Recent advances in radiosensitivity determinants in melanoma

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Purpose of review
Radiotherapy has been proven to be useful but insufficient in melanoma management due to the intrinsic radioresistance of melanoma cells. Elucidation of the molecular mechanisms and pathways related to resistance/sensitivity to radiotherapy in melanoma is of paramount importance. In this review, we will summarize and discuss the recent 'discoveries' and advances in radiosensitivity determinants in melanoma.

Recent findings
The different levels of radiosensitivity among the various melanoma tumors could be attributed to the DNA damage signaling and repair proteins, tumor microenvironment, hypoxia, cell metabolism, glutathione and redox balance, protein kinase signaling pathways as well as pigmentation and melanin content.

Summary
It is therapeutically important to elucidate the factors involved in radiation resistance/sensitivity of melanoma. More importantly, improving radiosensitivity may 'widen the clinical utility' in melanoma of this important therapeutic modality.

Keywords
DNA repair, hypoxia, immunological microenvironment, melanoma, metabolism, pigmentation, protein kinase signaling, radiosensitivity, redox balance

INTRODUCTION

Radiation therapy is a commonly used modality for the treatment of cancer. Distinct from other local (i.e., surgical) and systemic (i.e., biological or chemotherapy) treatments, radiation therapy is directed toward a specific body site that harbors gross or microscopic disease and exerts its effects through ionization events in the tumor cells [1]. The antitumor effect of ionizing radiation is mediated by the generation of reactive oxygen species (ROS), which damage DNA and induce apoptosis or/and senescence of cancer cells [2▪,3]. Ionizing radiation is a major pillar in the therapy of solid tumors. However, normal tissue toxicities and radioresistance of tumor cells can limit the therapeutic success of radiation therapy [3].

Despite advances in systemic treatment, with biological agents and immune checkpoint inhibitors playing a key role in the management of melanoma, radiation therapy is still used as a definitive treatment for cutaneous melanoma for patients unfit for surgery and in *lentigo maligna* [4]. Locoregional radiation therapy to the nodal basin should be considered after therapeutic lymph node dissection for high-risk nodal involvement [5]. Palliative radiation therapy significantly mitigates symptoms such as bone pain and central nervous system dysfunction induced by melanoma metastases [6]. Radiation therapy is also increasingly used for oligometastatic disease as a life-prolonging treatment in the form of stereotactic radiation therapy [7].

Melanoma is traditionally viewed as a radioresistant tumor, which may explain the limited role of radiation therapy in this disease as compared with many other tumors. However, improving radiosensitivity may widen the clinical utility of this important therapeutic modality. Combining radiation with immune modulators is one of the alternative strategies to achieve this goal [8].

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Curr Opin Oncol 2022, 34:131–138
DOI:10.1097/CCO.0000000000000818

KEY POINTS

- Resistance to DNA-damaging agents is a significant cause of treatment failure and poor outcomes in melanoma radiotherapy.
- The therapeutic efficacy of radiotherapy is not limited by the killing potential of melanoma cells but also involves the activation of antitumor immune responses.
- New links between hypoxic signaling and cell metabolism may provide insights into our fundamental understanding of radioresistance in melanoma.
- Inhibition of melanogenesis can improve radiotherapy modalities in melanoma.
- Combining radiation with immune modulators and/or kinase inhibitors has been postulated to have a promising effect.

In this review, we will summarize and discuss recent discoveries in melanoma radiosensitivity determinants. The different levels of radiosensitivity among the various melanoma tumors could be attributed to the DNA damage signaling and repair proteins, tumor microenvironment, hypoxia, cell metabolism, glutathione and redox balance, protein kinase signaling pathways as well as pigmentation and melanin content.

DNA DAMAGE SIGNALING AND REPAIR PROTEINS: SIGNIFICANT RADIOSENSITIVITY DETERMINANTS IN MELANOMA

Radiotherapy-induced DNA double-strand breaks (DSBs) are considered to be the most toxic and responsible for cell death. One of the major discernible hallmarks of the cellular response to DSBs is the accumulation and local concentration of a plethora of DNA damage signaling and repair proteins in the vicinity of the lesion, initiated by ataxia telangiectasia mutated-mediated phosphorylation of H2AX (γ-H2AX) and culminating in the generation of distinct nuclear compartments, so-called ionizing radiation-induced foci [9]. However, cancer cells that are not killed may acquire enhanced proliferative capacity and radioresistance via upregulation of DNA damage repair [10]. The p53 tumor suppressor pathway is a major mediator of the cellular response to radiation-induced DNA damage [11,12]. The inactivation of p53 observed in about 90% of melanomas [13] is involved in the complex response to ionizing radiation through transcriptional control of target genes that influence cell growth, DNA damage repair and apoptosis.

Nonhomologous end joining (NHEJ) represents the major DSB-repair pathway throughout the cell cycle, whereas homologous recombination repair (HRR) is essential for about 15% of ionizing radiation-induced DSBs in S/G2-phase [14]. Although NHEJ repairs about 80% of the DSBs through a fast kinetic process, the role of HRR in slow repair kinetics cannot be ignored. Targeting HRR is an attractive strategy for the radiosensitization of melanoma. Several classical clinically used radio-sensitizers (gemcitabine, nucleoside analogs, etc.) principally operate through inhibiting the homologous recombination process [15].

Besides overexpression, most of the tumors are known to harbor mutations affecting a specific DNA repair pathway, which may result in a hyperdependence on a compensatory DNA repair mechanism [16]. These observations suggest that DNA repair-deficient tumors should exhibit heightened radiosensitivity to inhibitors of such pathways [17]. Hence, identification of defective DNA repair processes is warranted for pharmacological targeting of cancers for better clinical outcomes. A better understanding of the mechanisms that enable melanoma cells to resist ionizing radiation-induced DNA damage might thus improve strategies for melanoma management.

Werner gene (WRN) is epigenetically downregulated in multiple cancers, it is imperative to understand the differential repair pathway and find a therapeutic target for radio-sensitization of WRN-deficient cancers [18]. Pharmacological inhibitors (UCN-01 and SCH90076/MK8776) of checkpoint kinase 1 cause hyper-radiosensitization of WRN-deficient cancer cells of different tissue origin (osteosarcoma, colon adenocarcinoma and melanoma). Thus, pharmacological inhibition of HRR but not NHEJ impairs a compromised, but effective HR process in the WRN-deficient tumor cells and makes them hyper-radiosensitive. Further, targeting HR-pathway by inhibiting p38-MAPK also led to hyper-radio-sensitization of WRN-deficient cancer cells – which makes it an attractive drug target for WRN-deficient cancers [19].

Overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) K has been associated with resistance to radiation therapy in melanoma, head and neck squamous cell carcinoma (HNSCC) and colorectal carcinoma [20,21]. hnRNP K is an important cofactor in the p53-mediated DNA damage response pathway upon ionizing radiation and exerts antiapoptotic effects also independent of p53 pathway activation [21].

Recent reports have suggested that 5-aminolevulinic acid (5-ALA), which is a precursor to protoporphyrin IX (PpIX), leads to selective accumulation of PpIX in tumor cells and acts as a radiosensitizer *in vitro* and *in vivo* in mouse models of melanoma, glioma and colon cancer [22–25]. Since PpIX can enhance ROS generation even in a hypoxic environment and can

induce DNA damage, combined radiation therapy treatment with 5-ALA is expected to improve therapeutic efficacy for radioresistant melanoma [26].

In B16 mouse melanoma, the intracellular biochemical processes that occur after irradiation have been recently reported [27]. ATP is released from B16 melanoma cells after gamma irradiation and the release is mediated by P2X7 receptor and connexin 43, and the inhibition of the P2X7 receptor had a radiosensitizing effect [28–30]. In addition, the adenosine A2B receptor contributes to radioresistance via its effect on DNA damage and could be a new target for the development of agents to increase the efficacy of radiation therapy [27].

The transient receptor potential melastatin 8 channel plays a role in the development and progression of tumors, but it is also involved in radiation-induced DNA damage repair and contributes to radioresistance [31].

In HNSCC, after a whole-genome CRISPR–Cas9 screen following exposure to ionizing radiation as a selective pressure, STING (stimulator of interferon genes) was identified as an intrinsic regulator of tumor cell survival [2▪]. STING regulates a transcriptional program that controls the generation of ROS, and that STING loss alters ROS homeostasis to reduce DNA damage and to cause therapeutic resistance. We hypothesize that this may be a major radiosensitivity determinant in melanoma too.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a neuroprotective factor produced in response to the endoplasmic reticulum stress-induced by various stressors [32]. It was indicated that MANF is released by irradiated melanoma cells, and contributes to radioresistance by promoting the cellular DNA damage response [33].

It is assumed that combining Poly ADP (Adenosine Diphosphate)-Ribose Polymerase (PARP) inhibitors with radiation therapy could be beneficial for cancer treatment. Recently, Jonuscheit *et al.* [34] found that both PARP inhibitors (talazoparib and niraparib) sensitize melanoma cells to ionizing radiation. Healthy tissue seems to be less affected than melanoma cells. However, the great heterogeneity of the results suggests prior testing of the tumor cells to personalize the treatment.

All together, resistance to DNA-damaging agents is a significant cause of treatment failure and poor outcomes in melanoma radiation therapy.

TUMOR MICROENVIRONMENT AS A RADIOSENSITIVITY DETERMINANT IN MELANOMA

The crosstalk between tumor cells and the tumor microenvironment has been regarded as an important factor among the tumor radioresistance factors that make radiation therapy challenging [35,36]. Several vascular, stromal and immunological changes that are induced in the tumor microenvironment by irradiation may promote radioresistance and tumor recurrence [35].

Macrophages can be polarized into antitumoral M1 or protumoral M2 populations depending on tumor-secreted factors, such as IL-4, IL-10 and M-CSF [37]. Unlike the M1 phenotype, the M2 phenotype has been reported to have resistance against γ-irradiation [38]. Therefore, functional inhibition of M2 macrophages in the tumor microenvironment may be an alternative strategy for effective radiation therapy [39].

Melanoma liver metastases co-opt host peripheral tolerance mechanisms to cause acquired immunotherapy resistance through $CD8^+$ T-cell deletion, and the combination of liver-directed radiation therapy and immunotherapy could promote systemic antitumor immunity [40▪]. However, radiation therapy only affects the microenvironment in combination with anti-Programmed death-ligand 1 (PD-L1), radiation therapy alone does not increase CD8+ number and activity. Recent analysis suggests that patients with treated liver metastases have improved time to failure of immune checkpoint blockade (ICB) compared with patients with active liver metastases at the time of ICB [40▪]. This confirms that irradiation of liver metastases may improve response to ICB and sheds light on the importance of liver-metastasis-directed radiotherapy (LRT) and ICB timing [41]. Prospective studies to further investigate the use and timing of LRT and ICB are warranted.

A recent study reports the time course of IFN1 activation *in vitro* and *in vivo* following both external beam radiation therapy and targeted radionuclide therapy in multiple tumor models [42]. The authors observed that IFN1 activation peak can be delayed by a week following radiation and this time course may vary considerably across tumor models. Specifically, they detected an IFN1 activation peak at day 1 following radiation therapy in the MOC2 HNSCC model, and this contrasts with delayed peak activation at day 7 after radiation therapy in the B16 and B78 melanoma models. This activation of IFN1 is dependent on the STING pathway, the time course of IFN1 activation correlates with the one in the kinetics of Cyclic GMP-AMP synthase (cGAS)/STING pathway activation but likely not due to susceptibility or response to DNA damage [42]. Thus, the STING-dependent activation of IFN1 following in-vitro radiation is dose, time and cell-line dependent [42].

A phase 2 clinical trial showed that the addition of stereotactic body radiation therapy (SBRT) to

nivolumab in patients with metastatic or locally advanced inoperable melanoma leads to an objective response rate of 45% without an increase in toxicity [43]. This observed response rate is similar to that noted for nivolumab monotherapy [44]. Thus, in this trial, the null hypothesis that the addition of SBRT does not lead to an increased response rate cannot be rejected [43]. Additional clinical trials investigating the combination treatment are therefore warranted. In patients with melanoma spine metastases, ICIs may enhance survival and tumor control after SBRT [45]. Post-SBRT immune checkpoint inhibitors (ICI) may improve systemic disease control and survival, while pre-SBRT ICI may augment local control [45]. However, in advanced mucosal melanoma patients, a prolonged survival benefit with radiation therapy in combination with ICIs was not observed, although radiation therapy may improve local control of the tumor and relieve local symptoms [46].

Traditionally, the effectiveness of radiation therapy has been attributed to the killing potential of ionizing radiation over melanoma cells; however, it has become clear that the therapeutic efficacy of radiation therapy also involves activation of innate and adaptive antitumor immune responses.

HYPOXIA AND CELL METABOLISM: A NEW LINK TO OVERCOME RADIATION RESISTANCE IN MELANOMA

Hypoxia, a common phenomenon in solid tumors, is a prognostic indicator for radiation therapy outcomes [35]. Hypoxia plays a significant role in radioresistance by inducing pleiotropic cellular adaptions, such as cell metabolism rewiring, epigenetic landscape remodeling and cell death weakening, with significant clinical repercussions [47,48]. In a hypoxic microenvironment, cancer cells switch to alternatives to promote glycolysis and the tricarboxylic acid (TCA) cycle [49]. Several glycolysis intermediates and related factors participate in the molecular mechanisms underlying radioresistance. Hypoxia-inducible factor 1α (HIF-1α) aggravates the transformation of normal melanocytes into melanoma [50]. Lactate dehydrogenase isoform A (LDHA), a key HIF-1α target, catalyzes the reduction of pyruvate to lactate and sustains cell survival under hypoxic conditions by compensating for the reduction in oxidative mitochondrial functions [51]. The HIF-1α/LDHA pathway is involved in tumor-protective responses against radiation therapy [52]. Targeting tumor glucose metabolism and HIF-1α could alter the tumor microenvironment, leading to metabolic alterations and sensitization of multiple solid cancers to radiation therapy [53]. In addition, previous studies have found that the levels of certain glycolysis-related proteins are closely associated with radiation therapy resistance; for instance, higher expression of glucose transporter 1 in cancers indicates poor prognosis of patients treated with radiation therapy [54].

A recent study shows that radiation-resistant cells possessed low glycolysis, mitochondrial respiration and TCA cycle but high glutamine anabolism [55]. Transcriptome analyses revealed that glutamine synthetase, an enzyme catalyzing glutamate and ammonia to glutamine, was responsible for the metabolic alteration. ChIP and luciferase reporter assays revealed that glutamine synthetase could be transcriptionally regulated by STAT5. Knockdown of glutamine synthetase delayed DNA repair, weakened nucleotide metabolism and enhanced radiosensitivity both *in vitro* and *in vivo* [55]. Altogether, these data show that glutamine synthetase links glutamine metabolism to radiation therapy response through fueling nucleotide synthesis and accelerating DNA repair.

The long noncoding RNALINC00518 was confirmed to be an oncogene in melanoma, which induces radioresistance by regulating glycolysis through a miR-33a-3p/HIF-1α negative feedback loop [56]. On the other hand, targeting the lactate/pyruvate metabolism breaks the radioresistance by impairing the stress response [57].

As for chemotherapy, cancer metabolism is an important determinant of cancer radiosensitivity, in that ionizing radiation is cytotoxic via ROS-dependent DNA damage and that mitochondria play a central role in cellular responses to redox stress. In addition, intriguing links between hypoxic signaling and cell metabolism have been recently discovered, which may provide insights into our fundamental understanding of radioresistance in melanoma.

GLUTATHIONE AND REDOX BALANCE ROLE IN MELANOMA RESISTANCE TO RADIOTHERAPY

Cellular radioresistance in numerous healthy and tumor cell lines is mediated by increased glutathione synthesis (GSH) leading to the scavenging of ROS produced by irradiation [58,59]. Intracellular GSH is the major component of nonprotein thiol and acts to combat oxidative stress in normal tissues. In cancerous tissues, different studies suggest that an increase in GSH levels is involved in cellular resistance to radiation therapy and chemotherapy, while the use of thiol-depleting reagents to enhance the radiosensitivity of hypoxic cells has been stimulated recently by several developments [60,61].

While the depletion of endogenous GSH levels is thought to increase the efficacy of treatments with ionizing radiation or chemotherapeutic agents [59,62,63]. However, in cancer tissue, various reports have demonstrated that GSH depletion increases the efficacy of chemotherapy and radiotherapy [60,61].

Studies on substances like rosmarinic acid could help clarify mechanisms allowing protection of healthy normal cells while exclusively injuring neoplastic cells [64]. Potentially, the simultaneous administration of this type of substance along with radiation at the same time could protect healthy cells, while allowing significant damage to melanoma cells [64].

Since the cystine-glutamate antiporter xCT promotes cystine uptake and glutathione biosynthesis, it may confer protection against oxidative stress and ferroptosis [65] and the inhibition of xCT may contribute to optimizing cancer radiation therapy [66]. Recently, mechanistic target of rapamycin (mTOR) inhibition using rapamycin was shown to promote ROS-mediated cell death via functional inhibition of xCT expression in melanoma under γ-irradiation [67].

Another study reported that ROS promotes an invasive phenotype in tumor-associated macrophages (TAMs) extracted from skin cancer (melanoma) through secretion of tumor necrosis factor α [68]. It has been observed that several key mitochondrial genes are highly expressed in TAMs obtained from melanomas, suggesting mitochondrial ROS is the major source of oxidative stress within TAMs. Now, it is very clear that ROS is not only involved in oxidative stress but also important in immune modulation in human malignancies.

However, numerous authors suggest that intracellular GSH level modulation affects both normal and tumor cells, making many selective treatments including radiation therapy ineffective in cancer patients [64,69].

PROTEIN KINASE SIGNALING PATHWAYS ROLE IN MELANOMA RESISTANCE TO RADIOTHERAPY

Molecular classification of melanoma has established four molecular subtypes: BRAF mutant (50%), RAS mutant (28%), NF1 mutant (10–15%) and triple-negative (10%) [70]. The vast majority of these mutations lead to constitutive activation of the MAPK/ERK pathway, which is known to be involved in tumor radioresistance [71]. The prevalence of such mutations opened new therapeutic perspectives so that targeting the MAPK pathway with BRAF and MEK inhibitors dramatically changed the management of metastatic melanoma patients and improved outcomes [72].

The association of targeted therapies and radiation has already shown positive results in preclinical [71,73] and clinical studies [74]. Significantly, the level of cross-resistance between combined MAPK inhibition and radiation therapy is dependent on the treatment sequence [75]. JARID1B/KDM5B (Jumonji AT-rich interactive domain 1B/lysine-specific demethylase 5B) was identified as a cellular marker for cross-resistance between BRAFi and radiation therapy. JARID1B high cells appeared more frequently under upfront BRAFi as compared with upfront radiation. JARID1B favors cell survival by transcriptional regulation of genes controlling cell cycle, DNA repair and cell death [75].

As the resistance of melanoma cells to radiation therapy may very well be in relation with the constitutive activation of MAPK pathway (including RTK, NRAS and BRAF mutations) and/or with the inactivation of p53 observed in about 90% of melanomas [13,76,77], we recently reported that combining MAPK inhibition to p53 reactivation significantly enhances the radiosensitivity of melanoma both *in vitro* and *in vivo* [71].

Centipedegrass extract (CGE) which contains phenolic and C-glycosyl flavone compounds, has been shown to have anti-inflammatory and anticancer effects. CGE extract enhances radiosensitivity in melanoma cells by inducing G2/M cell cycle phase arrest. Radiosensitizing effects of CGE were associated with MAPKs (ERK1/2, p38 and JNK) pathways in ionizing radiation-exposed melanoma cells [78].

Microarray analysis of two melanoma cell lines, SK-Mel5 and SK-Mel28, with different radiosensitivities, was employed to identify target proteins enriched in the more radioresistant cell line, SK-Mel28, among which integrin αvβ3 adhesion molecule as well as Akt, were found to contribute to radioresistance, supporting the therapeutic potential of targeting these molecules [79].

CC-115 is a dual inhibitor of the mTOR kinase and the DNA-dependent protein kinase (DNA-PK) that is currently being studied in phase I/II clinical trials [80]. DNA-PK is essential for the repair of DNA-DSBs. CC-115 showed radiosensitizing potential in seven out of nine melanoma cell lines, but not in healthy skin fibroblasts thus holding promise for an alternative to target repair mechanisms in melanoma [80].

miR-335 is identified as one of the differential expressions of miRNAs in recurrent melanoma biopsies postradiotherapy [81]. miR-335 is upregulated in melanoma, targeted Rho-associated kinase 1 (ROCK1) via direct binding to 3′-UTR of ROCK1,

resulting in reduced proliferation, migration and radioresistance of melanoma cells [81].

Altogether, combining radiation therapy with kinase inhibitors has been postulated to have a promising effect.

MELANOMA PIGMENTATION AND RADIOSENSITIVITY/RESISTANCE

Melanins, particularly eumelanins, possess high free radical scavenging abilities conferring significant radioprotective properties [82] and can attenuate radiation therapy efficacy [82]. In addition, radiation may also activate melanocytes, thereby promoting the formation of melanin and hyperpigmentation [83].

The expression of DOPAchrome tautomerase, also known as tyrosinase-related protein 2, an enzyme involved in eumelanin synthesis, is significantly elevated in radiation-resistant cells [58] probably as a result of the hyperactivation of the MAPK pathway.

Our group previously reported that the degree of pigmentation modulates the radiosensitivity of human melanoma cells [84]. Briefly, we found that a decrease in cell radiosensitivity was correlated with the type of melanin, with a clear involvement of eumelanin rather than pheomelanin. Increasing the intracellular content of both melanins promoted the growth of irradiated cells. Moreover, at a dose of 10 Gy, both tyrosinase activity and melanin cell content were significantly increased in the absence of any other melanogenesis promoter [84]. In another study, we showed that stimulating intracellular eumelanin content is inversely correlated with DNA damage even in a GSH-depleted background. It was concluded that increasing the intracellular eumelanin content by the melanin precursor tyrosine or by favoring the pheomelanin to eumelanin switch, compensates for the loss of the two intracellular radioprotectors that are glutathione and cysteine [85].

Thus, inhibition of eumelanogenesis may improve radiation therapy efficacy.

CONCLUSION

Melanoma is notoriously known as a radioresistant tumor; hence, understanding the underlying mechanisms may lead to new radiosensitizing approaches for the management of this difficult to treat disease. In addition, the same model can be exploited to uncover radioprotective mechanisms and propose radioprotective compounds for healthy tissues. On the other hand, melanoma as a model for intrinsic radioresistance appears ideal to study and propose such strategies. Altogether, the recent discoveries in melanoma radiosensitivity, demonstrate the possibility to use irradiation therapy not only in palliative settings but also in curative settings in combination with targeted therapy and/or immunotherapy.

Acknowledgements

None.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
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