

Review

Isabelle R. Miousse, Julia Tobacyk, Stepan Melnyk, S. Jill James, Amrita K. Cheema, Marjan Boerma, Martin Hauer-Jensen and Igor Koturbash*

One-carbon metabolism and ionizing radiation: a multifaceted interaction

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Abstract: Ionizing radiation (IR) is a ubiquitous component of our environment and an important tool in research and medical treatment. At the same time, IR is a potent genotoxic and epigenotoxic stressor, exposure to which may lead to negative health outcomes. While the genotoxicity is well described and characterized, the epigenetic effects of exposure to IR and their mechanisms remain under-investigated. In this conceptual review, we propose the IR-induced changes to one-carbon metabolism as prerequisites to alterations in the cellular epigenome. We also provide evidence from both experimental and clinical studies describing the interactions between IR and one-carbon metabolism. We further discuss the potential for the manipulation of the one-carbon metabolism in clinical applications for the purpose of normal tissue protection and for increasing the radiosensitivity of cancerous cells.

Keywords: DNA methylation; epigenetics; ionizing radiation; methionine; methyl donors.

*Corresponding author: Igor Koturbash, Department of Environmental and Occupational Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA, e-mail: ikoturbash@uams.edu

Isabelle R. Miousse: Department of Environmental and Occupational Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Julia Tobacyk: Departments of Environmental and Occupational Health, and Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Stepan Melnyk and S. Jill James: Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Amrita K. Cheema: Departments of Oncology and Biochemistry, Molecular and Cellular Biology, Georgetown University Medical Center, Washington DC 20057, USA

Marjan Boerma and Martin Hauer-Jensen: Division of Radiation Health, Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Introduction: ionizing radiation and epigenetics

Ionizing radiation (IR) is a ubiquitous environmental stressor and a widely-used tool in many spheres of human life. One of the largest sources of IR exposure comes from medical radiation when utilized as a diagnostic and treatment modality. Approximately 50% of all cancer patients receive radiotherapy and over 70 million computed tomography (CT) scans are performed annually in the US alone (1, 2), creating an ever-growing number of patients who are routinely exposed.

While it is generally accepted that the benefits of medical radiation outweigh the risks, there is considerable concern about unintended side-effects, as exposure to IR may result in a number of negative outcomes, including the development of cancer and degenerative diseases (3–5). Radiation-induced genomic instability and carcinogenesis are stochastic effects, where there appears to be no threshold dose and the risk of these effects increases with increasing dose. In addition, exposure to IR can have deterministic effects, short-term and long-term injury in normal (non-tumor) tissues for which, there appears to be a threshold dose below which, these effects do not occur (6). Normal tissue radiation injury can vary from acute radiation syndrome that is seen after large parts of the body have been exposed to relatively high doses of IR, typically within a few minutes (7, 8), to early and late injury and adverse remodeling in tissues that are exposed to IR during radiotherapy. Side effects of radiotherapy include skin erythema after breast cancer treatment (9), radiation enteropathy due to exposure of the intestinal tract in abdominal radiotherapy (10) and fibrosis in the lung and heart that may develop several years after thoracic radiotherapy (11, 12).

It is now generally accepted that both genotoxic and epigenotoxic properties of IR underline the mechanisms of those effects. At the same time, while the ability of IR to damage DNA (genotoxicity) is a well-known and well-characterized phenomenon, epigenetic effects (or those

that are not related to alterations in DNA sequence) of exposure were discovered relatively recently and are not well understood.

Epigenetics is the study of heritable changes in gene expression that are not associated with alterations in the primary DNA sequence. The epigenetic mechanisms of regulation include methylation of DNA, post-translational histone modifications, nucleosome positioning along DNA and non-coding RNAs. These mechanisms are vital for normal development and maintenance of cellular homeostasis. Specifically, DNA and histone methylation regulate the expression of genetic information in a cell, tissue and sex-specific manner (13, 14). They also play critical roles in controlling the expression of repetitive elements (RE) – transposable elements and satellite DNA – that together comprise over half of the mammalian genomes (15).

Alterations in DNA and/or histone methylation may substantially affect the cellular epigenome, leading to altered gene and RE expression and resulting in genomic instability and the development of pathological states, including cancer. In fact, loss of global DNA methylation was the first epigenetic alteration reported in virtually all human cancers (16, 17). Later, DNA hypermethylation at the promoter regions of tumor-suppressor genes was also reported in various cancers (18–20). Further studies have demonstrated that epigenetic alterations, primarily changes in DNA and histone methylation, are not simply the consequences of cancer but may often serve as the drivers of carcinogenesis and can be detected in early stages of cancer development (21–25).

Detection of epigenetic alterations in tumors associated with occupational exposure to radiation suggested that epigenetics may also contribute to IR-induced carcinogenesis. For instance, hypermethylation of *p16INK4a* (26) and *GATA5* (27) genes was observed in lung adenocarcinomas of occupationally exposed workers in comparison with adenocarcinomas from the cohort of non-exposed patients. Further studies using experimental rodent models have convincingly demonstrated that IR affects DNA and histone methylation in the target organs, such as bone marrow, thymus and spleen (28). The majority of the existing literature in the field indicates that exposures to doses of IR 1 Gy and above, are usually characterized by the loss of global DNA methylation in these organs (29–32). Subsequent studies demonstrated that the observed changes in DNA methylation stem primarily from RE, while the gene-specific alterations seem to be less obvious (33–36).

Less is known about the effects of IR on histone methylation, however, these changes are primarily characterized

by the loss of histone methylation from hours to several days after irradiation, similar to the effects on DNA methylation. For instance, histone marks that are responsible for the formation of transcriptionally silent heterochromatin structure – histone H3 lysine 9 (H3K9me3) and histone H4 lysine 20 (H4K20me3) trimethylation – are negatively regulated after exposure to both low and high-dose IR (30, 37). This relaxed chromatin structure may allow for easier access of repair complexes to the sites of DNA damage. At the same time, histone methylation is generally more labile than methylation of DNA and often changes observed shortly after irradiation are not detectable at later time-points (37).

Radiation-induced alterations in DNA and histone methylation

Despite significant progress in radiation epigenetics in the past decade, the mechanisms of radiation-induced changes in DNA and histone methylation remain largely unknown. A number of hypotheses have been proposed, suggesting different mechanisms including the affected function of DNA and histone methyltransferases, interference of DNA damage with the ability of DNA methyltransferases to methylate DNA, DNA damage and repair and radiation-induced proliferation, to name a few (reviewed in ref. 28).

DNA and histone methyltransferases are the key enzymes needed for methylation of the two abovementioned substrates. While there are a limited number of DNA methyltransferases (DNMT) which are primarily represented by the predominantly maintenance DNA methyltransferase DNMT1 and *de novo* methyltransferases Dnmt3a and Dnmt3b, histone methylation at different residues are facilitated by specific histone methyltransferases.

IR has been shown to affect mRNA and protein levels of DNA methyltransferases, as well as their enzymatic activity. In particular, the levels of the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* were found to be decreased 3 months after total body irradiation to low absorbed mean doses of either heavy ions (^{56}Fe) or protons in the mouse model (38). Similarly, exposure to low-dose X-rays resulted in simultaneous decreased protein levels of Dnmt1, Dnmt3a and Dnmt3b in the murine thymus (30). Interestingly, in the same study, the authors have also demonstrated the IR-induced loss in histone H4 lysine 20 trimethylation (H4K20me₃), however, the status of methylases and demethylases specific to this histone mark was not assessed.

In cell lines, nuclear DNA methyltransferase activity was found to be decreased up to 3 days after exposure to

10 Gy of γ -rays (39). At the same time, there was a parallel increase in DNA methyltransferase cytoplasmic activity. Sequestration of Dnmt1 in the cytoplasm in its active form may substantially contribute to IR-induced DNA hypomethylation (40).

In addition to affecting methyltransferases, accumulating evidence suggests that IR also affects the availability of methyl donors (38, 41). Both DNA and histone methylation require the donation of a CH_3 group from the universal methyl donor S-adenosylmethionine (SAM). Addition of those methyl groups modifies how proteins interact with a section of DNA or chromatin and in turn, influence the level of expression of that region. Any impact on the availability of methyl donors and in the enzymes responsible for their metabolism will consequently affect the level of DNA and histone methylation. Those methyl groups used for DNA and histone methylation originate from one-carbon metabolism.

One-carbon metabolism

The reactions surrounding the transfer of the methyl group from SAM to the acceptor molecules and the regeneration of SAM are the key components of folate-dependent one-carbon metabolism (Figure 1). The latter ties together gene regulation, amino acid synthesis, purine and pyrimidine synthesis, four vitamins and antioxidants, to name a few and over a hundred of biomethylation reactions (42). The ramifications of changes in that pathway affects nearly all cellular functions.

The direct precursor of SAM is the essential amino acid methionine. The adenylation reaction is catalyzed by the

enzyme methionine adenosyltransferase (MAT, Figure 1A). There are two forms of this enzyme: the hepatic form MAT1A and the ubiquitously expressed extrahepatic form MAT2A. SAM is utilized as a substrate for dozens of methyltransferases, including DNA methyltransferases (Figure 1B), yielding S-adenosylhomocysteine (SAH). In turn, SAH exerts substrate inhibition on methyltransferases because they have higher affinity to SAH compared to SAM (43, 44). SAH is further converted to homocysteine through the action of the enzyme S-adenosylhomocysteine hydrolase (SAHH, Figure 1C). Approximately, half of homocysteine can then be remethylated back to methionine using the 5-methyltetrahydrofolate through the enzyme methionine synthase (MTRR, Figure 1D) and another half converted to cystathionine by cystathionine beta synthase (CBS, Figure 1J) (45). Methionine synthase is ubiquitously expressed and necessitates the contribution of vitamin B_{12} , also known as cobalamin, in the form of methylcobalamin as a cofactor. The origin of the methyl group of SAM derives from 5-methylfolate which links methionine and SAM metabolism to folate-dependent one-carbon metabolism.

Homocysteine is an amino acid that is not used in protein synthesis (non-protein-forming). When elevated in blood, homocysteine level is an independent risk factor for neurological disorders, bone tissue damage and cardiovascular disease (46–48). While administration of B vitamins (see Figure 1) successfully lowers plasma homocysteine levels, it is unclear if this leads to any health benefits. It also remains unclear whether homocysteine itself is the cause of disease, or if it merely reflects other underlying health problems (49).

Through 5-methyltetrahydrofolate, the folic acid cycle provides another source of methyl groups for DNA

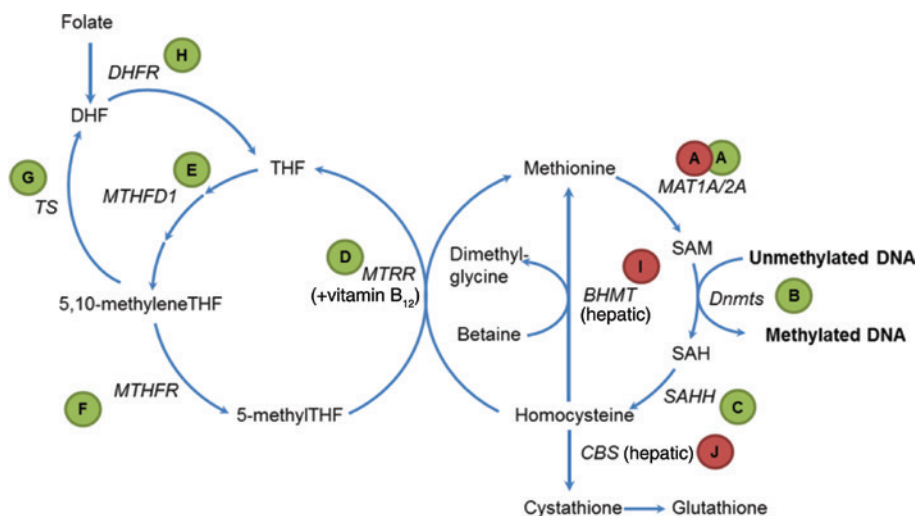


Figure 1: Overview of one-carbon metabolism.

methylation. Folic acid in the diet is reduced to dihydrofolate and tetrahydrofolate (THF) by the enzyme dihydrofolate reductase. THF is metabolized by the enzyme serine hydroxymethyltransferase (SHMT) to 5,10 methyleneTHF which is then metabolized to 5-methylTHF by methyltetrahydrofolate reductase, MTHFR (Figure 1F). MTHFR catalyzes the rate-limiting reaction to 5-methylTHF in the methyl cycle.

Alterations in one carbon metabolism and human disease

Two polymorphisms have been identified in human populations that influence MTHFR enzymatic activity. One is A1298C (Glu429Ala) and the other one is C677T (Ala222Val). This second is by far the more studied of the two. Compared to the ancestral C allele, the homozygous form of the T allele has 30% residual activity (50). It has been associated not only with variation in the blood levels of homocysteine (51), but also with elevated risks of vascular disease (52) and different types of cancers (53). However, publication bias is suspected and the effect may be significantly less than previously thought (54). A few more enzymes such as thymidylate synthase (TS, Figure 1G) and dihydrofolate reductase (DHFR, Figure 1H) serve additional roles in folate-dependent one-carbon metabolism.

In addition to common methionine-folate cycles in somatic cells, the liver expresses two enzymes that provide alternative pathways for the degradation of homocysteine. These are betaine homocysteine methyltransferase (BHMT, Figure 1I) and CBS (Figure 1J). BHMT utilizes trimethylglycine – also known as betaine and derived from the dietary precursor choline – to remethylate homocysteine to methionine. Conversely, CBS utilizes a transsulfuration reaction to produce cystathionine, which is later converted to cysteine and the antioxidant glutathione. A genetic defect in this enzyme is the cause for a rare inborn disorder called cystathionine β synthase deficiency that leads to abnormally high levels of homocysteine. Those levels are 5 to 10 times higher than those seen in patients with hyperhomocysteinemia associated with vascular disease.

Effect of radiation on one-carbon metabolism

Folate is susceptible to oxidative degradation and this process is exacerbated by exposure to IR (55). Irradiation

with γ rays led to reduced total folate levels by about half in the mouse liver 4 days after exposure (56). Folate was also found to be decreased in plasma (5 to 120 h) and in bone marrow (24 h) after total body irradiation with 3 Gy X-ray (57). As a consequence, enzymes in the folic acid cycle were also affected after exposure to IR. There was a dose-dependent increase in the gene expression for the enzymes TS, DHFR and methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1) that peaked 2 h after lymphoblastoid cell lines were exposed to 10 Gy of γ -rays (58). This is supported by the observation of an increase in enzymatic activity for TS and DHFR up to 96 h after total body irradiation with 2–7 Gy of γ -rays (56). Interestingly, the effect was opposite for the rate-limiting MTHFR, where there was a reduction in activity that was sustained over a week after exposure.

In addition to these observations in folate, cobalamin levels were also reported to be affected by IR. At the end of 5 weeks of radiotherapy to the pelvic area, patients showed significantly reduced mean serum levels of cobalamin (59). The levels continued to decrease up to 6 weeks after the end of the treatment period and remained significantly decreased a year later. Similar findings were identified after radiotherapy for cervical and bladder cancer (60–62). In the case of cobalamin, reduced absorption due to radiation damage to the intestinal lumen is the most likely reason for the changes in serum levels (61, 62).

Exposure to IR also seems to affect the distribution of methyl groups from methionine (63). The level of tracer for the methyl carbon was decreased in heart, brain, kidney and muscle and increased in the gastrointestinal tract, skin, thymus and thyroid. Concentrations of methionine, SAM, SAH and the ratio of SAM and SAH were also shown to be affected by exposure to protons and ^{56}Fe radiation in the mouse heart (38). It has been proposed that alterations in one-carbon metabolism may serve as driving mechanisms of epigenetic alterations, DNA and histone methylation, in particular (28). Indeed, decreases in methionine or synthesis of SAM would substantially affect the availability of universal methyl donor (SAM), needed for the maintenance of DNA and histone methylation. To our knowledge, there is only one study that simultaneously addressed, the one-carbon metabolism and methylation of DNA in response to IR. Interestingly, whole-body exposure to low absorbed mean doses of protons and ^{56}Fe resulted in increased cardiac tissue levels of methionine and SAM and was paralleled by the increase in DNA methylation of RE, suggesting an overall increase in global DNA methylation in the heart (38). It must be mentioned that both protons and ^{56}Fe are sources of high linear energy transfer (LET) radiation, biological effects of exposure (including

epigenetic effects), which are often very different from the exposure to terrestrial low-LET radiation (31, 64, 65). Some studies investigated the potential of methionine supplementation on DNA methylation in response to exposure to IR; these studies are discussed further in this review.

Methyl donor deficiency

Given the effects exerted by IR on one-carbon metabolism, it is not surprising that the response to IR can be modified by altering the supply of methyl group donors. Manipulations of folate, choline, cobalamin and methionine intake have been demonstrated to have impacts on birth defects, cognition and risk of cancer.

In *in vitro* models, folate deficiency was shown to sensitize cells to radiation-induced chromosomal instability (66, 67). The incidence of micronuclei formed, as a result of exposure to IR was inversely proportional to the concentration of folic acid in the culture medium. Homozygosity for the MTHFR C677T polymorphism further amplified the damaging effect of low folic acid concentration (67). *In vivo*, folate deficiency induced a change in the pattern of histone methylation (68). There was a suppression of H3K27 histone methyltransferase activity (a suppressive mark) with a parallel increase in H3K4 histone methyltransferase activity (an activating mark). IR also induced a decrease in the liver folate levels and depletion in the universal methyl donor SAM.

As suggested previously, IR leads to a decrease in liver folate levels (56). Combined with a folate-deficient diet, levels can be driven down even further (68). However, folate deficiency combined with IR also decreased liver and kidney choline levels (69). In parallel, exposure to IR led to an increase of choline into the circulation that peaked around 48 h and slowly decreased afterwards. Interestingly, the levels of choline in the brain were also elevated after the combination of folate-deficient diet and IR (69). A similar phenomenon was observed with a choline-free diet in combination with IR. The levels of choline in the liver were decreased while they were increased in the serum and brain (70). The combined insult is presumed to divert the choline reserves away from the liver, their storage point and into the serum to be directed towards other utilizing organs (69).

Methyl donor supplementation

Given the amplifying effect of a folate or choline-deficient diet on the effects exerted by exposure to IR, several

groups have explored the protective potential of methyl donor supplements. Indeed, betaine showed radioprotective properties on several fronts. Supplementation with betaine reduced the number of chromosomal aberrations in human lymphocytes exposed to either γ -rays or carbon ions (71). It also showed protection for the intestinal lumen by increasing crypt survival after irradiation with γ -rays, although this effect was not seen after irradiation with carbon ions (72). Overall, treatment with betaine supplementation also increased survival in animal models (71, 72).

Similar beneficial effects were observed using a supplementation mix of folic acid, choline and cobalamin. This treatment was able to preserve liver and serum folate levels after exposures up to 6 Gy (73). Homocysteine levels were also near baseline levels in the serum. In the liver, an IR-induced increase in homocysteine (2–2.5-fold) was observed at a 48 h time-point even on the supplemented diet, however this increase was ~4-fold on the normal diet. The supplemented diet also rescued DNA methylation and DNMT levels at this time-point.

The D isomer of methionine also shows promises at mitigating radiation effects. Survival of animals after irradiation was improved and there was an improvement in the mouth and tongue mucosal epithelial cells seen after D-methionine treatment (74, 75). To our knowledge, no studies have evaluated the radioprotective effects of L-methionine, which is the form used by cells. Although L-methionine is a methyl donor, it also displays toxicity at high levels. When given in excess, it interferes with urea-nitrogen balance (76). High dietary methionine intake also leads to increased levels of serum homocysteine (77) and may result in the formation of highly toxic methanethiolcysteine disulfides (78).

Methionine restriction

While long-term complete methionine deficiency leads to liver steatosis (79), methionine restriction has been associated with a number of health benefits in animal models. In a way that is postulated to be similar to caloric restriction, a reduction of 40%–80% of the levels of methionine in the diet leads to an increase in longevity in both rats and mice (80, 81).

Methionine restriction improves barrier function after exposure to IR, including in the gut (82–84). As irradiation results in reduced intestinal barrier integrity (85), methionine restriction may provide certain protection against gastrointestinal toxicity as a result of radiotherapy or

accidental exposure. It is possible that the positive effect of the D isomer of methionine after IR may actually be due to a competition with the protein-building form of the amino acid.

Cancerous cells are known for high sensitivity to methionine restriction. While normal cells tolerate methionine deficiency, the vast majority of cancer cells are methionine auxotrophs, meaning they exhibit irreplaceable nutritional requirements for methionine (86). A number of studies convincingly demonstrated that methionine deprivation holds substantial potential to increase the effects of chemotherapeutic agents both *in vitro* and in experimental models (86–90). Several clinical studies have also investigated the potential of the methionine-deprived diet in cancer therapy (91, 92). To our knowledge, however, no studies have addressed the effects of methionine deprivation combined with radiotherapy.

Expert opinion

It is increasingly recognized that exposure to IR affects the one-carbon metabolism. Alterations to methionine, one of the central molecules in the one-carbon metabolism and major substrate for methyl donors, may further impact normal DNA and histone methylation. Some experimental evidence supports this hypothesis, however further studies examining the link between IR-induced changes to the methionine cycle and epigenetic alterations are clearly needed.

Besides the numerous impacts IR exerts on one-carbon metabolism, it is evident that alterations to one-carbon metabolism influence the response to IR. This new understanding of the interaction between these two previously separated fields brings the possibility of using interventions on one-carbon metabolism as a strategy for modulation of both tumor and normal tissue responses to IR exposure. These applications are especially relevant for patients receiving radiotherapy to abdominal and pelvic areas, where gastrointestinal toxicity often appears to be the most frequent dose-limiting limiting factor (10, 93). For instance, methionine deprivation that may exert protective effects on the gut (i.e. by regulating the tight junction-related proteins and suppressing microflora) combined with extreme sensitivity of cancer cells to methionine starvation, may substantially increase efficacy of radiotherapy.

On the other hand, methionine has been proposed as a potential radiomitigator as methionine dietary supplementation may increase the availability of methyl donors for

DNA and histone methylation, as well as provide additional substrates for the synthesis of glutathione. It must be kept in mind, however, that despite its importance for normal homeostasis, methionine is also considered the most toxic amino acid that may potentiate IR-induced gastrointestinal toxicity – either via formation of toxic methanethiolcysteine disulfides, or by boosting the gut microbiome growth (development of the small intestine bacterial outgrowth syndrome) (reviewed in refs. 28, 94). Therefore, new research approaches must be established in order to learn how to benefit normal cells and cause maximal harm to cancer cells by this delicate methionine manipulation.

In these regards, metabolomics is one of the most promising and rapidly developing avenues. Metabolomics, an emerging technology, is the comprehensive study of the metabolome, the repertoire of small molecules or biochemicals present in cells, tissue and body fluids. Recent advances in mass spectrometry-based metabolomics technology have augmented the targeted quantification of metabolites with remarkable specificity and sensitivity. This is advantageous for rapid and reliable quantification of metabolites of one-carbon metabolism. The metabolites are quantified using stable isotope dilution – multiple reaction monitoring – mass spectrometry (SID-MRM-MS) using a triple quadrupole mass spectrometer wherein the quantitation is based on transitions (daughter ions) specific to the metabolite of interest. The ability to interrogate precise changes in one-carbon metabolism and related pathways is valuable in the context of biochemical alterations, as well as promises to gain more insights into the normal and cancerous cells response to IR.

Outlook

The last 10 years have seen significant progress in our understanding of the metabolic and epigenetic effects of exposure to IR. We predict that the next 10 years will be dedicated to using one-carbon metabolism to manipulate the cellular epigenome, as well as to modulate normal and cancerous tissues' response to IR.

The potential modulation of dietary methionine intake is of particular interest as it seems to be the most confronting. Some data suggests that methionine supplementation may have a radioprotective effect on the normal tissue, while other data suggests the potentiation of IR-induced normal tissue toxicity. Interestingly, beyond the generally accepted cancer cell-specific toxicity due to the methionine starvation, accumulating evidence also indicates that methionine overload can be equally toxic

to cancer cells (95–97). This duality will certainly require further investigations that we foresee to occur in the next decade.

One of the obstacles in the clinical application of methionine deprivation is its toxicity during the long duration of cancer therapy. Indeed, administration of a methionine-deficient diet causes substantial and often unaffordable weight loss in cancer patients (0.5 kg/week) (98). Furthermore, studies in cancer patients and *in vivo* models report methionine deprivation-induced thrombocytopenia and neutropenia, as well as the development of hepatosteatosis (91, 92, 99). However, recent advances in tumor imaging and irradiation techniques, along with the development of stereotactic body radiation therapy (SBRT), have begun to reveal increased local tumor control within the substantially decreased duration of radiation treatments compared to conventional fractionation (100–102). This opens a new horizon for methionine deprivation utilization in clinical practice, since a short-term methionine starvation can be expected to have significantly lower potential for toxicity and negative health outcomes. We anticipate the studies investigating the combined effects of methionine deprivation with radiotherapy in the near future.

Highlights

- Exposure to IR leads to epigenetic alterations, often exhibited as a loss of global and RE-specific DNA and histone methylation.
- Exposure to IR affects one-carbon metabolism and the tissue concentrations of methyl donors, suggesting a plausible mechanism for IR-induced epigenetic alterations.
- Supplementation in methyl donors can mitigate the genetic, epigenetic and apical effects of IR.
- Methionine dietary supplementation and deprivation may – positively and negatively – regulate both normal and cancerous tissue sensitivity to IR.

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List of abbreviations

BHMT	betaine-homocysteine methyltransferase
CBS	cystathionine beta synthase
DHFR	dihydrofolate reductase
DNMT	DNA methyltransferase
IR	ionizing radiation
MAT	methionine adenosyltransferase
MTHFD1	methylenetetrahydrofolate dehydrogenase, cyclohydro- lase and formyltetrahydrofolate synthetase 1
MTHFR	methyltetrahydrofolate reductase
RE	repetitive elements
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
THF	tetrahydrofolate
TS	thymidylate synthase

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