Treatment-related mutagenic processes in acute lymphoblastic leukemia

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Abstract

Karyotyping, SNP arrays, fluorescence *in situ* hybridization and next-generation sequencing techniques have greatly improved our understanding of the genetic drivers of acute lymphoblastic leukemia (ALL). In the past years, another layer of genetic data has been added by the study of mutational signatures, patterns of somatic mutations that represent specific mutational mechanisms. Mutational signatures can give insight into tumor development, but also reveal mutagenic side-effects of treatment in relapse samples. Multiple treatment-related mutational signatures have been detected in relapsed ALL that could play a role in therapy resistance and relapse development. In fact, multiple pathogenic driver mutations have been attributed to these treatment-related mutational processes, including a recurrent *TP53* mutation. Studies in childhood ALL revealed that thiopurine exposure is the most common source of therapy-related mutagenicity in ALL and presents differently when patients are DNA mismatch repair deficient. Thiopurine-induced DNA damage indicates that leukemic cells were able to survive thiopurine exposure. This could be due to metabolic defects, acquired mutations that induce thiopurine resistance during treatment, or resistance to drugs synergizing with thiopurines. In this review, we discuss the types and prevalence of treatment-related mutational signatures in ALL, and explore mechanisms of thiopurine cytotoxicity and mutagenicity.

Introduction

Over the past decades, our understanding of the genetic drivers of acute lymphoblastic leukemia (ALL) has greatly improved. Initially, karyotyping and fluorescence *in situ* hybridization allowed detection of aneuploidies and major genomic rearrangements like *ETV6::RUNX1*, *BCR::ABL1* and iAMP21.[1] In more recent years, analysis of larger cohorts and the use of next-generation sequencing techniques has helped distinguish subtypes driven by rare genomic rearrangements.[2,3] So far, over 30 subtypes of ALL have been identified, and many of these serve as important markers for prognosis and risk group stratification.[3,4]

Next-generation sequencing techniques were also of great importance in the identification of secondary drivers. In fact, some primary subtype-defining drivers, like *ETV6::RUNX1*, are insufficient for leukemogenesis and require secondary drivers to progress to malignancy.[5] Secondary drivers were initially identified through SNP arrays that detected commonly deleted genes like *ETV6* and *PAX5*,[6,7] or prognostically relevant relapse-enriched genes like *IKZF1*.[8-10] More recently, the number of known secondary drivers has expanded using whole genome sequencing to detect genome-wide pathogenic single base substitutions and deletions.[11-13]

Next-generation sequencing studies have also provided information about the frequency of somatic mutations. Overall, the mutational load in ALL is among the lowest in human cancer.[14,15] Nevertheless, subtype-specific differences have been observed, and also within subtypes there is large variety in the number of mutations.[13] To further study these somatic mutations, mutation types can be grouped based on their genomic context to distinguish so-called mutational signatures that correspond to various mutational processes.[14,15] Mutational signatures are commonly studied using single base substitutions (SBS), double base substitutions (DBS), or small insertions and deletions (indels).[14,15] For SBS signatures, the nucleotides upstream and downstream of the mutation are included, resulting in patterns of 96 trinucleotides that enable associations with mutational processes. Similarly, indel signatures account

for the indel size and whether the indel is in a repeat or microhomology region. In addition, there are 78 different DBS types that enable recognition of mutational processes.[14-16] So far, the majority of mutational signatures, and their associated mutational processes, have been identified based on patterns of SBS.[15,17]

Mutational signatures are commonly detected in cancer and can originate from acquisition of mutations during DNA replication as well as from abnormal activation of mutational processes or defects in DNA repair.[14,15] The normal replication-associated mutations accumulate in a more or less fixed number per cell division in both healthy and malignant cells, presenting as so-called clock-like mutational signatures.[18] Examples include the single base substitution signatures SBS1 and SBS5. In tumors, these signatures become apparent due to the clonal outgrowth of malignant cells. Moreover, the acquisition of these clock-like mutational signatures can be accelerated by chemotherapy.[19] Whereas clock-like mutational processes can result in pathogenic mutations, abnormal mutational processes generally have a much larger impact on malignant transformation. For example, aberrant intrinsic processes like DNA damage from reactive oxygen species or specific DNA repair defects can result in rapid accumulation of mutations.[15] Furthermore, DNA damage from exogenous mutagens is highly enriched in some cancer types, such as DNA damage induced by UV-light in skin cancer or tobacco smoke-associated DNA damage in lung cancer.[15,20]

In ALL, several mutational processes were shown to be active at time of diagnosis, often in specific subtypes. For example, a subset of patients with aneuploid karyotypes, including the hyperdiploid, near haploid and iAMP21 subtypes, are affected by SBS7a, a mutational signature that has been associated with UV-light induced DNA damage.[12,13,21] Furthermore, patients of the *ETV6::RUNX1*-rearranged and *ETV6::RUNX1*-like subtypes may present with SBS2 and SBS13-associated mutations.[12,13,22] SBS2 and SBS13 result from cytidine deaminase DNA editing activity of APOBEC3 family proteins which target and damage viral single-stranded DNA and RNA as part of the innate immune system, but are known to damage host DNA in various cancers.[23] This subtype-specific presentation of mutational signatures in ALL is still not completely understood.

Acute lymphoblastic leukemia is mainly a pediatric disease with approximately 80% of cases occurring in children.[24,25] However, prognosis worsens with increasing age.[26] This is partly explained by a higher incidence of poor prognosis subtypes and higher rates of adverse treatment effects in older age groups.[24,27] Over the past decades, pediatric ALL cure rates have greatly improved and the incidence of relapse has decreased.[28] These improvements can be attributed to more adequate risk stratification, more therapeutic options, and better supportive care. Nevertheless, relapse occurs across both low-risk and high-risk subtypes, and the processes that contribute to relapse formation are still poorly understood.[4] Overall survival for adult ALL patients has also improved, mainly through the use of pediatric-based treatment protocols, but prognosis is still dismal with high relapse rates.[24,29] Most research on mutational signatures in ALL has been performed on pediatric ALL samples. Thus, there is a lack of knowledge in the field of adult ALL.

The mutational load in ALL generally increases with every relapse, and in patients that experience multiple relapses, the mutational load may even reach a hypermutation threshold.[11,30] This observation suggests that certain drugs used in the treatment of ALL may have mutagenic side-effects that play a role in therapy resistance and relapse development. Such treatment-related mutational processes would leave mutational signatures that are not observed in the primary tumor, as has been described in other cancer types.[31,32] This review discusses the types, prevalence, and potential prognostic impact of therapy-related mutational signatures in ALL.

Treatment-related mutational signatures in acute lymphoblastic leukemia

Acute lymphoblastic leukemia treatment is extensive, consisting of an induction phase, a consolidation phase, and a maintenance phase. In total, treatment generally lasts two years, and involves a large variety of chemotherapeutics.[24,29,33-36] Whereas many chemotherapeutics, including cyclophosphamide, doxorubicin and etoposide, have been associated with genotoxicity, only a small subset is known to induce lasting mutagenicity.[15,37] Treatment-related mutational signatures can be expected in ALL relapses, particularly when prior treatment with a mutagenic drug showed reduced effectivity because of, for example, inadequate dose adjustments, ineffective drug delivery, or impaired bioconversion to a functionally active compound. Alternatively, resistance to other drugs administered in parallel may result in the survival of leukemic cells that contain the mutational footprints of mutagenic drugs. Which drugs induce increased mutational loads and what mutational signatures they produce is difficult to predict. Different approaches can be used to identify mutagenic properties, including prolonged sublethal exposure of cell lines to drugs *in vitro*, and whole genome sequencing of relapsed and metastatic patients that underwent chemotherapy. Both approaches have their drawbacks. The *in vitro* cellular exposures may reveal signatures that do not occur *in vivo*. On the other hand, the etiology of mutational signatures identified in cohorts of treated cancers can be difficult to determine. Treatment history can be unclear, and patients have often been treated with a plethora of different drugs. Therefore, the two approaches should ideally be combined

to reveal direct associations between drug exposure and mutational consequences that occur *in vivo*.

Regarding the *in vitro* approach, multiple studies have exposed cell lines to common mutagens and commonly used chemotherapeutics to characterize their mutational impact.[20,38,39] However, the tested drugs are not necessarily relevant to ALL. In fact, only three chemotherapeutics commonly used in ALL treatment have been investigated by cell line exposure experiments: the anthracycline doxorubicin, the alkylating agent cyclophosphamide, and the topoisomerase II inhibitor etoposide.[20,39] Doxorubicin and etoposide did not appear to be mutagenic, but cyclophosphamide induced a significant mutational load in two independent studies. However, the observed mutational patterns were different between the two studies and neither of these signatures has been observed in ALL. This could be due to the limited use of cyclophosphamide during ALL treatment or the previously mentioned discrepancies between *in vitro* and *in vivo* results.

The second strategy to identify therapy-related mutational processes is by studying cohorts after therapy. Mutational signature analysis in relapsed ALL has so far revealed six mutational signatures that can be associated with ALL treatment (Table 1). SBS87 and thio-dMMR can be allocated to thiopurine exposure, a class of chemotherapeutic drugs commonly used in ALL treatment. SBS87 appears to affect about 15% of relapsed patients across different subtypes,[40,41] but the incidence can increase to up to 52% in multiply relapsed patients.[30] In a subset of ALL patients that have a defect in DNA mismatch repair (MMR), thiopurines appear to induce a mutational signature that is distinct from SBS87, referred to as thio-dMMR. Thio-dMMR affects the same trinucleotide contexts of the C>T mutation spectrum but, unlike SBS87, it does not include C>G or C>A mutations. SBS86 is found in approximately 10% of relapsed ALL patients and seems to be enriched in patients of the hyperdiploid subtype.[40] The etiology of SBS86 is still unknown but its incidence was found to be far greater in Chinese patients (15%) than US patients (1%).[40] The US and Chinese treatment protocols used similar chemotherapy, with the exception of etoposide,[41] which was non-mutagenic in *in vitro* studies.[20,39] Perhaps, the source of SBS86 could be a form of supportive medication. In the cohort of multiply relapsed ALL patients, we found one ALL patient with mutations representing signature SBS17 in the first and second relapse.[30] Additionally, a recent study of adult ALL patients treated with inotuzumab ozogamicin (InO) identified a hypermutated patient with a novel indel signature consisting of large insertions.[42] The significance of these sporadic findings is as yet unclear. Nevertheless, rare mutational signatures can have clinical relevance as evidenced by the mutational signature linked to ganciclovir, an antiviral drug which is used to prevent viral reactivation after hematopoietic stem cell transplantation. This ganciclovir-associated mutational signature was found in non-malignant cells and a therapy-related acute myeloid leukemia after hematopoietic stem cell transplantation as part of ALL treatment.[43] With the exception of the potential InO signature, these mutational signatures have all been identified in pediatric ALL samples. Only one small study of 19 adult T-cell ALL patients has investigated the mutational signatures in relapsed adult ALL samples, and found no treatment-related mutational signatures.[25] However, it is worth mentioning that over half of the included patients relapsed early, thus limiting the exposure to potentially mutagenic drugs. As DNA damage by thiopurines is most common in ALL, and has been relatively well-studied, the remainder of this review will focus on thiopurine-induced mutational signatures.

Thiopurine metabolism

Thiopurines have been a core component of ALL treatment since the 1950s[44] and are still used throughout all phases of treatment. Thiopurines are used most extensively during maintenance therapy, in which the thiopurine analog 6-mercaptopurine (6-MP) is taken daily for at least six months.[24,29,33,35,36,45]

Table 1. Mutational signatures identified after acute lymphoblastic leukemia treatment.

Mutational signature	Disease type	Cause	Reported frequency	Validation	References
SBS87	Relapse & t-MN	Thiopurine exposure	~15%	*In vitro* assay	30, 40, 41
thio-dMMR	Relapse & t-MN	Thiopurine exposure in MMR-deficient context	~5%	*In vitro* assay	11, 41
SBS86	Relapse	Unknown	~10%	-	30, 40, 41
SBS17	Relapse	Reactive oxygen species	Sporadic	-	30
Potential InO signature	Relapse	Potentially Inotuzumab ozogamicin	Sporadic	-	42
Ganciclovir	t-MN	Ganciclovir exposure	Sporadic	*In vitro* assay	43

Reported frequency includes any studies that reported mutational signatures in large cohorts. SBS17 and the potential inotuzumab ozogamicin (InO) signature frequencies are sporadic as only one case of each mutational signature has been described in relapsed acute lymphoblastic leukemia (ALL). Ganciclovir signature frequency is noted as sporadic as it has not been studied in the context of larger ALL cohorts, but is likely to be rare as it can only affect patients who underwent a hematopoietic stem cell transplant. t-MN: therapy-related myeloid neoplasm; MMR: DNA mismatch repair.

6-MP is a pro-drug that requires metabolic activation to become cytotoxic. The first conversion step is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to produce thioinosine monophosphate (TIMP). TIMP is further metabolized to form thioguanine nucleotides (TGN) or methyl-thioinosine monophosphate.[46-48] TGN compete with guanine nucleotides for DNA incorporation opposite cytosines during DNA replication without a direct cytotoxic effect (Figure 1). During replication, incorporated TGN pair with thymines and cytosines equally, and TGN-thymine pairs are repaired by MMR.[46] Approximately 1:10,000 incorporated TGN become methylated, and pairing with cytosines can then trigger MMR depending on sequence context.[46,49,50] As a result, a cycle of repeated mismatch insertion and repair occurs. Eventually, DNA strand breaks accumulate and trigger apoptosis, the intended cytotoxic effect of 6-MP (Figure 1).[46,48]

Besides 6-MP, the thiopurine analog 6-thioguanine (6-TG) is also used in ALL treatment, but 6-MP is now preferred due to 6-TG hepatotoxicity.[51] Unlike 6-MP, 6-TG is directly converted to thioguanine monophosphate (TGMP) by HGPRT (Figure 1).[46] Consequently, 6-TG has a shorter half-life, potentially increasing toxicity. Furthermore, 6-MP is metabolized to methyl-thioinosine monophosphate (MeTIMP), while 6-TG

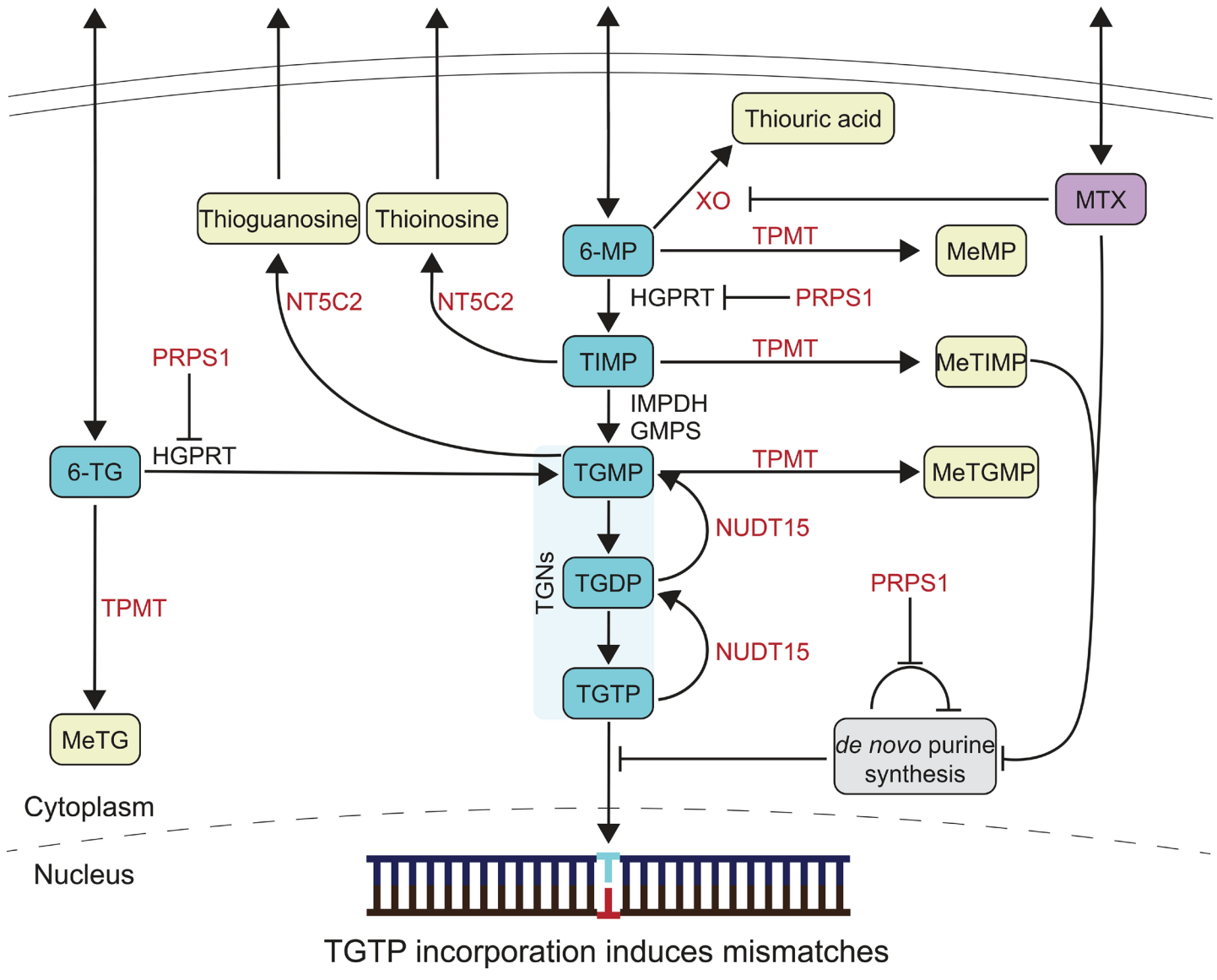

Figure 1. Pathway of thiopurine pro-drug conversion to cytotoxic thioguanosine triphosphate. The main pathway is shown in blue, and thiopurine metabolites that can no longer be converted to thioguanosine triphosphate (TGTP) are shown in yellow. 6-mercaptopurine (6-MP) is converted to thioinosine monophosphate (TIMP) by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). TIMP is then converted by inosine monophosphate dehydrogenase (IMPDH) and subsequently guanine monophosphate synthetase (GMPS) to thioguanine monophosphate (TGMP). HGPRT is also involved in the direct conversion of 6-thioguanine (6-TG) to TGMP. TGMP is phosphorylated to thioguanine diphosphate (TGDP) and TGTP by deoxynucleoside kinases and reductase. TGTP represents the toxic nucleoside analog that is transported to the nucleus and incorporated in DNA during replication. Methotrexate (MTX) is another drug used in the treatment of acute lymphoblastic leukemia (ALL) that synergizes with thiopurines through inhibition of xanthine oxidase (XO) and *de novo* purine synthesis. MTX also indirectly inhibits thiopurine S-methyltransferase (TPMT) by limiting the supply of methyl donor S-adenosyl methionine; we chose not to depict this indirect interaction here. Several enzymes counteract in this pathway, thereby reducing TGTP toxicity (depicted in red font). For example, XO converts 6-MP to thiouric acid. TPMT methylates 6-TG, 6-MP, TIMP and TGMP, which prevents cytotoxicity. Nudix hydrolase 15 (NUDT15) inhibits thiopurine toxicity by dephosphorylating TGTP and TGDP. Activating phosphoribosyl pyrophosphate synthetase 1 (PRPS1) mutations prevent TGMP formation from 6-MP and 6-TG through inhibition of HGPRT. Furthermore, these mutations impair the negative feedback loop of *de novo* purine synthesis, which leads to increased levels of purines that compete with TGTP for incorporation in DNA. Finally, NT5C2 reduces thiopurine toxicity by dephosphorylation of TIMP and TGMP, facilitating their export from the cell. MeMP: methyl-mercaptopurine; MeTG: methyl-thioguanine; MeTIMP: methyl-thioinosine monophosphate.

is not.[46] MeTIMP inhibits *de novo* purine synthesis, limiting the number of guanine nucleotides competing with TGN for incorporation in DNA, improving 6-MP efficacy (Figure 1).[46-48]

DNA mismatch repair in acute lymphoblastic leukemia and thiopurine metabolism

MMR plays a key role in the repair of thioguanine-thymine lesions, as well as in triggering apoptosis upon thiopurine exposure. MMR deficiency, caused by alterations (SNV, indels or deletions) in either of the key genes *MSH2*, *MSH6*, *MLH1*, or *PMS2*, is a well-known mutagenic mechanism leading to a hypermutator phenotype and recognizable mutational signatures.[15] MMR deficiency as a thiopurine resistance mechanism in ALL has been well studied, particularly in children. In fact, it has been shown that at initial diagnosis about 11% of pediatric ALL samples had excessive degradation of MSH2, resulting in low or undetectable MSH2 protein levels.[52] Additionally, *MSH2* or *MSH6* appear to be altered in up to 10% of relapsed patients.[11,53,54] These patients do not necessarily present with typical features of MMR deficiency as an intact allele is often still present.[53] Yet, apparently, MMR function is reduced sufficiently to increase thiopurine resistance, prevent apoptosis, and increase incorporation of thioguanines in DNA.[52,53] As a result, patients had an increased incidence of relapse and lower overall survival, which seemed independent of ALL lineage and subtype.[52] Interestingly, the MMR alterations primarily involve *MSH2* and *MSH6*, while *MLH1* and *PMS2* alterations only occur sporadically.[41,55-57] This biased enrichment suggests that binding of the MSH2-MSH6 dimer to thioguanine-thymine lesions is sufficient to trigger an apoptotic cascade in response to thiopurine-induced DNA damage, and recruitment of MLH1 and PMS2 to initiate repair of these lesions is not needed for cytotoxicity. Alternatively, ALL cells may require a complete deficiency of MLH1 or PMS2 to induce thiopurine resistance, while for MSH6 and MSH2, haploinsufficiency suffices. Altogether, MMR deficiency in ALL is a potent thiopurine resistance mechanism that is enriched at relapse.

Recently, it was noticed that MMR deficiency rarely co-occurs with mutations in the base excision repair pathway. Hence, the authors posited that base excision repair would be critical for survival of thiopurine DNA damage in an MMR-deficient context.[54] The base excision repair pathway would then represent a targetable vulnerability of MMR-deficient malignancies. Indeed, MMR-deficient cell lines and patient-derived xenografts were vulnerable to a combination of 6-thioguanine and oleanolic acid, an inhibitor of polymerase β which is critical for base excision repair.[54] However, the clinical feasibility and efficacy of base excision repair inhibition in ALL patients remains to be studied.

The etiology of thiopurine-associated mutational signatures

SBS87 and thio-dMMR have been identified in large cohorts of relapsed patients.[11,40,41] The prominence of C>T mutations in both signatures suggested thiopurines as a potential source of mutagenesis. The thiopurine origin of SBS87 was confirmed in a thiopurine-exposed cell line model.[40] Similarly, thiopurine exposure in an MMR-deficient cell line replicated the thio-dMMR signature.[41] Notably, thio-dMMR induced a far higher mutational load than SBS87, with some patients acquiring over 15,000 thio-dMMR-associated mutations. This high mutation load is in line with the highly increased tolerance of thiopurine-induced DNA damage in MMR-deficient ALL[52-54] as well as the lack of repair of thioguanine-thymine lesions. This lack of repair potentially explains why SBS87 and thio-dMMR present as different patterns (Figure 2). In an MMR-deficient context, the thymines that are incorporated opposite methylated TGN are systematically ignored, whereas in MMR-proficient cells a cycle of mismatch recognition and repair occurs. The exclusive presentation of C>G mutations seen in SBS87, in comparison to thio-dMMR, could indicate that MMR becomes more error-prone after repeated repair attempts, or that the repeated single strand breaks induced by MMR might attract alternative, more error-prone repair mechanisms.

Whereas TGN have no known preference for specific genomic regions or consensus sequences,[48,58] the trinucleotide contexts that define thio-dMMR (N [C>T] G) and SBS87 (N [C>G/T] G) are restricted to cytosines with a downstream guanine (CpG sites). CpG sites are preferentially bound and methylated by DNA methylases[59] that might also methylate the TGN that are incorporated at CpG sites. It is likely that this restriction to CpG sites also explains why thio-dMMR so clearly resembles SBS1, which underlies cytosine to thymine mutations at CpG sites due to deamination of methylated cytosines.[15,18,41]

While Yang *et al*. were the first to link the thio-dMMR signature to thiopurine exposure in an MMR-deficient context,[41] the thio-dMMR signature had already been noticed in an earlier study that identified an increased mutational load in five mismatch repair deficient samples.[11] In this study, the similarity to SBS1 was noted but, in contrast to SBS1, a significant bias for the transcribed strand was observed. This bias for the transcribed strand is also present in SBS87, and is thought to result from thioguanines triggering transcription-coupled nucleotide excision repair, thus often being removed from the transcribed strand.[40,41] In retrospect, the mutational signature extracted in the Waanders *et al*. study is a near perfect match with the thio-dMMR signature described by Yang *et al*.[11,41]

Possible mechanisms underlying thiopurine mutagenesis

Despite widespread use of thiopurines in ALL treatment,

the detection of SBS87 in only approximately 15% of first relapses may be unexpected.[40,41] The factors determining SBS87-associated damage remain unclear, but its selective occurrence in ALL relapses suggest that additional factors are involved. Since SBS87 indicates thiopurine damage without cell death, thiopurine dosage or efficacy may be crucial. Potential factors could be metabolic defects, acquired mutations that induce thiopurine resistance during treatment or resistance to drugs synergizing with thiopurines.

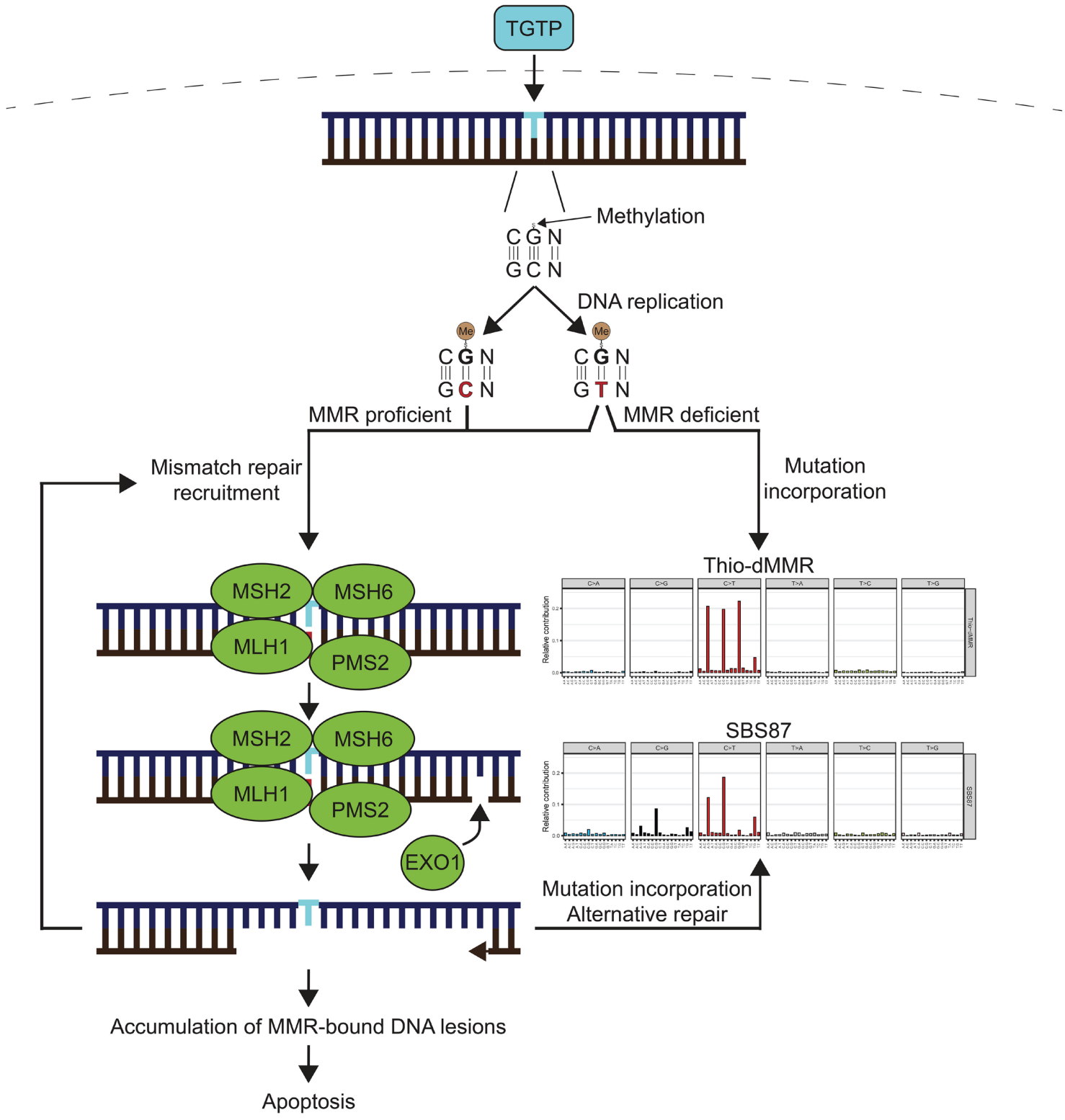

Figure 2. Cytotoxicity and mutagenesis by the thiopurine metabolite thioguanosine triphosphate. After incorporation in DNA thioguanosine triphosphate (TGTP) are normally paired with cytosines or thymines. Whereas TGN-thymine mismatches are repaired by mismatch repair (MMR), TGN-cytosine pairs are ignored. However, incorporated TGN often become methylated and then mismatch with both thymines and cytosines. In an MMR deficient context, these lesions are not repaired and cytosine to thymine mutations are incorporated in DNA, resulting in a mutational signature called thio-dMMR. In an MMR proficient context, both methylTGN-thymine and methylTGN-cytosine lesions are recognized by MMR, which removes a large portion of the coding strand, to enable resynthesis. However, after the mismatching nucleotide is removed, it is replaced with another mismatching cytosine and thymine, as the template strand still contains the methylated thioguanine. This can then induce a cycle of repeated MMR recruitment and failed repair. If these repair cycles remain unresolved, they are thought to incur double strand DNA breaks during the next round of DNA replication, resulting in apoptosis. If the cell does not commit to apoptosis, a cytosine to thymine mutation can be permanently established. Alternatively, a guanine or adenine can be inserted at the lesion, but it is currently unknown if this is due to the repeated action of the mismatch repair pathway or due to alternative repair pathways. Together, these cytosine mutations are recognized as mutational signature SBS87. EXO1: exonuclease 1; MLH1: MutL homolog 1; MSH2: MutS homolog 2; MSH6: MutS homolog 6; PMS2: postmeiotic segregation increased 2.

Numerous genes regulate thiopurine metabolism and, thereby, the efficacy and toxicity of 6-MP treatment (Figure 1). Alterations in these genes can cause sensitivity or resistance to thiopurines. This altered thiopurine metabolism complicates 6-MP use, and could be conducive for acquisition of SBS87. An increased sensitivity to thiopurines can result from germline alterations in *TPMT* or *NUDT15*.[60,61] TPMT prevents the formation of cytotoxic thioguanine nucleotides by methylating 6-MP, 6-TG and their downstream metabolites (Figure 1).[46] NUDT15 prevents incorporation of thioguanine nucleotides into DNA by dephosphorylating thioguanine nucleotides (Figure 1).[61] Patients with *NUDT15* or *TPMT* germline alterations experience increased adverse effects, are at increased risk of second malignancies and are treated with lower thiopurine doses.[60-62] Lower TPMT activity increases cytosolic thioguanines, but does not increase thioguanine incorporation in DNA.[63] A possible explanation is that thiopurine metabolites that are methylated by TPMT inhibit *de novo* purine synthesis (Figure 1). Thus, lower TPMT activity results in more guanines that compete with thioguanines for incorporation into DNA.[63] Therefore, lower thiopurine doses in *TPMT*-deficient patients could result in insufficient cell death, resulting in an increased burden of SBS87. In line with this hypothesis, Li *et al*. found significantly lower *TPMT* expression in SBS87-positive patients.[40] Thus, increased sensitivity to thiopurines may increase the risk of thiopurine-induced mutagenesis.

NT5C2, *PRPS1*, and *PRPS2* mutations can all induce 6-MP resistance by decreasing, but likely not completely abolishing, the incorporation of thioguanines into DNA. This low rate of thioguanine incorporation could allow cells to survive thiopurine treatment, but still acquire DNA damage. Activating *NT5C2* mutations cause increased dephosphorylation of thiopurine metabolites, which are subsequently exported out of the cell (Figure 1).[64-66] *PRPS1* mutations decrease 6-MP efficacy through two mechanisms. PRPS1 is essential for the first step of *de novo* purine synthesis, and is inhibited by the purine end products of this pathway, which is known as the negative feedback loop of *de novo* purine synthesis. Activating *PRPS1* mutations prevent PRPS1 inhibition by purines, thereby increasing *de novo* purine synthesis and thus the pool of purines competing with thioguanines for incorporation in DNA (Figure 1).[67] Another consequence of this increased purine pool is an excess of hypoxanthine that saturates HGPRT, thereby limiting conversion of thiopurines by HGPRT. Additionally, PRPS1 forms a hexamer structure with PRPS2, which can be destabilized by a specific *PRPS2* mutation. This destabilized hexamer prevents inhibition of PRPS1, which in turn increases PRPS1 activity.[68] *NT5C2* and *PRPS1* mutations have been described in relapsed ALL cases that showed SBS87.[30,41] However, not all cases with *NT5C2* or *PRPS1* mutations present with SBS87, and not all cases that present with SBS87 have *NT5C2* or *PRPS1* mutations. Another cause of SBS87 could be a more general 6-MP dosing issue. The most accurate measure of thiopurine efficacy during maintenance therapy is the amount of thioguanine nucleotides incorporated into DNA, called the DNA-TGN. As leukemic cells and circulating leukocytes are similarly exposed to thiopurines, the DNA-TGN of circulating leukocytes can be used as a proxy.[69] Leukemic cells of patients that show consistently low DNA-TGN will likely not commit to apoptosis and instead acquire SBS87. In fact, it is known that a higher DNA-TGN content correlates with a lower chance of relapse,[69,70] which suggests that low DNA-TGN correlates with reduced apoptosis. It would, therefore, be an interesting future subject of study to compare the DNA-TGN levels during treatment between patients who have and and those who have not acquired SBS87 at relapse. A method to increase the DNA-TGN is currently being studied as a subprotocol of the European ALLTogether1 trial. During maintenance therapy, 6-MP is complemented with a low dose of 6-TG. The hypothesis is that DNA-TGN levels will be increased, without introducing the hepatotoxicity associated with 6-TG.[71,72] As the DNA-TGN of the patients in this trial is closely monitored, this study represents an excellent opportunity to correlate DNA-TGN levels with SBS87 levels at relapse.

A third possible cause of SBS87 relates to the need for synergy with other drugs, as 6-MP alone might simply not eliminate all leukemic cells in a patient. ALL is treated with a multi-drug regimen and resistance to a single drug will lower treatment efficacy.[73] During maintenance therapy, daily 6-MP intake is combined with weekly methotrexate (MTX) administration.[35,36,45] MTX improves 6-MP efficacy in multiple ways. Firstly, MTX inhibits xanthine oxidase, which increases 6-MP bioavailability as xanthine oxidase converts 6-MP to thiouric acid (Figure 1).[47,74] Additionally, MTX inhibits *de novo* purine synthesis, thereby decreasing the pool of nucleotides competing with thioguanine nucleotides for incorporation in DNA (Figure 1).[47,74] Lastly, MTX inhibits dihydrofolate reductase, which is essential for the generation of tetrahydrofolate from folic acid. Tetrahydrofolate in turn is needed for S-adenosyl methionine generation, which is the methyl donor for methylation of 6-MP and its downstream metabolites by TPMT.[47,74] Thus, dihydrofolate reductase inhibition by MTX might shield 6-MP and its downstream metabolites from methylation by TPMT. While MTX is not essential for 6-MP cytotoxicity, it enhances its effects, and MTX resistance may lower 6-MP efficacy and promote SBS87 acquisition. MTX is a pro-drug and requires polyglutamation by folylpolyglutamate synthase to become cytotoxic, and loss of folylpolyglutamate synthase induces MTX resistance.[75,76] Low levels of polyglutamated MTX predict poor outcome.[77] Thus, MTX resistance represents another mechanism that decreases 6-MP cytotoxicity, and could initiate a switch from 6-MP induced apoptosis to 6-MP induced mutagenesis (SBS87).

Pathogenicity of mutational signatures

Mutational processes can affect highly specific DNA contexts and, as a result, some mutational processes have a high propensity of inducing specific driver (hotspot) mutations.[78] By comparing the affinity of each mutational process for each trinucleotide context, with the contribution of each mutational process to the mutational load of a sample, the odds of specific mutations having been caused by a mutational process can be calculated.[79,80] Additionally, it has recently been proposed that activity of mutational processes in cancer results in higher clonal heterogeneity, which increases the chance that a therapy-evading clone emerges.[81] This ability of mutational processes to induce driver mutations and increase clonal heterogeneity also applies to treatment-related mutagenicity. In other words, mutations induced by chemotherapy can cause chemotherapy resistance.

The role of treatment-related mutational processes in relapse development has also been studied in ALL. SBS86, SBS87 and thio-dMMR were all found to induce relapse-driving mutations.[30,40,41] Numerous therapy-induced mutations have been detected in *TP53* (broad chemotherapy resistance[82]), *NT5C2* and *PRPS1* (thiopurine resistance[66,67]), and *NR3C1* (glucocorticoid resistance[83]), as well as sporadic therapy-induced mutations in other relapse-associated genes.[30,40,41] Three mutations have been recurrently detected, of which the *TP53* R248Q hotspot mutation is most common (Table 2). This C>T mutation occurs in a CCG context, which is a prominent peak in the SBS87 and thio-dMMR signatures (Figure 2). Indeed, the majority of *TP53* R248Q mutations detected by Yang *et al*. were attributed to SBS87 or thio-dMMR.[41] Still, the question of cause and effect remains, since treatment-related mutagenesis can induce relapse-driving mutations in ALL and, conversely, chemotherapy-resistance mutations can potentially induce treatment-related mutagenesis.

Around 0.5-2% of children treated for cancer develop a therapy-related second malignancy.[5] ALL treatment most often incurs therapy-related acute myeloid leukemia or myelodysplastic syndrome. These second malignancies have a dismal prognosis and are harder to treat than equivalent primary malignancies.[84] Whole genome sequencing of such therapy-related myeloid neoplasms (t-MN) revealed treatment-associated mutational signatures similar to those found in ALL relapses. A study in which 16 pediatric t-MN were whole genome sequenced, of which 11 resulting from ALL treatment, revealed a high incidence of treatment-related mutational signatures.[32] Of the 11 t-MN resulting from ALL treatment, 4 presented with SBS87 and 2 presented with thio-dMMR. These mutational processes induced pathogenic mutations that could drive these t-MN, including the thiopurine-associated *TP53* R248Q mutation in a therapy-related acute myeloid leukemia.[32] Similarly, another study identified SBS87 in 3 out of 4 t-MN resulting from ALL treatment.[61] Finally, the largest whole genome sequencing study of t-MN to date included 44 t-MN and reported SBS87 in 10 out of 11 t-MN resulting from ALL treatment, with potentially SBS87-induced driver mutations in the majority of these patients.[86] As such, t-MN resulting from ALL treatment are frequently affected by thiopurine-induced mutational signatures, which often induce pathogenic driver mutations. The numerous thiopurine-induced driver mutations detected in t-MN may indicate that thiopurine-induced mutagenesis contributes to the development of t-MN.

Future perspectives

Insight into the mutational processes at play in ALL has helped understand the negative side-effects of chemotherapy and the process of relapse development. Nevertheless, much remains to be studied. For example, it is still not fully understood why treatment-related mutations are only found in a subset of patients, but metabolic defects and acquired therapy resistance likely play a role. Moreover, techniques to recognize those patients acquiring treatment-related mutations before a relapse-driving mutation is induced need further development. Additionally, there are rare mutational signatures that are only sporadically

Table 2. Recurrent pathogenic driver mutations induced by treatment-related mutagenesis.

Gene	Mutation (trinucleotide context)	Mutational signature	N of times reported	Disease type	References
TP53	R248Q (C [C>T] G)	SBS87 (4), thio-dMMR (5)	9	Relapse (8), t-MN (1)	30, 32, 40, 41
NT5C2	R367Q (T [C>T] G)	SBS87	4	Relapse	30, 40
PRPS1	S103R (G [C>G] C)	SBS86	2	Relapse	40

Mutational signature: the number in brackets denotes how often the mutation was attributed to each mutational signature. Disease type: the number in brackets denotes how often the mutation was detected in relapsed acute lymphoblastic leukemia or therapy-related myeloid neoplasms (t-MN). MMR: DNA mismatch repair.

identified. What causes patients to acquire mutagenesis that is not or rarely seen in other patients, and if such rare mutational signatures impact therapy and disease development, requires further study as well. Another key area of research is the role of treatment-related mutagenesis in second malignancies. Identifying which patients are at the greatest risk of second malignancies could enable effective monitoring and prevention strategies to reduce mutational burden during treatment. Furthermore, the presence of treatment-related mutational signatures in relapsed adult ALL is understudied, despite the high relapse rate of adult ALL. Adult treatment protocols are mostly based on pediatric treatment regimens.[24,29] Thus, similar mutational patterns to those observed in pediatric ALL could be expected in adult ALL, but the prevalence is still unclear.

To better monitor if and when patients acquire treatment-related mutations, studies are needed in which the effective drug dose is closely monitored throughout treatment. In the case of SBS87, the incorporation rate of thioguanines in DNA should then be monitored during treatment. It will be interesting to compare the rate of SBS87 acquisition between patients that had extensive periods of low thioguanine incorporation with patients that had high thioguanine incorporation levels. Such studies could help clarify whether it is thiopurine underexposure or overexposure that causes SBS87.

A major ongoing development in ALL treatment is the addition of various forms of immunotherapy.[87] Whether these novel drugs are mutagenic is still an underexplored topic. Immunotherapies like chimeric antigen receptor T cells and blinatumomab, which allow efficient killing of leukemic cells by T cells, are unlikely to be mutagenic. Immunotherapies relying on conjugated drugs, however, could be highly mutagenic depending on the conjugated drug. InO, for example, uses a calicheamicin-class conjugate that kills cells through DNA damage. Indeed, InO escape mechanisms not only rely on defects of the receptor protein CD22, as seen in resistance to other immunotherapies,[87] but also on deregulation of the G1/S DNA damage checkpoint through defects in *TP53*, *ATM* and *CDKN2A*.[42] Furthermore, one hypermutated sample presented with a novel indel signature, hallmarked by insertions >10bp, which could be the result of excessive error-prone double strand break repair. The accumulation of mutations resulted in the appearance of multiple subclones with CD22 mutations. Based on these findings, it appears worthwhile to study therapy-induced mutagenicity in cohorts treated with specific novel therapies, and InO in particular.

Over the past decades, multiple ALL relapse-drivers have been identified.[88] These genes stood out for carrying pre-existing mutations that were strongly selected during therapy, but also because mutations in these genes were frequently acquired in relapse. The treatment-related mutagenicity discussed in this review has now improved our understanding of the acquisition of relapse drivers after initial diagnosis. However, while it is clear that treatment-related mutagenesis can induce relapse-driving mutations, whether chemotherapy resistance contributes to treatment-related mutagenesis is still understudied. For example, *NT5C2* mutations, that induce thiopurine resistance, have been associated with SBS87. It has already been extensively proven that activating *NT5C2* mutations allow cells to tolerate thiopurines *in vitro*,[64-66] but it is still unknown if cells then also acquire SBS87. Therefore, it would be interesting to see if thiopurine doses that kill *NT5C2* wild-type clones, would instead induce SBS87 in *NT5C2* mutant clones.

The additional mutational load induced by mutational signatures is thought to result in higher clonal heterogeneity. This increased clonal heterogeneity may increase the chance that a therapy-resistant clone emerges, as recently observed in patients treated with InO.[42,81,89] As such, the high mutational load induced by thiopurines in mismatch repair deficient samples could represent a contraindication for thiopurine use in these patients. After all, the drug is well tolerated by the leukemic cells and the increased mutational load represents a risk for acquisition of additional pathogenic alterations. Thiopurine use would represent a risk especially in patients that carry a single copy loss of *TP53*, either somatically or in the germline. Patients with a single copy *TP53* loss present with broad therapy resistance and, as a result, a poor prognosis.[82] Loss of both *TP53* alleles, for example, by an additional SBS87-associated R248Q mutation, worsens prognosis further.[90] Another risk group is made up of patients with constitutional mismatch repair deficiency (CMMRD). CMMRD is a cancer predisposition syndrome caused by germline homozygous loss of any of the four key MMR genes,[91] and CMMRD patients are often diagnosed with hematologic malignancies.[92-94] A contraindication already exists for the methylating agent temozolomide, which is a core component of glioblastoma treatment, the most common malignancy in CMMRD patients.[95,96] Similar to thiopurines, temozolomide methylates guanine, resulting in O6-methylated guanine nucleotides that trigger a cycle of repeated failed mismatch repair, followed by apoptosis.[97] Consequently, in an MMR-deficient context, temozolomide induces hypermutation without therapeutic benefit. The controversial status of temozolomide use in CMMRD patients and MMR deficient tumors shows that thiopurine use in these scenarios also requires further scrutiny.[98]

Finally, mutational processes have mostly been defined using SBS, but this does not necessarily mean that other mutational signatures are absent. After all, rare or less distinct mutational signatures can easily be missed when lacking sufficient power. Moreover, the repertoire of mutational signatures is ever expanding, as mutational signatures have now been defined for copy number alterations,[99,100] structural variations,[101-103] and single base substitutions in RNA.[104] It has already been shown that structural variation breakpoints differ between ALL subtypes, which indicates that ALL drivers are generated by distinct mutational pro-

cesses.[12] Future studies, using novel methods of mutational signature detection and extended cohorts will surely help further delineate the mutational processes driving ALL at initial diagnosis and relapse.

Disclosures

No conflicts of interest to disclose.

Contributions

RPK conceptualized the study and supervised the review. CGH conceptualized the study, wrote the first version of the manuscript, and prepared the figures. All authors wrote the manuscript.

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