 phosphorylation and presents radiosensitization of pancreatic cancer cells *in vitro* and in xenograft models in vivo without toxicities affecting normal cells and tissues [49]. Given the fact that more than 50% of human tumors are defective in p53 tumor suppressor function and ATM/Chk1 activity, ATR inhibition might be an effective approach to induce radiosensitization through synthetic lethality. In tumor cells addicted to oncogenes such as K-RAS or Myc, activation of oncogenes leads to increased replication stress that is detected by histone H2AX phosphorylation at Ser-139. In such cancer cells, activation of ATR-Chk1-WEE1 protects cells with functional p53 from replicative catastrophe, a condition that leads to the hypersensitivity of cells to ATR targeting.

Phosphorylation of Chk1 at Ser-317 and Ser-345 by ATR leads to Chk1 activity. Exposure to ionizing radiation induces Chk2 activation through phosphorylation at Thr-68 as well as at Ser-19, Ser-33, and Ser-35 downstream of ATM and NBS1 [50]. Many cancers such as triple-negative breast cancer and colorectal cancer present Chk1 upregulation. Phosphorylation of Chk1 at Ser-317 is a predictive biomarker of radiotherapy resistance and early local recurrence shown in breast cancer [51]. It is believed that Chk1, but not Chk2 is the major drug target checkpoint kinase [52]. Additionally, since more than 50% of human cancers present p53 mutation, similar to ATR targeting, interfering with G2 arrest through targeting Chk1 activity might be one of the mechanisms to achieve enhanced radiotoxicity through synthetic lethality, with a minimum effect on normal tissue. To this end, a number of small-molecule inhibitors have been developed against Chk1. Pre-clinical *in vitro* as well as *in vivo* data demonstrate that abrogation of G2-M arrest by targeting ChK1 or its

substrate WEE1 induces mitotic catastrophe [53], leading to radiosensitization. Most studies with Chk1 inhibitors have been conducted using staurosporine analog UCN-01 (7-hydoxistaurosporine). Graves and colleagues demonstrated for the first time that Chk1 is the target of UCN-01 [54]. Later, a potential radiosensitizing effect of UCN-01 was shown in preclinical models for several cancer cell lines, e.g. HNSCC cancer stem-like cells [55], breast carcinoma cell lines [56], osteosarcoma and colorectal carcinoma cell lines [57]. Radiosensitization has also been achieved through abrogation of the G2 checkpoint by other inhibitors such as AZ7762 *in vitro* and inhibition of HR repair of DSBs [58, 59].

So far, UCN-01 as monotherapy has failed to show a high level of clinical activity, most likely through the proposed compensatory activation of ATM and ERK1/2 pathways [60]. Interestingly, the selective Chk1 inhibitor MK8776 presented chemoradiosensitization in HR-proficient pancreatic cancer cells and corresponding tumor xenografts [61]. The study by Engelke et al. [61] suggested that MK8776 in combination with gemcitabine and radiation is an effective approach in locally advanced pancreatic cancer. Since approximately 90% of pancreatic cancers present K-RAS mutation, constitutive ERK1/2 activation in K-RAS-mutated tumors might prevent its further activation of ERK1/2 through Chk1 inhibition and have a positive impact on radiosensitization. The radiosensitizing effect of Chk1 inhibitors depends on p53 status. TP53 mutation has a positive impact on the radiosensitizing effect of chk1 inhibitors as shown in tumor cells from lung, pancreas, breast and colon cancers *in vitro* as well as *in vivo* [58, 62, 63] without untoward toxicity in normal cells when administered alone or in combination with radiation [62]. Chk1 inhibition in combination with irradiation has been shown to be a potential approach to improve local tumor control and to prolong median survival of animals bearing lung cancer brain metastasis xenografts [64].

Many successful phase I and phase II clinical trials using a panel of Chk1 inhibitors with accepted levels of toxicity have been performed (Table 11.1). Surprisingly, UCN-01 presented no or limited antitumor activity after monotherapy [65] or in combination with other chemotherapy agents such as irinotecan [66] and topotecan [67]. According to the clinical trials presented in Table 11.1, checkpoint inhibitors in combination with chemotherapy have poor clinical efficacies, which might be owed to the compensatory activation of alternative pathways such as ATM and ERK1/2 [68].

Although the radiosensitizing effect of Chk1 inhibitors has been well documented in the preclinical models, no clinical data exist so far. However, given the well-described radiosensitizing effect of Chk1 inhibition by different inhibitors tested in several preclinical *in vivo* studies, Chk1 targeting seems to be an effective strategy for optimizing radiotherapy outcome.

Following DNA damage, replication stress is characterized by accumulation of single-strand DNA bound by RPA. Thereafter, RPA is replaced by RAD51 recombinase for initiating HR repair. Accumulated RPA also triggers activation of ATR leading to Chk1 activation and G2/M arrest. RPA needs to be phosphorylated by ATR and DNA-PKcs on RPA32 subunit [69, 70]. Thus, targeting the DNA-binding

Table 11.1 Checkpoint kinase inhibitors in phase I/II clinical trials

Drug	Combination/Trial	Cancer type/ Reference	Toxicity/Major findings
LY2603618	Pemetrexed/Phase I	Advanced solid tumors [224]	Efficacious dose ≥105 mg/m^2 with acceptable safety
	Pemetrexed and cisplatin/ Phase I	Advanced solid tumors [225]	Two NSCLC patients with partial response and 8 patients had stable disease. Recommended phase II: 275 mg/m^2
AZD7762	Gemcitabine/Phase I	Japanese patients with advanced solid tumors [226]	No objective responses
			5/20 (all lung cancer) had stable disease
			MTD: 25 mg/m^2 in combination with 1000 mg/m^2 gemcitabine
	Gemcitabine/Phase I	US patients with advanced solid tumors [227]	2/20 (Non-small cell lung cancer) had objective response
			MTD: 30 mg/m^2 in combination with 1000 mg/m^2 gemcitabine
			Unpredictable cardiac toxicity
MK-8776	Gemcitabine/Phase I	Advanced solid tumors [228]	Bioactivity was assessed by γ-H2AX ex vivo assay
			2/30 partial response
			13/30 stable disease
			MK-8776 was well tolerated
			Recommended phase II (200 mg/m^2) plus gemcitabine (1000 mg/m^2) on days 1 and 8 of a 21-day cycle
UCN-01	Cisplatin/Phase I	Advanced solid tumors [229]	Posttreatment tumor biopsies: UCN-01 active against Chk1
			Potentiating response to Cisplatin
			Cisplatin, 30 mg/m^2 dose limited
	Irinotecan/Phase II	TNBC [66]	Limited activity
	Monotherapy/Phase II	Metastatic melanoma [65]	Insufficient activity
	Topotecan/Phase II	Recurrent ovarian cancer [67]	No significant activity

activity of RPA not only interferes with DNA replication, but it also causes cell cycle arrest and impairs repair of DSBs through HR. Since cancer cells with activated oncogenes such as RAS and Myc generate increased levels of replication stress [71], targeting RPA as a multi-potent protein in combination with radiotherapy might be an opportunity to preferentially kill cancer cells. To this end, small-molecule RPA protein interaction inhibitors have been identified and proof-of-concept preclinical studies have been performed *in vitro* and *in vivo* [72]. More studies are necessary to prove the efficacy of RPA targeting as a molecular targeting strategy in combination with radiotherapy. Potential druggable target molecules and the related inhibitors to block DNA repair by hampering cell-cycle arrest after exposure to ionizing radiation have been summarized in Fig. 11.1.

11.3.2 Targeting SSBs Repair Pathway

BER is the major repair pathway for radiation-induced SSBs. AP endonuclease 1 (APE1), polynucleotide kinase (PNK) and DNA polymerase beta (polβ) are the important components in this pathway, and their role has been investigated in cellular radiation response [73, 74]. APE endonuclease activity promotes resistance to radiation plus chemotherapy in medulloblastomas [75]. APE1 is elevated in human cancers such as gliomas [76] and in pediatric ependymomas [77]. Overexpression of APE1 results in resistance to the alkylating agent temozolomide [78] as well as to radiotherapy [79] while downregulation of APE1 leads to tumor cell radiosensitivity in preclinical models *in vitro* and *in vivo* [80–82]. Along with studies of the role of APE1 in radiation response using knockdown strategies *in vitro* and *in vivo* [83], a study using the relatively specific APE1 inhibitors lucanthone and CRT004876 in combination with irradiation has been performed [79]. Data from this study and studies using genetic models demonstrate that APE1 may be a useful target in combination with irradiation to improve radiation response. Since APE1 inhibitors are synthetically lethal in HR-deficient cells [84], APE1 targeting might be a specific approach to induce radiosensitization, especially, in tumor cells with mutation in the HR pathway. TRC102, a small-molecule inhibitor of DNA repair that binds to AP sites has been tested in phase I trials in patients with advanced solid tumors [85]. Combination of TRC102 with pemetrexed, that induces BER, led to stable disease in more than 50 % of patients under study [85], indicating a potential benefit of this inhibitor in combination with other therapeutic approaches inducing base damages.

Ionizing radiation-induced 5′-hydroxyl and/or 3′-phosphate termini in SSBs must be converted to 5′-phosphate and 3′-hydroxyl termini in order to allow the function of DNA polymerases and ligases in strand rejoining. Polynucleotide kinase (PNK) with 5′-kinase activity and a 3′-phosphatase activity is the key enzyme involved in this end-processing [86]. The structure and function of PNK in SSBs repair offers the possibility to design specific inhibitors. To this end, a novel PNK inhibitor A12B4c3 as a potent inhibitor of PNK phosphatase activity has been developed and its specificity as well as the positive effect on post-irradiation cell

survival has been investigated in lung cancer and breast cancer cell lines [87]. A12B4c3 also sensitizes acute myeloid leukemia to radioimmunotherapy [88]. A summary of above-described potential targets to interfere DNA repair through BER pathway after irradiation has been presented in Fig. 11.2.

Among the components of the BER pathway, Polβ is one of the most common altered genes in human cancers such as gastric [89, 90], colorectal [91], uterine, ovarian and prostate cancers [89]. Patients with high Polβ expression have poor prognosis [92]. Inversely, downregulation of Polβ expression by siRNA resulted in an increased sensitivity to cisplatin [89, 92]. Vens *et al.* showed that expression of the dominant negative form of polβ radiosensitizes human carcinoma cells [74]. Human cancers commonly express aberrant Polβ, which leads to the inhibition of BER. Under this condition, cells use HR to repair DNA damage after irradiation [93]. Thus, downregulation or inhibition of base excision repair may constitute a benefit for patients deficient in HR pathways through synthetic lethality.

Poly (ADP-ribose) polymerase (PARP) regulates different cellular functions including gene regulation, transcription and replication. Among the different PARP family members, PARP1 and PARP2 are known to be involved in DNA repair and to be activated by ionizing radiation. The enzyme PARP1 is an abundant nuclear protein with a high affinity for SSBs as well as DSBs [94]. PARP1 in the early response to DNA damage modulates ATM signaling [95]. Although PARP activity is mainly involved in BER, varieties of genes in the other repair pathways, i.e. HR, NER and MMR, present a PARP1 binding motif [94].

PARP1 binds to oxidative DNA damages and produces poly-ADP ribose, followed by the attraction of repair proteins to the DNA damage site [96]. Inhibition of PARP1 leads to accumulation of SSBs, which if not repaired are converted to DSBs during DNA replication. Thus, PARP1 inhibition can be used as a synthetic lethal approach to specifically kill cancer cells that are dependent on a compensatory DNA repair such as BRCA1/2 mutated HR deficient ovarian cancers [97]. This concept can also be applied in BRCA1 proficient tumors by BRCA1 knockdown or after exposure to ionizing radiation that mediates cytoplasmic accumulation of BRCA1 [98]. Likewise, targeting the epidermal growth factor receptor also leads to nuclear depletion of BRCA1 [98]. According to this concept PARP1 inhibition in combination with ionizing radiation generates persistent DNA double-strand breaks leading to radiosensitization *in vitro* [99–101] and *in vivo* [102]. This approach has been shown to be applicable in the context of radiotherapy with low and high linear-energy-transfer irradiation [103]. The majority of PARP1 inhibitors have been studied in phase I/II trials [104]. Phase I trials using the PARP inhibitor veliparib (ABT-888) in combination with radiotherapy as well as phase II trials of the inhibitors veliparib, olaparib (AZD2281, Ku-0059436), iniparib (BSI-201) and rucaparib (AG-014699, PF-01367338) alone or in combination with chemotherapy are summarized in Table 11.2. The safety and efficacy results obtained from these trials and, especially, from the trials combining inhibitor treatment with radiotherapy are encouraging [105, 106]. They provide a rationale to investigate the potential benefit of these drugs in combination with radiotherapy in terms of improving outcome in further multicenter trials.

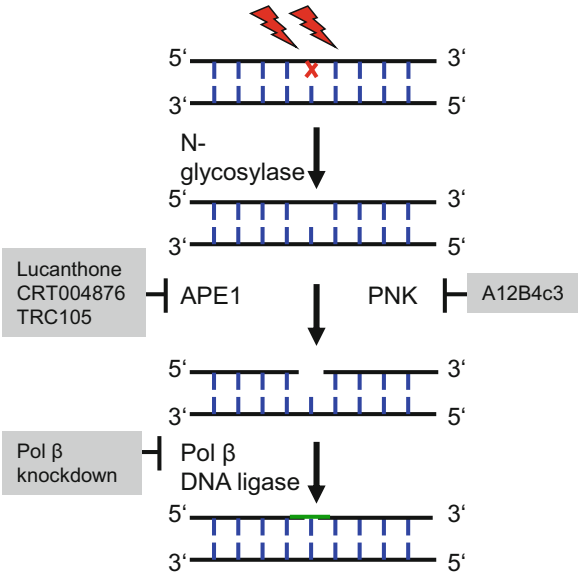

Fig. 11.2 Potential targets to interfere DNA repair through BER pathway after irradiation. Please see text for detailed information

11.3.3 Targeting DSBs Repair Pathways

DSBs are the most deleterious damage induced by radiotherapy. Novel strategies have become subjects of extensive research to enhance the effect of radiotherapy in tumor cells by inhibition of DSBs repair and to limit its cytotoxic effect in normal tissue. Several components in NHEJ and HR repair of DSBs can be considered as key targets for radiosensitization. In principle, targeting HR and NHEJ repair pathways is either based on application of inhibitors of specific enzymes known to be involved in DNA repair (direct targeting) or based on the inhibition of a cellular phenotype that is dependent on HR or NHEJ (indirect targeting).

11.3.3.1 Targeting HR

Because of the accessibility of undamaged DNA molecules with a homologous sequence, HR functions during the late S and G2 phases of the cell cycle. Therefore, this is a significant repair pathway in dividing cancer cells while non-dividing normal cells are in G0 or G1 and cannot undergo HR after DNA damage [107]. Additionally, the HR deficiency that is a common phenomenon in many cancers, e.g. breast cancers with BRCA1/2 mutations, leads to higher sensitivity to the inhibitors of other DNA repair pathways such as inhibition of BER and SSBs repair by PARP1 inhibitors [108] through the concept of synthetic lethality. More than half of human tumors are p53 mutated that causes a lack of G1 arrest after irradiation. In such tumors inhibition of HR leads to tumor cells specific synthetic lethality while

Table 11.2 Clinical trial PARP1 inhibitors in patients with solid tumors

Drug	Combination/Phase	Cancer type/Reference	Major findings
Veliparib	w/WB radiotherapy/phase I	NSCLC and breast cancer with brain metastasis [105]	Encouraging safety and prolonged median survival
	w/LDFWA radiotherapy/ phase I	AST and peritoneal carcinomatosis [106]	Prolonged disease stability for some patients particularly in the OFCS
	w/o/phase II	Recurrent ovarian cancer (germline BRCA1 or BRCA2 mutation) [230]	Single agent efficacy and tolerability
	Topotecan/phase I–II	Persistent or recurrent carcinoma of the uterine cervix [231]	A combination with minimal activity
			Low PARP1 expression associated with longer PFS
Olaparib	w/o/phase II	Castration-resistant prostate cancer [232]	High response rate in DNA repair-deficient patients
	Standard chemotherapy/ phase II	Ewing's sarcoma [233]	No significant responses or durable disease control
	w/o/phase II	Platinum-sensitive recurrent SOC [97]	SOC with BRCA mutation have greatest benefit
	w/o/phase II	BRCA1/2 wildtype poorly differentiated OC or TNBC [234]	Objective response in OC No response in TNBC
	w/o/phase II	BRCA1 or BRCA2 mutations and recurrent OC [235] and advanced breast cancer [236]	Positive proof of concept of the efficacy and tolerability
Iniparib	GCis/phase II	Stage IV NSCLC [237]	No improved ORR over GC alone
	GCar/phase II	Metastatic TNBC [238]	Improved PFS and OS
Rucaparib	Temozolomide/phase II	Metastatic melanoma [239]	Safe combination with longer PFS

WB whole brain, *LDFWA* low-dose fractionated whole abdominal, *AST* advanced solid tumors, *OFCS* ovarian and fallopian cancer subpopulation, *PFS* progression-free survival, *ORR* overall response rate, *GCis* gemcitabine/cisplatin, *SOC* serous ovarian cancer, *OC* ovarian carcinoma, *TNBC* triple-negative breast cancer, *GCar* gemcitabine and carboplatin, *OS* overall survival

the majority of normal cells arrest in the G1 phase to repair DNA damage by alternative pathways such as NHEJ repair of DSBs. Moreover, HR can be inhibited by targeting molecules such as MRN complex, ATM, ATR and Chk1 that are involved in the activation of the components of this pathway as discussed in Sect. 11.3.1.

Direct Targeting of HR

The inhibition of proteins that directly catalyze HR seems to be a more effective approach to inhibiting DSBs repair. RAD51 is a key element of the HR repair pathway, which is overexpressed in many cancers [96], in association with increased rate of HR. Thus, RAD51 targeting might be an efficient way to interfere with genomic stability and increase radiation response. As summarized by Ward et al. [96] several small-molecule inhibitors of RAD51 including B02 [109], halenaquinone [110], RI-1 [111] and IBR2 [112] have been tested for inhibition of RAD51 activity. Proof-of-principle preclinical studies have been performed using Rad51 inhibitors and the data are convincing [113, 114]. However, further proof-of-concept clinical studies are necessary to show the benefit of these inhibitors in combination with radiotherapy.

Indirect Targeting of HR

There are signaling pathways that indirectly regulate HR activity as reviewed by Ward et al. [96]. For example, accumulation of Rad51 to DSBs is stimulated by PI3K activity [115]. Moreover, PI3K inhibition leads to downregulation of BRCA1/2 and consequently to inhibition of HR repair. The cABL tyrosine kinase inhibitor imatinib mesylate (Gleevec) in combination with irradiation leads to radiosensitization and tumor growth delay through reduced expression of RAD51 and impaired HR [116]. In several phase I/II trials application of imatinib was shown to be safe and tolerable, and demonstrated evidence of anti-tumor activity [117, 118].

Histone deacetylase (HDAC) activity together with histone acetyl transferases controls critical functions of the cell such as gene expression and DNA repair through regulation of the acetylation of histone proteins and chromatin remodeling. HDAC targeting is one of the promising strategies for cancer treatment. HDAC inhibitors have entered clinical trials for both hematologic malignancies and solid tumors. Among many of the inhibitors investigated, vorinostat (SAHA), panobinostat (LBH-589) and romidepsin (FK228) have been approved by the US Food and Drug Administration (FDA) for treatment of different cancers [119]. Suppressing transcription of HR-associated genes such as RAD51 and BRCA1 is the major mechanism of radiosensitization by HDAC inhibitors [120, 121]. These reports and existing data from early-phase clinical studies suggest that indirect targeting of HR using HDAC inhibitors might be a beneficial approach in combination with radiotherapy [122, 123].

11.3.3.2 Targeting NHEJ

Activation of DNA-PKcs is the key step for DSBs repair by NHEJ. Likewise, the expression level of DNA-PKcs correlates with therapy resistance and overall survival. Therefore, direct targeting of DNA-PKcs has been thought to be an effective

strategy for sensitizing cells to radiotherapy. Several approaches have been proposed for blocking DNA-PKcs activity. These approaches include the DNA-PKcs inhibitor, small inhibitory peptides that can target autophosphorylation of DNA-PKcs [124] and single-chain variable antibody fragments specific to DNA-PKcs [125]. A number of potent DNA-PKcs inhibitors, e.g. notably NU7026 [126] and NU7441 [127], have been developed and tested for radiosensitization. The radiosensitizing effect mediated through DNA-PKcs inhibition has been shown when cells were exposed with photons [128] as well as carbon ions [129, 130]. However, the inhibitors of the NHEJ pathway in general and, especially, DNA-PKcs inhibitors have not been investigated in clinical studies so far. One of the major reasons for that is the importance of this pathway for DSBs repair in both tumor cells and normal cells with the risk of increased normal tissue toxicity and a potentially reduced therapeutic ratio.

DNA-PKcs presents a phosphytidylinositol 3-kinase (PI3K) domain structurally similar to classical PI3K. In a wide range of tumor types the PI3K/Akt pathway is hyperactivated and stimulates repair of DSBs through both NHEJ and HR [131]. Thus, simultaneous targeting of the single component in the PI3K/Akt pathway and DNA-PKcs might be a more effective strategy for radiosensitizing cancer cells to irradiation and overcoming the disadvantage of DNA-PKcs inhibitors. Preclinical data reported for these inhibitors in combination with chemotherapy agents are promising. KU-0060648, a dual inhibitor of DNA-PK and PI3K, sensitizes colon and breast cancer cells to topoisomerase II inhibitor etoposide *in vitro* and in xenograft models without increasing etoposide toxicity to unacceptable levels [132]. Thus, dual targeting of PI3K and DNA-PKcs might be an alternative approach for improving the toxicity of irradiation, especially, in those tumors with alteration in the PI3K pathway.

11.3.3.3 Indirect Targeting DNA Damage Repair Through Interference with Cytoplasmic Signaling Pathways

Cytoplasmic signaling pathways can stimulate components of DDR proteins in the classical NHEJ repair pathway. Receptor tyrosine kinases (RTKs) are the major regulators of ionizing radiation-induced activation of cytoplasmic signaling pathways. Among the 20 families of RTKs so far described [12], members of the erbB family similar to insulin-like growth factor 1 receptor (IGF-1R) are activated not only by stimulation with ligand binding, but also through exposure to ionizing radiation in a ligand-independent manner. Ionizing radiation induces several downstream pathways of these receptors; e.g. the PI3K/Akt pathway is one of the important cascade. In the following paragraphs, some of the mechanisms by which RTKs, especially, EGFR and the downstream pathway PI3K/Akt regulate DSBs repair will be discussed. Likewise, the current status of the preclinical and clinical studies of molecular targeting approaches in combination with radiotherapy will be summarized.

Targeting EGFR-Mediated DNA Repair for Radiosensitization

Among the families of RTKs, the erbB receptor family with four members (EGFR or erbB1, erbB2, erbB3 and erbB4) is the major one, which is dysregulated in different cancers leading to the activation of downstream signaling pathways. EGFR/erbB1 is overexpressed or mutated in about 50 % of human solid tumors [133, 134] and is activated following exposure to ionizing radiation in a biphasic manner [135]. Additionally, in many cancers EGFR is hyperactivated through enhanced autocrine secretion of the related ligands. Translational studies have demonstrated that hyperactivation of EGFR leads to both chemo- and radiotherapy resistance and, consequently, to a poor prognosis [136–139].

It is well accepted that EGFR is also present and active in the nucleus and its expression is related to therapy resistance [140–142]. Resistance to ionizing radiation in EGFR overexpressing tumor cells is believed to be achieved mainly through stimulating DSBs repair. Several mechanisms have been demonstrated by which the nuclear form of EGFR regulates DNA repair potentially through NHEJ. It is proposed that following irradiation EGFR contributes to tumor radioresistance, through a functional interaction with DNA-PKcs [143, 144] as a core enzyme in the NHEJ repair pathway. There are several other mechanisms by which EGFR activity stimulates repair of DSB. BRCA1 as an essential component of HR repair machinery can be found in the same protein complex as EGFR [145]. Likewise, EGFR regulates phosphorylation of ATM at Tyr-370 leading to Chk2 activity and stimulation of HR [146]. Additionally, phosphorylation of the proliferating cell nuclear antigen (PCNA) as an essential protein for DNA replication and damage repair is regulated by EGFR [147]. EGFR also mediatesTyr-72 phosphorylation of H4, which leads to H4-K20 methylation and acceleration of DNA synthesis and repair [15]. These and other lines of evidence strongly indicate that EGFR is an indirect regulator of DNA repair through stimulation of DSBs repair pathways (Fig. 11.3).

Given the fact that the EGFR expression inversely correlates with radiation sensitivity [148] and overall survival after radiotherapy [136], this receptor is considered as an important target in combination with radiotherapy. For this purpose, several strategies have been developed. Among them EGFR tyrosine kinase inhibitors (TKIs) and monoclonal antibodies against extracellular domain of the receptor are the most prominent ones. Gefitinib and erlotinib are EGFR inhibitors with the potential to impair repair of DSBs through interference with NHEJ and HR [149, 150].

Until now the majority of clinical trials addressing the combination of EGFR targeting strategies with chemoradiotherapy have been performed using the anti-EGFR monoclonal antibodies such as cetuximab, panitumumab and nimotumumab. In a multinational phase III clinical trial [16] it was reported that adding cetuximab to primary radiotherapy increases overall survival in patients with locoregionally advanced squamous cell carcinoma of the head and neck 3 years after treatment [16] as well as after 5 years [17]. After the trial by Bonner et al. [16] cetuximab was approved for the first-line treatment of non-metastatic head and neck cancer in combination with radiation therapy and in combination with platinum and 5-FU or

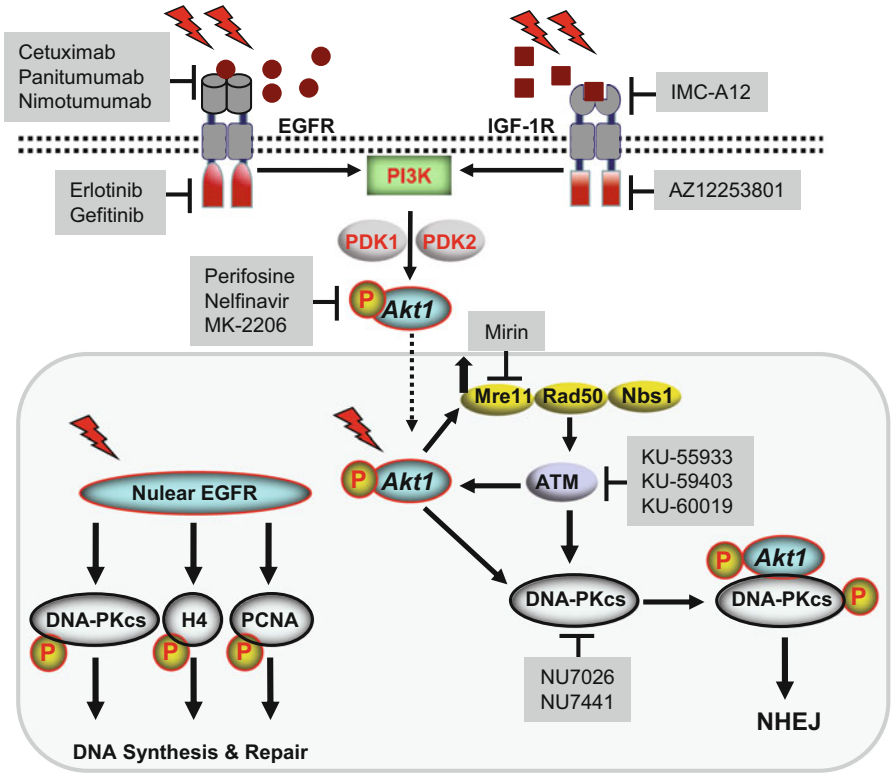

Fig. 11.3 Overview of targeting of EGFR, IFG-1R, ATM and Akt/DNA-PKcs pathway that interfere with repair of ionizing radiation-induced DSB repair. All the inhibitors have been reported to induce radiosensitization in preclinical studies. Clinical data also exists for the combination of cetuximab with radiotherapy of patients with squamous cell carcinoma of head and neck. Please see text for detailed explanation of the pathways

as a single agent in the treatment of recurrent and/or metastatic HNSCC [151] in the USA and Europe. Clinical data indicate the better survival benefit of a combination of cetuximab with radiotherapy in patients with EGFR positive tumors than EGFR negative tumors [152].

HPV infection and p16 positivity are associated with a favorable outcome in oropharyngeal squamous cell cancer patients treated with radiotherapy and cetuximab. The cellular gene expression profiles of HPV-positive and HPV-negative are different and among the upregulated genes are those involved in cell cycle regulation and DNA repair [153]. However, no direct clinical data exist, which support inhibition of DNA repair as the mechanism of improved survival of HNSCC patients by cetuximab after radiotherapy. Therefore, other mechanisms such as accumulation of cells in the more radiosensitive cell cycle G2-M phases and inhibition of angiogenesis might be an alternative mechanism of radiosensitization by cetuximab [154, 155].

For the combination of EGFR-TKI, so far, several phase II clinical trials have been performed. Combination of EGFR-TKI erlotinib with radiotherapy or chemoradiotherapy may be beneficial in terms of progression-free survival or progression-free survival and overall survival of patients with different tumors, such as metastatic non-small cell lung cancers [156–160], locally advanced cervical cancer [161] and head and neck cancers [162], compared with historical values. So far, no results from randomized phase III clinical studies exist to demonstrate the absolute benefit of the combination of EGFR-TKI with radiotherapy or chemoradiotherapy.

Combination of Insulin-Like Growth Factor-1 Receptor Targeting with Radiotherapy

Among the family of transmembrane receptor tyrosine kinases, stimulation of DSBs repair does not seem to be a specific phenomenon of EGFR. Dysregulation of insulin-like growth factor-1 receptor (IGF-1R) is linked to several cancer hallmarks [163]. Evidence from preclinical studies indicates that activation of IGF-1R can also accelerate repair of radiation-induced DSBs through NHEJ and HR [164, 165]. In line with this effect, it was shown that targeting IGF-1R either by a knockdown approach [166] or using an anti-IGF-1R monoclonal antibody IMC-A12 [167] as well as IGF-1R tyrosine kinase inhibitor AZ12253801 [164] enhance the radiosensitivity of human tumor cells *in vitro* and *in vivo*. However, the underlying mechanism and role of IGF-1R in DSBs repair is not fully understood so far. An interaction between EGFR and IGF-1R [168] as well as activation of pro-survival signaling pathways such as the PI3K/Akt pathway (Fig. 11.3) are likely to be involved in the enhanced repair of DSBs initiated by IGF-1R.

Akt Regulates DNA Repair and Is a Potential Target for Radiosensitization

Aberrant pathways downstream of growth factor receptors are specific to cancer cells. Thus, it is expected that targeting such pathways may offer a tumor-specific approach to improving radiotherapy outcome associated with minimal normal tissue toxicity. Among the different pathways downstream of RTKs, so far the PI3K/Akt pathway is the most important one for regulating DNA damage repair. Its activity status depends on the pattern of expression and mutational status of membrane-bound receptors and of the downstream components such as PTEN, PI3K and RAS. Similar to stimulation with growth factors, exposure to ionizing radiation can induce PI3K/Akt activity [169–174]. A significant correlation exists between activation of EGFR and Akt phosphorylation [175]. Thus, EGFR seems to be the major upstream regulator of PI3K/Akt activity. The impact of PI3K/Akt activity on radioresistance has been reported by several laboratories using cancer cell lines from different origins, including head and neck, colon, lung, and brain cancers *in vitro* and *in vivo* [169, 170, 174, 176–179]. Although stimulation of PI3K regulates the activation of many other substrates such as SGK [180] with a pro-survival effect as

well, activation of Akt plays a key role in the radioresistance of tumor cells. Akt activity regulates DSBs repair, mediating radioresistance in tumor cells with different entities [171, 181–186]. Phosphorylated Akt1 at S473 is necessary for full Akt activity and is a prognostic marker for response of patients with head and neck cancer and cervical cancer to radiotherapy [187, 188]. Since DSBs are the most lethal type of DNA lesions and lead to cell death following exposure to ionizing radiation [189], the predictive value of Akt in radiotherapy outcome supports the preclinical data on the role of Akt regulating DSBs repair (Fig. 11.3).

Akt1 directly interacts with DNA-PKcs through its C-terminal domain immediately after irradiation. Akt1 and DNA-PKcs form a functional complex in the nucleus [186, 190]. DNA-PKcs accumulation in the DSBs site partially depends on Akt1. Likewise, Akt1 plays a role in DNA-PKcs kinase activity and its phosphorylation in amino-acid positions Thr-2609 and Ser-2056 are essential for repair of DSBs during NHEJ [191, 192] as well as the release of DNA-PKcs following efficient repair [186]. The role of Akt in DNA-DSBs repair is further evidenced by the co-localization of γH2AX foci as a marker of DSBs with P-Akt after irradiation [181, 193, 194]. Given the role of Akt in phosphorylation of DNA-PKcs and the timing of localization of these two proteins [185, 186, 195], it can be suggested that Akt is involved in the fast component of DSBs repair.

Akt has been described to upregulate the expression of Mre11 in the MRN complex through the GSK3β/β-catenin/LEF pathway and, thus, elevating DSBs repair capacity in cancer cells [196]. The MRN complex recruits ATM, which is in turn activated to phosphorylate members of MRN complex itself and stimulates phosphorylation of Akt at Ser-473 [197] through RNF168 [181]. Given the role of Akt in MRE11 expression [196], targeting Akt leads to the downregulation of MRE11 at the transcriptional level and interference with ATM signaling, leading to impaired HR. Thus, the role of Akt1 in DNA repair under this mechanism might be owed to regulating the slow component of DNA repair, a complementary mechanism to the Akt/DNA-PKcs-dependent fast repair process. Akt in addition to its role in DSBs repair is involved in activating DDR signaling. Akt directly phosphorylates Chk1 kinase at Ser280 [198] that is essential for G2/M arrest. Based on current knowledge, a detailed mechanism for the role of Akt in stimulating DSBs repair has been summarized in Fig. 11.3.

The PI3K/Akt pathway is the major survival pathway in tumor and normal cells; nevertheless this pathway presents the most common alterations in human cancers, e.g. in head and neck cancers [199, 200] and glioblastomas [201]. PI3K/Akt is theoretically activated by all the families of the RTKs as well as other upstream regulators such as G-protein coupled receptors. It is known that targeting of PI3K increases radiosensitivity [202] through impaired DSBs repair [183, 203]. Given this observation and the crucial role of Akt in DSBs repair it is assumed that the indirect inhibition of DNA repair by PI3K targeting can be an efficient approach for increasing the radiation response of tumor cells. There are three classes of PI3K of which class IA PI3K isoform has been strongly implicated in cancer. This isoform of PI3K with a p85 regulatory subunit and p110 catalytic subunit has become a potential target in oncology. So far, application of PI3K inhibitors as single-agent treatment has not

been successful. This is mainly because the majority of the PI3K inhibitors have cytostatic and cytotoxic effects. The cytostatic effect of these inhibitors can limit their therapeutic effect. Likewise, mutation in the downstream components of this pathway will limit outcome after targeting of PI3K. Alternatively, extensive cross-talk at different levels between the PI3K/Akt pathway and other pathways such as the MAPK/ERK pathway [204] is an obstacle to single-targeting PI3K in oncology. This suggests that combined inhibition of both pathways may be an efficient way to indirectly target DNA repair [205, 206].

Activation of Akt downstream to PI3K as well as its amplification and overexpression has been reported in many human cancers. Akt activity is strongly correlated with the inactivation of phosphatase and tensin homolog (PTEN), associated with poor prognosis as shown in patients with NSCLC [207]. PTEN activity is lost following deletion, mutation and silencing of promotor methylation in primary as well as metastatic human cancers [208]. Mutational activation of Ras and PI3K leads to constitutive activation of Akt and is accompanied by resistance to chemoradiotherapy [183, 209–211]. According to the function of Akt in stimulating DNA repair and cancer cell survival, an indirect targeting of DNA repair by direct targeting of Akt can be an alternative strategy to a PI3K inhibitor approach to overcome radiotherapy resistance. So far, the majority of phase I studies with Akt inhibitors have been performed with the allosteric inhibitor perifosine alone or in combination with chemotherapeutic drugs with a favorable safety profile and potential beneficial effect on different solid tumors [212–216]. Perifosine interacting with the PH domain of Akt isoforms inhibits binding of Akt to cell membrane [217]. A potential radiosensitizing effect of perifosine in head and neck squamous cell carcinoma has been shown *in vitro* and *in vivo* [218]. Similar to its combination with chemotherapy, perifosine in combination with fractionated radiotherapy of patients with advanced solid tumors was reported to be safe and recommended for phase II study [219]. Phase I studies of Akt inhibitors in combination with chemoradiotherapy have been performed by using the protease inhibitor nelfinavir. Nelfinavir in combination with chemoradiation for locally advanced rectal cancer [220], pancreatic cancer [221], NSCLC [222], and glioblastoma multiforme [223] was also reported to be safe, with promising activity. Further studies are urgently needed for better knowledge on the efficacy of this treatment regimen in solid tumors generally and in those tumors with mutations in the components of the PI3K/Akt pathway such as mutations in PTEN, PI3K and K-RAS. An overview of druggable targets and related inhibitors that inhibit DSB repair indirectly (EGFR, IFG-1R, Akt) or directly (MRE11, ATM and DNA-PKcs) has been presented in Fig. 11.3.

11.4 Concluding Remarks

Radiotherapy is used to destroy malignant cells by inducing DNA damage. The curative potential of radiotherapy is affected by intrinsic radiosensitivity (e.g. repair capacity), tumor cell proliferation (e.g. repopulation), tumor microenvironment

(e.g. reoxygenation) and cell cycle phenomenon (e.g. redistribution). Evidence for radiosensitization of tumor cells owed to the targeting of DNA repair is growing rapidly. The efficacy of DDR targeting strategies to enhance radiosensitization is however mainly challenged by the issue of tumor heterogeneity. Tumor heterogeneity can be caused by activation of multiple oncogenic processes and mutations in certain DNA repair pathways. Both conditions can dictate the sensitivity of tumor cells to radiotherapy and the effectiveness of molecular targeting strategies in combination with radiotherapy. Several early phases of clinical studies testing potential targeting approaches through inhibiting DNA repair have been performed. So far, except for the anti-EGFR antibody cetuximab in combination with radiotherapy, which has become standard therapy for a subset of oropharynx cancer patients, none of the agents that directly or indirectly inhibit DNA repair have been applied in phase III clinical studies in combination with radiotherapy. Thus, more predictive biomarkers are needed to design rational combinations. The evaluation of such biomarkers through more translational studies will help to select those patients for whom DNA repair targeting strategies may function efficiently in tumor cells. In the light of the lessons learned from PARP inhibitors, targeting DNA repair might be a more effective therapeutic strategy to improve radiotherapy outcome, if the targeting strategies are based on the concept of synthetic lethality.

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Chapter 12
Radiosensitizing Glioma by Targeting ATM with Small Molecule Inhibitors

Amrita Sule and Kristoffer Valerie

Abstract Malignant glioma is a devastating and incurable brain cancer. Current standard treatment of malignant glioma is surgery followed by chemotherapy and radiation. Progress during the past few decades in improving long-term survival has been painfully slow with a median overall survival currently at a little more than 1 year. New strategies targeting the DNA damage response, including the ATM (ataxia telangiectasia mutated) kinase, are currently being pursued. ATM is a master regulator of cell cycle checkpoints, DNA repair, and cell death in response to radiation. Pre-clinical studies using novel small molecule inhibitors of the ATM kinase are in progress and results from these look promising for future testing in humans. In fact, one ATM kinase inhibitor is currently in a Phase I trial in combination with chemotherapy of advanced solid cancers. This chapter focuses on discussing recent advances in developing and testing highly specific inhibitors targeting the ATM kinase for cancer therapy with focus on malignant glioma.

Keywords Ataxia telangiectasia mutated (ATM) • Convection-enhanced delivery (CED) • DNA damage response (DDR) • Glioblastoma multiforme (GBM) • Ionizing radiation (IR) • Malignant glioma • Mitotic catastrophe • Phosphatidylinositol 3-kinase-related kinase (PIKK) • p53 • Radiosensitizer • Radiotherapy • Temozolomide (TMZ)

12.1 Introduction

Nearly 80,000 new cases of malignant glioma (classified by the World Health Organization (WHO) as Grade III and IV glioma) are diagnosed each year in the United States with 17,000 people dying from the disease. Grade IV is also referred

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to as glioblastoma multiforme (GBM). GBM is a highly lethal brain tumor presented as one of two subtypes with distinct clinical histories and molecular profiles. Hallmark characteristics of GBM include uncontrolled cell proliferation, diffuse infiltration, and resistance to apoptosis. These features account for GBM's poor prognosis and resistance toward radio- and chemotherapy, and a median patient survival of only 12–15 months [1]. In older individuals, the most common form is of the primary subtype which arises de novo with no prior symptoms or evidence of progression from low-grade tumors. The secondary subtype of GBM occurs in younger patients from lower grade glioma. GBMs are classified into four different subgroups based on gene expression profiling; (1) classical, (2) mesenchymal, (3) neural, and (4) pro-neural [2, 3]. Primary GBM is mostly found in the classical subgroup with EGFR mutation/amplifications and mutations in CDKN2A and PTEN. On the other hand, secondary GBMs are usually found in the pro-neural subgroup with frequent mutations in PDGFR, IDH1/2, and p53 [2]. The frequency of p53 mutation in this sub-group is 65 % or greater whereas classical GBM harbors p53 mutations 30 % of the time [4, 5]. Recently, a new more reliable molecular classification based on IDH status and specific TERT promoter mutations was proposed [6, 7].

Standard treatment of GBM is surgery followed by temozolomide (TMZ), an alkylating drug, and radiation [8, 9]. However, little improvement has been seen in the long-term survival of patients with GBM during the last several decades. Thus, new treatments and approaches are urgently needed. As the understanding of the molecular mechanisms associated with GBM continues to expand, and more specific and potent drugs are developed, efficient delivery of therapeutic agents to the brain becomes very important and remains a challenging clinical problem. In particular, both the blood–brain barrier (BBB) and blood–tumor barrier hamper the successful treatment of brain tumors by severely limiting access of therapeutic agents to the brain and tumor [10, 11]. These obstacles have made the efficient delivery of anticancer drugs to the brain a major technical hurdle, and therefore this area of research is lagging behind the development of the drugs themselves. Because surgery is standard treatment for GBM, the delivery of therapeutic agents directly to the brain during surgery, e.g. GLIADEL® wafers, or post-surgery by convection-enhanced delivery (CED) via a cannula and positive pressure does not deviate significantly from current treatment practice. For the obvious reasons of being easier to administer and lower cost, an orally bioavailable and BBB-penetrable ATM inhibitor would be preferable over CED-based delivery. However, specific circumstances might favor the latter route of drug administration, e.g. if radiomimetic drugs, such as etoposide, and camptothecin, etc., that either are too toxic when administered systemically or are BBB-impermeable, CED could be the most efficacious and appropriate mode of delivery [11].

There have been significant advances in the development and pre-clinical testing of radiosensitizers for high grade gliomas during the past few years with focus on targeting the DNA damage response (DDR) (see [12–14] for recent reviews). Despite the identification of exciting new targets and the development of drugs

against these targets, their clinical use is still under evaluation. One of the earliest targets identified and pursued is the protein mutated in ataxia telangiectasia (ATM) and its intrinsic protein kinase [15]. ATM is mutated in the human autosomal recessive disorder, ataxia-telangiectasia (A-T) [16]. The extreme radiosensitivity of cells from A-T patients has been known since the 1970s [17]. ATM, a serine-threonine kinase and member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, is a major regulator of the DDR. ATM is activated in response to DNA double strand breaks (DSBs) induced by DNA damage such as ionizing radiation (IR) or spontaneously during replication and cell growth. Once activated, ATM phosphorylates numerous proteins involved in cell cycle regulation, DNA repair, apoptosis, etc. [18, 19]. ATM-mediated phosphorylation and other subsequent post-translational modifications affect the stability, sub-cellular localization, and the interaction of proteins involved in these processes, thereby masterminding the DDR [20]. ATM is also known to regulate insulin and other growth factor signaling responses resulting from the stimulation with non-classical DDR agents suggesting a much broader role for ATM in regulating cell growth and homeostasis in addition to the DDR [16].

During the past 10 years the ATM inhibitor KU-55933 has extensively been used in tissue culture experiments by numerous laboratories to demonstrate the involvement of the ATM kinase in various capacities. KU-55933 was developed by KuDOS Pharmaceuticals, Ltd, in the United Kingdom, and shown to be a highly specific ATM kinase inhibitor competitively binding to the ATP-pocket [21]. The KU-55933 IC_{50} for ATM (13 nM) is at least 200-fold lower than for the other PIKKs, including DNA-PKcs and ATR. Around 2007, at the time KuDOS was acquired by AstraZeneca, we were offered an improved analog, KU-60019, to test as a radiosensitizer in our mouse glioma models. We extensively characterized KU-60019 in vitro with glioma cells to assess its impact on the DDR [22]. Briefly, in addition to the improved radiosensitization seen with KU-60019, we documented high specificity toward the ATM kinase with no effect on 229 other kinases in vitro. Radiosensitization was observed with all cell lines tested, whether tumor or normal, except for A-T cells, strongly suggesting that the ATM kinase was the target for KU-60019. Furthermore, KU-60019 has high stability and is quickly reversible in vitro in wash out experiments. Additionally, we carried out limited in vitro combination testing of KU-60019, temozolomide (TMZ), and radiation [23]. When U87 glioma cells were co-treated with KU-60019 and TMZ a slight increase in radiation-induced cell killing was noted although TMZ alone was unable to radiosensitize the cells. In addition, without radiation, KU-60019 with or without TMZ reduced glioma cell growth but had no significant effect on the survival of human astrocytes [23]. Another study showed a beneficial interaction of KU-55933 and TMZ in vitro but only with inherently TMZ-sensitive glioma cell lines [24]. Thus, there is no reason to believe that an ATM kinase inhibitor would be counter-effective with current standard care of glioma. Other ATM inhibitors, such as CP466722 [25] and KU-59403 [26], have been developed with only the latter evaluated in a pre-clinical setting and neither one tested against glioma.

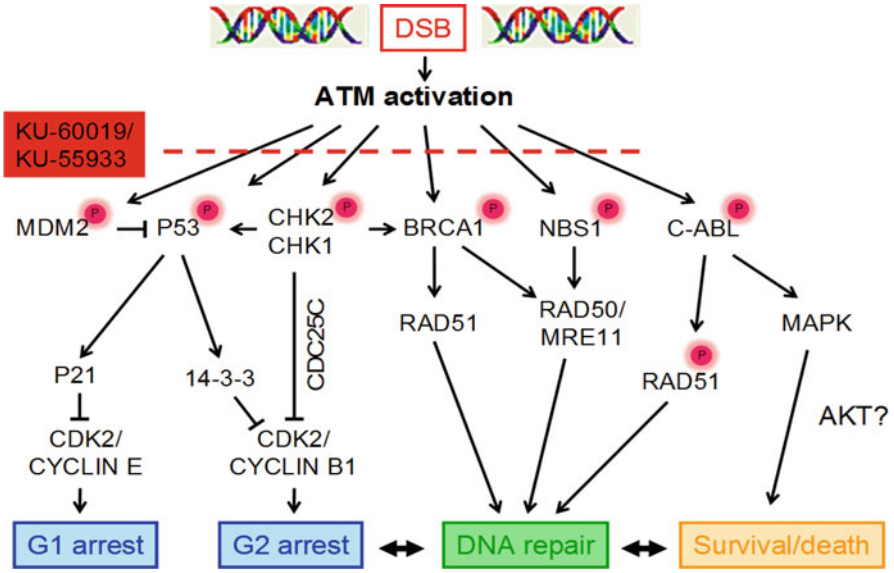

Fig. 12.1 Potential impact of an ATM inhibitor in combination with a DNA damaging agent on cell cycle checkpoints, DNA repair, and cell death. The ATM kinase phosphorylates >700 proteins, some at multiple sites, that is necessary for fully triggering the DDR [27]. Blocking the DDR including G1 and G2 arrest, DNA repair, and apoptosis/cell death with an ATM inhibitor is expected to affect many cellular responses to radiation and chemotherapy and kill tumor cells. Descriptors; →, activation/phosphorylation (℗); inhibition, ⊥

12.2 Rationale for Targeting the ATM Kinase

12.2.1 Advantages of ATM Kinase-Directed Therapy

It was realized early on that an ATM inhibitor would likely serve as an excellent radiosensitizer based on the radiosensitivity of A-T patients [17]. The basic idea behind this notion is that an ATM kinase inhibitor, such as KU-55933 or KU-60019, would be expected to block cell cycle checkpoints and DNA repair so that tumor cells would die from apoptosis or other cell death (Fig. 12.1). Many proteins regulating cell cycle checkpoints (e.g., p53, MDM2, and CHK2), DNA repair (BRCA1, NBS1), cell death/apoptosis (cABL) are directly phosphorylated by ATM [16, 27, 28], so an ATM inhibitor would effectively block signaling and prevent all downstream DDR-associated processes from taking place with fatal consequences to the tumor cell.

Cancer-specific targeting is a long-sought-after goal in cancer therapy. We demonstrated for the first time that a small molecule ATM kinase inhibitor, KU-60019, efficiently radiosensitized orthotopic gliomas with a much greater response seen with mutant p53 relative to matched glioma with normal p53 [29]. Briefly, human glioma U87 cells (p53 wild type) transduced with a retrovirus expressing a p53-281G mutant were grown intra-cranially in nude mice in parallel with mice

injected with parental U87 cells. The mutant p53 acts a dominant-negative in this situation and imposes a mutant p53 phenotype on the cells. Treatment with KU-60019 prior to radiation repeated three times 3 days apart resulted in a significantly improved ($p = 0.00011$) survival of U87-281G mice, whereas mice with parental U87 p53 wild type gliomas did not respond under these conditions of relatively low total radiation dose (Fig. 12.2). The radiation dose was purposely set low so that KU-60019 radiosensitization would be more easily discerned. With parental U87 tumors, radiation alone and KU-60019 alone showed a trend toward longer survival, whereas a significant effect of KU-60019 alone versus untreated was noted with U87-281G tumors [29].

The U87 parental and U87-281G cells were analyzed in vitro in their response to KU-60019 with or without radiation (Fig. 12.3). We found that indeed the U87-281G cells had a compromised G1/S checkpoint, as expected, grew substantially faster and were more responsive to KU-60019 treatment alone in growth assays. Additionally, the cells were more radiosensitive, and responded more robustly to KU-60019 and radiation, resulting in more cell death than with U87 parental cells. The results from this work laid the foundation for the notion that mutant p53 gliomas might respond to ATM inhibitor radiosensitization more robustly than p53 wild type gliomas.

It has been reported that high grade glioma cells show signs of elevated replicative stress compared to lower grade brain tumor cells [30], perhaps favoring a highly responsive phenotype to radiation and inhibition of ATM, which is then enhanced by mutant p53. Our own work suggests that mutant p53 glioma cells die by increased mitotic catastrophe (apoptosis in or subsequent to mitosis) when challenged by radiation in the presence of an ATM inhibitor [31]. It is likely that the consequence of ATM inhibition and interference with DDR signaling in p53 mutant glioma cells occur at multiple levels, e.g., abrogation of cell cycle checkpoints and inhibition of DNA repair, blocking signaling through the TAO kinases and p38-MK2-CDC25A, ultimately leading to mitotic catastrophe [32, 33]. The more proximal mechanism causing mitotic catastrophe is possibly through PLK1 and Aurora A controlling the G2/M transition into M resulting in elevated mitotic failure in p53 mutant glioma cells exposed to radiation in the presence of an ATM inhibitor [34–36]. Since p53 is mutated in about a third of all gliomas and the notion that mutant p53 gliomas are more responsive to ATM inhibitor-based radiation therapy suggests that an ATM inhibitor could be a promising adjuvant therapy that would fit well with current standard of care [9].

The molecular weight of KU-60019 is >500 Da and does not cross the BBB. Therefore, in our initial attempts to radiosensitize gliomas KU-60019 was administered intra-tumorally by CED or osmotic pump in order to document inhibition of the DDR in tumor and surrounding brain tissue resulting in a survival benefit to mice transplanted with p53 mutant gliomas [29]. Consequently, an orally bioavailable ATM inhibitor would simplify and reduce the technical aspects of ensuring efficient glioma radiosensitization. Further in this chapter we will discuss efforts that our group and others have made toward bringing an ATM inhibitor closer to clinical testing.

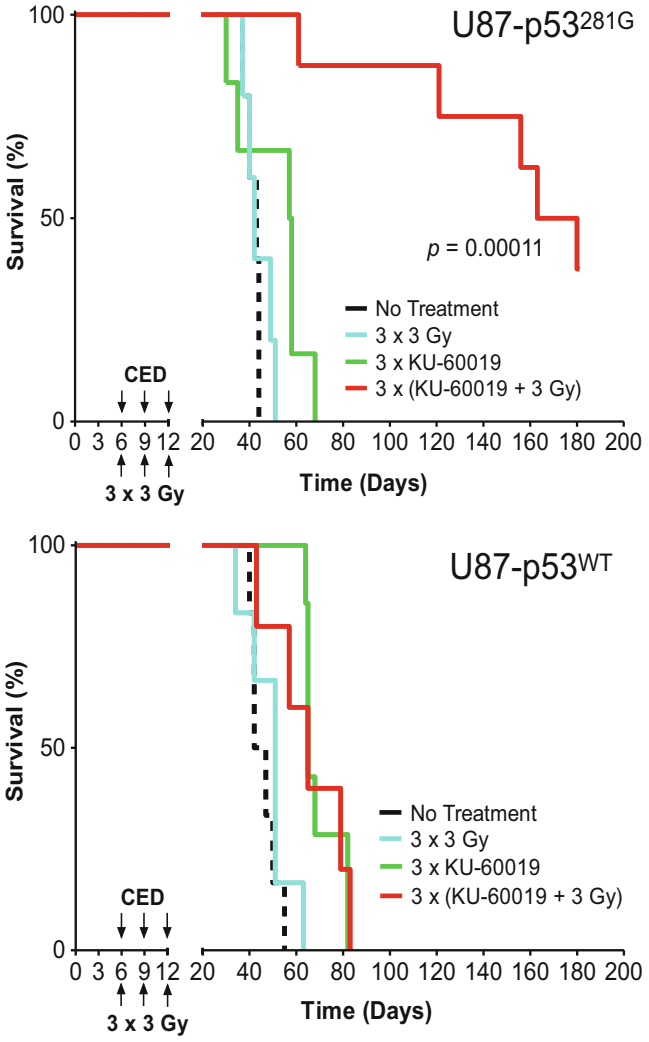

Fig. 12.2 KU-60019 radiosensitizes p53 mutant but not p53 wild type intra-cranial U87 tumors. Human glioma U87 cells were transduced with a retrovirus expressing mutant p53-281G. ATM inhibitor was administered by CED immediately followed by 3 Gy radiation on day 6, 9, and 12 (3×3 Gy) after intracranial injection of tumor cells. (*Top*) U87-281G tumors are highly responsive to KU-60019 radiosensitization whereas parental U87 tumors (*bottom*) are not (p=0.00011). Whereas mice injected with parental U87 cells survived 60–80 days regardless of treatment, 50% of the mice injected with U87-281G cells and treated with both KU-60019 and radiation survived for at least 160 days. Radiation dose was purposely set at 3×3 Gy in order to see survival benefits with KU-60019 and radiation. Survival is plotted as Kaplan-Meier curves. Adapted from Biddlestone-Thorpe et al. with permission [29]

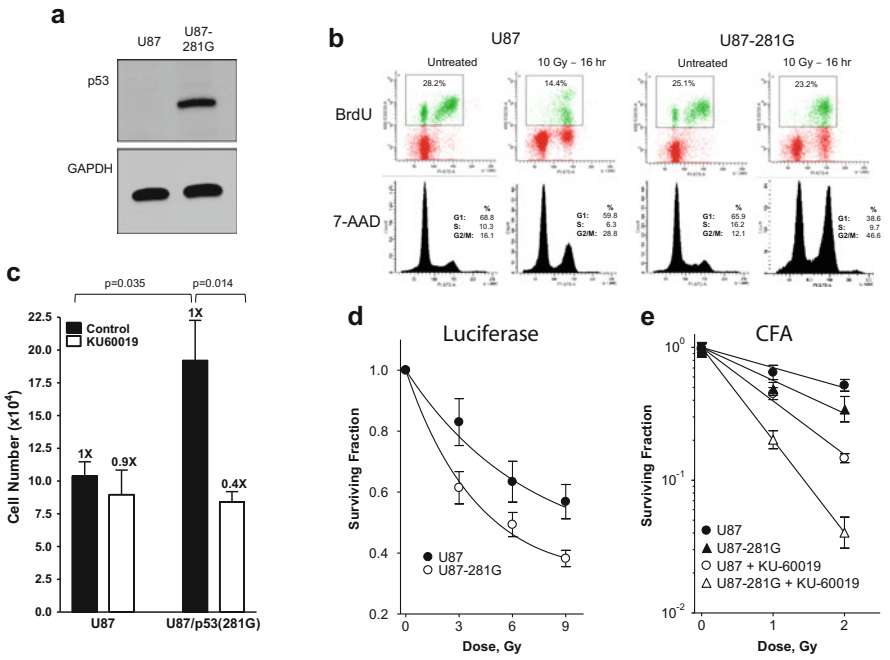

Fig. 12.3 Genetically matched glioma cells differing in p53 status demonstrate significantly different responses to ATM inhibitor and radiation. (**a**) Over-expression of mutant p53-281G from a retrovirus in p53 wild type human U87 glioma cells produces a dominant p53 effect on cell cycle checkpoints and DNA repair. Western blotting with anti-p53 antibody of extracts from U87 or U87-281G cells shows expression of mutant p53 whereas endogenous wild-type p53 is undetectable in these unirradiated cells (**b**) U87-281G cells have a defective radiation-induced G1/S checkpoint and an intact G2/M checkpoint. U87 and U87-281G cells were irradiated with 10 Gy and BrdU added immediately for 16 h to detect cells entering S to accumulate in G2/M. Both U87 and U87-281G cells entered S after radiation with 10 Gy but whereas parental U87 showed a 50% decrease, U87-281G traversed into S unperturbed relative to unirradiated cells suggesting that the G1/S checkpoint is compromised in the latter cells. Additionally, more U87-281G cells arrest in G2/M than U87 cells because more cells go through the G1/S checkpoint. Altogether, U87-281G cells have a compromised G1/S checkpoint whereas the G2/M checkpoint is still relatively intact (**c**) In line with the finding that U87-281G cells lack the G1/S checkpoint, the cells demonstrate higher proliferation rate and are more responsive to KU-60019 growth inhibition (**d**) U87-281G cells are more radiosensitive than parental U87 by luciferase assay (**e**) In a colony-forming assay, U87-281G cell are more radiosensitive and responsive to KU-60019 than parental U87 cells. Adapted from Biddlestone-Thorpe et al. with permission [29]. Colony-forming assay, CFA

12.2.1.1 ATM Regulates Pro-survival Signaling at Multiple Levels

Interestingly, in our initial studies we found that KU-60019 not only inhibited the DDR, but also reduced AKT phosphorylation and pro-survival signaling, and inhibited migration and invasion in vitro [22]. AKT needs to be phosphorylated on both

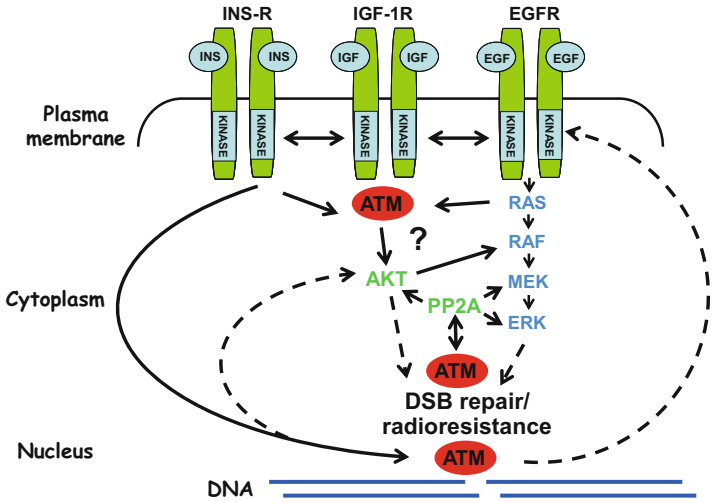

Fig. 12.4 AKT and MEK/ERK signaling are subsets of the ATM signaling network. ATM is known to interact with insulin, IGF-1R, as well as EGF growth factor signaling [22, 38, 39, 68]. Also central to ATM activation and regulation is the yin-yang relationship with PP2A, a phosphatase known to bind to ATM and intimately partake in the reversal of the DDR by dephosphorylating many proteins phosphorylated by ATM and other kinases [69–71]

S473 and T308 in order to become fully activated and able to phosphorylate downstream target proteins necessary for eliciting a proliferative response. Since the AKT S473 and T308 residues are not followed by an asparagine (-S/Q- or -T/Q-), i.e., consensus ATM phosphorylation sites, and thus would not likely be direct ATM kinase targets suggests that ATM might regulate AKT phosphorylation/activation indirectly [22]. On the other hand, the more promiscuous DNA-PKcs is known to phosphorylate AKT S473 in response to DNA damage [37]. We favor a mechanism by which DNA-PKcs directly phosphorylates AKT and ATM negatively regulates AKT dephosphorylation in response to radiation and growth factor signaling thus implicating a critical role for ATM in AKT signaling [22].

The fact that insulin-mediated AKT S473 phosphorylation is substantially reduced (~50 %) by ATM inhibition suggests a role for ATM in AKT pro-survival signaling and tumor growth that overlaps with the DDR [22, 38]. Interestingly, it has been shown that overexpression of insulin growth factor 1 (IGF-1) receptor in A-T cells increases radioresistance [39]. Growth factor receptors such as insulin, IGF-1R, and EGFR, are intimately associated with ATM and the DDR (Fig. 12.4). It is possible that ATM interacts with receptor signaling at multiple levels including the plasma membrane, cytoplasm, and nucleus. The observation that both RAS-RAF-MEK-ERK as well as AKT signaling are affected by ATM manipulation has been reported by a number of laboratories including ours [22, 40–42]. From these studies it is clear that ATM exerts its control at multiple levels including growth factor receptors, cytoplasmic signal transduction, and dephosphorylation of AKT. It makes sense that a key DDR regulator such as ATM would serve as the gate-keeper

between cell survival and death. Importantly, one would expect ATM inhibitors to act a multiple levels to enhance tumor radiosensitivity and inhibit tumor growth.

In addition to controlling the DDR, ATM also seems to regulate glioma migration and invasion which is not surprising given its association with ERK and AKT signaling. We first demonstrated that an ATM inhibitor reduced the migration and invasion of human glioma cells in vitro [22]. In support of this early finding, other groups have since shown that reducing the ATM protein by genetic means generates a blockade to AKT phosphorylation/activation downstream of HER2 which, if left unperturbed, promotes breast cancer dispersal [43]. Thus, ATM promotes HER2-dependent tumorigenicity and its expression correlates with reduced time of recurrence diagnosed with invasive HER2+ breast cancer. This suggest that HER2+ tumors have a selective advantage in retaining ATM expression and, therefore, ATM inhibition might counter metastatic potential of HER2+ breast cancers. In a separate study, it was demonstrated that ATM acts via IL-8 to enhance breast cancer metastasis to the lung [44]. The induction of IL-8 occurred as a consequence of oxidative stress which is known to activate ATM [45]. Knocking down ATM or inhibiting with KU-55933 resulted in reduced oxidative stress and IL-8 expression suggesting that IL-8 was under control of ATM [44]. Most importantly, blocking ATM reduced breast cancer migration and invasion in vitro and in vivo. Thus, ATM might play a role in breast cancer metastasis and progression in addition to its well-established role in tumor suppression [44].

Our own follow-up studies that an ATM kinase inhibitor reduces glioma cell migration and invasion in vitro [22], have now provided evidence that glioma dispersal in mouse brain is under control of ATM (in preparation). Briefly, a matched human glioma cell pair, one with ATM levels reduced by shRNA and the other a mock knockdown, showed significant reduced ability of the shATM glioma cells to migrate and invade in vivo when presented as intra-cranial tumors. Therefore, an ATM inhibitor might prevent glioma dispersal in between radiation fractions as well as enhancing the killing of tumor cells when irradiated as we have proposed previously [23]. Altogether, ATM might exert control over cell growth, survival, and death signaling at multiple levels; distal via growth factor receptors at the plasma membrane and more proximal at the level of cytoplasmic signal transduction via the ERK and AKT pathways, and a more direct involvement in the dephosphorylation of AKT resulting in reduced tumor cell growth. In summary, an ATM inhibitor may limit tumor growth, migration, and invasion by inhibiting ERK and AKT signaling in addition to acting as a very potent radiosensitizer.

12.2.1.2 ATM-EGFR-ERK and ATM-AKT Signaling Modulate DNA DSB Repair

As expected, there is a close relationship between pro-survival signaling and proficient DNA repair—at low levels of DNA damage occurring during clinically relevant radiation doses, DNA repair is operating optimally whereas apoptosis and related death mechanisms are suppressed [46, 47]. Using KU-55933, we demonstrated that

ATM was critically involved in regulating homologous recombination repair (HRR) in human glioma cells via MAPK signaling and that the ERK pathway appears to form a regulatory feed-back loop with ATM [40]. We and others have shown that the stimulation of cellular growth with growth factors such as epidermal growth factor (EGF), or, alternatively, blocking growth signaling by small molecule inhibitors or by genetic means modulate DSB repair [48–53]. In regards to GBM, it is particularly relevant that EGFRviii-mediated signaling promoted DSB repair both via both non-homologous end joining (NHEJ) and HRR [48, 54]. EGFRviii is a mutant form of EGFR that acts in a ligand-independent and auto-stimulatory manner through multiple pathways including ERK and AKT in many primary GBMs [55]. Altogether, an ATM inhibitor will directly inhibit DSB repair resulting from radiation.

12.2.1.3 ATM Is Required for Neuronal Cell Death

The brain consists mostly of non-proliferating, terminally differentiated cells such as neurons, astrocytes, and oligodendrocytes. Proliferating cells are mostly limited to neural progenitors and stem cells able to reconstitute in part cell populations after traumatic brain injury. Since ATM is required for radiation-induced neuronal apoptosis [56], transiently inhibiting ATM in the brain is expected to protect neurons from cell death. However, this has yet to be demonstrated. Almost 20 years ago using mouse genetics it was elegantly demonstrated in a string of very significant reports that ATM-dependent apoptosis in the CNS, and specifically in neurons, is mediated by p53 since p53−/− mice showed a similar lack of radiation-induced cell death in the developing nervous system as ATM−/− mice [56]. In addition, the ATM-dependent apoptotic pathway in neurons required BAX, a p53-dependent effector and critical participant in apoptosis [57]. Furthermore, ATM- and BAX-dependent apoptosis also required caspase-3 activation. However, in contrast to radiosensitive ATM−/− fibroblasts and radioresistant ATM−/− neurons, survival of ATM−/− astrocytes after irradiation was similar to wild-type astrocytes suggesting that in this type of CNS cells, ATM functions in controlling cellular growth and radiosensitivity by distinct mechanisms [58].

Altogether, based on these earlier findings and our own unpublished results, we speculate that an ATM inhibitor would have a substantial protective effect on irradiated CNS (p53 wild type) at low radiation doses that would result in cell cycle arrest rather than apoptosis in cells with proliferative capacity such as neural progenitors and stem cells, and prevent p53-dependent apoptosis in terminally differentiated neurons that would also require BAX and caspase 3. In contrast, gliomas, and in particular those with defective p53 signaling, would die by mitotic catastrophe when exposed to ATM inhibitor and radiation (Fig. 12.5). However, it is currently unclear whether a small molecule ATM kinase inhibitor would result in the same phenotype as the complete absence of ATM in the mouse (as in ATM−/− mice) [59], and whether the mouse phenotype can be recapitulated in humans and be fully applied to the human situation during cancer therapy. Future clinical trials will address these issues.

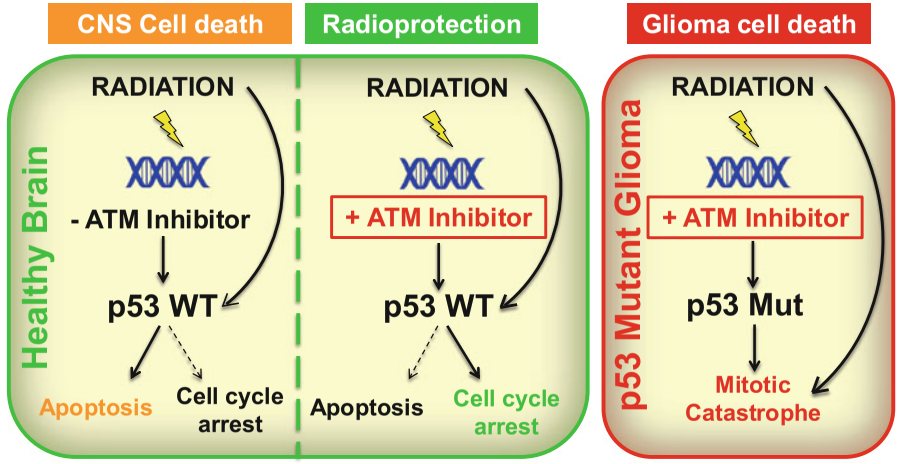

Fig. 12.5 Proposed model for the enhanced response of mutant p53 gliomas to ATM inhibitor radiosensitization and the protection of normal healthy brain. The model is based on the findings by McKinnon and co-workers using ATM, p53, BAX, and ARF KO mice [56–58]. Healthy brain not exposed to an ATM inhibitor is expected to undergo apoptosis in a radiation dose-dependent manner. In the presence of an ATM inhibitor a shift from apoptosis to cell cycle arrest occurs after radiation due to an intact p53 response. On the other hand, in a glioma p53 mutant environment ATM inhibition and radiation would kill cells by mitotic catastrophe

12.2.2 Limitations of ATM Kinase-Directed Therapy

The adverse effect of an ATM inhibitor on HRR and triggering of carcinogenesis has been put forward cautioning against its use in patients [60, 61]. However, it is important to realize that any chemo- or radiotherapy regimen will potentially have the unfortunate side-effect of causing secondary cancers. Clinical dose-findings will reveal whether benefits outweigh toxicity of ATM inhibitor-based therapies. Clearly, one of the advantages with combining an ATM inhibitor with radiation therapy is the ability to reduce systemic toxicity by applying conformal radiation only targeting the tumor. Chemotherapy in combination with an ATM inhibitor would not have that benefit since drugs alone would potentially show increased systemic toxicity to organs such as liver, kidneys, and blood. Thus, a strong case for using an ATM inhibitor in combination with conformal radiation can be made.

12.2.3 Cancer-Specific Targeting

Almost 50 % of all cancers have mutated or defective p53 [62]. For gliomas the overall proportion is about 30 % with slower-progression secondary GBM having much more frequent p53 alterations than primary GBM [5]. Since p53 is mutated in about a third of all gliomas and our discovery that mutant p53 gliomas are more responsive, ATM inhibitor-based radiation therapy could be a promising adjuvant therapy that would fit well with current standard of care to treat this subset of patients [9]. It would be very exciting to see whether the results from our pre-clinical testing of an ATM inhibitor translate into a greater response in patients with p53-mutated gliomas. If so, a third of all GBM patients might have a greater chance of survival past 1 year.

12.3 Pre-clinical Testing

Our studies showing that orthotopic human xenografts are responsive to ATM inhibitor radiosensitization are supported by similar findings by several other groups. It is now well-established that glioma stem cells or glioma-initiating cells (GICs) are the important tumor population responsible for treatment failure [63]. In fact, CD133+ GICs isolated from both human glioma xenografts and primary patient GBM preferentially activate the DDR in response to radiation, and the repair of radiation-induced DNA damage was more effective than in CD133- tumor. In addition, the radioresistance of CD133+ cells was reversed with a specific inhibitor of the CHK1 and CHK2 checkpoint kinases. An ATM kinase inhibitor would probably result in the same effect as the CHK inhibitors. In fact, we have shown that mouse glioma cells isolated from a spontaneous tumor from a genetically

engineered mouse with high incidence of glioma-formation responded well to KU-60019 radiosensitization in vitro [29]. In support of these findings, Vecchio et al. showed a p53-dependent response to ATM inhibitor and radiation in that low expressing p53 GICs responded better to KU-60019 and radiation than higher expressing cells [64], in agreement with our earlier report using matched laboratory glioma cell lines only differing in p53 [29]. It was also demonstrated that KU-60019 appeared to be safe when administered alone without resulting in any detectable toxicity or mutation in the various mouse tissues examined [65]. Furthermore, similar radiosensitization was also seen with pediatric GICs suggesting that an ATM kinase inhibitor could be a safe and effective radiosensitizer of both adult and pediatric gliomas. In another study, Lim et al. demonstrated that KU-55933 was able to radiosensitize GICs and prolong the survival of mice with orthotopic gliomas [66]. However, it appears as if this study pretreated the GICs with the ATM inhibitor prior to intra-cranial injection and radiation. Nevertheless, the authors' conclusion was that HRR was the predominant type of DSB repair in the GICs which was reduced by the ATM inhibitor and, in turn, resulted in radiosensitization. On the other hand, using a small molecule inhibitor of DNA-PKcs, important for classical NHEJ, did not affect DSB resolution and radiosurvival in vitro suggesting that NHEJ is less critical for DSB repair in GICs and might be less effective for therapeutic intervention toward GBM.

As all these studies have indicated, KU-60019 does not cross the BBB. Therefore, new more BBB-penetrable ATM inhibitors are needed. We have tested one such orally bioavailable ATM inhibitor and presented preliminary data from several mouse orthotopic glioma models [67]. Briefly, both a mouse syngeneic glioma grown in immune-competent mice as well as human orthotopic xenografts in nude mice were radiosensitized after the mice were given oral gavage of the ATM inhibitor (manuscript in preparation). Continued research on the efficacy and safety in the next year or so will demonstrate whether any such ATM inhibitor could move forward toward clinical testing.

12.4 Clinical Testing

AZD0156, a clinical ATM inhibitor compound developed by AstraZeneca, is currently undergoing testing in patients with advanced malignancies (ClinicalTrials.gov ID: NCT02588105). The goal of this trial is preliminary assessment of the antitumor activity of AZD0156 either as monotherapy or in combination with Olaparib (PARP inhibitor), cytotoxic chemotherapies, or novel anti-cancer agents. Planned enrollment in this multi-national trial is 225 patients. A more effective, BBB penetrating ATM inhibitor for glioma is currently being tested in pre-clinical models. It will be important to soon as possible also test AZD0156 together with radiation since the conformal nature of this modality is expected to result in a greater therapeutic index than any combination with DNA damaging drugs.

12.5 Conclusions

The development and testing of ATM inhibitors for brain cancer therapy is proceeding and is expected to enter the clinical testing arena in the next few years or so. The potential benefits of an ATM inhibitor as an adjuvant to radiotherapy go beyond just killing the tumor with a radiosensitizer. It is possible that significant clinical benefit might be seen in patients with mutant p53 gliomas with cancer-specific killing whereas normal, healthy brain tissue is protected or at least a reduction in toxicity seen. Regardless, one should be able to lower the total radiation dose to the brain in combination with an ATM inhibitor thereby reducing long-term sequela and cognitive impairment of patients. In fact, today with a median survival of only little more than 1 year for GBM patients the long-term consequences of surgery and chemoradiation are not fully considered because of the anticipated short life expectancy of these patients. Once long-term survival rate improves treatment side-effects would have to be addressed to also increase the quality of life. In fact, it is possible that ATM inhibitors could also be beneficial in the recovery of radiation damage to neurons and the brain as a whole when provided post-treatment.

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