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Acute myeloid leukemia

Therapy-related leukaemias with balanced translocations can arise from pre-existing clonal haematopoiesis

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To the Editor:

Therapy-related leukaemia is a life-threatening complication of cancer treatment. The prognosis is poor and incidence is increasing, mainly due to the increasing survival from primary cancers, leading to an emerging major healthcare problem [1].

Currently two groups of tMN are recognised: the first, accounting for ~80% of cases, is characterised by exposure to alkylating agents, a latency period of 5–6 years, a complex or monosomal karyotype and/or mutations in TP53 [2]. These mutations have been detected in samples pre-dating therapy exposure [3] and presence of clonal haematopoiesis (CH) prior to cancer treatment is associated with a markedly increased risk of tMN with estimated hazard ratios of 5.8–13.7 [4, 5], indicating that cancer therapy may drive the expansion of pre-existing mutated clones, eventually leading to malignant transformation.

The second group of tMN accounting for ~20% of cases is characterised by exposure to topoisomerase II (topo2) targeting agents, a latency period of 1–2 years and presence of recurrent fusion genes identical to those seen in de novo AML [6, 7]. In these cases, there is evidence that drugs targeting topo2 directly initiate chromosome rearrangements: in the presence of mitoxantrone or etoposide, topo2 cleaves the PML and RARA loci at the precise base pair positions observed to be fusion breakpoint junctions in patients who develop therapy-related acute promyelocytic leukaemia (tAPL) after exposure to these agents [8, 9].

Fusion-gene associated chromosome translocations are widely regarded as primary leukaemia-initiating events in AML. Genome-wide studies show striking exclusivity between fusion genes and other mutations shown to be leukaemia-initiating events. For example, mutations in DNMT3A are frequent in AML and are associated with a preleukaemic state [10–12]. These mutations either do not occur or are rare in leukaemias with fusion genes [11–13]. Thus, two distinct processes that lead to the generation of tMN appear to mirror two separate mechanisms of de novo leukaemogenesis, with two distinct types of primary leukaemia-initiating event (fusion genes and preleukaemic mutations, respectively).

The relationship between CH and therapy-related leukaemias with recurrent chromosome translocations has not previously been investigated. Here, we focussed on a molecularly defined group of patients with PML-RARA+ tAPL and present evidence that in these patients, in contrast to de novo APL, chromosome translocations may be secondary events, arising on a background of pre-existing CH.

We first performed whole-exome sequencing of 55 cases of de novo (dnAPL) and 13 cases of therapy-related APL (tAPL, exposure details shown in Supplementary Table 1). Information regarding library preparation, sequencing and analysis is provided in the Supplementary data. We identified

Supplementary information
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a mean of 17.1 and 15.3 somatic mutations per exome, respectively ($p = 0.59$). There were no significant differences in the numbers of non-synonymous single nucleotide variants (SNV, mean 8.8 vs. 8.2, $p = 0.69$) or synonymous SNV (7.0 vs. 5.8, $p = 0.49$) or InDel mutations (1.3 vs. 1.4, $p = 0.72$).

To identify recurrent APL-associated mutations, we combined this data set with three other whole-exome sequencing studies of APL [12–14] for a total of 164 sequenced cases, and identified 70 recurrently mutated genes (i.e. mutated in two or more cases of APL). We identified a mean of 2.0 and 2.2 recurrent mutations per patient for dnAPL and tAPL, respectively (median 2 range 0–6 and median 2 range 0–5, $p = 0.74$, Supplementary Fig. 1). There was no clear difference in the pattern of mutated genes. There were three genes that were mutated in tAPL but not dnAPL (AP3D1, DNMT3A and GPR158, Fisher’s exact test, $p = 0.035$).

We next analysed samples taken in molecular complete remission (mCR, defined as the absence of PML-RARA or RARA-PML fusion transcripts at a sensitivity of at least 1:10⁴) from all 13 tAPL patients and a subset of 22 dnAPL patients who were matched in age and other clinical characteristics (Supplementary Table 2). Targeted, error-corrected next-generation sequencing (EC-NGS) was performed using the HaloPlexHS system (Agilent Technologies, Santa Clara, California) using probes designed to capture all somatic mutations detected at diagnosis (i.e. APL-associated mutations). The mean depth of sequencing was 27,111× with 97 and 84% of bases covered at >1000× and >10,000×, respectively. In the tAPL group, we identified persistence of a median of 2.5 APL-associated mutations in mCR (range 1–8) in four patients, with a median variant allele frequency of 6% (range 0.4–13.9%, Fig. 1a). Hereafter we refer to these as persistent APL-associated mutations. One potential driver mutation was present in each case, these were DNMT3A (two patients) PPM1D and MYCN (Fig. 2a, b). We did not find the persistence of APL-associated mutations in any patient with dnAPL (Fisher’s exact test, $p = 0.01$). We did not observe an association between persistence of APL-associated mutations and patient age, type of primary cancer, type of treatment received or latency period, however this small cohort provided limited power to detect such associations.

We then performed EC-NGS using a separate HaloPlexHS library designed to capture the entire coding region of all genes identified as bearing persistent mutations in mCR, together with those of other genes previously associated with CH (a total of 87 genes, listed in Supplementary Table 3). Mean sequencing depth was 3378× with 98 and 85% of bases covered at >100× and >1000×, respectively. This method identified APL-unrelated mutations (i.e. mutations not present in the major clone at diagnosis) in both the tAPL and dnAPL groups (3/21 dnAPL and 2/13 tAPL, $p = 1.0$, Fig. 1). These mutations either emerged or

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**Fig. 1** Presence of somatic mutations during molecular complete remission. Mutations identified by error-corrected next-generation sequencing performed on bone marrow samples taken during molecular complete remission after treatment for de novo ($n = 22$) and therapy-related APL are shown. All remission samples tested negative for PML-RARA fusion transcripts at a sensitivity of at least 1:10⁴. Variant allele frequencies (%) at diagnosis and remission are shown. Mutations present at a frequency of <1% at APL diagnosis (i.e. APL-unrelated mutations) are highlighted in blue. Mutations which were present with a frequency of >30% at APL diagnosis (i.e. APL-associated mutations) are highlighted in red.
increased in frequency after treatment and were mainly in genes previously linked with CH, consistent with previously described therapy emergent or rising clones [15].

In order to determine whether persistent APL-associated mutations were present prior to cancer therapy, tumour biopsies were obtained from time of primary cancer diagnosis for three patients (UPN T2, T10 and T11) and analysed by EC-NGS. Using a VAF cut-off at 0.1% we identified APL-associated mutations in four samples from two patients in whom these remained detectable. Predicted driver (PD) mutations are highlighted in blue. B Browser views showing the presence of PD mutations in mCR samples. C Change in VAF of APL-associated mutations (red line) and PML-RARA fusion transcript levels (blue line) over time, with date of tAPL diagnosis plotted as time 0. D VAF of APL-associated PD mutations in sorted cell populations obtained from samples taken in molecular complete remission (the relevant sample is marked with an asterisk in C). CD34+= CD34+ stem and progenitor cells, B = CD19+ B lymphocytes, T = CD3+ T-lymphocytes, M = CD33+ myeloid cells.

To identify the cell lineage(s) harbouring persistent APL-associated mutations, we obtained cryopreserved mononuclear cell preparations from bone marrow samples taken during mCR from the same three patients. Flow sorted T and B lymphocytes, monocytes, granulocytes and CD34+ progenitors were analysed using amplicon-NGS (further details are provided in the Supplementary data). In each case, the predicted driver mutation was present in the haematopoietic stem and progenitor cell containing CD34+ fraction at a median VAF of 3.2% (range 0.5–5.2%) and was present in both the myeloid and lymphoid lineages, consistent with origin in a multipotent haematopoietic stem or progenitor cell (Fig. 2d).

Finally, to establish whether persistent APL-associated mutations were present in functional haematopoietic stem cells, we injected cryopreserved mononuclear cells taken at diagnosis of tAPL from patients T2 and T8 into irradiated
NSG mice. A total of $1 \times 10^6$ cells were injected into three mice for each patient. We detected human engraftment in 1/3 recipient mice at 18 and 22 weeks from injection for patient T2 and T8, respectively. After sacrifice, flow cytometry analysis demonstrated human myeloid and B-lymphoid engraftment in both mice (Supplementary Fig. 2). Sorted human CD45+ cells tested negative for PML-RARA and RARA-PML fusion transcripts by RT-qPCR and were also negative for the fusion by fluorescence in-situ hybridisation. DNA extracted from this population was analysed by amplicon-NGS and identified the t(15;17)-associated predicted driver mutation in each case (T2 PPMID exon 6, 1 bp del, VAF 2.9%; T8 MYCN exon 3, 6 bp ins, VAF 1.4%). Interestingly, the observed allele frequencies indicated that the clone harbouring the somatic mutation contributed a minor component to the human engraftment in each case, suggesting that the mutation did not provide a significant selective advantage in this situation.

Taken together these results indicate that in therapy-related but not de novo APL, the PML-RARA fusion may be a secondary event, occurring on a background of pre-leukaemic CH. It is intriguing that this phenomenon was only observed in tAPL. We cannot exclude that this mechanism may rarely underlie de novo APL and speculate that this observation may be accounted for by differences in prevalence, clone size and mutational spectrum of CH between patients exposed to chemotherapy and individuals with no history of cancer.

These findings contribute to the current understanding of the relationship between CH and leukaemia and challenge the previous assumption that fusion genes are always primary transforming events. It will be of interest to establish the role of CH in other types of tMN with balanced translocations, particularly for patients with KMT2A rearrangements, where co-operating mutations are not always necessary. Finally, the high prevalence of CH after treatment for tAPL (observed in 4/13 or 31% of patients studied, with the presence of multiple independent clones in 2/13) also has important implications for treatment of tAPL, a condition where chemotherapy-free treatment in the form of arsenic trioxide and ATRA is already available.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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