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REVIEW ARTICLE

Acute myeloid leukaemia: recent data on prognostic gene mutations, in relation to stratified therapies for elderly patients

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Summary

TET2 protein is encoded by the gene TET2 which specifically catalyses the demethylation of 5-methylcytosine to cytosine. Mutations in TET2 have been identified in a number of haematological malignancies, including leukaemias and lymphomas. In acute myeloid leukaemia (AML), loss of TET2 function drives DNA methylation and gene silencing, contributing to disease pathogenesis and progression, making it an interesting target.

Although such mutations are considered rare, there is an increasing body in the literature identifying them as unfavourable prognostic markers in AML.

The hypomethylating agent nucleoside analogue 5-azacytidine is used in the treatment of AML and other haematological malignancies i.e. myelodysplastic syndrome (MDS). It functions by re-activating silenced genes is responsible for cytosine methylation, thereby driving differentiation and also promoting apoptosis of dysfunctional haematological cells.

The present review article deals with the consequences of DNA methylation in relation to TET2 in AML, focusing on the potential prognostic effect of TET2 gene mutations, along with demethylating epigenetic strategies towards prediction of therapeutic response.

The necessity for personalized therapeutic regimes, especially for older patients suffering from AML with mutated TET2 and/or other genetic alterations, along with its prognostication are also underlined.

Key words: *acute myeloid leukaemia, DNA methylation, TET2 , TET2 mutation, 5-azacytidine*

Introduction

Broadly, the myeloid and lymphoid paths of the haematopoietic cell production define the leukaemias that may arise [1]. Acute myeloid leukaemia (AML) is a disease of the bone marrow and specifically the progenitor cells of the myeloid line. It is characterized by clonal expansion of immature white blood cells (WBCs), which leads to progressive accumulation of blasts in the bone marrow (BM), with ultimate BM failure and death if not treated [2,3]. In the majority of cases with progression of AML, spleen and other organ tissue are affected, leading to increased oxidative stress in the area. Production of normal components in the blood (platelets, white blood cells for immune response, red blood cells) are also massively affected during AML, ultimately resulting in bone marrow failure. Without treatment AML is rapidly fatal [4].

The outlook and prognosis for AML varies widely. Identification of subtype (using specific classification protocols) is vital. The prognosis of the patient can vary between high likelihood of full remission (90 % of cases) to a relatively poor response and survival rate from diagnosis. Type of AML, age of patient, mutations associated with disease, response to treatment (poor performance status or intolerance to intensive chemotherapy),

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co-morbidities, secondary leukaemia status and other patient-specific factors play a significant role in prognosis.

Following assessment and first line treatment response, outcome could be characterised as favourable, intermediate or unfavourable [5,6]. Cytogenetic findings are traditionally employed to help establish the foundation for prognostication and identify therapeutic strategies with the ultimate aim of treatment stratification. Appelbaum et al. demonstrated that older patients over 65 years old, had more commonly unfavourable-risk cytogenetics, than favourable-risk disease and were more prone particularly to abnormalities in chromosomes 5, 7 and 17 [7].

Since 2013, the application of advances in whole genome sequencing technology, analysis, and interpretation has revolutionized our understanding of AML biology. Evidence of AML somatic mutations has demonstrated clinical relevance by improving our knowledge to determine prognosis using pre-treatment risk stratification and/or high-sensitivity measurements of disease burden [8,9]. This information, however, has had minimal impact therapeutically, as the typical AML treatment in both initial induction and remission settings remains unchanged for almost 50 years now. This intensive cytotoxic chemotherapy has been a combination of a topoisomerase II inhibitor (e.g. daunorubicin or idarubicin) and cytosine arabinoside, sometimes with the addition of a third agent [10, 11]. If patients enter remission after the induction treatment and if they are considered as 'low risk' for remission, they usually stay on high dose of cytarabine (ara-C), which has been proven relatively effective, with up to 65% of patients achieving remission [12,64]. Autologous or allogeneic stem cell transplantation is reserved as an option for intermediate and high risk patients. In addition, patients that follow an allogeneic stem cell transplantation show a lower risk of relapse. A 42% of low-risk patients on an allogeneic stem cell transplantation succumb to remission compared to patients on an autologous stem cell transplantation, where the remission percentance is 20%. It is worth, however, to be noted that there is a potential risk of death due to allogeneic stem cell transplantation in the first 10 years post-transplantation reaching up to 15% of patients [10,13-15].

The genetic data regarding the origin and frequency of mutations seen in AML and the clonal heterogeneity, although being of utmost importance, have not yet been incorporated in standard diagnosis schemes, as they can be quite variable especially in non-recurring mutations. Non-recurring mutations are the non-repeating ones, that do not follow measurable and consistent frequencies. The differences between presentation and relapse in each patient has, however, opened up the possibility for truly personalized therapy based on direct targeting of aberrant pathways within each individual's AML clone and genotype or at least within each cluster/group of patients with similar variants [10,16].

The aim of present article is to provide a sort overview on the crucial consequences of DNA methylation concerning *TET2* and other related genes in older AML patients, along with their possible prognostic value, with regard to stratified (personalized) therapeutic approaches.

Methylation of DNA

Epigenetics refer to the heritable changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence (a change in phenotype without a change in genotype). These alterations may or may not be heritable, although the use of the term "epigenetic" to describe processes that are not heritable is controversial [17].

It is well known that epigenetic mechanisms are involved in carcinogenesis. Epigenetic changes, such as DNA methylation, histone modifications and post transcriptional gene regulation by non-coding microRNAs (miRNAs) are easily influenced by dietary and environmental factors. These pro-

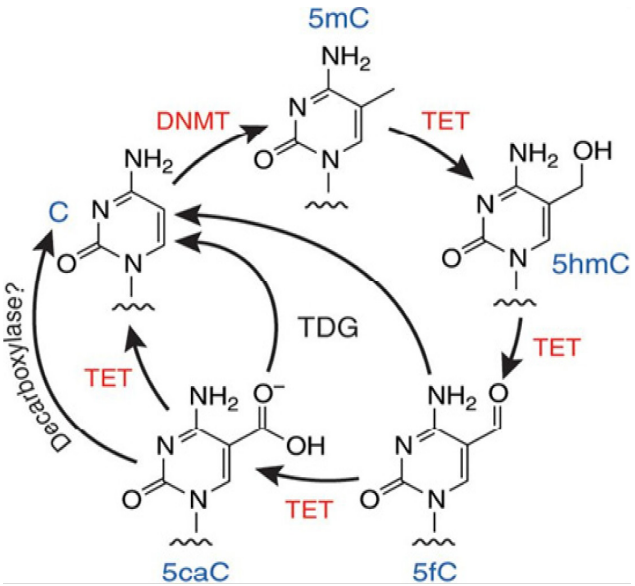

Figure 1. DNA demethylation cycle and mechanisms. α-ketoglutarate (α-KG) dependent dioxygenase TET catalysing histone methylation, inhibition of lineage specific gene expression and astrocyte transformation. The conversion from 5-methyl-cytosine (5mC) to 5-dihydroxy-methyl-cytosine (5hmC) by TET proteins.

cesses affect transcript stability, DNA folding, nucleosome positioning, chromatin compaction, and complete nuclear organization of the genetic material. Disruption of the epigenome certainly underlies disease development [18,20].

DNA methylation is the most well studied epigenetic modification in mammals. Briefly, a methyl (-CH3) group is covalently attached to the 5' carbon of cytosine (5mC) [21]. Cytosine-guanine dinucleotide (CpG) methylation mainly encompasses regions rich in CpG sequences, known as CpG islands, but could occur anywhere in the genome (Figure 1). The hydroxylation of 5mC to 5hmC, and later to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) as described in Figure 1, is considered as a key step in the process of DNA demethylation at CpG islands and other sites. Generally, DNA methylation seems to play a critical role in cellular processes, one of the most important/promising being regulation of gene expression (Figure 1). Methylated cytosine (5mC) can be demethylated into unmodified cytosine through several, passive or active, modifications mechanisms. 5-methylcytosine (5mC) is a minor base in mammalian DNA constituting approximately 1% of all DNA bases.

Despite this, methylation undergoes massive dynamic changes during early embryogenesis and prevails in more than half of human promoters. During early development the majority of methylation is dynamically altered by specific regulatory factors such as NANOG (Transcription factors in embryonic stem cells). These are crucial for keeping embryonic stem (ES) cells and early-stage embryos pluripotent [21]. Certain genes are considered 'imprinted' when they display highly stable methylation patterns, which are also retained across the majority of the organism's genome. The imprinting process has a certain and rather specific type of expression regulation. It is also known that imprinted regions are essential for X-inactivation and non-selective expression of those genes [22,23]. Multipotent adult cells are as affected by methylation as all other cells across the differentiation branches (myeloid, lymphoid).

Continuous genetic and epigenetic transformation can be seen in leukaemic cells as well as in healthy ones. Specifically, methylation leads to clonal diversification and the observation of many highly heterogeneous subpopulations of leukaemic cells which can be characterised uniquely. It is worth mentioning that several genes, including *p15, MDR1, ER*, and *HIC1*, have been shown to be inactivated by methylation in AML cases 6,25].

DNMTs are the main family of enzymes known to control and actively regulate methylation. Although DNA is methylated in many regions across the genome, CpG islands are of great interest when it comes to gene regulation [26]. Methylation can be characterised using global analysis, pyrosequencing, and array technology, according to whether it affects single CpG sites, multiple independent CpGs or multiple linked CpGs [27]. Areas affected by methylation are split into those including specific structures over 200 bp in length, known as 'island' where the observed CG ratio can be over 60%, 'shore', an area approximately 2kb from an island, 'shelf' at 2-4kb from the island, and 'open sea' which refers to methylation affecting CpGs isolated in various spots across the genome [28].

The most common model of DNA methylation is the one affecting promoter regions of gene, where hypermethylation can cause transcriptional silencing and *vice versa*. However, Brenet et al. argue that sometimes DNA methylation in the region of the first exon of a gene, is more tightly linked to transcriptional silencing than the one in the upstream promoter region [6]. DNA regions can acquire or lose methylation, depending on age and other environmental factors.

There is an increasing interest in epigenetic events and their role in pathogenesis of human malignancies [29]. As mentioned, methylation of gene promoter regions located in CpG islands plays a crucial role in epigenetic silencing of genes. Specifically, during development, progression and relapse of leukaemias genes, such as *ESR1, IGSF4,* and *CDKN2B/p15*, seem to be affected by transcriptional silencing [24]. Additionally, relatively recent findings suggest that promoter DNA methylation patterns could provide important data regarding risk and outcome, when it comes to leukaemias [30,31]. Although the aforementioned studies seem promising, the prognostic value of individual DNA methylation biomarkers has not been assessed especially in a context of cytogenetic subgroups.

DNA methylation in cancer

The role of methylation in early embryonic development and cell differentiation is known and studied broadly. There is great interest, however, to explore the aberrant methylation's role in many cancer types [32,33]. When normal cells progress to leukemic, global genomic methylation is seeing a decrease [32]. For instance, decreased expression of *MAGE-1*- a gene hypomethylated in malignant cells- is of the plenty examples for gene specific methylation in cancer [34]. However, in AML, gene-specific hypermethylation seems to follow a specific pattern with inactivation of the *p15* tumor suppressor gene being the most usual outcome [24].

In AML and other malignancies, aberrant DNA methylation patterns include global hypomethylation, gene-specific hypermethylation/hypomethylation, and loss of imprinting (LOI). LOI occurs during leukaemogenesis (as an early event of epigenetic alterations) and has a significant role in the correlation of methylation patterns and AML evolution. Specifically, LOI was found only in blood from subjects with AML and MDS but not in samples from healthy individuals [15].

The complete mechanism of DNA demethylation and consequential pathways remain unclear, but they have mostly been linked to alterations in cell proliferation and cell cycle regulation. Mounting evidence on the latter suggests that gene-specific hypomethylation can lead to overexpressed genes contributing to the neoplastic phenotype in leukaemia [5,29,35,36].

Conversely, hypermethylation of DNA mismatches repair genes. It can, for example, drive microsatellite instability with simultaneous inactivation of multiple genes in myelodysplastic syndrome (MDS). Hypermethylation is associated with quick evolution of the leukaemic burden, blast infiltration in peripheral blood (PB) and increase in BM and shortened overall survival [37,38]. Hypermethylation of cancer patients' genome is mostly observed in CpG rich parts, specifically in gene promoter regions, affecting cell cycle related genes such as tumour suppressors ones [24,32]. Aside from DNA methylation affecting promoter function, hypermethylation has also been seen to affect regulation of noncoding RNAs expression (those of microRNAs and others). Subsequently, expression of downstream target genes and transcripts is also altered [24]. Optimistically enough however, DNA methylation is a reversible process, unlike many other genetic modifications, and can therefore be considered a promising approach for therapy [39,40,50].

TET2 and AML

The *TET2* protein is encoded by a gene (*TET2*) residing on the long arm of chromosome 4 and is mutated or deleted in a significant proportion of patients with myeloid malignancies, including a ~10% of AML patients [2]. The *TET2* gene comprises 11 exons reaching a 150 kb in length and amongst being present in the phenotype of normal

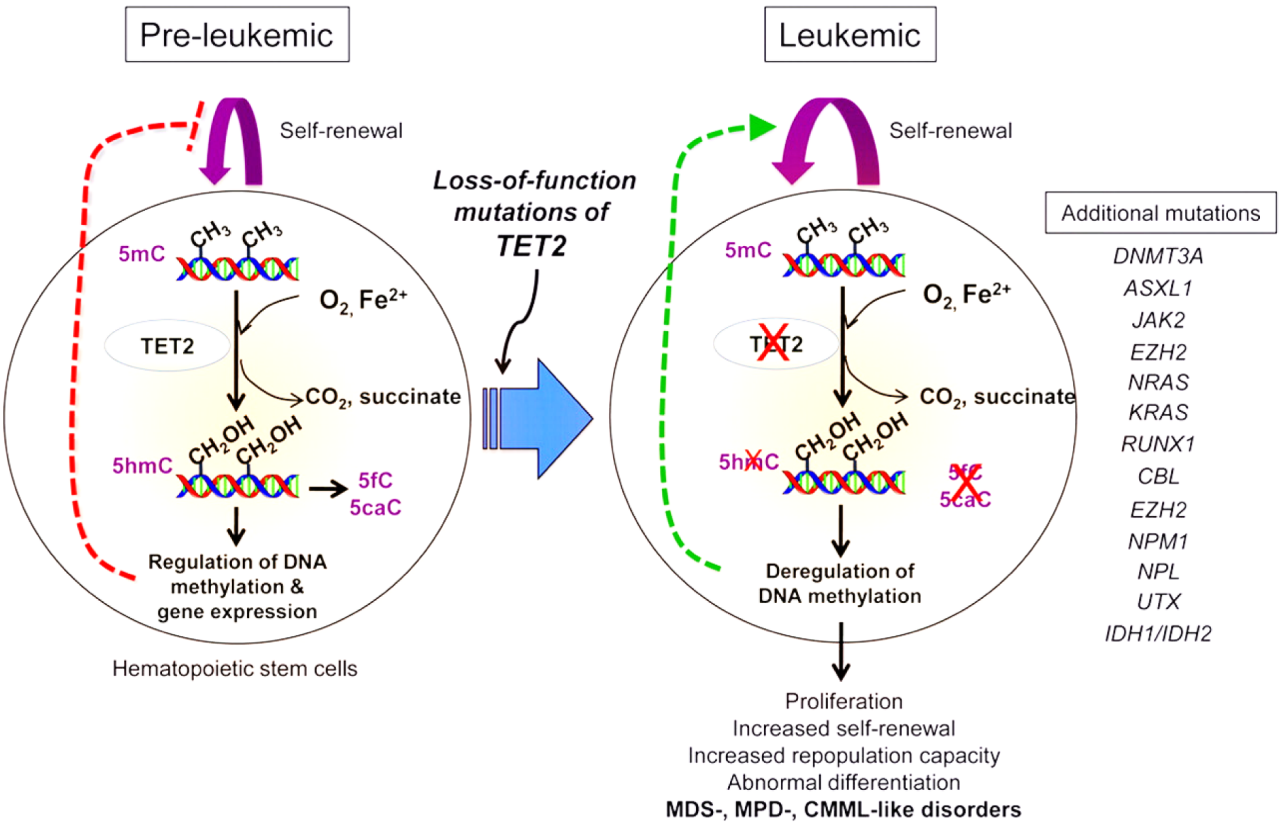

Figure 2. TET2 mutations in myeloid leukaemogenesis. TET2 catalyses the oxidation of 5mC to 5hmC, 5fC and 5caC mostly by regulating gene expression via DNA methylation. TET2 mutations frequently coexist with other mutations, possibly co-operating in tumour initiation, progression and other phases of tumourigenesis. Figure from Ko et al. (2011) [47].

and abnormal haematopoietic cells, it promotes DNA demethylation. *TET2* shares a conserved domain region with TET1 and TET [2].

Mutations in *TET2* were first discovered in myeloid malignancies by high-resolution single nucleotide polymorphism (SNP) and comparative genomic hybridization arrays [11]. Although such mutations are considered rare, there is an increasing body in the literature identifying them as unfavourable prognostic markers in AML, increasing the interest for their in depth study and characterisation [4,9,40,41].

A 5-25 % of adult AML cases, with a highest frequency of older patients have at least one mutation of *TET2* [4]. The most common *TET2* aberrations include deletion and base substitution mutations, most of which seem to affect only one allele with only 30% being bi-allelic [42,43]. More specifically, heterozygous mutations in *TET2* present in BM cells of AML patients, may actively contribute to malignant transformation. *TET2* mutation also drives transformation demonstrated to occur more in cells with it being present in both *TET2* alleles in AML [4]. This transformation, which can be present and in other genes, has a main limitation: it cannot directly lead to full cellular transformation and it is harder to discern as heterozygous mutations and/or mutations. Additionally, its function is not yet fully understood [7,28,44]. Bi-allelic *TET2* mutation also appears to confer survival advantage to the transformed cells over normal tissue and this can endorse malignant cells survival and/or resistance to treatment [63].

The prevailing evidence suggests that loss of *TET2* can result in global reduction of 5hmC with a respective increase in 5mC, giving rise to a hypermethylated genotype, illustrated in Figure 2. It is hard to distinguish a specific pattern for the *TET2*'s role in aberrant methylation during oxidation of 5mC in the presence of other gene aberrations, however, prevailing evidence show a slightly stronger hypermethylated phenotype is observed when loss affects both copies of the gene, when compared to heterozygous *TET2* loss, although both states show increased overall methylation [2,28].

Recent studies have associated the loss of *TET2* with poorer outcome in intermediate risk AML [2,4,11]. This was supported *in vivo* as conditional mouse models proposed that *TET2* loss can occur in progenitor cells, making them more prone to myeloid malignancies [48]. However, *TET2* mutations, as mentioned, are not adequate for complete conversion to the leukaemic phenotype [47,49]. Furthermore, production of 2-hydroxyglutarate present in patients with mutated *IDH1* and *IDH2*, and WT1 mutations lead to reduced *TET2* function and/or inhibition and reductions in 5hmC [30,44].

Mutations that 'co-operate' with *TET2* have been found to aid the transformation of normal cells to leukaemic ones. These include, but are not limited to, *NPM1, FLT3* and *RUNX1* [17,24]. Collectively, the *TET2* hydroxymethylation pathway is altered by somatic mutations in a median of 30 % of AML and other haematological and/or epithelial malignancies [30,50].

The effects of *TET2* mutations in primary AML remain unclear. The main reason for this uncertainty stems from the *TET2* mutations and its association with other genetic alterations not being fully unravelled. To date, not many studies have identified a positive link between *TET2* and *NPM1* mutation in AML patients achieving complete remission (CR) [41]. However, reports from other authors do not seem to confirm this association. The *TET2* gene is a homeostatic factor of normal haematopoiesis, and loss of its function impairs myeloid differentiation. Thus, somatic mutations in the *TET2* gene result in abnormal or increased proliferation of myeloid cells with or without the development of leukaemia or of other myeloid disorders. It is worth noting that *TET2* mutations occur in all of the 11 exons of the gene, prompting Euba et al. to suggest that mutational studies of *TET2* should include the entire coding region of the gene [51-53].

There are relatively few studies that have evaluated the frequency and clinical role of *TET2* mutation within large cohorts of patients with AML, including participants of prospective/scheduled clinical trials in the near future [25,51]. Mutations of *TET2* have been reported in more than a fourth of MDS or secondary AML cases [11,47], in 14 % of MPNs [11], and in 7-23 % of *de novo* AML [25,54]. The prevalence of homozygous *TET2* mutation was 26% in patients with *TET2*-mutated MDS, and 9% in patients with *TET2*-mutated AML. The prognostic value of this in the context of treatment outcomes has not been fully investigated [4,45,55].

Similarly, a study reported a 27% of *TET2* mutation frequency in patients with AML who did not achieve a CR, in comparison to 17% in those who achieved CR, with Nibourel et al. using a cohort enriched with elderly patients [56]. Overall, prevalence of *TET2* mutations in patients up to the age of 60 is slightly less than 10% [30]. A *TET2* mutation prevalence of 23% in one study might be a result of the age distribution that ranged from 18 to 83 years (median 66 years) for patients with AML [57]. Taken together, the evidence suggests that approximately 7% of younger adult patients have missense, nonsense or frameshift mutation

in the *TET2* gene with most heterozygous [25]. For older patients these rates are proposed to be at or around 23% [4].

TET genes and methylation

As analysed above, *TET2* protein contributes to methylation/demethylation homeostasis, and reductions in the levels of *TET2* in mice have been associated with genome-wide hypermethylation [45].

Various studies report that *TET2* is involved in gene expression/silencing however no study has reported to date the precise mechanism of this. The role of *TET2* in myelopoiesis mostly revolves around maintaining the hematopoietic stem cell (HSC) pool and in controlling HSC differentiation (when the genes function and expression are normal) [15,32,58]. Although previous methylation profiling studies in AML have attempted to identify drivers of disease to be altered epigenetic regulators and specifically the association between somatic and epigenetic aberrations, this has been yet unclear [30]. Nonetheless, although genetic and *in vitro* studies have suggested a role for *TET2* in regulating haematopoietic differentiation and stem/progenitor cell expansion, the *in vivo* effects of *TET2* loss in mouse models have not yet been fully elucidated either, as this review underlines in a later section.

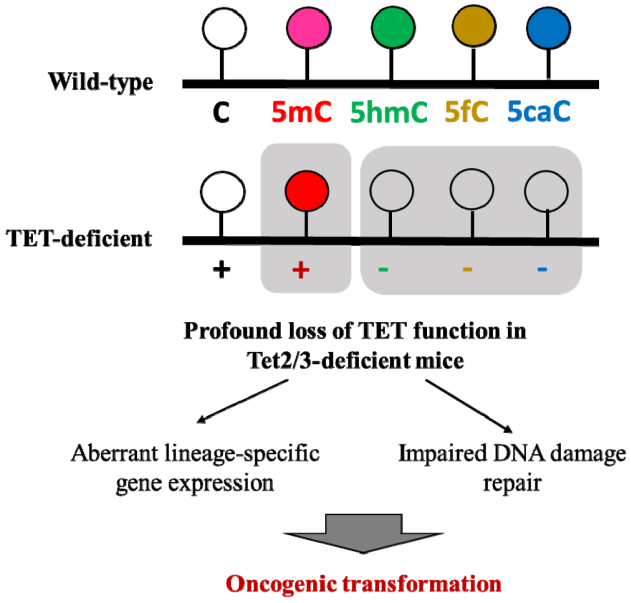

Figure 3. TET proteins help maintain genome integrity. In mice lacking Tet2 and Tet3 a development of an aggressive form of myeloid leukaemia was observed. In the Tet deficient model, the methylation process is ceased and leads to an increase in 5mC and interruption of the production of the rest of the products 5hmC, 5fC, 5caC.

Prognostic effect of TET2 gene mutations

As for the prognostic impact of *TET2* alterations in myeloid malignancies, findings have been contradictory. This appears to depend on the disease under study and interactions with other mutations within the different cytogenetic profiles [59]. *TET2* mutation was seen to have a negative prognostic effect in patients with CN-AML and genetic aberrations linked to favourable groups such as *NPM1*-mutated/*FLT3 ITD* –ve AML, or *CEBPA* mutated AML, in patients receiving intensive therapy [4], although this finding was not confirmed in subsequent studies [25,56,60]. Chou et al. (2011) reported the possibility that *TET2* mutation might be linked to poor outcome in patients with intermediate cytogenetic risk [52], however, when other mutations were encompassed in the analysis model, this association weakened [10].

In conclusion, mutated *TET2* does not need to be a linked event, even though this is seen in some patients. *TET2* proteins help to maintain genomic integrity, and mice with *TET2* deficiency are observed to develop aggressive myeloid leukaemia as shown in Figure 3.

Whole genome sequencing [61,62] has identified over 20 recurrent driver somatic mutations in AML genome. The aforementioned studies identified lesions with the potential for therapeutic targeting, including mutations in *FLT3, NPM1* and epigenetic modifier genes (*DNMT3, IDH1/2* and *TET2*). Apart from standard remission induction regimens, low-dose ara-C and hypomethylating agents (HMAs), anti-proliferative and demethylating agents are also in the spectrum of current treatment options for older patients. As an example, 5-azacytidine and decitabine are both HMAs and are affecting methylation, mainly through the inhibition of DNA methyltransferases (DNMTs), although other mechanisms are involved [59].

Therapy

Although standard chemotherapy is well tolerated in younger patients, it has been associated with increased morbidity in elderly AML patients. In patients younger than 60 years remission rates reach 70%, however these rates appear to be lower, varying from 30 to 50%, in older ones. In a glimpse, one third of elderly patients succumb to complications of treatment, one third remains leukaemic and the other third enters remission. Even those who enter remission tend to relapse typically within 6-12 months with poor survival thereafter [57,63]. Overall, less than a tenth of adult patients over 60 survive five years after standard remission induction [57,64]. The mapping of the

genomic landscape of diseases and other advances in determining minimal residual disease (MRD), will allow AML patients, and especially older ones, to benefit greatly [45]. Consequently, there is an urgent need to develop novel therapies aimed more specifically at targeting pathways disrupted by mutations in AML, such as *TET2* which is associated with genome-wide hypomethylation [1,65].

Other genes

MDS in general and AML in particular can be a result of a very wide range of possible genetic mutation, however to date only mutations concerning the *TET2* gene have been reported to offer some prediction of response to 5-azacytidine or other chemotherapeutic demethylating agents [47,66]. Furthermore, when low variant allele fraction (VAF) was taken into consideration, *TET2* mutation status and response rate association weakens, showing that response might not be predicted if the *TET2* mutation is present in a minor leukaemic clone [6,67].

The largest AML sub-group is AML with normal karyotype (NK-AML) and is linked with intermediate risk. In NK-AML, distinct genomic abnormalities of the *NPM1, CEBPA* and *FLT3* genes provide prognostic information. Somatic mutations targeting *IDH1, IDH2, TET2* and *WT1* occur frequently in AML, and there is potential interference between lesions in these genes and response to demethylating agents [4,70]. Many studies have investigated mutation in other genes (such as *ASXL1, RUNX1* and *NRAS*) that may also affect response to demethylating chemotherapy; specifically have an adverse prognosis in AML and could be used as biomarkers of response to treatment with specific anti-proliferative agents.

Firstly, association studies with *ASXL1*-mutated and wild-type *TET2* as well as *TET2*-mutated and wild type *ASXL1* have been carried out to evaluate the prognostic effect and response of treatment with demethylating chemotherapy [68]. For example, Metzeler et al. [41] identified that patients with mutated *TET2* and wild type *ASXL1* (10% of all participants) were the group with the most favourable response to treatment with demethylating agents. Potential explanations for looking into *ASXL1* mutations include partial resistance to HMAs caused by them. Conversely, *ASXL1* mutated with intact *TET2* had lower likelihood of response in two studies without major statistical significance [4,67]. Figueroa et al. [23] and Metzeler et al. [41] found no common gene expression patterns when comparing *TET2* mutated AML and *IDH* mutated AML, however *TET2* mutated AML displayed a hypermethylation mark similar to that of patients with *IDH* mutations [4,30]. Other factors that could play a role are recently discovered mutations in *EZH2, NPM1* and *DNMT3A*, which could be associated with epigenetic deregulation [30,69]. These data suggest that the classification as intermediate risk could be further refined with the addition of information on other mutations. These could particularly be in the context of specific therapies such as demethylating agents [71].

A large study from the New European Leukaemia Net (ELN) proposed a reporting system that included cytogenetic and molecular abnormalities to predict outcome in patients with AML [41]. This study concluded that *TET2* mutations affect different sets of microRNAs and genes in favourable-risk and intermediate-risk patients with normal cytogenetics. ELN also reported that a subset of patients with mutated *TET2* had poor response when administered conventional post-remission treatment [71]. Other studies concerning mutations in other genes that potentially play a role in AML showed that the *NPM1* mutation was observed more commonly in patients with heterozygous *TET2* mutations than in patients with homozygous *TET2* mutations. This was fairly statistically significant as well (p=0.017) [51,72]. However, there was no distinct difference regarding the clinical features and other mutations between patients with concurrent mutations. Although *TET2* mutations frequently coexist with other mutations, possibly co-operating in tumour instigation, evolution and other phases of malignant development, mutations targeting *IDH1, IDH2*, and *TET2* genes specifically can be mutually exclusive, leading to a need for further investigation [24].

Demethylating therapy in myeloid malignancy

Risk classification based on age, cytogenetics and presence of more than average white blood cells is taken into consideration prior to prescription of demethylating therapy. The current controversy about the conditional use of demethylating agents in AML treatment, although these agents are generally well tolerated, is focused on the high degree of heterogeneity in efficacy and the need to identify which subgroups of older patients would benefit the most [25,31]. Considerable attention has recently been drawn to the development of regimens containing 5-azacytidine and decitabine. There have been suggestions that five days of 5-azacytidine may be as effective as the 7+3 course of treatment, and both subcutaneous, oral and intravenous schedules are equivalent [48].

Several randomized trials with either 5-azacytidine or decitabine have shown a response rate between 40 and 60% in patients with MDS. Additionally, a wide safety profile alongside improvements in clinical benefit was reported. These benefits include, but are not limited to, eliminating transfusion dependence, complete or partial normalization of blood counts, and significant reductions in bone marrow blast percentages in responding patients [42,43,73].

Azacytidine (5-azacytidine, Vidaza™, Celgene) is a derivative of the naturally occurring pyrimidine nucleoside cytidine. The structure of 5-azacytidine is shown in Figure 4. Half a century ago, 5-azacytidine and its deoxygenated derivative, decitabine [74] were characterised as classical cytostatic agents and debuted at high doses as novel treatments for acute leukaemias, but were soon replaced by other drugs such as ara-C [75,76]. Subsequently, the differentiation-promoting effects of 5-azacytidine were discovered and later associated with DNA hypomethylation [76,77]. Further studies, also investigating the properties and effects of these agents in methylation, when used at lower doses to treat MDS (so called "non-cytostatic" schedules) led to the hypothesis that demethylation could have an anti-leukaemic effect [37,64].

Effect of demethylating agents on tumour suppressor genes

As it is well known, the two major classes of genes most recurrently mutated in cancer are oncogenes and tumour suppressor genes; the former play an important role in tumorigenic evolution and establishment, while the latter demonstrate a critical role in suppressing uncontrolled proliferation, immortality, and tumourigenicity. Knudson's retinoblastoma tumour suppressor gene observation has led to discovery of new genes with tumour suppressing activity [49,51].

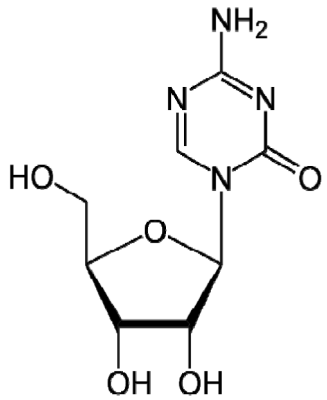

Figure 4. The structure of 5-azacytidine.

When first discovered, it was believed that 5-azacytidine was introducing its cytotoxic effects through incorporation into the genome and RNA. 5-azacytidine acts through random decrease of methylation levels at promoters regions of tumour suppressor genes where function is counterbalanced via increased gene expression. It is well established that leukaemias are blood disorders where different genes and mutations can be involved prior to onset and loss of tumour suppressor function is often associated with an increased oncogene activity. However, restoring tumour suppressor activity may not be sufficient to reverse the malignant phenotype in most cases. There are various well-studied ways through which tumour suppressors can be inactivated: mutation, loss of function, epigenetic inactivation and dominant negative inhibition. Historically, epigenetic inactivation was not sufficiently proven to be targetable, but while our understanding on epigenetics increases, novel cancer treatments including demethylases or other targeted molecules are being employed and could be coupled with other existing or more targeted therapies [49,78].

On the other hand, oncogenes can have a dominantly negative effect-compared to tumour suppressor genes and can drive transformation to the malignant phenotype even when only one mutant allele is expressed. *TET2* performs like a tumour suppressor but it has not been yet shown to consistently behave like a canonical tumour suppressor and as such it is not officially clustered as one.

Sensitivity to demethylating chemotherapy in TET2-mutated MDS and AML

5-azacytidine has been approved for the treatment of MDS [48,69], and has similar efficacy in the treatment of leukaemias as decitabine, another demethylating agent and cytidine analogue [79]. Lately, focus has shifted to identifying patient subgroups defined by somatic genetics, which may particularly benefit from therapy with demethylating agents with an increased overall survival [42,70]. In an international randomized phase-III AZA-001 clinical trial, the two-year overall survival of MDS patients treated with 5-azacytidine was almost 15% more than the conventional treatment approaches. These conventional treatments included best supportive care, low-dose cytarabine or intensive chemotherapy as appropriate [25]. Smaller clinical trials and single-centre non-randomised observational studies suggest that the presence of *TET2* and/or DNMT3A mutations may be associated with a positive response to treatment with demethylating agents [11,26,41,69]. However, other studies did

not confirm these findings and presence of *TET2* as a prognostic marker of response to 5-azacytdine remains unresolved [3,24].

A molecular basis which could be connected with poor response or resistance to treatment with hypomethylating agents and eventual relapse have previously been proposed [5]. Itzykson et al. worked on behalf of the Groupe Francophone des Myelodysplasies (GFM) studying the prevalence of multiple gene mutations in single-cell colonies of chronic myelomonocytic leukaemia (CMML) patients. The work covered in this study used predominantly next generation sequencing (NGS). Early clonal prevalence and a lack of influence of treatment on the expansion of the more mutated clones was observed. Furthermore, fifteen MDS and AML patients entered complete remission following a combination treatment of 5-azacytidine and valproic acid. Nevertheless, Craddock and Owen indicated that leukaemic stem progenitors were never entirely eradicated although were undeniably substantially reduced at first [80]. Morphological relapse was only seen with the prior deterioration of these stem cells and blasts still in circulation.

The necessity for a continuous treatment even post-response increase is underlined by the reversible nature of methylation. Further to this, treatment intermission was indeed found to be associated with relapse further increasing the importance of continuity [81].

However, a recent study from Ross Levine's group focusing on epigenetic regulatory genes (*TET2, IDH1, FLT3ITD*) showed that the absence of epigenetic function post-knockdown seems to lead to an increased response to treatment with 5-azacytidine. In further support of this, a significant sensitivity to the anti-leukaemic effects of 5-azacytidine in a *TET2*-mutant murine model was also observed [82]. These two studies demonstrate that there is increased sensitivity of leukaemic cells to 5-azacytidine if *TET2* mutations are present.

In conclusion, demethylating agents such as 5-azacytidine can be of great benefit in MDS and potentially in AML, but continuous treatment seems to be necessary due to the reversible nature of DNA methylation. Haematopoietic stem cells can become leukaemogenic in the absence of treatment, potentially causing relapse.

TET2 knockout mice and models of TET2-mutant AML

As human AML can be preceded by mutations in *TET2*, a recent novel mouse model of the human genetic variation producing AML (deletion of *TET2*) has been used to demonstrate DNA hypermethylation in pre-leukaemic cells [83]. This was done both in itself and in combination with deletion of *DNMT3A* (combination of these mutations has been shown to precipitate the development of human AML) [84].

In addition to mice, other animal models were employed for the study of *TET2* deletion in haematological malignancies, with recent zebrafish mutants being generated as viable and fertile, and develop progressive clonal myelodysplasia as they age [13,85]. Whilst zebrafish can be exploited in the genetic research, sometimes its results should be treated with caution, as genetic activity in zebrafish does not always correlate with the mammalian counterpart [46,86]. Subtle differences between the animal models may however exist.

During 2011, multiple research groups generated *in vivo* studies on *TET2*-deficient mouse mod-

Table 1. Animal models using Tet2 function as an epigenetic regulator demonstrating very similar features upon deletion.

Group	*Quivoron et al., 2011*	*Moran-Crusio et al., 2011*	*Ko et al., 2011*	*Li et al., 2011*
Model	Exon 9 (gene trap) Exon 10-11 (conditional deletion)	Exon 3 (conditional deletion)	Exon 8-10 (conditional deletion)	Tet2 disruption 6pb upstream to the transcription start
Development	Normal growth and organ development	Normal growth and organ development	Normal growth and organ development	Normal growth and organ development
5-hmC levels	Decreased	Decreased	Decreased	Decreased
Extramedullary haematopoiesis	Spleen Liver	Spleen, 20 weeks	Spleen, 3 months	Spleen 2-4 months
BM progenitors /Repopulation capability in vivo	Increased	Increased	Increased	Increased

els [44,47,87,88] (Table 1), *TET2* gene knockout in mice leads to a progressive immature haematopoietic compartment with stem, blast and other myeloid progenitors infiltrating BM and other tissues [88]. Irregularities in monocytic, erythroid and megakaryocytic maturation as well as T- and B- lymphoid cell development were identified after close examination. It is worth noting that mice were most likely to develop a CMML-like disease rather than AML or MDS. Deletion of the *TET2* gene induced splenomegaly alongside a tremendous increase of circulating WBCs, myeloid dysplasia and infiltration of blasts within the bone marrow and stem cell compartment. A more specific example can be seen in the work of Li et al. who studied the *TET2* germline knock out murine model. Extramedullary haematopoiesis (EM-AML) was the main observations, as well as a drift towards monocyte production and myeloid proliferation *in vivo*, enlarged spleen and increased haematopoietic stem cell self-renewal. Furthermore, Moran-Crusio et al. engineered mice with no *TET2* function and observed that they displayed characteristics similar to patients with MPNs [44,88].

Although genetic and *in vitro* studies have suggested participation of *TET2* in regulating haematopoietic differentiation and stem/progenitor cell expansion the *in vivo* effects of *TET2* loss have not yet been fully elucidated [87]. Quivoron et al. [47] found that knock down of the murine *TET2* gene disturbs all haematopoietic steps, launching a competitive advantage to the cells and allowing for malignancies to develop, in an effort to test *TET2*'s function as an immediate regulator of homeostasis [87]. Generation of two *TET2* null mouse lines using gene-trap and a conditional knockdown of the *TET2* allele led to two comparable engineered mouse models in which the 5-hydroxymethylation was impaired.

In addition, Kameda et al. used murine models to demonstrate that loss of *TET2* by reduction in mRNA expression due to epigenetic silencing contributes towards the progression of myeloid neoplasms [89]. Thus, reduced *TET2* function due to loss of function mutations, may either result due to epigenetic silencing or to not yet elucidate mechanisms that may contribute to the malignant phenotype of haematological malignancies although decreased *TET2* transcript levels observed in myeloid neoplasms are not always associate with *TET2* mutation status [88]. *TET2* deficient animals as observed by Moran-Crusio et al. seem to develop CMML-like disease, alongside blast cell infiltration into the liver and spleen, with consequent splenomegaly. Their model was based on a conditional deletion within exon 3 by insertion of targeting vector sites (a more detailed comparison can be found on [44].

Generally, most of the analyses of *TET2* gene mutation status by several groups [44,47,87,88] have an initial step on their mouse modelling approach in common, in that their research was based on the known role of *TET2* as a regulator of normal haematopoiesis [44,47,87,88]. Following the observation that deletion of the *TET2* gene brings on myeloid/lymphoid transformation and/or amplification of the monocytic lineage, the evolution of the malignancies in the mice was highly dependent on the mutational background and induction of the deletion. Nonetheless, heterozygous mutated *TET2* and *TET2* null clones seem to behave similarly towards induction of variable malignancies in the mice.

Conclusions

Stratified medicine could be of particular value in the older AML patient group. Elderly patients often have co-morbidities that are dose-limiting for cytotoxic chemotherapies such as daunorubicin and cytarabine. Many patients have heart problems and treatment with daunorubicin, a cardiotoxin, can increase the risk of heart failure.

Adopting a more stratified view, would include screening AML patients for specific mutations that are found in the literature to be more sensitive to 5- azacytidine or other drugs which would then be tested in a clinical trial setting. Proving such a hypothesis, while leaving space for co-existing mutations correlation investigation, would require a cohort of elderly AML patients carrying *TET2, NPM1* and/or *IDH1* and *FLT3ITD* mutations who would then be treated with a selected drug, thus personalising their treatment. A demethylating therapy could for example be standardised across a dosage range to determine the optimal dose for remission induction and alleviation of leukaemic symptoms should they persist.

Specifically, Dombret et al. in their 5-azacytidine clinical trial review, demonstrated an overall survival benefit in patients with higher-risk MDS in the absence of complete remission, which was validated in a subsequent clinical study [36]. Their results, the authors concluded, suggest that 5-azacytidine may provide an important additional treatment option for older patients with newly diagnosed AML and that combination treatment regimens may further improve outcomes and prognosis [59]. Larger studies need to be completed to further support these findings [90].

Previously published evidence suggests that mutations of *TET2* confer cellular sensitivity to

demethylating agents [5,51], although the extent of the sensitisation has not been fully elucidated and it is still unclear whether specific lesions in *TET2* qualify as a genetic predictor of outcome and response to treatment. Nevertheless, there is some evidence supporting this model where heterozygous and homozygous deletions in *TET2* were associated with a higher rate of response than in *TET2* mutations in general. In a prior study by Itzykson et al. mutations of *TET2* detected by Sanger sequencing were found to predict a nearly two-fold greater response rate in the treatment with 5-azacytidine. Consistently with this finding, Ravandi's et al. investigation utilised aza-treated mice engrafted with human AML cells, resulting in significant differential sensitivity based on *TET2* genotype, with *TET2* null cells being more sensitive to the growth inhibitory effects of the agent [91].

Concluding, some crucial points towards AML personalized therapy are presented in short below:

- Stratified medicine could be of particular value in the older AML patient group, as evidenced by the literature review reported here. Elderly patients often have co-morbidities that are dose-limiting for cytotoxic chemotherapies such as daunorubicin and cytarabine (the standard chemotherapeutic regime of 7+3). Many patients above a certain age have heart problems and treatment with daunorubicin, a cardiotoxin, can increase the risk of heart failure.
- More studies need to be assembled to test the potential effect of *TET2*-null presentations of AML are more sensitive to certain demethylating agents in a clinical trial setting. Proving such a hypothesis would require a cohort of elderly AML patients carrying *TET2* mutations as well as deletions (so complete lack of *TET2*) who would then be treated with a demethylating agent, thus personalising their treatment.
- Demethylating therapy could be standardised across a dosage range to determine the optimal dose for remission induction and alleviation of leukaemic symptoms.
- The subsequent step would be for AML patients with mutated and/or deleted *TET2* to be treated alongside another cohort of patients with concurrent mutations on other genes such as *IDH1, 2, NPM1* and others to test for synergies and/or antagonisms between the gene expression and the treatment.
- Ultimately AML as well as MDS-affected patients should participate in clinical trials focusing on personalising treatments, for more information to be collected regarding the strategies of effectively treating AML in older patients, while reducing treatment-induced morbidities and thus increasing survival.

Conflict of interests

The authors declare no conflict of interests.

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