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Defining The Translocation Mechanism In Therapy-Related Acute Promyelocytic Leukaemia

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Acute myeloid leukaemia (AML) occurring in children and younger adults is commonly associated with reciprocal balanced chromosomal translocations, which lead to the formation of chimaeric proteins that play a key role in mediating the leukaemic phenotype (reviewed 1). Whilst a substantial body of information has been collected with respect to the functional activity of leukaemia-associated oncoproteins such as PML-RARα generated by the t(15;17)(q22;q21) translocation in acute promyelocytic leukaemia (APL)[1,2] relatively little is known about the mechanisms that give rise to chromosomal translocations which represent a critical step in the development of AML. However, insights can potentially be gained through the investigation of therapy-related leukaemias, which invariably have de novo counterparts. For many years it has been appreciated that exposure to drugs that target the enzyme DNA topoisomerase II predisposes to the development of secondary leukaemias with balanced translocations, particularly involving *MLL* at 11q23, *NUP98* at 11p15, *AML1* at 21q22 and *RARA* at 17q21.[3-8] Intriguingly, the nature of the prior drug exposure has a bearing on the molecular phenotype of the secondary leukaemia, with translocations involving 11q23 being particularly associated with exposure to epipodophyllotoxins such as etoposide,[9] whilst recent studies have drawn attention to an association between exposure to epirubicin or mitoxantrone used in breast cancer regimens and the development of therapy-related APL (t-APL) with the t(15;17).[10-13]

The association between exposure to drugs targeting DNA topoisomerase II and the development of chromosomal translocations has naturally implicated this enzyme in the DNA damage process, but how this occurs has been a matter of conjecture and debate. One hypothesis proposed by Betti *et al.*, is that drugs targeting DNA topoisomerase II lead to the formation of chromosomal translocations through an indirect mechanism involving induction of apoptotic nucleases which cause double strand breaks in DNA.[14,15] These are aberrantly repaired to generate leukaemic fusion genes, which in turn confer a relative survival advantage to progenitors following the original chemotherapy insult. According to this hypothesis, Betti *et al.* propose that topoisomerase II binding effectively stabilises DNA, rendering regions (e.g. within the *MLL* locus) lying between these binding sites and neighbouring scaffold attachment regions susceptible to cleavage by apoptotic nucleases.[15] However, such models derived from *in vitro* analysis of transformed cell lines, do not take into account the normal function of DNA topoisomerase II, which serves to relax supercoiled DNA by transiently cleaving and religating both strands of the double helix via the formation of a covalent cleavage intermediate.[16] Chemotherapeutic agents that target topoisomerase II such as mitoxantrone, epirubicin and adriamycin, cause DNA damage by disrupting the cleavage/re-ligation equilibrium, leading to an increase in the concentration of DNA topoisomerase II covalent complexes.16 This process has been examined in the context of leukaemias involving the *MLL* locus using an in vitro assay, which entails trapping of the cleavage complexes and mapping of the cleavage sites at the sequence level.[17-19] We have applied this approach to investigate mechanisms involved in generating the t(15;17) in t-APL. Interestingly, long-range PCR assays and sequence analysis revealed that while genomic breakpoints associated with *de novo* APL or

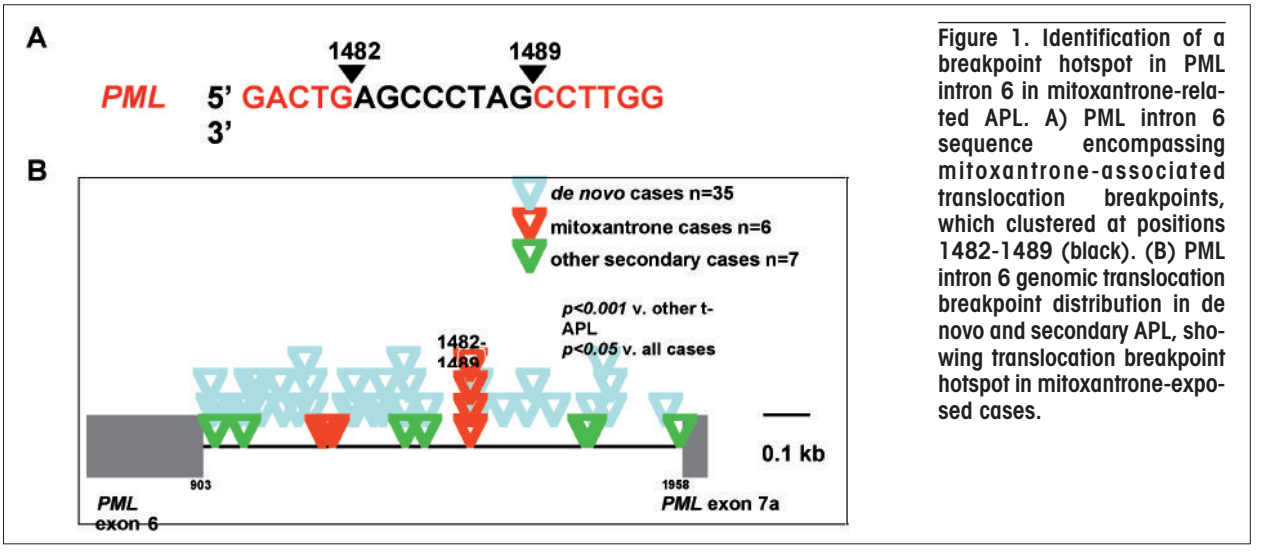

Figure 1. Identification of a breakpoint hotspot in PML intron 6 in mitoxantrone-related APL. A) PML intron 6 sequence encompassing mitoxantrone-associated translocation breakpoints, which clustered at positions 1482-1489 (black). (B) PML intron 6 genomic translocation breakpoint distribution in de novo and secondary APL, showing translocation breakpoint hotspot in mitoxantrone-exposed cases.

arising secondary to radiotherapy are generally dispersed, breakpoints for cases arising following mitoxantrone exposure were found to be remarkably restricted.[20] Indeed all cases occurred within *PML* intron 6, with 4 of 6 cases tightly clustered within a breakpoint hotspot region of only 8bp (Figure 1). Given that *PML* intron 6 is over 1kb in length, this clustering of breakpoints within the hotspot was highly unlikely to have occurred by chance ($p<0.001$ vs breakpoint location in 7 t-APL following radiotherapy, $p<0.05$ vs breakpoints in 35 cases of *de novo* APL). Functional assays undertaken using the normal homologue of *PML*, revealed that the breakpoint hotspot region of *PML* intron 6 corresponded precisely to a preferential site of mitoxantrone-induced topoisomerase II-dependent DNA cleavage site (Figure 2). Although the respective der(17) translocation breakpoints in RARA intron 2 were not clustered, each was also found to correspond to a mitoxantrone-induced topoisomerase II-dependent site of DNA cleavage. In all cases, cleavage at the translocation breakpoints persisted following heating of the reaction, indicating stability of the cleavage complexes (Figure 2). Sequence analysis of the der(15) and der(17) genomic junctions revealed short sequence homologies, consistent with double-strand DNA break repair mediated by the non-homologous end-joining (NHEJ) pathway. Taking into account the translocation breakpoints defined in functional assays, experimental data indicating that topoisomerase II cleaves DNA by introducing 4bp staggered breaks and known mechanisms of repair of double-strand DNA breaks, it is possible to generate models for the formation of the t(15;17) translocation following chemotherapy leading to the development of t-APL (Figure 3).

We are interested to establish whether similar mechanisms underlie formation of the t(15;17) in t-APL secondary to other chemotherapeutic agents and whether these are also related to distinct breakpoint *hotspots*. To date genomic breakpoints have been defined by long-range PCR in 4 cases, arising following exposure to epirubicin (n=2), adriamycin alone (n=1) or adriamycin+ etoposide. PML breakpoint locations were distinct in the epirubicin-exposed cases; one occurred in PML intron 3 (bcr3) and the other within intron 6 (bcr1), in a region outside the mitoxantrone hotspot. The adriamycin-exposed cases were observed to have distinct PML breakpoints within intron 3. Amongst the whole of group of t-APL patients characterised, no clustering of der(17) breakpoints was observed. Functional assays undertaken in the case exposed to both adriamycin and etoposide for laryngeal carcinoma, revealed induction of DNA cleavage at the t(15;17) translocation breakpoints with both agents in a topoisomerase II-dependent manner, precluding determination of which of the two was causative of the t-APL in this particular patient.

Taken together, these data clearly implicate topoisomerase II in mediating DNA cleavage at the translocation breakpoints in t-APL, as opposed to a more indirect process involving induction of apoptotic nucleases.[14,15]

Similarly, functional topoisomerase II cleavage sites identified at translocation breakpoints in MLL-associated t-AML indicate that the former mechanism coupled with aberrant repair by the NHEJ pathway is likely to be relevant to the for-

Figure 2. Demonstration of mitoxantrone-induced topoisomerase II dependent DNA cleavage at translocation breakpoints in therapy-related APL. A) *In vitro* DNA topoisomerase II cleavage assay carried out for a *PML* substrate containing the breakpoints of 4 treatment-related APL (t-APL) cases (F-8,-24,-25,-27) within a 8bp breakpoint hotspot (positions 1482-1489). Patients received combination chemotherapy including the topoisomerase II inhibitor mitoxantrone for breast carcinoma. Control reactions were carried out in the absence of DNA topoisomerase II (Lanes 1-4), and in the presence of etoposide (VP16), etoposide catechol (VP16-OH), etoposide quinone (VP16-Q) and mitoxantrone (Mit). Dideoxy sequencing reactions of the substrate are shown in lanes 5-8. Cleavage reactions were carried out by exposure to human DNA topoisomerase IIα in the absence of drug (Lane 9), and in the presence of etoposide (Lane 10), etoposide catechol (Lane 11), etoposide quinone (Lane 12) and mitoxantrone (Lane 13). Additional cleavage reactions were carried out to evaluate the heat-stability of cleavage complexes formed by incubation at 75°C for 10 min (Lanes 14-18). The nucleotide shown by the dash is the 5' side of the cleavage site (-1 position), which corresponds to the der(15) and der(17) translocation breakpoints in 4 cases of mitoxantrone-related APL (far right). The cleavage site at position 1484 was observed in the absence of drug, and in the presence of etoposide, both etoposide metabolites and mitoxantrone (Lanes 9-13). Cleavage at this position was the strongest site observed in the presence of mitoxantrone (Lane 13). Furthermore, the complexes formed at this site were shown to be heat-stable in the presence of mitoxantrone (Lane 18). Interestingly, a cleavage site at position 1502 is also observed, which corresponds to a breakpoint detected in a case of *de novo* APL. B) DNA topoisomerase II cleavage assay of normal homologue of der(15) and der(17) RARA translocation breakpoints in APL of one of the mitoxantrone-related cases (F-8). The substrate spanning positions 2603 to 2871 of RARA intron 2 contained the translocation breakpoints. Dash at right shows (-1) position of cleavage site corresponding to der(15) and der(17) translocation breakpoints (arrow far right).

mation of translocations that disrupt other genes that are commonly involved in t-AML. Moreover, the identification of the preferential mitoxantrone-induced topoisomerase II dependent cleavage site in the PML locus and the recent documentation of an etoposide-induced breakpoint hotspot within MLL,[19] provide a plausible molecular explanation for the propensity to development of different subtypes of AML according to the nature of the particular chemotherapeutic agent used. Whilst these studies have provided considerable insights into mechanisms leading to the development of therapy-related leukaemias a number of key questions remain to be addressed. Importantly, it is uncertain as to whether individuals are predisposed to development of leukaemia following exposure to a particular agent, providing a potential opportunity for delivery of risk-adapted treatment of primary tumours. Furthermore, definition of the translocation mechanism in therapy-related APL carries much broader implications, raising the possibility that environmental and dietary exposure to agents targeting topoisomerase II and aberrant DNA repair could account for the formation of the balanced chromosomal rearrangements that represent a primary event in the development of AML arising *de novo*.

Acknowledgements

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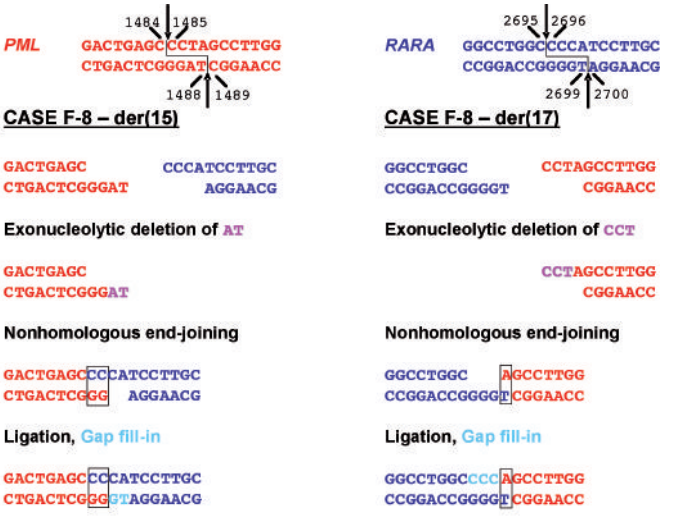

Figure 3. DNA topoisomerase II cleavage sites at *PML* position 1484 and *RARA* position 2695 with 4-base 5'overhangs, and processing to form der(15) and der(17) breakpoint junctions in a case of mitoxantrone-related APL (case F-8). Native *PML* and *RARA* sequences are red and blue, respectively. The processing includes exonucleolytic nibbling to form two-base (der(15)) or single-base (der(17)) homologies and creation of both breakpoint junctions by error-prone NHEJ. In formation of the der(15), positions 1487-1488 on the antisense strand of *PML* are lost by exonucleolytic nibbling (pink) before NHEJ joins the indicated bases. Positions 1485-1487 on the sense strand of *PML* are lost by exonucleolytic nibbling (pink) and the der(17) forms by NHEJ. Template-directed polymerization of the relevant single-stranded overhangs fills in any gaps (light blue). Each *RARA* overhang is preserved com

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