DNA Repair

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Abstract

Cellular chromosomal DNA is the principal target through which ionising radiation exerts it diverse biological effects. This chapter summarises the relevant DNA damage signalling and repair pathways used by normal and tumour cells in response to irradiation. Strategies for tumour radiosensitisation are reviewed which exploit tumour-specific DNA repair deficiencies or signalling pathway addictions, with a special focus on growth factor signalling, PARP, cancer stem cells, cell cycle checkpoints and DNA replication. This chapter concludes with a discussion of DNA repair-related candidate biomarkers of tumour response which are of crucial importance for implementing precision medicine in radiation oncology.

Keyword

Ionising radiation · DNA damage response · DNA strand breaks · DNA double-strand break repair · Molecular targeting · Biomarkers

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1 Introduction

DNA is a vitally important biomolecule which stores the genetic information required to create the molecular building blocks for cells, tissues and whole organisms. At the same time, it is surprisingly prone to damage and decay, even in the absence of any exogenous stressors. To counteract endogenous processes such as oxidation and hydrolysis as well as exogenously induced lesions, cells have devised a range of pathways for repairing damaged DNA, without which higher life forms would not have been able to evolve.

Not surprisingly, cellular DNA is also the principal target through which ionising radiation exerts its main biological effects, whether cell killing, neoplastic transformation, mutation induction, growth arrest or cellular ageing (UNSCEAR 2000). Whilst the main aim of radiotherapy is tumour cell inactivation, one needs to consider all of these biological responses to get the full picture of how radiotherapy affects the tumour and the surrounding normal tissue. And in order to understand these cellular responses, it is important to know about the molecular machinery that cells employ to repair DNA that has been damaged by radiation.

Apart from furthering our understanding of the basic mechanisms that govern the cellular radiation response, research into DNA repair also opens up opportunities to (i) learn why some individuals may react more severely to radiotherapy than others, (ii) identify potential markers of individual tumour and patient response to support a move towards personalised treatment and (iii) establish biological targets that can be used for tumour radiosensitisation.

2 Radiation-Induced DNA Damage and Early Cellular Responses

Radiation can damage chromosomal DNA either directly or indirectly via the production of free radical species (such as the hydroxyl radical) in the immediate vicinity of the DNA. The breakage of chemical bonds in the sugar–phosphate backbone of the DNA may result in the formation of strand breaks. Other types of lesions induced by ionising radiation include altered base and sugar moieties as well as cross-links which may form between proteins and DNA. All these modifications are not randomly distributed across the cell nucleus; instead, they form along the track of each ionising particle, as it deposits its energy. The resulting clustered lesions consist of multiple DNA lesions which are closely spaced within a volume of several nanometres, corresponding to up to about 20 base pairs. Occasionally, two strand breaks may be induced on opposite strands within one clustered lesion. These may cause the DNA molecule to break up, if there is insufficient overlap to maintain the DNA double helix via the weak hydrogen bonds between the paired, complementary bases. The resulting DNA double-strand breaks (DSBs) are very

deleterious. If left unrepaired, they may cause DNA degradation and loss of chromosomal fragments during the next mitosis. Furthermore, their faithful repair is complicated by the fact that, in contrast to lesions that affect only one of the two DNA strands, there is no template immediately available that could be used to correctly restore the original DNA sequence. For this reason, DSB ends are prone to be misrepaired, causing either small-scale mutations at the break point or chromosomal rearrangements such as translocations if break ends from multiple breaks interact and get misrejoined (Rothkamm and Lobrich 2002). It is for these reasons that DSBs are believed to be the most important DNA lesions induced by ionising radiation. For the same reasons, the remainder of this chapter will focus mainly on the cellular response to DSBs.

Cells respond to ionising irradiation through a highly interactive functional network of partially overlapping DNA damage response pathways (Sulli et al. 2012; Jackson and Bartek 2009). Upon detection of DSBs or extended stretches of single-stranded DNA, molecular DNA damage sensors such as the MRE11-RAD50-Nibrin (MRN) and the KU70-KU86 complexes or RPA, ATRIP and the RAD9-RAD1-HUS1 complex will activate the apical kinases ATM, DNA-PK or ATR, respectively, which belong to the family of phosphatidyl inositol 3′ kinase-related kinases. These will in turn phosphorylate a plethora of DNA damage mediators and downstream kinases. Examples of mediators—which frequently accumulate at or in the vicinity of DSBs to form microscopically visible DNA damage foci—include MDC1, 53BP1, H2AX, BRCA1 and TOPBP1. Downstream kinases include the checkpoint factors CHK1 and CHK2. Figure 1 exemplifies some of the known and predicted protein interactions for the key DNA damage kinase ATM, according to STRING 10 database (Jensen et al. 2009).

The main outcomes of the DNA damage response include the following:

(i) Transcriptional activation or repression of DNA damage-responsive genes.
(ii) Restriction of cell cycle progression at DNA damage-induced checkpoints in order to allow DNA repair to proceed before the cell enters S phase or mitosis. CDC25 and p53 are important effectors to facilitate this outcome.
(iii) Post-translational modification (phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation) of chromatin constituents and associated proteins around the site of the DNA lesion to facilitate repair (Bekker-Jensen and Mailand 2011; Brown and Jackson 2015).
(iv) Repair of DNA lesions, possibly resulting in mutations and chromosomal aberrations.
(v) Induction of apoptosis in radiation-damaged cells, which may occur via p53 or in a p53-independent manner.
(vi) Induction of autophagy, probably via inhibition of mammalian target of rapamycin complex 1 (mTORC1) (Czarny et al. 2015).

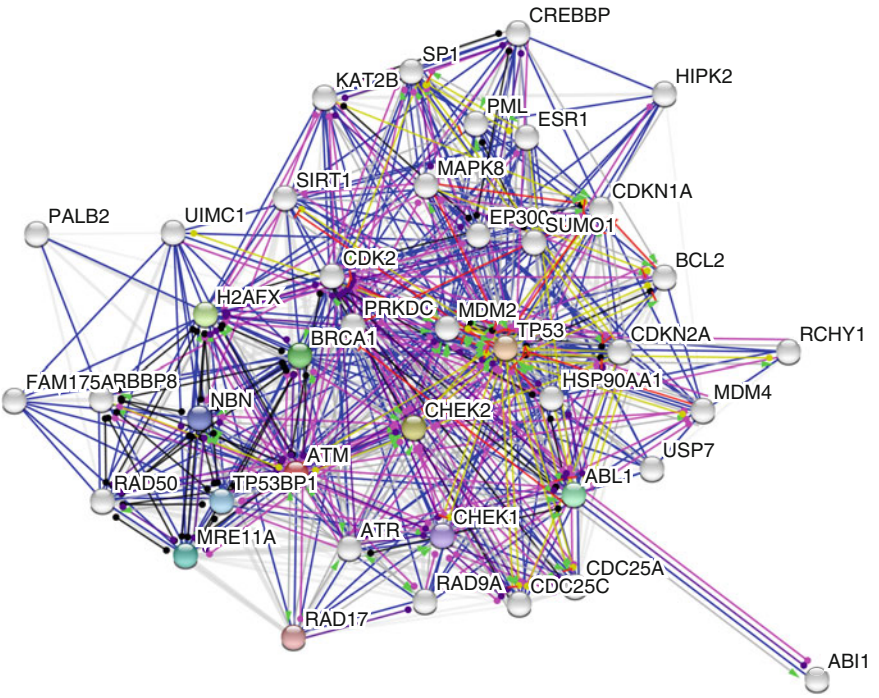

Fig. 1 Network of some of the proteins that interact physically or functionally with ATM, according to STRING 10 database (http://string-db.org; accessed 4 November 2015). *Line colours* indicate the nature of the physical or functional interaction: *green*—activation; *red*—inhibition; *blue*—binding; *turquiose*—phenotype; *purple*—catalysis; *pink*—post-translational modification; *black*—reaction; *yellow*—expression

3 Repair of Single-Stranded DNA Lesions

Several mechanisms for repairing ionising radiation-induced DNA damage exist in human cells which have some overlapping functions and may act as backup pathways for each other to minimise the risk of any damage being left unrepaired. Single-strand breaks and damaged bases such as 7,8-dihydro-8-oxoguanine or thymine glycol are the most common lesions induced by ionising radiation and also form very frequently endogenously, thus requiring efficient repair mechanisms to maintain genome integrity. Thanks to the availability of the complementary sequence on the intact opposite strand which serves as a template for repair, these lesions do not normally give rise to any mutations.

Single-strand breaks caused by damage to the sugar moieties in the DNA are detected by and activate poly(ADP-ribose)polymerase 1 (PARP1) which subsequently modifies itself and other proteins with chains of hundreds of ADP-ribose units. These recruit XRCC1 protein complexes (probably containing DNA polymerase beta,

ligase 3 and one of PNKP, APTX or APLF) which process DNA ends. Two alternative options exist for the final two steps, gap filling and DNA ligation, namely short-patch and long-patch repair during which either one or 2–10 nucleotides are incorporated by polymerase beta and/or polymerase delta/epsilon, resulting in the displacement of the damaged strand in the latter case which is then removed by FEN1/PCNA, possibly also with support from PARP1. Repair patches are sealed by the XRCC1/ligase III alpha or PCNA/ligase 1 complexes (Caldecott 2014).

Base lesions are essentially repaired by the same base excision repair (BER) process described above for single-strand break repair, except that this pathway initially employs lesion-specific glycosylases to remove altered bases and then converts the abasic sites into single-strand breaks via an AP endonuclease activity (Brenerman et al. 2014). These breaks are then processed in the same way as those directly induced by radiation.

In global nucleotide excision repair, bulky lesions which distort the helical DNA structure can be recognised by the XPC-hRAD23B-centrin 2 complex which then melts the DNA around the lesion and recruits the TFIIH complex. Subsequently, XPB and XPD unwind the DNA to form a bubble of about 30 nucleotides. XPA then binds the DNA near the 5′ end of the bubble, and RPA binds the single-stranded DNA opposite the lesion to protect it from degradation. The first incision is then made by ERCC1-XPF and repair synthesis commences, displacing the damaged strand. Finally, XPG makes the second incision to remove the oligonucleotide contained the lesion, and the newly synthesised DNA patch is sealed by ligase I or ligase III alpha-XRCC1 (Spivak 2015).

In contrast to the above process, which only detects bulky lesions, a transcription-coupled excision repair pathway can repair any DNA lesion located within an actively transcribed gene. In this case, it is the stalling of RNA polymerase II-mediated transcription which detects the lesion and triggers the recruitment of transcription-coupled repair factors. The polymerase is then backtracked or removed to make space for repair which proceeds as described above for global nucleotide excision repair (Spivak 2015).

DNA strand breaks in cellular DNA are commonly measured using alkaline single-cell gel electrophoresis, also called the comet assay (Ostling and Johanson 1984; Olive 2009). In brief, cells are embedded into low-melt agarose and spread onto microscopy slides, lysed, electrophoresed in alkaline running buffer, neutralised and stained with a fluorescent DNA dye, such as ethidium bromide or Sybr Gold, and imaged using a fluorescence microscope. Whilst cells with intact, unirradiated DNA will show round nuclei (‘heads’) without any DNA leaking out of the nucleus into a tail, cells containing strand breaks will show a comet-like tail of fragmented DNA which migrated out of the nucleus towards the anode during electrophoresis. The percentage of DNA in the tail and the tail length can be measured, and the tail moment be calculated by multiplying the two as a robust indicator of the amount of damage present in the sample. As ionising radiation induces about 30 times more single-strand breaks than DSBs, the initial signal

directly after irradiation will be dominated by single-strand breaks. However, the kinetics for repairing single- and DSBs differ considerably, with the former being repaired faster, so that residual damage remaining several hours after irradiation will be greatly enriched for DSBs. In that respect, any signal remaining after many hours in an alkaline comet assay will largely reflect the level of residual DSBs, at least in cells that are BER-competent.

One strategy to measure base lesions utilises glycosylases/AP endonucleases that convert specific base lesions into single-strand breaks (Collins 2014). To this end, lysed cells embedded in agarose slides are incubated with commercially available purified enzymes such as formamidopyrimidine DNA glycosylase or Nth (endonuclease III) to detect oxidised purines or pyrimidines, respectively, prior to electrophoresis. The additional tail moment measured on top of that observed in a parallel sample without glycosylase treatment is then a measure of the amount of base damage. Detailed protocols have been developed in recent years for the application of comet-based assays for various BER and NER substrates in different types of biological material (Azqueta et al. 2013).

4 Pathways for Repairing DNA Double-Strand Breaks

DSBs can be repaired by non-homologous end-joining (NHEJ) processes as well as homology-dependent recombination pathways. Whilst excision repair pathways for single-stranded DNA damage generally use the intact complementary strand as a template, ensuring that repair is mostly error-free, DSB repair is generally more challenging and frequently error-prone. This is especially the case in situations when several DSB ends are sufficiently close to be misaligned and exchanged during repair, giving rise to chromosomal deletions or rearrangements. Promiscuous repair in the presence of multiple DSBs is also responsible for the quadratic increase of deleterious chromosome aberrations with gamma- or X-ray dose, despite a dose-linear increase of DSBs (Barnard et al. 2013). It is therefore important to appreciate that DSB repair efficiency is an important determinant of genome stability and radiosensitivity.

Gel electrophoretic studies of double-stranded DNA fragments following irradiation and immunofluorescence microscopy-based scoring of DNA damage-associated protein ‘foci’ forming at the sites of DSBs have shown that initial radiation-induced DSB yields do not in general vary very much between genomic loci, organs or individuals. However, some variation has been reported for different tumour cells which cannot be explained by other confounding factors such as DNA content but which may be associated with cellular radiosensitivity (El-Awady et al. 2003; Rube et al. 2008; Rothkamm et al. 2003; Cheng et al. 2015).

X- or gamma-ray-induced DSBs are repaired with biphasic exponential kinetics with half-lives of 5–30 min and several hours, respectively. Repair may reach a plateau of residual, unrepaired and potentially also misrepaired, DSBs (Rothkamm and Lobrich 2003; Dahm-Daphi and Dikomey 1996). Longer half-lives and more residual breaks have been observed in cells exposed to densely ionising particles.

Whilst the time course of DSB repair is generally comparable between tumour cells, lymphocytes or normal fibroblasts, more variability seems to exist between different tumour cell lines. These may reflect bigger differences in the efficiency of DSB processing in tumours, likely caused by deficiencies or upregulation of particular DNA damage response pathways.

Chromatin structure and cell cycle stage also affect both efficiency and kinetics of DSB repair. DSBs in heterochromatic DNA regions are much more difficult to access by repair factors, require additional chromatin relaxation steps mediated by KAP1 and tend to be repaired more slowly (Goodarzi et al. 2008). Repair pathway utilisation depends very much on cell cycle position as it is affected by the availability of a sister chromatid (Rothkamm et al. 2003; Bauerschmidt et al. 2010). Consequently, the radiation response of a normal tissue or a tumour may change during a course of fractionated radiotherapy if cells accumulate in a specific cell cycle phase. One example is the loss of fraction size sensitivity in the basal epidermal layer of irradiated skin which may be explained by the accumulation of cells in the S/G2 phase and consequential increase in the utilisation of homology-dependent repair mechanisms (Somaiah et al. 2012).

Repairing a DSB is of utmost importance for a cell because, if left unrepaired, it would lead to the loss of the affected chromosome fragment and cell death (Helleday et al. 2007). Furthermore, incorrect repair of a DSB may produce deletions or chromosome rearrangements which are hallmarks of cancer genomes. For these reasons, efficient DSB repair is of crucial importance for the survival and genomic stability of a cell. Human cells employ two fundamentally different types of DSB repair mechanisms, one relying on the joining of break ends with little or no regard for sequence homology, and the other heavily dependent on the availability of sequence homology. These will be discussed in more detail in the following sections.

4.1 End-Joining Mechanisms

The main DSB repair pathway in mammalian cells is classical non-homologous end-joining (NHEJ). It is active in all phases of the cell cycle (Lieber et al. 2003) except mitosis (Orthwein et al. 2014; Terasawa et al. 2014). NHEJ is initiated by the binding of the Ku70/Ku80 heterodimer to DSB ends, followed by recruitment and activation of the catalytic subunit of the DNA-dependent protein kinase to form the DNA damage kinase DNA-PK. Depending on the nature of the break, the ends may need to be trimmed before they can be ligated, e.g. by nucleolytic resection or by DNA polymerase-mediated fill-in. Then, the ligase complex consisting of ligase IV and its cofactors XRCC4 and XLF binds to the DNA ends and seals the break (Davis and Chen 2013). Intriguingly, whilst the basic NHEJ mechanism has been known for over twenty years, its regulation in human cells is still far from being fully understood.

Cells deficient in NHEJ can repair DSBs via an alternative end-joining mechanism (Alt-EJ) (Wang et al. 2003; Audebert et al. 2004) which is less efficient than

NHEJ and depends on PARP1 for DSB recognition and on ligase III and its cofactor XRCC1 for the ligation step (Mansour et al. 2010, 2013). It is associated with deletions of DNA at the break site, probably caused by the slower speed of the pathway which prevents nucleolytic degradation less efficiently than classical DNA-PK-dependent NHEJ (Mansour et al. 2013). Whilst Alt-EJ does not strictly require microhomology between overlapping bases at the break ends, it still preferentially uses it if available (Mansour et al. 2010). Interestingly, these features, i.e. deletions and microhomologies at breakpoint junctions, are frequently found in human cancer cells (Welzel et al. 2001; Weinstock et al. 2007; Jager et al. 2000).

4.2 Homology-Dependent Repair Pathways

The main homology-dependent repair pathway, homologous recombination (HR), operates at the replication fork as well as in post-replicative DNA, i.e. during S and G2 phase when the sister chromatid can serve as an intact template to facilitate repair of a DSB by restoring the original sequence. Repair via HR is therefore largely error-free. HR is initiated by a nucleolytic DNA end resection step during which single-stranded overhangs are generated. This is achieved by Mre11-CtIP, followed by Exo1, DNA2 and the BLM helicase (Helleday et al. 2007; Sung and Klein 2006). RPA protects the single-stranded DNA overhangs until RAD51 binds to form a nucleofilament, supported by the RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 as well as by BRCA2 and RAD52. Following a search for DNA with extensive sequence homology, the RAD51 nucleofilament invades the double helix of, typically, the sister chromatid to form a so-called displacement or D loop. Using the complementary strand as a template, replication polymerases then synthesise DNA until ligase 1 seals the ends. The resulting branched structure of four double-stranded DNA arms joined together, called the Holliday junction, can be resolved either through symmetrical cleavage by GEN1/Yen1, through asymmetrical cleavage (Mus81/Eme1) or through dissolution via the BLM-TopIII-alpha complex to complete the repair process (Andersen et al. 2011; Sarbajna et al. 2014; Wyatt and West 2014).

A different homology-dependent repair mechanism for DSBs does not use a homologous copy such as the sister chromatid but instead utilises homologies between repetitive sequences on the broken chromosome. It is called single-strand annealing (SSA) and is also initiated by a nucleolytic end resection step to produce long single-stranded DNA overhangs to which RPA binds. Subsequently, heptameric rings of RAD52 form at the DNA overhangs and facilitate homology search and annealing of the repeat sequences. Next, the DNA sequences between the repeats are flapped out and cleaved off, most likely be the ERCC1/XPF endonuclease (Shinohara et al. 1998), and an as yet unknown ligase seals the remaining gap. It is clear from the above that SSA is a non-conservative process which results in the deletion of the DNA flanking the breakpoint, including one copy of the repeat sequences used by this mechanism.

4.3 DSB Repair Hierarchy

In normal cells, a functional hierarchy, regulated by an extensive cellular signalling network including cell cycle and DNA damage response factors, determines the use of the DSB repair pathways described above in order to ensure the efficient and faithful processing of DSBs (Mansour et al. 2008). NHEJ usually predominates and suppresses Alt-EJ, HR and SSA pathways. In situations of NHEJ deficiency, most DSBs are repaired by Alt-EJ, although HR and SSA also contribute more strongly to DSB repair (Mansour et al. 2008). Interestingly, DSB repair has been found to be frequently switched to Alt-EJ in tumour cell lines of various origin (Kotter et al. 2014) as well as in primary bladder (Bentley et al. 2004) and head-and-neck tumour cells (Shin et al. 2006). It is tempting to speculate that this switch to a more mutagenic repair mechanism enables tumours to accelerate their genetic diversification in order to overcome further barriers to growth. In any case, this switch to Alt-EJ in tumours offers opportunities for their specific targeting (Kotter et al. 2014).

5 The DNA Damage Response as a Target for Tumour Radiosensitisation

Treatment outcomes could be improved for many tumours treated with radiotherapy if it were possible to selectively enhance tumour cell radiosensitivity without affecting normal tissue responses. Our progress in understanding the cellular radiation response over the past years sets the scene for the development of such strategies, based on targeting signalling and repair pathways that tumours have become addicted to.

5.1 The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a very important signalling factor in many tumour cells. It is frequently mutated or over-expressed in tumours and can be targeted therapeutically using antibodies such as cetuximab or tyrosine kinase inhibitors such as erlotinib or gefitinib, either as a monotherapy or combined with irradiation (Krause and Van Etten 2005). Combined treatment with cetuximab and radiotherapy has been demonstrated to improve local tumour control in patients with advanced head-and-neck cancer (Bonner et al. 2006). Interestingly, it was especially patients suffering strong cetuximab-associated side effects who benefitted most from the combined treatment. This suggests that there are subgroups of patients with a differential response to this targeted therapy (Bonner et al. 2010).

Cetuximab is thought to radiosensitise tumours at the cellular level (Harari et al. 2007). Although the exact mechanisms are not yet completely understood, DSB repair seems to be involved as it has been shown to be regulated by EGFR and to be suppressed by treatment with cetuximab or tyrosine kinase inhibitors which appear to block both NHEJ and HR (Kriegs et al. 2010; Myllynen et al. 2011). However,

the observed repair inhibition was not always associated with enhanced cellular radiosensitivity or improved tumour response (Myllynen et al. 2011; Kriegs et al. 2015; Stegeman et al. 2013). One possible explanation is that these treatments inhibit DSB repair only partially or transiently. Other responses observed following EGFR inhibition in combination with irradiation include the induction of apoptosis (though not confirmed in subsequent studies), semi-permanent cell cycle arrest and premature senescence which correlated with radiosensitisation (Kriegs et al. 2015; Wang et al. 2011). However, recent clinical trials report no improvement of therapy outcome for patients treated with tyrosine kinase inhibitors or cetuximab in combination with radiochemotherapy (Ang et al. 2014; Giralt et al. 2015; Martins et al. 2013; Mesia et al. 2015).

Apart from targeting EGFR function, its sheer abundance in tumours makes it also an ideal target for radioimmunotherapy, in order to selectively irradiate tumour cells (Cai et al. 2008; Saker et al. 2013), thereby allowing tumour control to be achieved with only moderate additional doses given with external beam radiotherapy (Koi et al. 2014). Other cell signalling factors that could be promising targets for DSB repair modulation include MAPK and AKT signalling (Kriegs et al. 2010; Toulany et al. 2006). Furthermore, EGFR-independent targets are being investigated, such as the proto-oncogenes Myc and Ras, and the therapeutic potential of multi-kinase inhibitors such as imatinib, dasatinib or sorafenib is being explored, with promising early results (Laban et al. 2013; Möckelmann et al. 2016). However, it is not at all clear at this stage whether DNA repair plays a significant role in these treatment strategies.

5.2 Poly (ADP-Ribose) Polymerase-1 (PARP1)

PARP1 contributes to a number of cellular tasks, such as DNA repair, replication, transcription and cell cycle regulation. In cancer therapy, it has been identified as an interesting druggable target because of its role in detecting DNA single-strand breaks and facilitating their repair via BER. Following PARP inhibition, single-strand breaks accumulate, resulting in the formation of DSBs especially during replication, which in turn require HR to be efficiently repaired. For this reason, HR-deficient tumours such as those carrying mutations in BRCA1 or BRCA2 have been targeted using PARP inhibitor. The logic behind this ‘synthetic lethality’ approach is that normal cells will tolerate PARP inhibition because they are HR-proficient, whereas tumour cells which are already compromised in HR, the backup repair pathway for unrepaired single-strand breaks encountered during replication, will struggle to survive upon loss of their main single-strand break repair pathway. PARP inhibition using drugs such as olaparib has been and is being used as a monotherapy in a number of studies which focus mostly on BRCA-deficient tumours (Bryant and Helleday 2004; Benafif and Hall 2015; Mateo et al. 2015). However, the combination of PARP inhibition with DNA-damaging chemo- or radiotherapy is even more promising, due to their synergistic interaction which has been shown in vitro as well as in vivo (Bryant et al. 2005; Fong et al. 2009; Tutt et al. 2010). Given that tumour sensitisation by PARP inhibition

is achieved mostly through the formation of DSBs at collapsed replication forks following their collision with unrepaired single-strand breaks, one would expect the best radiosensitising effects of PARP inhibition in tumours with a large S-phase fraction (Noel et al. 2006).

In addition to the 'BRCAness'-dependent effects described above, it is becoming increasingly clear that also tumours that have switched from DNA-PK-dependent NHEJ to PARP-dependent Alt-EJ may also be promising candidates for tumour-specific radiosensitisation by PARP inhibition (Wang et al. 2003; Audebert et al. 2004; Mansour et al. 2010; Kotter et al. 2014).

5.3 Cell Cycle Checkpoint Signalling

Following irradiation, cells arrest at the G1/S and G2/M cell cycle checkpoints to allow time for DNA repair before cells enter the most critical phases of the cell cycle, namely replication and mitosis. In addition, an intra-S-phase checkpoint functions to postpone DNA replication in the presence of DNA damage (Morgan and Lawrence 2015).

DNA damage-induced cell cycle checkpoints are regulated by the kinases ATM and ATR via phosphorylation of the checkpoint kinases Chk2 and Chk1, respectively, which inhibit CDC25 phosphatases and thereby block cyclin-dependent kinase-mediated cell cycle progression. The initiating signals for these signal cascades are either a DSB which activates ATM or an extended stretch of single-stranded DNA coated with RPA (often associated with replication fork stallage and HR intermediates) which activates ATR (Marechal and Zou 2013). Accordingly, the G1/S and G2/M checkpoints are regulated by ATM/Chk2, whilst ATR/Chk1 are essential for the intra-S checkpoint and also contribute to the G2/M arrest. The G1/S checkpoint also requires an intact p53 response and downstream p21/CDKN1A induction and is therefore frequently compromised in tumours. In the absence of a functional G1/S arrest, p53-deficient tumours are very reliant on the ATR/Chk1 pathway to prevent entry into mitosis with too much DNA damage and may therefore be targeted using ATR or Chk1 inhibitors in combination with DNA-damaging agents such as radiotherapy, exploiting the synthetic lethality concept. Indeed, early studies have demonstrated enhanced sensitivity to radio- or chemotherapy of human cell lines derived from various tumour entities (Garrett and Collins 2011; Dillon et al. 2014; Busch et al. 2013).

However, toxicity and lack of specificity of the first generation of inhibitors, such as the Chk1 inhibitor UCN01, has been a serious drawback. More specific and better tolerated inhibitors for the checkpoint kinases, such as SCH 900776/MK-8776 or LY2606368, have been developed more recently and are now being tested in the clinic. Inhibitors for ATR and Wee1—which inhibits CDK1 and thereby delays entry into mitosis—are also being tested in phase I and II trials in combination with chemotherapy. Unfortunately, there are to date only two trials testing inhibitors for the G2/M arrest in combination with radiotherapy (NCT02223923, NCT01922076). As the targeted proteins are also involved in other

important aspects of DNA maintenance, such as repair and replication (Sorensen and Syljuasen 2012), their inhibition is also being explored as a monotherapy (McNeely et al. 2014). Furthermore, one could argue that any risk of systemic toxicity of these inhibitors when used in combination with chemotherapy may potentially be avoided or at least reduced when combining them with local radiotherapy treatments.

5.4 Cancer Stem Cells

The concept of cancer stem cells (CSCs) is based on that of normal tissue stem cells. Accordingly, CSC have the potential to self-renew, differentiate and maintain tumour growth and repopulation following radio- or chemotherapy. Two models exist for the organisation of CSC: one that assumes a hierarchically organised system in which only a small proportion of tumour cells actually have any tumourigenic potential whilst most tumour cells are unable to induce tumours (Lapidot et al. 1994; Al-Hajj et al. 2003), and a clonal evolution model in which random changes enable subclones to emerge with new functions and treatment responses within the tumour. In fact, both models may have some merit and could coexist, assuming that clonal evolution may shape the make-up of the small proportion of stem-like cells (Maugeri-Sacca et al. 2014).

Similar to tissue stem cells, CSC are generally thought to be resistant to DNA-damaging agents thanks to upregulated DNA damage response and repair pathways (Maynard et al. 2008; Mandal et al. 2011). Examples include CD133+ glioma stem cells which were shown to have an enhanced Chk1-dependent checkpoint response (Baumann et al. 2008; Bao et al. 2006) and upregulated expression of Nibrin, one component of the MRN complex which is involved in DSB sensing (Cheng et al. 2011). Enhanced DNA damage responses and repair gene expression were also observed in CD133+ CSC in A549 human lung carcinoma cells (Desai et al. 2014), mammary tumour-initiating cells (Zhang et al. 2014), pancreatic putative CSC (Mathews et al. 2011) and patient-derived non-small-cell lung cancer CSC (Bartucci et al. 2012). However, a number of studies showed no difference or even deficient DNA damage responses in CSC (McCord et al. 2009; Ropolo et al. 2009; Lundholm et al. 2013). These conflicting observations suggest that an enhanced DNA damage response may not be a general feature of all CSC and that inter-tumour and possibly even intra-tumour heterogeneity in the DNA damage response functionality may need to be considered (Magee et al. 2012). One important factor that may affect the composition and treatment response of CSC is epithelial–mesenchymal transition (EMT) which has been associated with enhanced radioresistance of mammary CSC. ZEB1, a zinc finger transcription factor which is phosphorylated and stabilised by ATM following DNA damage and then stabilises Chk1, appears to be an important player linking EMT to the DNA damage response (Zhang et al. 2014).

Interestingly, PARP1 inhibition was reported to radiosensitise glioma stem cells (Venere et al. 2014) and Chk1 inhibition reduced the CSC pool in non-small-cell

lung cancer cells, suggesting that DNA damage response inhibitors are a promising targeting strategy for CSC (Bartucci et al. 2012). However, as multiple DNA damage response pathways may be simultaneously upregulated in CSC, as shown in glioma stem cells, a multi-targeting approach involving combined inhibition of DNA repair and cell cycle checkpoint mechanisms may be a more promising strategy for overcoming CSC resistance (Signore et al. 2014; Ahmed et al. 2015).

5.5 Replication

One of the most exciting emerging strategies for improving tumour control is the replication-dependent sensitisation of tumours to radiation and chemotherapeutic drugs. It exploits the fact that DNA damage response pathways are of critical importance for replication fork stability, control of origin firing and the resolution of collapsed forks caused by DNA damage (Zeman and Cimprich 2014) (Fig. 2). They serve to counteract replication stress and the formation of secondary DSB induced by cytotoxic cancer therapies (Kotsantis et al. 2015).

The molecular targeting approaches for a range of DNA damage response pathways that are now becoming available for clinical testing provide the opportunity to enhance the toxicity of radio- and chemotherapy during replication (Pearl et al. 2015). Inhibition of the DNA damage response kinases ATR, CHK1 and WEE1 increases the activity of cyclin-dependent kinases, which leads to uncontrolled replication origin firing and depletion of the nucleotide pool, resulting in the

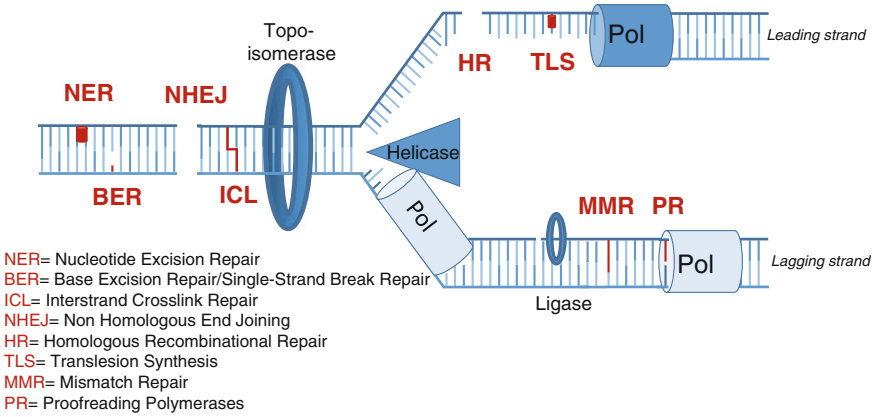

Fig. 2 DNA repair mechanisms active at replication forks to maintain genomic stability. Cells are constantly exposed to insults from endogenous and exogenous agents that can introduce DNA damage and generate genomic instability. Many of these lesions cause structural damage to DNA and can alter or eliminate fundamental cellular processes, such as DNA replication. To counteract harmful effects, cells have developed a higly specialised DNA repair system, which can be subdivided into distinct mechanisms including base excision repair/single-strand break repair, nucleotide excision repair, mismatch repair, interstrand cross-link repair and double-strand break repair, together with the proofreading activity of polymerases and translesion synthesis

accumulation of replication-associated DSBs (Syljuasen et al. 2015). HR proteins which are not only involved in the repair of replication-associated DSBs but also support replication fidelity are also interesting targets (Huang and Mazin 2014). Examples include Rad51-, NUCKS- and RAD51-associated protein 1 whose disruption compromises replication fork progression, increases the firing of replication origins and negatively affects genome stability (Parplys et al. 2014, 2015a, b).

Inhibition of the DNA single-strand break repair factor PARP was first described as a promising strategy for BRCA-deficient mammary cancers, which, due to the associated HR deficiency, cannot efficiently deal with unrepaired DNA damage present during replication. However, PARP1 also senses stalled replication forks and recruits Mre11 which degrades the stalled forks to enable HR-dependent repair and replication fork restart (Ying et al. 2012).

ATR, CHK1 and PARP1 have been shown to be prime targets for radiosensitisation of S-phase cells (Ahmed et al. 2015; Dungey et al. 2008; Pires et al. 2012; Dobbelstein and Sorensen 2015). Furthermore, irradiated cells undergoing replication form secondary DSBs (Groth et al. 2012) and suffer from blocked replication elongation (Parplys et al. 2012). For these reasons, radiation-induced tumour cell killing could potentially be enhanced by enriching the fraction of cells that are in S phase during irradiation, e.g. through the use of replication inhibitors (Dobbelstein and Sorensen 2015).

6 Biomarkers of Treatment Response

Radiotherapy treatment decisions are currently made based on clinical parameters such as tumour size, site and grade. The individual response of patient and tumour, based on their biological make-up, is typically not taken into account, simply because it has not been possible to reliably predict either the tumour or the normal tissue response of a particular patient, except for very rare cases of patients with radiation hypersensitivity syndromes such as ataxia telangiectasia. However, it would be of great benefit if it could be determined prior to the treatment which patients would actually benefit from radiotherapy, what dose should be given and whether the chances of tumour control could be improved using a particular molecular-targeted approach in combination with radiotherapy. Biological markers including mutational or single nucleotide polymorphism (SNP) signatures, gene expression profiles and functional assays for core radiation response mechanisms such as DSB repair capacity are slowly gaining relevance and are starting to pave the way for a precision medicine approach in radiation oncology.

We have reviewed the use of chromosome and DNA damage/repair assays for assessing individual radiation exposures and for predicting normal tissue responses quite extensively in the recent past (Chua and Rothkamm 2013; Pernot et al. 2012; Manning and Rothkamm 2013; Rothkamm et al. 2015) and will therefore focus on biomarkers of tumour response here.

Early attempts to use functional assays for tumour radiosensitivity prediction involved cell suspensions obtained from tumour biopsies which were irradiated in vitro and plated for colony formation to produce survival curves. Cell survival measured in vitro with such an approach was shown to correspond with clinical outcome in cervical cancer (West et al. 1993) and other tumour entities (Bjork-Eriksson et al. 2000), thus demonstrating that therapy outcome can be linked to cellular radiosensitivity. However, due to the amount of effort required for this assay and the long delay before colony counts are available, this method proved unsuitable for routine use in a clinical setting.

A quicker method for assessing the radiation response of tumour cells exploits the fact that cellular radiosensitivity is closely linked to DSB repair capacity. Immunofluorescence microscopic scoring of gamma-H2AX and/or 53BP1 nuclear protein foci, which form at the site of a DSB, is widely used as a surrogate marker of DSBs (Rothkamm et al. 2015) and can be performed in cell lines as well as in frozen or formalin-fixed paraffin-embedded tissue sections (Somaiah et al. 2012; Chua and Rothkamm 2013; Qvarnstrom et al. 2004; Barber et al. 2006; Crosbie et al. 2010; Rothkamm et al. 2012). This method has recently also been applied to determine residual DSBs in xenografts or patient-derived tumour biopsies following in vivo as well as ex vivo irradiation and repair incubation (Menegakis et al. 2015). A recent study demonstrated that residual foci levels obtained with this assay are consistent with the known differences in radioresponsiveness of different tumour types, thus providing proof of concept for this strategy (Menegakis et al. 2015). Additional information about the functionality of DNA damage response pathways in a tumour can be obtained using other biomarkers. For example, Rad51 foci formation following ex vivo treatment can indicate the functionality of the HR pathway and has been used to identify individual breast tumours with HR deficiencies (Naipal et al. 2014). As already mentioned above, patients with such tumours could then benefit from targeted therapy using PARP inhibitor. Biomarkers for other pathways, e.g. for a switch from classical to alternative end-joining, are currently being investigated.

In summary, functional ex vivo assays of biological radiation effects and pathway functions in tumour biopsies are rapid indicators of treatment response which enable treatments to be tailored to the individual characteristics of a tumour. Assaying function, rather than just genetic or expression profiles of a tumour, has the clear advantage of an integrated approach that will register effects, such as epigenetic or post-translational alterations, that may well be missed when using a non-functional method. However, for the assays to be fit for routine use in the clinic, robust, standardised procedures for sample logistics, processing and analysis need to be established and regularly validated.

Aberrant expression of a protein can also indicate whether a particular tumour will respond to a specific treatment strategy. Examples include HPV-positive oropharyngeal cancers which over-express p16/INK4a (Lassen et al. 2009), head-and-neck cancers deficient in the DNA damage response due to downregulated ATM (Mansour et al. 2013), over-expression of EGFR associated with head-and-neck cancer radioresistance (Ang et al. 2002), prostate cancer cells which

over-express Bcl2, causing them to use alternative instead of classical end-joining (Catz and Johnson 2003; Wang et al. 2008), or colorectal carcinoma with deregulated HR and over-expression of RAD51 (Tennstedt et al. 2013). Also, over-expression of Ku, but possibly not of other players involved in NHEJ, was found to be associated with radioresistance in head-and-neck cancer (Lee et al. 2005; Moeller et al. 2011).

As gene expression profiling and whole-exome sequencing are becoming more affordable as well as accessible, there is increasing interest in identifying signatures that could be used to predict treatment response and individualise treatment. However, to date, no reliable signatures are available for routine use, although some candidate profiles have been reported (Ahmed et al. 2015; Tinhofer et al. 2015; Spitzner et al. 2010; Pramana et al. 2007).

7 Future Perspectives

Major progress has been made in recent years in the investigation and characterisation of cellular and molecular DNA damage response processes occurring in irradiated tumours and normal tissues. One important driver has been the development of new cellular and molecular methods and techniques. These have facilitated exciting discoveries at the cellular level, especially in the fields of signal transduction, cell cycle regulation and DNA repair. The recent discoveries should not only further our understanding of the cellular response to ionising radiation, but they may also help us develop and refine cancer treatment strategies. For instance, we now understand much better the opportunities—but also the potential caveats—that need to be considered when targeting signalling cascades, such as those involving EGFR, MAP kinase or the mTOR/AKT pathway, to selectively inactivate and/or sensitise tumour cells.

New discoveries have especially been made in the field of DNA repair. They now provide the exciting opportunity to understand the biochemical basis which underpins the large variation of tumour cell radiosensitivity that we have grappled with for so long. Tumour cells frequently suffer from deregulated DNA double-strand break repair, such as a switch from classical to PARP-dependent alternative end-joining or a ‘BRCAness’ phenotype of HR deficiency, which provides new targets for the selective sensitisation of tumours. The first successful steps in this direction involve the blocking of PARP or the checkpoint factors Chk1 and Chk2.

Gene, miRNA or protein expression profiling as well as functional assays should provide the means to assess the individual radiosensitivity, DNA repair capacity and susceptibility of a tumour to specific targeted therapies. However, overall, one should not underestimate the complexity of the interrelationship between DNA repair, other cellular processes and microenvironmental factors which will necessitate a careful evaluation of any initial findings.

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