MECHANISMS OF THERAPY-RELATED CARCINOGENESIS

James M. Allan and Lois B. Travis‡*

Abstract | Therapy-related cancers, defined as second primary cancers that arise as a consequence of chemotherapy and/or radiotherapy, are unusual in that they have a well-defined aetiology. Knowledge of the specific nature of the initiating exposure and exactly when it occurred has made it easier to identify crucial genetic events and to model these *in vitro* and *in vivo*. As such, the study of therapy-related cancers has led to the elucidation of discrete mechanisms of carcinogenesis, including DNA double-strand-break-induced gene translocation and genomic instability conferred by loss of DNA repair. Unsurprisingly, some of these mechanisms seem to operate in the development of sporadic cancers.

RELATIVE RISK
The observed number of cases reported after chemotherapy and/or radiotherapy as a function of the expected number of cases based on established incidence rates in the general population.

**Epidemiology and Genetics Unit, Department of Biology, University of York, Heslington, York, YO10 5YW, UK.*
‡Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, 6120 Executive boulevard, Bethesda, Maryland 20892-7238, USA.
Correspondence to J.M.A.
e-mail: Jim.Allan@egu.york.ac.uk
doi:10.1038/nrc1749
Published online
18 November 2005

Over the past 30 years, the number of cancer survivors in the United States has increased from 3 million to 9.8 million, representing 3.5% of the population[1]. The anticipated 5-year relative survival rate for adult patients who were diagnosed with cancer in 1995–2000 is now 64%, reflecting the results of earlier diagnosis, more effective treatment, prevention of secondary disease and decreases in mortality from other causes[1].

Although the acute toxicity of cancer therapy — which can cause symptoms such as nausea, myelosuppression and alopecia — is well defined, the late complications of treatment, including the development of dysplastic or fibrous tissue, continue to evolve because patients are now surviving longer. However, one of the most severe side effects following successful cancer therapy is the diagnosis of a second primary cancer (reviewed by van Leeuwen and Travis in REF. 2). This is defined as a new cancer that is distinct from the original disease and presents an independent picture of malignancy, and for which the possibility of it being a metastatic tumour has been ruled out[3]. The number of second primary cancers has steadily increased to the point where they now account for approximately one in six of all new cancer diagnoses in the United States[4].

Second primary cancers reflect not only the late effects of cancer therapy, but also the influence of aetiological factors that were shared with the initial cancer, such as tobacco and alcohol use, diet, immune function, hormonal status and environmental exposures[2,5,6]. Constitutional genetic variation is also likely to impact on an individual's risk of developing a second cancer. Indeed, polymorphic variation in carcinogen metabolism/detoxification and DNA repair pathways (BOX 1) have been associated with the risk of developing a second cancer (reviewed by Allan and Rabkin in REF. 7). So, as with other cancers, it is very difficult to identify with absolute confidence the specific causes of a second primary cancer in any individual patient. However, based on the relative increase in cancer incidence in survivors of a first cancer, documentation of statistically significant associations between increasing dose of chemotherapy or radiation and the risk of developing a second cancer, and the carcinogenicity of various cytotoxic drugs and radiotherapy in animal models, we are able to determine with reasonable accuracy the contribution of these exposures to cancer incidence at the population level, both in relative and absolute terms. The high RELATIVE RISK of developing a second cancer that is associated with specific treatments often exceeds the risk that is associated with other factors, including those that might have contributed to the development of the first cancer. Despite this, a lack of molecular genetic markers that are specific for therapy-induced cancer makes it almost impossible to say with absolute confidence whether a second cancer in any given individual is the result

ALKYLATING AGENTS
A large group of anticancer agents (for example, cyclophosphamide, procarbazine and cisplatin) that kill cells by the transfer of an alkyl group (for example, methyl or chloroethyl groups) to DNA, causing inhibition of replication and transcription.

TOPOISOMERASE INHIBITORS
Chemotherapeutic topoisomerase inhibitors (for example, etoposide and doxorubicin) prevent the re-ligation of enzyme-induced DNA double-strand breaks by stabilizing complex formation between the protein and its DNA substrate.

ANTI-METABOLITES
Anti-metabolite anticancer agents (for example, 6-thioguanine) share structural similarities with naturally occurring compounds, including nucleotides, and can be incorporated into DNA or RNA, causing inhibition of cell proliferation.

ABSOLUTE EXCESS RISK
Used as a measure of the actual number of excess cancers owing to previous therapy occurring in a defined population; usually expressed as a function of person-years of follow-up.

CLASTOGENIC
A substance or process that causes chromosome damage such as breaks, duplications or deletions.

Summary

- Treatment for a first cancer is associated with a significantly increased risk of developing a second primary cancer compared with the general population. Such cancers are termed 'therapy-related' and can represent a significant source of mortality for cancer survivors.
- Therapy-related cancers have been reported after structurally and mechanistically diverse treatments, in which the risk of developing these cancers is often dose dependent (such as for radiotherapy and alkylating agents).
- Molecular, cellular and epidemiological evidence indicates the existence of discrete mechanisms of carcinogenesis. These could involve either direct targeting of crucial transforming genes and relatively short latency of disease onset, or indirect targeting by the acquisition of a predisposing cellular phenotype (genomic instability) in which disease latency is longer.
- DNA double-strand breaks that are induced by chemotherapeutic topoisomerase inhibitors can lead to translocation of the mixed lineage leukaemia gene, as well as other crucial transforming genes.
- Chemotherapeutic methylating agents and thiopurines can promote the emergence of cells with dysfunctional DNA-mismatch repair and concomitant genomic instability.
- Radiotherapy and chemotherapy interact with other factors, such as hormonal status, cigarette smoking and genetic makeup, to modify the risk of developing a second cancer.
- By understanding the risk factors for developing therapy-related cancers, and the mechanisms by which they develop, we might be able to prevent them or identify patients at high risk who might benefit from surveillance.

of previous therapy. One possible exception to this is genomic microsatellite instability (MSI), which has been reported with high frequency in therapy-related myeloid leukaemia (discussed below) and is rare in sporadic myeloid leukaemia.

The administration of chemotherapy and radiotherapy represents one of the few situations in which humans are carefully exposed therapeutically to known amounts of potential carcinogens, and constitutes a source of risk that is more easily controlled than other influences. This produces a potentially useful setting in which to define dose–response and agent-interaction relationships with second cancer occurrence. Apart from increasing incidence rates, it should also be recognized that second cancers are an increasingly important cause of mortality among some cancer survivors; second cancers now constitute the leading cause of death in patients who have been cured of Hodgkin lymphoma[8–10].

Box 1 | Repair of DNA damage

The carcinogenicity of ionizing radiation and many chemotherapy agents is partly dependent on their ability to induce mutagenic and CLASTOGENIC DNA damage, including base adducts, replication errors, strand breaks and crosslinks. As such, the repair of DNA damage is an important mechanism that protects against the deleterious effects of carcinogenic therapies. The activation of DNA repair pathways is intimately linked with other cellular pathways, including transcription, cell-cycle checkpoint arrest and apoptosis, such that damaged cells respond appropriately to DNA damage and are either repaired or eliminated. Several interacting DNA repair pathways exist[133,134], including: direct repair of DNA damage, in which the integrity of the DNA duplex is restored in a single-step reaction; base excision, nucleotide excision and mismatch repair, in which damaged or mispaired bases are repaired by excision of a tract (between one and several hundred base pairs depending on the pathway) of single-stranded DNA and subsequent gap filling; strand-break repair involving either direct re-joining of damaged ends or repair by annealing to the homologous duplex; and crosslink repair, which shares components with both nucleotide excision repair and homologous recombination repair. Loss or attenuation of DNA repair can lead to genomic instability, and is predicted to be a mechanism that operates in the pathogenesis of some therapy-induced cancers.

Increased risks of developing a second cancer have been reported after treatment with radiotherapy and with structurally and functionally diverse chemotherapy agents, including ALKYLATING AGENTS, TOPOISOMERASE INHIBITORS and ANTI-METABOLITES. Furthermore, therapy-related cancers have been reported at many, but not all, sites in the body. However, research efforts have concentrated on the genetic and molecular mechanisms of therapy-induced leukaemia, particularly those that develop after chemotherapy, with relatively little work that addresses therapy-induced solid cancers. One reason for this imbalance might be that the relative risk of developing leukaemia after therapy is considerably higher than the risk of developing a solid cancer (FIG. 1), which is a reflection of the inherent sensitivity of the bone marrow to the toxic and mutagenic effects of several groups of chemotherapy agents. By contrast, the tissue specificity of radiotherapy-induced carcinogenesis not only reflects different organ sensitivities[11], but also reflects the physical boundaries of the exposure field. Despite the low relative risk of developing solid cancer after radiotherapy, the high ABSOLUTE EXCESS RISK highlights the need for further research in this area.

In addition to site and causative exposure, therapy-related cancers can also be characterized by acquired genetic abnormalities, phenotype and time between therapeutic exposure and diagnosis (latency), with each providing clues to the underlying mechanisms of carcinogenesis. For example, cases of therapy-induced acute myeloid leukaemia (AML) can be broadly classified into two groups; those that develop after treatment with topoisomerase-targeting chemotherapy, and those that develop after alkylating chemotherapy or radiotherapy. The former group is characterized by a short latency period — from as little as a few months to approximately 5 years (FIG. 1) — and often presents with only one

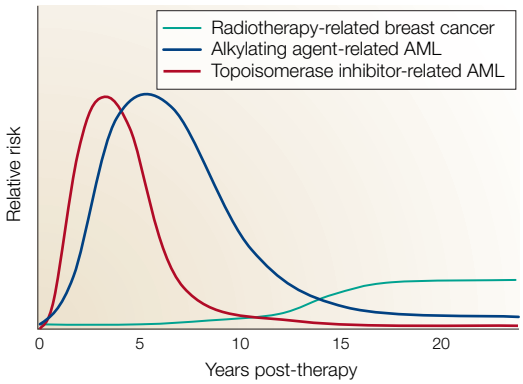

Figure 1 | **Relative risk of developing a therapy-associated cancer after Hodgkin lymphoma.** Using data from patients who have been treated for Hodgkin lymphoma, this figure compares the approximate temporal pattern and the relative risk of developing acute myeloid leukaemia (AML) after treatment with either alkylating agents or topoisomerase inhibitors with the risk of developing breast cancer after radiotherapy. The relative risk of developing radiogenic breast cancer is age dependent, but generally peaks 15–20 years post-therapy and decreases relatively little thereafter[123,126,135,136]. By contrast, the relative risk of developing AML after chemotherapy for Hodgkin lymphoma is higher and peaks earlier, between 2 and 7 years post-therapy[126,135,137], and decreases thereafter. Despite a lower relative risk of developing breast cancer after Hodgkin lymphoma, the higher background incidence of breast cancer in the population translates into a higher absolute excess risk of developing therapy-related breast cancer than leukaemia[8,135].

gross genetic abnormality. These features indicate a relatively simple mechanism of carcinogenesis; quick development after therapy that involves direct targeting of crucial transforming genes. Indeed, the epidemiological, biological and genetic data are broadly compatible with a limited 'one- or two-hit' genetic model for this type of leukaemia (FIG. 2), in which gene translocation, for example, provides the first hit, but subsequent mutational hits might be required for complete transformation. By contrast, the latter group is characterized by a longer latency period of 2–10 years post-therapy. Patients often present with numerous and complex genetic abnormalities and a pre-malignant dysplastic phase of disease. These features indicate a mechanism involving the acquisition of genomic instability and subsequent indirect targeting of crucial transforming genes.

Although we can make a direct comparison between leukaemias that arise after different exposures, we must be cautious of drawing parallels between cancers that arise in different tissues. Inherent differences in sensitivity to chemotherapy and radiotherapy, and the influence of other risk factors such as tobacco use, might affect different tissues in different ways[2,5]. Although the available data are limited, the general characteristics of therapy-induced solid cancers, which can include multiple genetic lesions[12,13] and an extended latency period (FIG. 1), indicate a multistage mechanism of carcinogenesis and indicate that genomic instability of some form might also be operating.

Therapy-induced DNA double-strand breaks

DNA double-strand breaks (DSBs) are difficult to repair and are extremely cytotoxic as a consequence, particularly to rapidly proliferating cells. Therefore, exposures that generate DSBs, such as ionizing radiation, can make very effective anticancer agents. Unfortunately, the difficulty in processing DSBs means that when they are repaired there is a possibility that they are repaired incorrectly and contribute to therapy-induced carcinogenesis. Gene translocations — which are often reported in therapy-induced cancers, particularly leukaemia — represent one form of DSB misrepair. The approximate linear relationship between radiotherapy dose to the lung in patients who are treated for Hodgkin lymphoma and the risk of subsequently developing lung cancer illustrates the carcinogenic potential of DSB-inducing exposures[14] (TABLE 1). Similar to radiotherapy, some chemotherapy agents are also effective at inducing DSBs, including those that inhibit the function of DNA topoisomerases.

Inhibition of DNA topoisomerases. DNA topoisomerases unknot and relax supercoiled DNA, and are therefore crucial to normal DNA metabolism. DNA topoisomerases carry out these functions through the ordered transient generation and re-ligation of DSBs. Chemotherapeutic topoisomerase inhibitors trap the protein following DNA cleavage but before re-ligation. This generates DSBs that then have the potential to participate in gene translocation[15].

The induction of DSBs and the translocation of several genes that are affected in therapy-related leukaemia, such as *AF4* and *AF9*, is postulated to occur by inhibition of DNA topoisomerase II (REFS 16–18). AF9 is a component of the t(9;11) translocation and is one of the most common aberrations seen in therapy-related leukaemia after chemotherapy with topoisomerase inhibitors. Other translocations that are seen in therapy-related leukaemia, such as t(8;21) and t(15;17), are also postulated to occur through topoisomerase-induced strand cleavage[19,20]. Indeed, Mistry *et al.*[20] demonstrated that *PML* translocation breakpoints in patients who developed t(15;17)-positive leukaemia after chemotherapy with the topoisomerase inhibitor mitoxantrone were hot-spots for mitoxantrone-induced topoisomerase inhibition and DNA cleavage *in vitro*.

However, it is the mixed lineage leukaemia gene (*MLL*, also known as *HRX* or *ALL1*) at chromosome 11q23 that has served as the definitive model for the study of translocation induction in leukaemia (both myeloid and lymphoid) that develops after treatment with topoisomerase inhibitors. *MLL* encodes a transcription factor with a role in the regulation of haematopoietic development[21,22], and it is often the only gross genetic aberration that is apparent in these cancers[23,24].

Consistent with a direct role in mediating *MLL* gene translocations, topoisomerase inhibitors, such as the chemotherapeutic epipodophyllotoxins and anthracylines, cause DSBs *in vitro* that map to translocation break points *in vivo*[16,25] (FIG. 3), including regions that

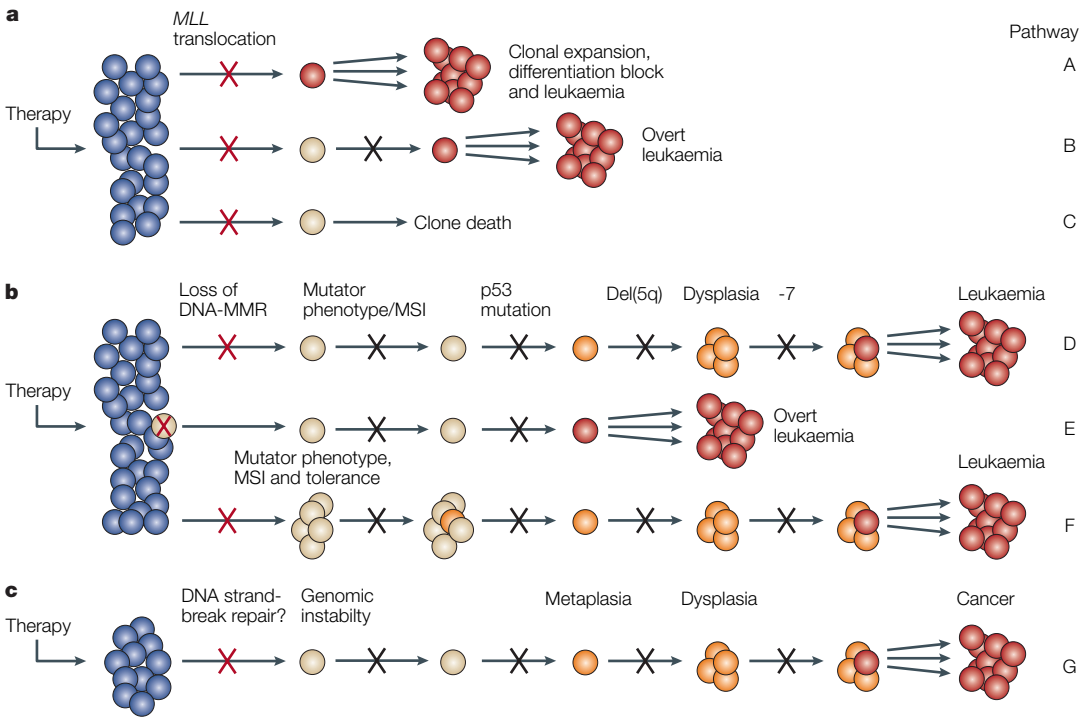

Figure 2 | **The one- or two-hit and mutator phenotype models of therapy-related cancer.** This figure illustrates the two basic models of transformation that are predicted to be operating in therapy-related carcinogenesis. **a** | First, a limited one- or two-hit model typified by mixed lineage leukaemia gene (*MLL*)-translocation-positive leukaemia. In this model, translocation to the appropriate partner gene might be sufficient for development of leukaemia (pathway A), or translocation to other partner genes might require additional mutations for transformation (pathway B). The fate of initiated cell clones that do not acquire a second mutation is not known, but one possible outcome is clone death (pathway C). Transformation by this mechanism develops relatively quickly after therapeutic exposure, and leukaemia usually presents without a preceding dysplastic phase. **b** | Second, the mutator phenotype model typified by microsatellite instability (MSI)-positive leukaemia. In this model, therapy is hypothesized to promote the generation of (pathways D and F), or emergence of a pre-existing (pathway E) DNA-mismatch repair (DNA-MMR)-defective cell clone. If methylating, thiopurine or platinating chemotherapy agents are involved, then loss of DNA-MMR might also confer tolerance to the killing effects, giving rise to clonal enrichment (pathway F). The mutator phenotype associated with loss of DNA-MMR is predicted to result in the accrual of multiple mutations in secondary genetic targets, ultimately leading to leukaemia. Transformation by this mechanism develops relatively slowly after therapeutic exposure, and leukaemia often presents with a preceding dysplastic phase, but can also present overtly. **c** | This figure illustrates a general model for transformation through acquisition of genomic instability involving abrogation of other cellular pathways such as DNA strand-break repair (pathway G). For all pathways, red crosses indicate a primary mutational event, black crosses indicate secondary or subsequent mutational events.

contain DNA topoisomerase-binding sites[16–18,26–30]. The demonstration by Libura and colleagues[31] that etoposide, a chemotherapeutic epipodophyllotoxin, can induce *MLL* rearrangement in cultured human haematopoietic progenitor cells provides conclusive evidence of a causal link between chemotherapy exposure and *MLL* translocation in what is the crucial target cell for leukaemogenesis (FIG. 3).

MLL-translocated leukaemia has also served as a model for the one- or two-hit mechanism of leukaemic transformation (FIG. 2). So far, more than 30 genes have been described as translocation partners of *MLL*[32], including many that have been described in therapy-related leukaemia. Despite the heterogeneity of these partner genes, all translocations studied so far have demonstrated deletion of the 3′ component of *MLL* and translocation, in frame, of the remaining 5′ component. For leukaemogenesis to occur, expression of the chimeric gene must give rise to a protein with dominant-transforming properties. The transforming potential of translocated *Mll* was illustrated by Wang *et al.*[33] using a mouse conditional knock-in mutant carrying *Mll* fused to the gene encoding cAMP-responsive-element-binding protein (*Cbp*) — one of the *Mll* partner genes that is almost exclusively reported in therapy-related leukaemia[34]. Within days of gene activation, the resulting chimeric protein caused selective expansion of myeloid cells (granulocytes and macrophages) in the bone marrow and gave rise to haematopoietic stem cells with an enhanced repopulating/proliferation potential[33]. Other mouse models expressing chimeric *MLL* genes that have been reported in therapy-related leukaemia, including *MLL–GAS7* (REF. 35), *Mll–Enl*[36] and *Mll–AF9* (REFS 37,38), also demonstrate the powerful transforming potential of translocated *MLL*.

Although it is clear that some *MLL* fusion genes are sufficient to cause leukaemia (the one-hit model)[36],

Table 1 | **Relative risks of developing lung cancer after radiotherapy for Hodgkin lymphoma**

Radiation dose to affected site in the lung (Gy)	Relative risk (95% confidence interval)	*p**
0	1 (reference)	Not applicable
0–4.9	1.6 (0.5–5.2)	0.39
5–14.9	4.2 (0.7–21)	0.11
15.0–29.9	2.7 (0.2–15)	0.4
30.0–39.9	8.5 (3.3–24)	<0.001
≥40.0	6.3 (2.2–19)	<0.001

This table illustrates that the relative risk of developing radiogenic lung cancer after Hodgkin lymphoma is compatible with a linear relationship to radiation dose to the affected area of the lung (adapted from Gilbert *et al.*[14]). *Two-sided *p* value based on the likelihood ratio test of the null hypothesis that relative risk = 1.

for most others the acquisition of additional genetic alterations seems to be required for complete transformation to leukaemia (the two-hit model)[38] (FIG. 2). Indeed, transformation to a fatal leukaemia-like disorder in the *Mll–Cbp* mouse model was dependent on treatment with a powerful mutagen[33]. Mouse models expressing other therapy-associated translocations have also indicated a requirement for additional mutations. For example, the t(8;21) translocation, which fuses the *AML1* gene with the *ETO* gene, is required but not sufficient for leukaemogenesis, and treatment with a powerful mutagen again demonstrates a requirement for one or more additional mutations for complete transformation[39]. The identity of putative secondary mutations remains elusive, although Ono and colleagues[40] identified a mutation in the FLT3 tyrosine kinase receptor as one that could complete the leukaemogenic process following initiation by *MLL* gene fusion. The two-hit model is in accordance with the conclusion by the International Agency for Research on Cancer that there is limited evidence in humans for the carcinogenicity of the topoisomerase inhibitor etoposide when given alone, whereas sufficient evidence exists for the carcinogenicity of etoposide given in combination with cisplatin and bleomycin[41].

The development of translocation-positive, therapy-induced leukaemia in humans follows a similar temporal pattern to that seen in many of the mouse models discussed above. For example, an *MLL–GAS7* fusion was first detected in the bone marrow of a paediatric patient with neuroblastoma just 6 weeks after the start of anti-topoisomerase chemotherapy. However, cytopaenia and therapy-related leukaemia did not develop until 4.5 and 15.5 months, respectively, after the fusion gene was first detected[42]. It remains unclear to what extent the intervening latency period reflects time for clonal expansion or a requirement for additional mutations. Further work will be required to establish the contribution of secondary mutations to translocation-positive leukaemogenesis and whether they represent a rate-limiting step in the development of therapy-related leukaemia. Nevertheless, these studies clearly demonstrate that translocation of *MLL*, and other genes, has a direct role in abrogating normal haematopoiesis and contributing to therapy-related leukaemogenesis.

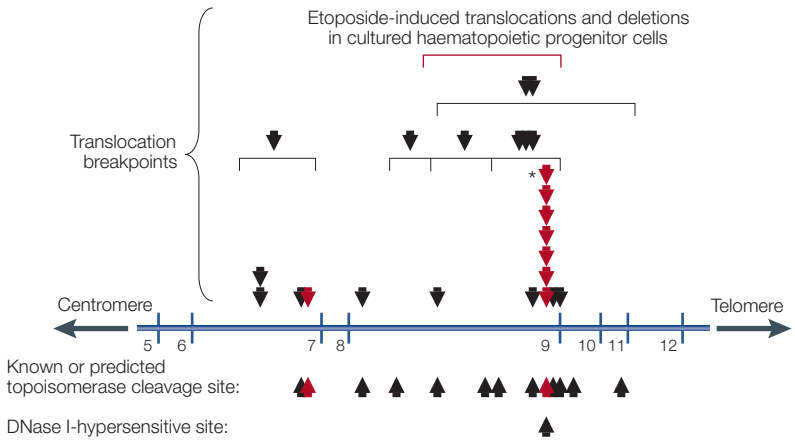

Figure 3 | **DNA topoisomerase cleavage sites and *MLL* translocation breakpoints in therapy-related leukaemia.** This figure shows a schematic representation of the translocation breakpoint cluster region of the *MLL* gene (blue horizontal bar). Exons 5–12 are indicated by blue vertical bars (not to scale). Approximate *MLL* translocation breakpoints for 24 patients with therapy-related leukaemia are shown by downward arrows[16–18,27–29,34,42,47,138], which includes a single patient who had not been previously treated with topoisomerase inhibitors[47] (indicated by the asterisk). Upward arrows indicate known or predicted topoisomerase binding sites, or DNase I-hypersensitive sites. Opposing downward and upward red arrows indicate therapy-related leukaemia cases in which translocation breakpoints co-localize precisely with topoisomerase cleavage sites[16,18,29,47]. The horizontal red line indicates the distribution of *MLL* translocation and deletion breakpoints in cultured haematopoietic progenitor cells after treatment with etoposide[31].

Apoptotic endonucleases. Although there is compelling evidence for the involvement of topoisomerase inhibition in the aetiology of some chemotherapy-induced translocations, the co-localization of DNase I-hypersensitive sites with some translocation breakpoints, including some in *MLL*[19,30] (FIG. 3), indicated an alternative mechanism of translocation induction that is independent of topoisomerase inhibition. Cleavage of the *MLL* gene in apoptotic cells has indicated that non-specific endonuclease activity can cause translocations[28,43]. The observation that mechanistically diverse exposures, including activation of CD95 and serum starvation, could initiate *MLL* translocation confirmed the association with apoptosis. This observation also indicated that DNA strand cleavage occurred early during apoptosis in cells that had initiated, but were not fully committed to executing, apoptosis[44–46]. These observations

DNA-MISMATCH REPAIR (DNA-MMR). Protects against mutation by correcting base-misinsertion errors made by DNA polymerases during replication. Loss of DNA-MMR confers a mutator phenotype, with microsatellite instability being one component of this.

provide an attractive model to explain the development of *MLL*-translocation-positive, therapy-related leukaemia in patients without any history of anti-topoisomerase therapy[47] (FIG. 3). DNA cleavage and translocation of other leukaemogenic proto-oncogenes — including *TEL*[48] — in cells exposed to pro-apoptotic signals, again indicates that this mechanism of translocation induction is not unique to *MLL*.

Therapy-induced genomic instability

DSB-induced chromosome damage (translocation, deletion, duplication, and so on) as a result of previous therapy contributes to the pathogenesis of therapy-related solid cancers. In contrast to translocation-positive leukaemias, however, the one- or two-hit model does not seem to be an important feature of therapy-induced solid cancers. Instead, limited data indicate that genomic instability might be a characteristic of such cancers[12,13]; a phenotype that has also been reported in leukaemias that develop after alkylating chemotherapy.

The genomic-instability model of multistage carcinogenesis predicts that a high underlying mutation rate, or 'mutator phenotype', predisposes the target cell to accumulate mutations in multiple genes that will eventually lead to transformation[49] (FIG. 2). The acquisition of a mutator phenotype is postulated to be caused by initiating mutations in key genes and pathways, such as DNA replication and DNA repair[50], that normally function to maintain genomic stability, and that this is an early event in the transformation process. Mutations in these pathways might not necessarily be directly transforming by themselves. Rather, they could promote the acquisition of mutations in genes and pathways that do have the potential to contribute directly to the transformation process. There is now considerable evidence to indicate that a mutator phenotype or genomic-instability mechanism might be operating in at least some therapy-induced cancers, particularly alkylating-chemotherapy-induced leukaemia (FIG. 2).

High-grade MSI, defined as expansion or retraction of repetitive microsatellite sequences, is very rare in *de novo* leukaemia, but common in therapy-related leukaemia; it has been reported in up to 90% of cases[51–56]. MSI is diagnostic of abrogated DNA-MISMATCH REPAIR (DNA-MMR), which, in other cancers, often involves loss of function of either MLH1 or MSH2. Indeed, abrogation of *MSH2* gene expression and point mutations have also been reported in MSI-positive therapy-related leukaemia[52,55].

There are at least two mechanistically distinct classes of therapeutic agents that can promote loss of DNA-MMR function, ostensibly by the same cellular process. Both O^6-guanine methylating and thiopurine anti-metabolite chemotherapy agents are postulated to drive carcinogenesis by promoting the emergence of cells with a DNA-MMR defect and genomic instability. Exactly how this might occur *in vivo* remains unclear. Mouse studies indicate that therapeutic exposure can promote the emergence of a pre-existing DNA-MMR-defective bone marrow progenitor cell population in a background of DNA-MMR-competent cells[57]. However, whether methylating agents or thiopurine metabolites can initiate generation of a DNA-MMR-defective cell has yet to be determined experimentally, although this seems a reasonable hypothesis given the mutagenicity of these agents (discussed below).

O^6-guanine methylating chemotherapy agents. Numerous chemotherapy agents, including procarbazine, dacarbazine, streptozocin and temozolomide, induce tumour cell death by methylating several atoms in DNA, including the O^6 atom of guanine. The resultant O^6-methylguanine DNA lesions are initially recognized and repaired by the O^6-methylguanine DNA methyltransferase (MGMT) protein in a process by which the methyl lesion is transferred to a cysteine residue within the active site of the protein[58] (FIG. 4). When successfully repaired by MGMT, O^6-methylguanine is neither mutagenic nor toxic. However, when MGMT is either absent or depleted, the toxicity of O^6-methylguanine DNA lesions is dependent on cellular processing by DNA-MMR. This processing initiates apoptosis in response to mispaired lesions through a mechanism that is thought to involve attempted, but failed, DNA repair[59,60] (FIG. 4). O^6-methylguanine efficiently causes mispairing during DNA replication, often with thymine[61] (FIG. 4). It is these mispaired base pairs, generated after two rounds of DNA replication, that elicit a DNA-MMR response.

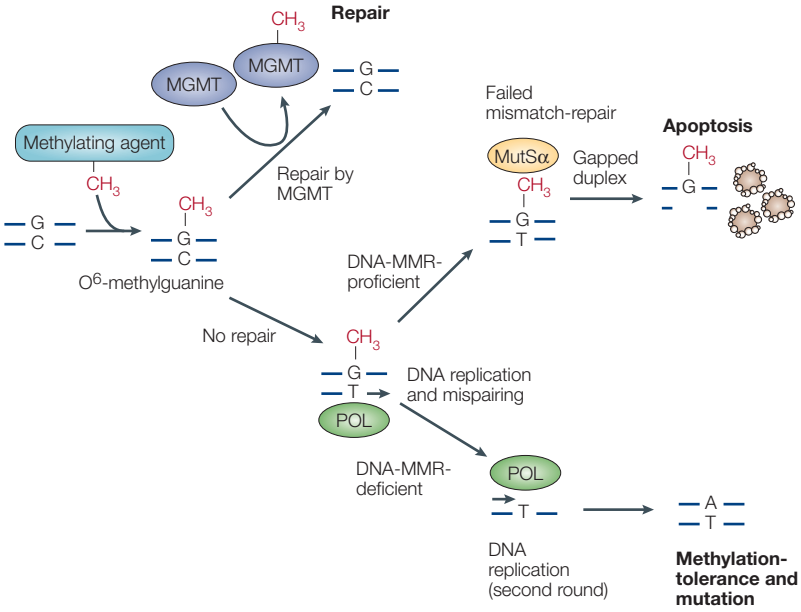

Figure 4 | **Cellular processing of a chemotherapy-induced O^6-methylguanine DNA lesion by MGMT and DNA-MMR.** This figure shows the induction of an O^6-methylguanine lesion in DNA by a chemotherapeutic methylating agent, such as procarbazine, and the possible outcomes after cellular processing determined by DNA-mismatch repair (DNA-MMR) status. Possible outcomes include: repair by O^6-methylguanine methyltransferase (MGMT); apoptosis; or methylation-tolerance and induction of mutations. The latter outcome is predicted to drive transformation. This basic model, excluding the involvement of MGMT, might also be applied to loss of DNA-MMR induced by exposure to thiopurine nucleotides and possibly by cisplatin chemotherapy. MutSα is a heterodimer of MSH2 and MSH6, and is an important component of the DNA-MMR system. POL, DNA polymerase.

However, it is the presence of the methylated base in the parent strand, which DNA-MMR cannot cleave, that is predicted to result in failed repair, ultimately leading to cytotoxicity (FIG. 4).

As might be predicted, the loss of functional DNA-MMR confers tolerance to the killing effects of methylating agents[59,62–64] (FIG. 4). Furthermore, the O^6-methylguanine lesions that persist in the genome of DNA-MMR defective cells are clastogenic and can also be fixed to mutations during DNA replication[61,65,66]. Indeed, the clastogenicity and mutagenicity of the O^6-methylguanine lesion is thought to contribute to the carcinogenicity of the methylating chemotherapy agents[67] (FIG. 4).

Crucially, *in vitro* treatment with O^6-guanine methylating agents can drive the emergence of cells with DNA-MMR deficiency[68]. It was this ability to generate DNA-MMR-defective cells in the laboratory that aided the characterization of the DNA-MMR pathway during the 1990s. It is also conceivable that exposure to methylating chemotherapy agents might promote the emergence of cells that have low expression of DNA-MMR proteins, by virtue of their greater tolerance, and that this would increase the risk of subsequent point mutation (non-disabling DNA repair defects are discussed later in more detail). Loss of DNA-MMR is often seen and is more readily induced by methylating agents in cells that are deficient in MGMT[62,68]. So, in MGMT-deficient cells, DNA-MMR has an important role in mediating cellular responses to the unrepaired methyl lesions induced by chemotherapeutic methylators[66,69]. Intriguingly, bone marrow cells, and particularly the CD34-positive haematopoietic progenitor target cell for leukaemic transformation, express low levels of MGMT[70]. These observations go some way towards explaining the extreme sensitivity of the bone marrow to the toxic, mutagenic and leukaemogenic effects of chemotherapeutic methylating agents[58]. Taken together, these data indicate a model whereby chemotherapeutic methylating agents select for cells with dysfunctional DNA-MMR *in vivo*, which might initiate the leukaemogenic process through the acquisition of genomic instability (FIGS 2,4).

Procarbazine causes solid tumours, including lung cancer in laboratory animals[71], and a significant ($p < 0.001$) relationship was recently found between the cumulative dose of procarbazine given to treat Hodgkin lymphoma and the risk of developing lung cancer[14,72]. It remains to be determined, however, whether abrogated DNA-MMR is a significant feature of solid cancers after methylating chemotherapy, although reports of MSI in solid tumours after chemotherapy for paediatric cancer[13], and development of lung cancer after Hodgkin lymphoma[12], indicate that this is a distinct possibility.

Thiopurine anti-metabolite chemotherapy. Similar to methylating agents, exposure of cells *in vitro* to the thiopurine chemotherapy agent 6-thioguanine can also induce loss of DNA-MMR[54]. Furthermore, when incorporated into DNA, 6-thioguanine lesions are also recognized by the DNA-MMR system and are postulated to initiate apoptosis by essentially the same DNA-MMR-mediated mechanism as O^6-methylguanine[73,74]. Indeed, tolerance to O^6-methylguanine is associated with cross-tolerance to 6-thioguanine[75].

Azathioprine, an immunosuppressant anti-metabolite that is used extensively as therapy for transplant patients to minimize the risk of organ rejection, is metabolized to 6-thioguanine nucleotide. Given this information, it is perhaps not surprising that the use of azathioprine is associated with a significantly increased risk of developing leukaemia, and that these leukaemias show extensive MSI and loss of DNA-MMR[54], which is indicative of a mutator phenotype. Moreover, non-Hodgkin lymphomas that develop after azathioprine treatment also show extensive MSI[76]. It remains to be seen whether other tumours that arise after thiopurine chemotherapy, including brain tumours and leukaemia in children treated for lymphoblastic leukaemia[77,78], also display MSI and DNA-MMR defects. It should be noted that other components related to therapy-induced immunosuppression, including T-cell depletion and susceptibility to infection, can also increase the risk of developing a second cancer in transplant patients and other immunocompromised patients. These indirect mechanisms of therapy-induced carcinogenesis have been comprehensively reviewed by others[6,79].

Radiotherapy, cisplatin and other chemotherapy agents. The requirement for functional DNA-MMR as a mediator of cell death after exposure to both methylating and thiopurine chemotherapy agents provides a basis by which cyclical chemotherapy dosing with these agents might select for cells with dysfunctional DNA-MMR and genomic instability, ultimately leading to therapy-related cancer. However, microsatellite-unstable leukaemia, and solid cancer, has also been reported after therapy that did not include either O^6-guanine alkylating or thiopurine agents[13,53,80]. This indicates that other therapeutic exposures might also be able to select for DNA-MMR-defective cells *in vivo*. Consistent with this hypothesis, the resistance of DNA-MMR-deficient cells to cisplatin has been documented in numerous studies[81–86], although the associated resistance phenotype tends to be very modest compared with that seen following exposure to methylating agents or thiopurines. This phenotype has been attributed to abortive DNA-MMR-mediated processing of cisplatin adducts; a hypothesis supported by the binding of the DNA-MMR MutSα complex (an MSH2–MSH6 heterodimer) to cisplatin-damaged oligonucleotides *in vitro*[82,87]. Crucially, cisplatin treatment has been shown to select for DNA-MMR-deficient cells both *in vitro* and *in vivo*[85], and can also induce MSI in relapsed human ovarian tumours[85,88], presumably through loss of DNA-MMR. So, despite the modest phenotype associated with loss of DNA-MMR, the available evidence would indeed indicate that

platinum-based therapy can promote the emergence of cells with a mutator phenotype *in vivo*. It remains to be seen whether the increased risk of developing leukaemia after platinum-based chemotherapy for testicular or ovarian cancer involves loss of DNA-MMR[89,90].

The relationship between the status of DNA-MMR and the transforming effects of radiotherapy is less clear. Some studies have reported resistance to the toxic effects of ionizing radiation in DNA-MMR-defective cells[91,92], whereas others report no difference or sensitivity[93–97]. However, consistent reports of sensitivity to the mutagenic and clastogenic effects of ionizing radiation in DNA-MMR-defective cells[95,98] indicates an alternative mechanism by which transformation might be promoted. So, although loss of DNA-MMR might not confer a survival advantage to radiation-exposed cells (as it does for methylating agents, thiopurines and cisplatin), it might predispose to radiotherapy-induced mutations. Highly mutagenic oxidized DNA bases, an important product of ionizing radiation exposure in living cells, accumulate to a greater extent in DNA-MMR-defective cells compared with competent cells[92,99], and also contribute significantly to the mutator phenotype of these cells[100]. As such, exposure to ionizing radiation might augment the already high spontaneous mutator phenotype of DNA-MMR-defective cells. Indeed, this model provides a plausible mechanism by which any mutagenic therapy might promote transformation of DNA-MMR defective cells, and might explain why MSI-positive leukaemia and solid cancer are reported after mechanistically diverse therapies, such as radiotherapy and the alkylating nitrogen mustards[12,13,53]. This model, however, does not exclude the possibility that radiation might induce genomic instability that is independent of DNA-MMR (discussed later).

Secondary targets in the mutator phenotype model. The genomic-instability model predicts that although the initiating genetic targets might be limited to a few specific pathways, such as DNA replication or DNA repair, there might be multiple different downstream mechanisms by which transformation might ultimately occur. Consistent with this model, numerous genetic defects have been reported in MSI-positive leukaemias, including chromosome deletions, translocations and point mutations[53,80,101]. Of particular significance is the loss of chromosomes 5 and/or 7, deletions in 5q, 7q or 17p, mutation of *RAS*, inactivation of *CDKN2B* (cyclin-dependent kinase inhibitor 2B), mutation of *TP53* and balanced translocations, all of which are proposed to operate in semi-independent pathways of leukaemogenesis that are not yet fully defined[102] (FIG. 2). A similarly diverse plethora of genetic lesions are also reported in therapy-related solid cancers[12,13]. Specifically, Behrens *et al.*[12] demonstrated that deletion and microsatellite alteration on the long arms of chromosomes 6 (6q13–14 and 6q22–27) and 17 (17q21) was more prevalent in therapy-related breast cancer than sporadic breast cancer, consistent with frequent alterations at these regions in non-transformed breast cells exposed to ionizing radiation *in vitro*[103]. These data indicate the presence of one or more tumour-suppressor genes at these regions that might be important in radiogenic breast carcinogenesis.

Many of the lesions described above are predicted to be directly transforming, including deleted chromosome 5q, which is thought to host at least one leukaemia-specific tumour-suppressor gene. Of particular interest are the cellular pathways in which abrogation in an MSI-positive, DNA-MMR-negative cell might predispose to the acquisition of gross genetic lesions. The prevalence of chromosome deletions and translocations, which are generated through the formation of DSBs, has implicated abrogated DSB-repair as a possible secondary target in therapy-induced cancers. Consistent with this, the genes that encode MRE11 and FANCD2, both of which have a role in the repair of DSBs, are targets for mutation in therapy-related leukaemias that show MSI[80,104] and DNA-MMR-defective sporadic cancers[105]. Moreover, the expression of several key DNA strand-break-repair genes, including *Mre11a*, is deregulated in mouse stem cells that are deficient in DNA-MMR; a phenotype that can be recapitulated in wild-type cells by treatment with a methylating agent[106].

Other models of genomic instability. There is considerable evidence supporting the induction of genomic instability in cells that are exposed to ionizing radiation *in vitro*[107–110], although the exact mechanisms underlying this phenotype remain unclear. Moreover, the findings of genomic instability in neighbouring 'bystander' cells that are not directly exposed are provocative (reviewed by Little in REF. 111). However, extending these *in vitro* findings to the human situation with the aim of identifying causal pathways in radiotherapy-related cancer has been limited by a paucity of data. Nevertheless, we can speculate that DNA-repair pathways *per se* might represent primary targets in therapy-related carcinogenesis, in which abrogation might confer a genomic-instability phenotype. However, whereas MSI serves as a useful surrogate for loss of DNA-MMR, which has no doubt aided research, similar phenotypic markers that can readily be applied in the laboratory have yet to be identified and developed for other putative forms of genomic instability that might be associated with DNA-repair defects.

We must also consider the possibility that genomic instability might be conferred on cells by attenuation, but not complete loss, of such pathways. Indeed, the DNA-MMR-mediated G2/M cell-cycle checkpoint that is activated in response to DNA-methylation damage is lost when expression of MLH1, a key component of DNA-MMR, is attenuated but not completely abolished[112]. Minimal expression of MLH1 was sufficient to restore functional DNA-MMR and protect against MSI[112], but the associated loss of cell-cycle checkpoints is predicted to confer other forms of genomic instability. If true, the transformation process might actually begin before complete loss

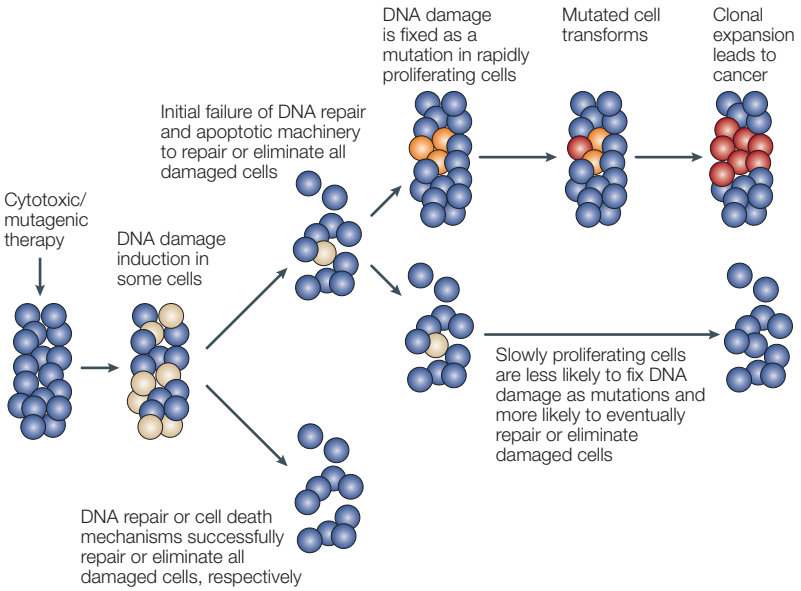

Figure 5 | **The role of cell death and proliferation in therapy-related carcinogenesis.** This figure illustrates the predicted roles of cell death and proliferation in therapy-related carcinogenesis. Exposure of non-target normal tissue to radiotherapy and/or chemotherapy can induce mutagenic and cytotoxic DNA damage. DNA repair and cell death pathways operate to repair or eliminate damaged cells, respectively. Failure to repair or eliminate damaged cells can result in fixation of DNA damage into mutation. Rapidly proliferating cells are more likely than slowly proliferating cells to fix DNA damage into mutations. A mutated cell has the potential to transform and can ultimately give rise to a cancer after clonal expansion. The proliferative state at the time of therapeutic exposure and also post-therapy (as tissues repopulate) are both predicted to impact on the risk of developing a therapy-induced cancer.

of DNA-MMR, perhaps with reduced gene expression conferred by loss of a single allele. Consistent with this notion, *Msh2* heterozygosity in mouse cells does not give rise to MSI, but does lead to accelerated tumorigenesis in animals, often with retention of the wild-type *Msh2* allele[113,114]. Extending this hypothesis, we might predict that attenuation, but not complete loss, of DNA-MMR in humans might predispose to therapy-induced cancer. Consistent with this model, Worrillow *et al.*[53] demonstrated an association between a polymorphism in *MSH2* — which does not abolish DNA-MMR function but is predicted to affect RNA splicing — and the risk of developing therapy-related leukaemia in patients who had previously been treated specifically with O^6-guanine alkylating agents.

Genomic instability and the mutator phenotype in sporadic cancers. The genomic-instability model of carcinogenesis is not unique to therapy-related cancer, and was initially developed in the context of sporadic carcinogenesis[49]. Indeed, loss of DNA-MMR, with its concomitant mutator phenotype, is seen in approximately 15% of sporadic colon cancer cases, a process thought to be partly driven by chronic exposure of the gut to endogenous and exogenous methylating agents (reviewed by Povey *et al.* in REF. 115). Loss of DNA-MMR is also reported in numerous other sporadic cancers (reviewed by Peltomaki in REF. 116). As such, there seem to be considerable parallels between the molecular mechanisms underpinning therapy-related and sporadic carcinogenesis.

Avoidance of cell death

The ability of multicelled organisms to selectively remove genetically unstable cells by apoptosis protects against cancer. Indeed, acquisition of an anti-apoptotic phenotype is considered to be fundamental to carcinogenesis, and might occur relatively late during transformation. Given the toxic doses that are associated with chemotherapy and radiotherapy, the avoidance of cell death at the time of exposure is probably also important in therapy-related carcinogenesis.

The importance of cell killing in the aetiology of therapy-induced cancer is illustrated by the relationship between radiation dose and the subsequent risk of developing leukaemia; at low doses there is a linear dose–response relationship with respect to the risk of developing cancer, but at high doses there is a downturn in risk[117]. Moreover, high doses of radiation to a limited exposure field confer little excess risk of developing leukaemia[118,119], whereas lower doses to a wider field are more leukaemogenic[117]. Taken together, these data indicate that the induction of cell death at high radiation doses attenuates its carcinogenic effects, presumably by eliminating heavily damaged cells. Consistent with this hypothesis, we would expect that the inappropriate avoidance of cell death would confer susceptibility to therapy-induced cancer (FIG. 5). Indeed, this principle has been elegantly demonstrated using transgenic mice that overexpress the anti-apoptotic protein BCL2. Following a 2 Gy dose of radiation, less than 2% of wild-type mice developed leukaemia. By contrast, almost 50% of *BCL2*-transgenic mice developed leukaemia following the same dose[120]. Similarly, attenuation of the p53-mediated apoptotic response significantly reduces the latency of radiation-induced lymphoma and sarcoma in both *BCL2*-heterozygous and *BCL2*-homozygous mice[121]. Furthermore, a common polymorphism in the *XPD* DNA-repair gene — which seems to protect myeloid cells from chemotherapy-induced death, as indicated by an association with poor prognosis — is also associated with an increased risk of developing chemotherapy-induced AML; both observations are consistent with an inability to appropriately trigger cell death after chemotherapy[122]. As discussed earlier and illustrated in FIG. 4, loss of DNA-MMR confers tolerance to the killing effects of O^6-guanine methylating and thiopurine chemotherapy agents, thereby promoting cell survival, which is predicted to contribute to the pathogenesis of therapy-related leukaemia.

Myelosuppression provides direct evidence of therapy-induced cell death in the bone marrow. Indeed, bone marrow toxicity is dose limiting for several chemotherapeutic alkylating agents. By contrast, however, direct evidence of cell death occurring in non-target solid tissues as a consequence of either chemotherapy or radiotherapy is less apparent. Dissimilar to the risk of developing leukaemia, the relative risk of developing lung cancer after Hodgkin lymphoma would seem

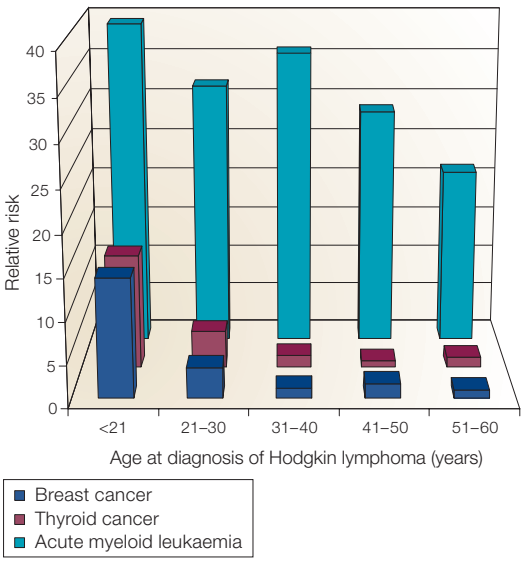

Figure 6 | **Relative risk of developing a second cancer according to age at diagnosis of primary Hodgkin lymphoma.** This graph illustrates that the relative risk of developing breast cancer after Hodgkin lymphoma is highest when diagnosed early in life and decreases with age, approaching background during the later years of life[126]. A similar age pattern, with the highest risks in the young, is observed for thyroid cancer. By contrast, the relative risk of developing acute myeloid leukaemia after Hodgkin lymphoma, although considerably higher than that for developing breast and thyroid cancer, remains elevated at all treatment ages[126], possibly indicating that the proliferative index of the target cell for transformation might be relatively constant throughout adolescence and adulthood.

not to be attenuated at high doses of either methylating chemotherapy, nitrogen-mustard chemotherapy or radiotherapy[72] (TABLE 1). Similarly, the subsequent risk of developing breast cancer after Hodgkin lymphoma is not attenuated at high radiotherapy doses[123]. Nevertheless, given the often systemic nature of exposure and the high doses that are used, we cannot exclude the possibility that there might be some therapy-induced cytotoxicity in these and other non-target solid tissues at the time of exposure that might affect the risk of developing subsequent cancer.

Cell proliferation

The induction of cell death means that cell proliferation is then required to facilitate repopulation. This phenomenon is particularly apparent in the bone marrow after alkylating chemotherapy, as the peripheral blood neutrophil count is routinely used to monitor bone marrow recovery after ablative therapy. Significantly, proliferating cells are more likely than non-proliferating cells to fix DNA damage into mutations[124] (FIG. 5), a phenomenon that might at least partly explain the very high relative risk of developing leukaemia after bone marrow-ablative therapy.

There is considerable evidence indicating that steady-state cellular proliferation at the time of therapeutic exposure is also a crucial determinant of the risk of developing a therapy-induced cancer. In some tissues, such as the breast, cellular proliferation is inversely correlated with age. As such, we might predict the relative risk of developing radiogenic breast cancer to be higher in younger pre-menopausal women than older or post-menopausal women. Indeed, this seems to be the case[123,125–127] (FIG. 6). Radiogenic breast cancer rates are also lower in women who were treated concomitantly with chemotherapy or in women who received a significant radiation dose to the ovaries[123,127]. This indicates that these exposures abrogate oestrogen production or induce premature menopause and attenuate the carcinogenic effects of radiotherapy, presumably by downregulating cell proliferation in the breast. Calaf and Hei[128] have partially recapitulated these observations *in vitro* and provide evidence indicating a role for oestrogen in driving cellular transformation by radiation. Using an immortalized, but non-transformed, human breast cell line they show that radiation exposure, in combination with oestradiol (the most abundant circulating oestrogen), induces cellular neoplastic transformation and breast tumorigenesis when injected into immunocompromised mice.

The unique sensitivity of the young to radiogenic breast and thyroid cancers has been reviewed[11] and recently highlighted in patients with Hodgkin lymphoma[126] (FIG. 6). In reviewing the results of any study in which comparisons with the general population are presented, it should be kept in mind that decreases in relative risk with increasing patient age might, in part, reflect the parallel increase in underlying cancer incidence rates.

Interactions between risk factors

The use of combination chemotherapy and combined-modality therapy has made it difficult to assess the specific carcinogenic effects of individual agents. However, these approaches can indicate how mechanistically diverse exposures interact to drive carcinogenesis; a model that is more relevant to the complex exposures that are associated with the development of sporadic cancers. For example, co-treatment with radiotherapy and alkylating chemotherapy for Hodgkin lymphoma seems to operate in a simple additive model with respect to the risk of subsequently developing lung cancer[14,72]. By contrast, cigarette smoking and radiotherapy interact multiplicatively with respect to the risk of developing lung cancer[14,72,129], as do tobacco use and chemotherapy[14,72]. Indeed, more than 60% of lung cancers in survivors of Hodgkin lymphoma are the result of the strong pro-carcinogenic interaction between therapy and smoking[72]. Tobacco smoke contains high concentrations of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent lung carcinogen in laboratory animals[130]. Similar to procarbazine, NNK is a methylating agent that efficiently induces O^6-methylguanine lesions in DNA[130]. As discussed earlier, the high frequency of MSI in lung cancer after Hodgkin lymphoma[12] is consistent with a role for methylating agents in

disease pathology. Given a common mode of action, it is intriguing that alkylating-chemotherapy-agents and smoking interact so differently with radiotherapy to modify the subsequent risk of developing lung cancer, indicating that other factors might be important or that several mechanisms might be operating. Indeed, one model predicts that chemotherapy acts to promote the expansion of pre-malignant cell clones that have been initiated by exposure to cigarette smoke.

Similar to lung cancer that has developed following Hodgkin lymphoma, the excess risk of developing bladder cancer following treatment with radiotherapy and cyclophosphamide for non-Hodgkin lymphoma was as would be expected if the individual risks were added together[131]. By contrast, radiotherapy and chemotherapy for Hodgkin lymphoma seem to act multiplicatively with respect to the development of gastrointestinal cancer[132], indicating that interactions between therapies are not only complex, but are also likely to be tissue and agent specific.

Conclusions

The development of novel therapeutic agents and regimens to treat cancer over the past 30 years has clearly led to significant improvements in long-term survival. This remarkable success, however, has been accompanied by considerable concern with regard to the late effects of treatment, particularly the development of second primary cancers, which can be associated with high mortality. Given this, the risk of developing a second cancer should be taken into consideration such that alternative therapies are offered if available. The replacement of MOPP (mechlorethamine, vincristine, prednisone and procarbazine), which has been associated with a relatively high risk of developing leukaemia, with other less leukaemogenic but equally effective chemotherapeutic regimens wherever possible is one example of this approach that is being followed at present. Of course, successfully treating the primary cancer should always remain the first priority. So, it is unlikely that the risk of developing a second cancer would ever completely contraindicate treatment. A better understanding of the risk factors for therapy-induced cancers might allow for the identification of patients at high risk who will benefit from post-therapy surveillance.

Treatment-related cancers provide a unique opportunity to study the molecular underpinnings of carcinogenesis, given the meticulous measurement of potentially cancer-inducing treatments. Importantly, the knowledge gleaned from careful genetic and molecular investigations of second primary malignancies will enhance our understanding of carcinogenic mechanisms *per se*, including those that might also be operating in some sporadic cancers. This understanding will therefore provide a stronger foundation for efforts aimed at preventing both sporadic and therapy-related cancers.

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Acknowledgements

J.M.A. acknowledges the support of Leukaemia Research and Yorkshire Cancer Research. We would also like to thank Tracy Lightfoot (Epidemiology and Genetics Unit, University of York) for critically reviewing this manuscript. This research was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Division of Cancer Epidemiology and Genetics.

Competing interests statement

The authors declare no competing financial interests.

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