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Review

Epigenetics in radiotherapy: Where are we heading?

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ABSTRACT

Radiotherapy is an important component of anti-cancer treatment. However, not all cancer patients respond to radiotherapy, and with current knowledge clinicians are unable to predict which patients are at high risk of recurrence after radiotherapy. There is therefore an urgent need for biomarkers to guide clinical decision-making.

Although the importance of epigenetic alterations is widely accepted, their application as biomarkers in radiotherapy has not been studied extensively. In addition, it has been suggested that radiotherapy itself introduces epigenetic alterations. As epigenetic alterations can potentially be reversed by drug treatment, they are interesting candidate targets for anticancer therapy or radiotherapy sensitizers. The application of demethylating drugs or histone deacetylase inhibitors to sensitize patients for radiotherapy has been studied *in vitro*, *in vivo* as well as in clinical trials with promising results. This review describes the current knowledge on epigenetics in radiotherapy.

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Background

Although radiotherapy (RT) is an important and effective element of current cancer treatment [1], a subgroup of patients does not respond to RT and has progressive disease or a recurrence shortly after treatment ends. As maximal RT dose on the tumor is determined by normal tissue tolerance, increasing radiation dose is often unfeasible as it also increases adverse effects in healthy tissues [2]. Also other efforts to optimize RT, such as precision in dose delivery and optimization of treatment plans, are not beneficial for some patients and large inter-individual differences in treatment response are observed [3]. Cellular RT response is (partly) dependent on the molecular composition of cells, but there is limited evidence that predictive biomarkers can use this altered molecular composition to predict radiosensitivity [3,4]. The ability to predict RT response would be a valuable asset to physicians for several reasons [5]. At the moment, prediction of RT outcome is based on clinical parameters, such as tumor stage and grade [6]. Combining several clinical characteristics has led to the development of publicly available predictive models for several cancer types (www.predictcancer.org) [7–19]. However, variation in response between patients with identical clinical characteristics indicates that these models can be improved, for example by adding blood-based (e.g. protein), DNA-based/molecular (e.g. epigenetic modifications) or imaging (e.g. hypoxia-imaging) biomarkers [20].

Epigenetic modifications – general introduction

Gene expression can be influenced by many different aberrations. Among the most studied epigenetic aberrations are DNA methylation and histone modifications which have been shown to have a crucial function in carcinogenesis and tumor progression and are considered potential targets for anticancer therapy and/or treatment sensitizers (Fig. 1). Epigenetics is a rapidly growing research field, and several in-depth reviews on epigenetic alterations are available (e.g. [21–27]).

DNA methylation

DNA methylation consists of the introduction of a methyl-group (CH3) at the 5′ position of the cytosine base in the DNA, established by DNA methyltransferases (DNMTs) (Fig. 1). Although global hypomethylation is frequently observed in cancer, the best characterized epigenetic modification in malignant cells is

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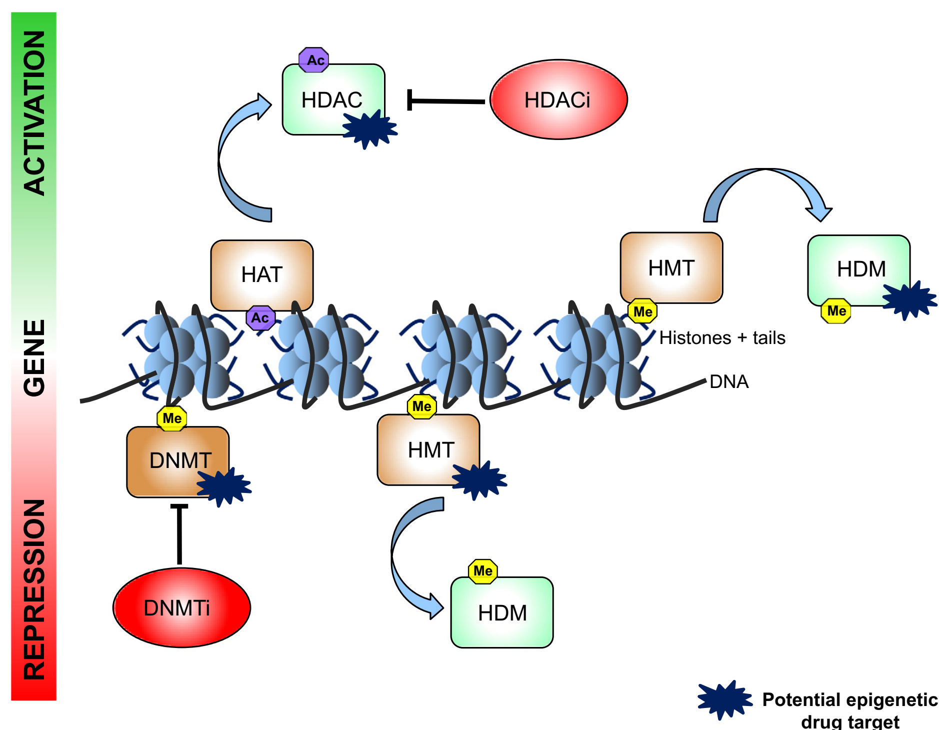

Fig. 1. Epigenetic regulation of gene expression. DNA is stored in the nucleus as chromatin. The building unit of chromatin is the nucleosome, which consists of eight histones (two each of H2A, H2B, H3 and H4) with 147 base pairs of DNA wrapped around it. The histone tails protrude from the nucleosome and are available for post-translational modification. Epigenetic regulation includes several mechanisms including DNA methylation, histone acetylation, and histone methylation that can, depending on the combination of modifications, either lead to gene repression or gene activation. Me: Methylation; Ac: Acetylation; DNMTs: DNA methyltransferases; DNMTi: DNMT inhibitors; HAT: histone acetyltransferases; HDAC: histone deacetylases; HDACi: HDAC inhibitors; HMT: histone methyltransferases; HDM: histone demethylases.

hypermethylation in CpG islands (genomic regions containing high amounts of cytosine bases followed by guanine bases) which are present in 70% of all mammalian promoters [21,22]. However, aberrant methylation has also been found within gene bodies and CpG shores (conserved sequences upstream or downstream of CpG islands) [21,28,29]. Recently, it was recognized that the position of methylation is crucial for the consequences on transcriptional levels [21,30], and the dynamic nature of DNA methylation was confirmed [31,32]. Moreover, other forms of DNA methylation (oxidation derivatives), such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosin (5caC), have been identified, emphasizing the complex nature of these epigenetic alterations.

Our knowledge about the methylome has been greatly expanded by whole-genome approaches and it seems clear that both function and influence on transcription vary with the context. These recent developments reveal complex interactions between different molecular markers but will also improve the use of epigenetic biomarkers in cancer [33,34]. Indeed, recent reports have demonstrated that DNA methylation marks can be found in circulating DNA from prostate cancer patients, and can be used to generate accurate, minimally-invasive epigenetic biomarkers [35].

Histone modifications

Histone modifications can alter electrostatic charges and recruit binding proteins that are frequently part of chromatin remodeling complexes, thereby making the chromatin more or less accessible for other proteins, such as transcription factors. This influences transcription and other DNA-based processes [33]. Currently, 16 types of histone modifications have been detected, the most common ones are acetylation and methylation [33]. To catalyze the addition and removal of histone modifications, several enzymes are involved, e.g. histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Fig. 1). Aberrant histone modification patterns have been reported in several cancer types [36,37], and are suggested as biomarkers of recurrence [38] and survival [39–42].

Cancer genetics and epigenetics have long been considered two separate mechanisms but nowadays it is generally accepted that both act together and take advantage of each other [34]. DNA methylation can be detected and quantified by numerous technologies including genome-wide screening methods as well as locus- or gene-specific high-resolution analysis in different tissue samples and body fluids [43–46] obtained through non-invasive procedures making DNA methylation a very suitable biomarker. O^6-methylguanine methyltransferase (*MGMT*) methylation (to predict treatment response in glioblastoma) and glutathione S-transferase P1 (*GSTP1*) methylation (to detect prostate cancer) are two examples of promising epigenetic biomarkers proving the applicability of DNA methylation as a biomarker [47–50].

Hypoxia – general introduction

Low tumoral oxygen levels (hypoxia) are known to decrease the effectiveness of RT [51,52], however, the precise mechanisms causing radioresistance are not yet completely understood [53,54].

RT causes DNA damage either by direct ionization or indirectly by DNA interaction of radicals formed by ionization of water surrounding DNA [55] resulting in DNA single- or double-strand breaks. Oxygen molecules react with these radicals, thereby changing the chemical composition of DNA strand breaks, causing them to be recognized by enzymes of the DNA damage repair (DDR) pathways [56]. Under hypoxic conditions, radicals undergo a chemical reaction with free protons, restoring their original form. This phenomenon counteracts the fixation of DNA damage and is therefore a major cause of radioresistance [56–58].

Other possible causes of radioresistance under hypoxic conditions can result from the induction of oxygen-sensitive signaling pathways, such as pathways mediated by (1) the hypoxia-inducible factor family of transcription factors (HIFs, and HIF-1 in particular), (2) unfolded protein response (UPR) or, (3) mammalian target of rapamycin (mTOR) pathway [59]. These pathways affect many biological processes, including mitosis, apoptosis and angiogenesis, some of which are known to influence radiosensitivity [55].

First, the HIF pathway is activated during moderate hypoxia activating cellular processes such as angiogenesis and cell survival. The interplay between HIF-1 and radiosensitivity appears to be complex; some HIF-1-mediated effects enhance radioresistance, others enhance radiosensitivity [55].

Second, under hypoxic conditions, the UPR program is activated by endoplasmatic reticulum (ER) stress sensors in the ER membrane (protein kinase-like ER kinase (PERK)/eukaryotic initiation factor 2α (eIF2α), inositol requiring kinase 1 and activating transcription factor 6) leading to inhibition of translation and activation of signaling pathways involved in protein folding [60,61]. Recently, especially eIF2α has been shown to be important for cell survival, and its inhibition was associated with treatment response improvement. eIF2α-deficient cells failed to produce enzymes needed for glutathione synthesis and cysteine uptake leading to elevated reactive oxygen species that are toxic for cells [61]. These results indicate the importance of this pathway in determining treatment response and as a possible target for radiosensitizers [61].

Third, during hypoxia, mTOR activity is reduced preventing the formation of eIF4F translation initiation complex; eventually causing repression of protein synthesis. eIf4E, a limiting factor in the eIF4F complex, has previously been associated with malignancy and poor outcome if overexpressed. Paradoxically, this overexpression is also associated with increased sensitivity to hypoxia-induced cell death and RT *in vitro* and *in vivo*, possibly due to a co-overexpression of 4E-BP1 [62].

Epigenetics in radiotherapy

Despite promising results in other cancer types [21,49,63–67], the usefulness of epigenetic alterations (single marker, marker panels or pathways) as potential prognostic or predictive biomarkers in RT has not been studied extensively (Table 1); available evidence on the connection between epigenetics and radiosensitivity is scarce and mostly based on *in vitro* or *in vivo* data [6,68].

Molecular markers

A well-known example of a predictive epigenetic biomarker is *MGMT* methylation in glioblastoma [48,49,65]. *MGMT* encodes for a DNA repair enzyme that counteracts the effect of alkylating treatment by removing alkyl groups from guanine. Moreover, hydroxyl radicals that are generated by radiation induce *MGMT* expression [69]. Patients with a methylated *MGMT* promoter have a better survival following adjuvant chemotherapy or RT [70–72]. A recent meta-analysis reports hazard ratios of approximately 0.7 for patients with high-grade glioma receiving RT after surgery. As no survival effect was observed in patients treated with surgery alone, *MGMT* is probably a predictive biomarker, not a prognostic [70]. In other solid tumors, *MGMT* methylation has been studied with varying results. In some tumors, such as cervix, *MGMT* methylation seems to be associated with poor prognosis after (chemo)radiotherapy [63,73–77] whereas no influence on RT outcome was found for other cancers [78]. Although evidence for *MGMT* methylation as a predictive biomarker seems convincing, the optimal method to assess *MGMT* methylation has not been established yet and the Level of Evidence is insufficient (below Ia; the level corresponding to evidence from meta-analyses and randomized clinical trials) for its use in current routine clinical practice [47,79].

In addition to *MGMT* as a possible methylation biomarker in RT, several other markers have been suggested to have a prognostic role. However, evidence is scarce, results often inconsistent, and clinical studies in patient populations are often lacking. For these reasons, none of these markers is expected to be useful in routine clinical practice in the near future. Ataxia Telangiectasia Mutated (*ATM*) [80,81] methylation has been associated with increased radiosensitivity *in vitro* [82], but expression is highly heterogeneous and its contribution to radiosensitivity remains questionable [83,84]. The impact of *ATM* methylation on toxicity has not been studied yet [85]. In addition, some evidence suggests false-positive incidence results for *ATM* methylation due to non-specific primer design [86]. Other single methylation markers suggested to be associated with poor RT response include *RPRM* (esophageal cancer patients) [87], *RUNX3* (esophageal cancer cell lines) [88], *TP73* (cervical cancer patients) [89] and *BRCA1* (cervical cancer patients) [73]. In contrast, *TIMP3* and *CDH1* methylation was associated with a better response to RT in head and neck squamous cell carcinoma

Table 1
Epigenetic modifications associated with radiotherapy (response).

RT sensitivity	RT resistance		RT induced alterations
DNA hypermethylation			*DNA hypermethylation*
TIMP3 [90]	BRCA1 [73]		CDKN2A [121]
CDH1 [90]	S100A6 [83]		SKOR2 [122]
	RUNX3 [87,88]		IRX1 [122]
	CDKN2A [87]		EBF3 [122]
	RPRM [87]		SLC5A8 [122]
	CDKN1C [87]		SEPT9 [122]
	TP73 [87,89]		ADAMTS9 [120]
	CHFR [87]	Radiotherapy (RT)	*DNA hypomethylation*
	MGMT [63,70–78,87]		Global [112–114]
	TIMP3 [87]		FOXC1 [120]
	HPP1 [87]		TRAPPC9 [120]
	SERPINB5 [83]		AMIGO3 [120]
DNA hypomethylation			*Loss of expression*
	CAT [83]		DNMT1, 3a and 3b [112–114]
	BNCI [83]		MBD2 [112–114]
Pathways			MeCP2 [112–114]
	PTEN/pAkt/p53 [94–96]		
	NRF2-Keap [92]		

patients [90]. Some of these single markers have been studied within a marker panel (*CDKN2A*, *RPRM*, *CDKN1C*, *TP73*, *RUNX3*, *CHFR*, *MGMT*, *TIMP3* and *HPP1*) showing decreased methylation in RT responsive esophageal cancer patients [87]. Although these single-marker studies indicate a role of specific epigenetic biomarkers in determining RT response, the prognostic value of these markers has not been validated in large patient populations yet, making them inappropriate for clinical use at the moment.

Only one study has been published using a non-candidate marker approach. In this study, the RT-resistant non-small cell lung cancer H1299 cell line showed a higher proportion of hypermethylation (18.7%) as compared to the RT-sensitive H460 cell line (15.9%), 1091 genes were identified as differentially methylated genes, of which 747 genes were hypermethylated and 344 hypomethylated in H1299. Hypermethylated genes were involved in multiple processes, such as cell–cell communication and signal transduction, while hypomethylated genes were mostly involved in transcriptional regulation. Four genes with the most significant differences in methylation between cell lines were studied more extensively, suggesting a role for *SERPINB5* and *S100A6* hypermethylation and *CAT* and *BNCI* hypomethylation in radioresistance. These results suggest that RT response is highly dependent on overall methylation profile of the patient's tumor [83]. Although the results of this study indicate that epigenetic alterations (prior to RT) might be associated with radioresistance, results are derived from *in vitro* studies, have not been validated in patients yet. In addition, the correlation between methylation and expression has not been studied. It remains uncertain whether observed differences between cell lines are indeed related to radiosensitivity, and not merely the result of comparing two different cell lines.

Molecular pathways

A recent review indicated that the majority of hypermethylated genes related to RT or chemotherapy outcome, were involved in DDR, WNT-signaling or PI3K/MAPK signaling [68]. Each of these can be activated through many mechanisms, including aberrant DNA methylation, point or structural mutations or inactivation of pathway negative regulators. Nevertheless it can be questioned whether single-gene modifications alone can be responsible for radiosensitivity [91]. Probably, (several) disrupted pathways determine a radiosensitive or -resistant phenotype. Following this hypothesis, several studies investigated dysregulation of specific pathways due to epigenetic events, and their influence on RT response.

In prostate cancer cell lines, the Nrf2-Keap1 pathway, which is activated upon UPR [61], has been reported to be deregulated due to Keap1 inactivation by loss-of-function mutations or promoter CpG hypermethylation, leading to increased Nrf2 expression. Inhibition of the Nrf2-Keap1 pathway has been shown to sensitize radioresistant DU-145 cells [92]. Radioresistant and doxorubicin-resistant MCF-7 breast cancer cells (MCF-7/DOX) show lower overall methylation levels as compared to radiosensitive MCF-7 cells. Treatment with methyl-donor S-adenosyl methionine (SAM) resulted in increased radiosensitivity. In contrast, SAM treatment led to a decreased radiosensitivity in MCF-7 cells, possibly due to hypermethylation of specific genes. These results emphasize the fine balance between overall methylation levels, methylation of specific genes and the subsequent radiosensitivity [93]. In oral squamous cell carcinoma, a gene-dosage effect was reported in patients treated with surgery and adjuvant RT; patients had a poorer disease-free survival with increasing activity of the Ras/PI3K/AKT pathway [94]. Activation of this pathway has been associated with radioresistance *in vitro* and *in vivo* [95,96]. The results of these studies clearly indicate that specific pathways (partly) regulated by epigenetic events, can have an effect on radiosensitivity. The clinical implications of these findings are however less clear at the moment, as it is difficult to manipulate specific pathways in order to improve radiosensitivity.

Despite increasing knowledge on other epigenetic events as histone modifications and micro-RNAs, these phenomena have not yet been assessed for their role in determining RT response.

Hypoxia and epigenetics

Recent literature describes four views on the extensive interaction between epigenetics and hypoxia [97]. First, expression of *von Hippel-Lindau (VHL)* and *prolyl 4-hydroxylase domain protein 3* (*PHD3*), two genes that are responsible for the ubiquitination and degradation of HIF in the presence of oxygen, are epigenetically regulated. Silencing of these genes can lead to the formation of transcriptionally active HIF under normoxia.

Second, epigenetic mechanisms maintain a transcriptionally active chromatin confirmation around HIF binding site regions, either through HIF-1α co-activation or through direct modifications of binding sites, thereby regulating HIF binding. During the initial hypoxic response, epigenetic modifying enzymes, e.g. histone acetyltransferase enzyme CBP/p300, are in direct contact with HIF-1α participating in the co-activation of hypoxia-inducible genes [97]. SRC-1 and TIF2, both members of the HIF-1α co-activation complex were also found to have HAT activity [98]. (Functional) DNA methylation within consensus hypoxia response elements has been reported, and it has been shown that hypoxia-induced expression depends on the tissue-specific methylation status [99–101]. As global methylation changes may result from chronic hypoxic conditions, the HIF-dependent transcriptional profile may be determined by the intensity and duration of hypoxia [97].

Third, several histone demethylase genes, including some of the Jumonji family (induced as a consequence of HIF binding during hypoxia) are direct HIF-1 target genes [102]. These findings reveal the possibility of direct hypoxic regulation of histone demethylases, resulting in both active and inactive chromatin states.

Finally, global changes in histone modifications and DNA methylation are observed in response to hypoxia, resulting in transcriptional activation or repression. For example, hypoxia has been shown to increase H3K4me3 (an active mark), decrease H3K27me3 (a repressive mark) and increase H3K27me3 (a repressive mark) [103]. Exposure to anoxia has been described to induce a 15–20% reduction in DNA methylation [104]. These latter findings indicate that epigenetics has a further important role in the adaptation and survival of cells that is not solely dependent on interaction with HIF. As hypoxia is regarded a major component of determining RT response, growing knowledge on the influence of epigenetics in the hypoxic response emphasizes the importance of epigenetics in RT.

Radiation as a cause of epigenetic alterations

RT effects on genetic alterations (or vice versa) have been studied extensively [105–111]. The effects on epigenetic alterations however, have been studied less often [1] even though these alterations potentially lead to changes in transcriptional activity and thereby to altered cellular resistance to radiation [112] (Fig. 2, Table 1).

RT causes global hypomethylation *in vitro* and *in vivo*, possibly due to a decreased expression of DNMT1, DNMT3a/3b, MeCP2 and MBD2 [112–114]. This effect seems more pronounced after fractionated RT, appears sex- and tissue-specific [115] and is persistent, even after repair of radiation-induced DNA damage [113,116–119]. As global hypomethylation has been linked to

malignant transformations, radiation-induced DNA hypomethylation may be a marker of oncogenic transformation [116,119]. In addition to global changes, locus-specific alterations, hypo- as well as hypermethylation, have also been reported after radiation [120–122]. An increase in methylation was observed in *ADAMTS9*, *FOXC1*, *TRAPPC9* [120] and *CDKN2A* [121] whereas *AMIGO3* [120] showed a decrease in methylation. Interestingly, *in vitro* studies show that after a recovery period in which cells overcome the radiation-induced growth-arrest, *FOXC1* and *TRAPPC9* showed a significant methylation loss compared to mock-treated cells. As both genes are involved in apoptosis, this hypomethylation might indicate reduced apoptotic signaling resulting in regrowth of cells after radiation [120].

Other locus-specific methylation changes after radiation have been reported in *SKOR2*, *IRX1*, *EBF3*, *SLC5A8* and *SEPT9* [122].

Although these results indicate global and locus-specific changes in DNA methylation after radiation, it was only recently revealed that these alterations were indeed enriched in pathways directly involved in radiation responses such as cell cycle regulation, DDR and apoptosis. A recent study using a more epigenome-wide approach identified 15 genes and 23 genes that were differentially methylated after radiation with 2 and 6 Gy respectively. Strikingly, overall methylation patterns appeared to be dose-dependent; cell cycle pathways tended to be hypermethylated directly after 2 Gy radiation, but hypomethylated at later time points. These patterns were opposite after 6 Gy radiation and in this case, the moment of switch from hypo- to hypermethylation was associated with a significant arrest in the G_2 phase of the cell cycle. This suggests a direct correlation between methylation patterns and the biological response to radiation, and implies that epigenetic alterations after radiation are not random. Similarly, a higher radiation dose was associated with higher hypermethylation in apoptosis pathways and an increased senescence-like phenotype. Dose-dependent differences in methylation were however not seen for genes involved in DDR; hypomethylation was the predominant alteration after both low- and high-dose radiation, particularly in NER, HR and NHEJ pathways [112].

As radioresistance has especially been reported in cancer stem cells [123], radiation-induced methylation levels in these cells seem particularly interesting. However, a recent study on mouse embryonic stem cells did not show any changes in DNA methylation levels after radiation [124]. Epigenetic alterations were also observed in offspring of mice exposed to radiation [125,126] indicating that they might be transmitted through the germline, leading to genomic destabilization, and a possible precursor for carcinogenesis [125].

The use of epigenetic drugs

Based on the previously described results, epigenetic alterations may be considered as potential targets for radiosensitization. This may be achieved by the regulation of chromatin structure modifications, or by epigenetic manipulation of genes involved in cell cycle, apoptosis or DNA repair. Over the past years, many epigenetic drugs have been studied *in vitro* or *in vivo*, but with varying results.

HDAC inhibitors

HDACs remove acetyl groups on histone tails and influence the interaction between DNA molecules, histone proteins and chromatin-associated complexes, resulting in the formation of heterochromatin and transcriptional deactivation. The FDA approved two HDAC inhibitors (HDACi); vorinostat (also known as suberoylanilide hydroxamic acid; SAHA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma [127,128]. Additionally, valproic acid (VPA) was FDA approved for the treatment of epilepsy and other seizure disorders, bipolar disorders, anxiety disorders, schizophrenia and migraine headaches [129].

HDAC inhibitors can be divided in four structural classes (short-chain fatty acids, hydroxamic acids, cyclic peptides and benzamides) and affect mostly HDAC class I or II. HDACi predominantly act by inducing differentiation, apoptosis and cell cycle arrest with a preferred cytotoxicity for tumor cells [130]. For all HDACi, radiosensitizing effects have been reported that may either be explained by chromatin conformation or a decreased repair capacity for double-strand breaks [129].

Several studies have suggested that VPA causes radiosensitization *in vitro* and *in vivo* [131,132], and clinical trials are currently ongoing. Histone tails in euchromatin undergo hyperacetylation and hypermethylation after VPA treatment, leading to decondensation of these compartments and to an increased number of sites for

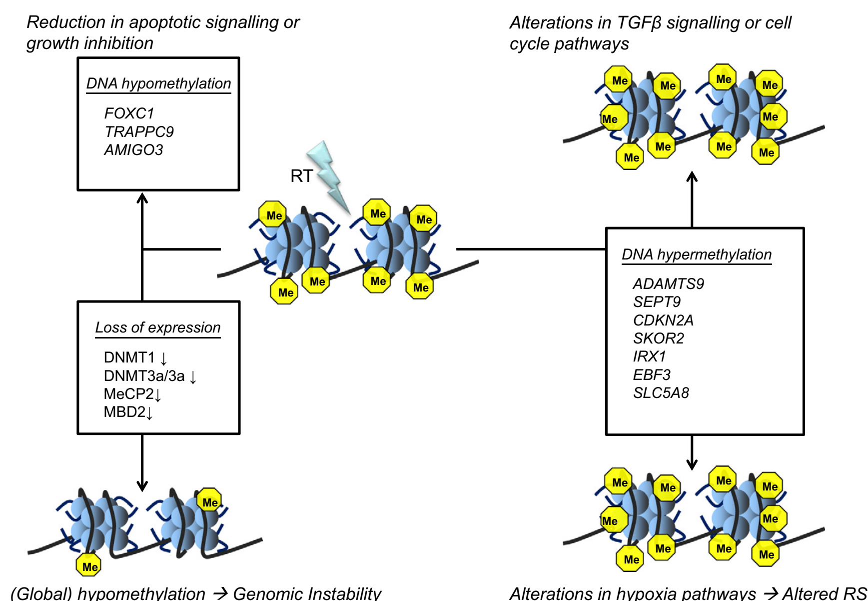

Fig. 2. Radiotherapy-induced epigenetic alterations.

radiation-induced DNA damage [132]. However, histone-acetylation and methylation-independent effects are likely to also contribute to radiosensitization [132]. Recent experiments suggest that the radiosensitizing effect of VPA is limited to differentiated cancer cells; in cells expressing cancer stem cell features, VPA even had radioprotective properties [133,134]. Psammaplin A, a natural marine product with cytotoxic effects in several cancer cell lines, also has HDAC activity and enhances radiosensitivity in cell lines possibly by an increase in radiation-induced apoptosis [135]. Other radiosensitizing HDACi include sodium butyrate (NaB), phenylbutyrate, tributyrin, MS275, PCI-24781, AR-42, LBH589 and trichostatin A. These agents sensitize radioresistant cell lines originating from melanoma [136,137], glioma/glioblastoma [138,139], squamous cell [140–142], prostate [138], colon [143,144], cervical [144], breast [145] and hepatocellular carcinoma [146]. However, some HDACis (e.g. SAHA, MS275 and NaB) induce a reduction in double-strand break repair capacity in human fibroblasts obtained from healthy skin emphasizing their potential long-term hazards [129].

In contrast, HDACi have been suggested as radioprotectors for normal tissue when administered topically, possibly due to a decrease in tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β [147,148]. Although evidence is limited, it indicates that HDACi might be used as a radioprotector under specific conditions [149].

DNMT inhibitors

As depletion of DNMTs results in global demethylation [150], different DNMT inhibitors (DNMTi) have been studied as radiosensitizers [128]. Two, 5-aza-cytidine (AZA; azacitidine) and 5-Aza-2′-deoxycytidine (DAC; decitabine), have been FDA-approved for treating myelodysplastic syndromes, acute myeloid leukemia and other myeloid syndromes in adults. DNMTi are nucleoside analogs that are incorporated during the S-phase and that irreversibly bind DNMTs to DNA thereby inhibiting them [128]. Because of this, DNMTs are depleted and genes silenced by methylation can be re-expressed. DNMTi are hypothesized to influence radiosensitivity through several mechanisms. First, as DNA synthesis inhibitors, DNMTi can inhibit the repair of RT-induced DNA damage. In addition, they may reduce the number of tumor clonogens by a preferential cytotoxicity to proliferative cells and slow down cell repopulation during RT. Finally, they may trigger apoptosis [2].

Increased radiosensitivity after AZA was observed *in vitro* for several colorectal [91] and gastric cancer cell lines [151]. Removing AZA returned radiation sensitivity to previous levels, except for HCT116 cells. HCT116 cells deficient in *DNMT3b* and double-knock-out HCT116 cells deficient for *DNMT1* and *DNMT3b* showed a trend toward increased RT sensitivity, but this was not seen in cells deficient for *DNMT1* alone. As methylation patterns between these cell lines differ, this may indicate differentially active methylation-regulated genes associated with radiosensitivity [91]. However, not all *in vitro* studies show radiosensitizing effects of AZA [135].

DAC treatment has been associated with increased radiosensitivity *in vitro* as well, but there was no association between drug-induced epigenetic alterations and radiosensitization. This could indicate that DAC works through mechanisms other than demethylation to influence radiosensitivity [152]. As DAC and AZA are relatively toxic to normal cells, unstable in aqueous solutions and cannot be taken orally, other (less toxic) DNMTi, such as zebularine and 5-fluoro-2′-deoxycytidine have been developed [128] that have also been associated with radiosensitivity [2,135,153].

Despite promising results, caution should be exercised. Most results were generated in *in vitro* studies and should be validated *in vivo*. Moreover, the effects of DNMTi combined with RT in healthy tissue have not been assessed and there is conflicting evidence on the (long-term) safety of DNMTi. Demethylating agents also lead to normal tissue hypomethylation and there are indications that this effect can influence radiation-enhanced bystander effects, secondary tumor risk and reactivation of silenced viruses such as Epstein-Barr [91,154]. *In vitro* and *in vivo* studies suggest increased mutation frequencies, chromosomal rearrangements, decreased fertility and loss of offspring after azacitidine or decitabine [2]. Interestingly, normal cells seem to have reduced incorporation of epigenetic drugs relative to cancer cells, likely due to their slower dividing rate. This may imply that even low doses will have clinically relevant effects [2,151]. In an attempt to overcome some of the problems of nucleoside DNMT inhibitors, non-nucleoside inhibitors of DNMTs have recently been developed directly binding to DNMT without being incorporated into the DNA. These compounds have demethylating activities *in vitro* and *in vivo* and, for some agents, clinical trials are underway [153].

Future studies, *in vivo* as well as clinical studies are needed to evaluate multiple remaining problems. The optimal treatment schedule for either DNMTi or HDACi has not been established yet. Several schemes are used in studies and therefore it is difficult to compare results. It has been suggested that combining HDACi and DNMTi is more effective in increasing radiosensitivity as both DNA methylation and histone acetylation are inhibited [150,155]. Even though this approach has been studied in non-RT settings, studies evaluating a combination of epigenetic drugs as radiosensitizers are scarce [135,155,156]. Further studies need to elucidate the biological mechanisms of radiosensitivity caused by HDACi and DNMTi in order to select the most effective radiosensitizer (or perhaps combinations thereof).

The use of epigenetic biomarkers in radiotherapy: future considerations

Despite growing interest and the growing evidence of an actual link between epigenetic alterations and RT response, there are currently no epigenetic biomarkers for RT response ready for use in daily clinical practice, or even ready for extensive clinical testing. An increasing number of studies focus on epigenetic drugs, either DNMTi or HDACi, as radiosensitizers but it is not known which agent, or which combination is most effective and which dose regimen should be used. Studies evaluating RT-induced normal tissue toxicity after DNMTi or HDACi are scarce. Epigenetic drugs might be effective at low doses only, but these regimens have not been thoroughly tested and the mechanisms of action for many compounds remain unclear [157,158]. Further, given the extensive interaction between hypoxia and epigenetics, the microenvironment might be crucial in determining the success of epigenetic drugs. Recent studies suggest an increased efficacy of HDACi and DNMTi under hypoxic conditions [159–162], thereby emphasizing the need to consider the tumor's microenvironment when studying epigenetic drug effects.

Although epigenetic biomarkers encounter some of the same problems as compared to general biomarker studies, they are also confronted with several specific challenges.

In general, similar to genetic biomarker studies, epigenetic biomarker studies are often small and lack validation [6,163]. Single-gene approaches disregard the highly multifactorial nature of radiosensitivity and candidate-marker studies might miss unknown, but highly relevant, biomarkers [6]. Recent technological developments, such as epigenome-wide sequencing, may overcome these problems [64] as no prior assumptions are made on which genes should be studied [6] and several techniques are currently available (for an extensive review, see Ref. [164]), each with

their corresponding (dis)advantages in terms of costs and covered genomic regions. Without exception, all available whole-genome methylation analyses methods require extensive bioinformatic analysis; a growing number of tools for this are being developed [164,165] but well-qualified and experienced bio-informaticians are crucial to distinguish useful patterns from noise. Indeed, major analytical questions remain unsolved, including the best way to handle heterogeneity in tumor cellularity [166] or to integrate diverse data-normalization protocols [167].

As the probability of false-positive findings is high, genome-wide studies need extensive validation and careful experimental design – including up-front power-analyses. For example, methylation profiles are strongly confounded with age, making properly controlled cohorts of particular importance. Internal validation of the utilized sequencing approach and corresponding computational methods is self-explanatory, and is incorporated in most studies. Validation of identified genes or signatures in large, independent study populations, and (if possible) in randomized clinical trials has also been accepted as crucial [165], but the correct protocols remain controversial even in the better-characterized field of mRNA-based biomarkers [168,169]. But studying the biological role of a potential biomarker *in vitro* and *in vivo* should also be an important part of biomarker development before use in patient care [64].

A structured approach to identify clinically relevant markers and constructing the most relevant prediction model, as well as the use of whole-epigenome sequencing techniques, might overcome some of the previous, more general problems current studies are facing. But other problems remain. Studies aiming to enhance radiation response by studying epigenetic alterations prediction also encounter several specific challenges. To obtain the most relevant prediction model, not only clinical or epigenetic predictors should be included, but genomic, proteomic and imaging biomarkers should be included as well. As different alterations reflect different tumor development or progression pathways, biomarker signatures monitoring all these different aspects may be more accurate than signatures focusing on one biomarker type exclusively.

It is becoming increasingly clear that methylation within promoter CpG islands is not random, only methylation of specific loci (core regions) can be regarded as functional, *i.e.* crucial for transcriptional repression [170]. Identifying the core regions regulating gene expression is therefore crucial when studying the clinical value of DNA methylation.

Along this line, the location of the primers used to measure DNA methylation seems critical for the final conclusions of a study [64,170]. Optimal primer design to ensure evaluation of the most relevant core regions is therefore one of the most important tasks when designing epigenetic biomarker studies that aim to obtain reliable clinical results. Core regions have also been observed outside the transcription start site region suggesting that larger regions should be evaluated when studying the methylation status of genes [170]. In addition, relevant methylation can occur in regions with low CpG density, CpG island shores [171] and gene clusters can become silenced by long-range epigenetic silencing. This global gene silencing can simultaneously inactivate large regions of the genome [170,172]. These recent discoveries indicate that the mechanism of gene silencing by DNA methylation is much more complex than initially thought and genes previously disregarded as potential epigenetic biomarkers may in fact be relevant when taking the location of methylation into account.

Recent discoveries of alterations such as 5hmC, 5fC and 5caC add another layer of complexity as current techniques cannot easily distinguish between different cytosine modifications. Nevertheless, epigenetic biomarkers could act as important complementary markers that, combined with clinical variables, blood-based and imaging biomarkers, can greatly improve prediction models for RT.

Conclusion

Despite the increasing number of studies describing an association between DNA methylation and RT response, many questions remain unanswered. There is currently no DNA methylation marker, or marker panel, that can predict RT response. Other epigenetic markers, such as histone modifications and miRNAs have not yet been evaluated for their influence on RT response. In addition, radiation can also cause epigenetic alterations. Although several studies have reported this, the clinical impact (if any) of this observation is not clear. It can be hypothesized that RT-induced epigenetic aberrations influence treatment outcome and should therefore be monitored. Even though underlying biological mechanisms have not been elucidated yet, a growing number of studies focused on clinical applicability of epigenetic drugs as anticancer treatments or radio sensitizers. None of these drugs have been approved for this application yet but results seem promising and it may only be a matter of time before the first epigenetic radiosensitizer is introduced.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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