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XXII

Investigation on the mechanisms underlying the chromosomal translocations in therapy-related acute myeloid leukemias

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Chapter 1

Introduction

Introduction

Therapy-related leukemias are well-recognized clinical syndrome occurring as a late complication following cytotoxic therapy. The term "therapy-related" leukemia is descriptive and based on a patient's history of exposure to cytotoxic agents. Although a causal relationship is implied, the mechanism remains to be proven. These leukemias are thought to be the direct consequence of mutational events induced by cytotoxic therapy, or via the selection of a myeloid clone with a mutator phenotype that has a markedly elevated risk for a mutational event. These types of leukaemias are becoming an increasing healthcare problem as more patients survive their primary cancers. The nature of the causative agent has an important bearing upon the characteristics, biology, time to onset and prognosis of the resultant leukaemia. Agents targeting topoisomerase II induce acute leukaemias with balanced translocations that generally arise within 3 years, often involving the *MLL*, *RUNX1*, *PML* and *RARA* loci at 11q23, 21q22 15q22 and 17q21 respectively. Chromosomal breakpoints have been found to be preferential sites of topoisomerase II cleavage, which are believed to be repaired by the nonhomologous end-joining DNA repair pathway to generate chimaeric oncoproteins that underlie the resultant leukaemias. Therapy-related acute myeloid leukaemias occurring after exposure to antimetabolites and/or alkylating agents are biologically distinct with a longer latency period, being characterised by more complex karyotypes and loss of p53. The treatment of therapy-related leukaemias represents a considerable challenge due to prior therapy and comorbidities, however, curative therapy is possible, particularly in those with favourable karyotypic features. Although the transforming function of leukemia-associated fusion proteins has been widely studied, little is known about the mechanisms that cause the underlying translocations. In this respect, insights can be gained from investigations of secondary leukemias, all of which have counterparts in primary leukemias[1].

1.1 Acute promyelocytic leukemia (APL) and therapy-related APL t(15;17)(q22;q21)

In 1977, Rowley et al[2] from the University of Chicago reported on the consistent occurrence of a chromosomal translocation t(15;17)(q22;q21) in APL. This aberration was subsequently found to be uniquely associated to, and therefore pathognomonic of the disease. Upon cloning of the translocation in the late '80s, it was shown that chromosome breakpoints lie within the *RARα* locus on chromosome 17 and the *PML* locus on chromosome 15, resulting in the fusion of the two genes[3,4]. APL is a particular leukemia subset characterized by a unique genetic lesion, i.e the *PML-RARα* fusion, and an exquisite response to differentiating agents. Until the late 80's, APL was considered the most aggressive and rapidly fatal form of acute leukemia. Over the past two decades, important advances have been made into the understanding of APL pathogenesis as well as in its treatment, such that it has been nowadays converted into the most frequently curable leukemia in adults. APL is regarded as a model disease for innovative tailored treatment of human leukemia including differentiation therapy and the use of chromatin remodeling agents and antibody-directed therapy.

The occurrence of APL as a second tumor (sAPL) has been increasingly reported in recent years, most commonly developing in patients receiving chemotherapy and/or radiotherapy for breast cancer or, less frequently, for other primary tumors (including prostate, uterus, ovary)[5-8]. Chemotherapy associated with development of sAPL usually induces DNA damage through targeting of topoisomerase II (topoII), with mitoxantrone, etoposide and epirubicin being the most commonly implicated agents[5-10]. More recently, a number of reports have been published describing the occurrence of sAPL in patients with Multiple Sclerosis (MS) most of whom received mitoxantrone given as an immunosuppressive agent for their primary disease [11-16]. Because only case reports have

been published and no systematic analysis has been performed so far, the true incidence of sAPL in MS patients treated with mitoxantrone is unknown. In addition, it is unclear at present whether factors other than chemotherapy may play a role in sAPL development in patients with MS.

In a study reported by Mistry et al[10] breakpoints in sAPL cases arising following mitoxantrone exposure for prior breast carcinoma were found to be clustered in an 8bp region within *PML* intron 6. In functional assays, this "hotspot" was found to correspond to a preferential site of mitoxantrone-induced topoII-dependent cleavage at *PML* nucleotide position 1484. These findings suggest a direct causative role of mitoxantrone in stimulating topoII-mediated cleavage within the *PML* gene thus implying a direct link between this agent and the formation of the t(15;17) chromosome translocation that is the hallmark of APL. However, the precise mechanisms leading to this aberration and therefore to APL development needs to be further clarified. In fact, several reports have described the occurrence of sAPL in patients treated with surgery alone for their primary tumor[6]. Neither in these cases nor in patients developing sAPL after treatment of MS has the t(15;17) translocation been investigated at the molecular level. Finally, no studies have analysed in details the response to endogenous or exogenous DNA damage in newly diagnosed APL and in sAPL.

DNA double-strand breaks (DSBs) are critical lesions that can result in cell death or in a wide variety of genetic alterations including large or small-scale deletions, loss of heterozygosity, translocations, and chromosome loss. Mitoxantrone creates exogenous DNA double strands breaks (DSBs) and interferes in the cleavage-religation equilibrium of the topoII enzyme[17]. The maintenance of genomic integrity and the prevention of tumor progression depend on the co-ordination of DNA repair mechanisms and cell cycle checkpoint signaling in an overall DNA damage response. It is believed that double strand

breaks (DSBs) are initially detected by specific sensors (e.g. MRN complex) that trigger the activation of transducing kinases (e.g. ATM, ATR, DNAPK). These transducers in co-ordination with mediators (e.g. H2AX) amplify the damage signal, which is then relayed to effector proteins (e.g. p53, SMC1, Kap1, CHK2, CHK1), that directly regulate the progression of the cell cycle and DNA repair. DSBs are normally repaired by non-homologous end-joining (NHEJ) and homologous recombination (HR) pathways.

Disruption of the repair proteins may lead the NHEJ pathway to function inappropriately and rejoin DNA ends incorrectly resulting in translocations[18]. The NHEJ is an error prone pathway in which rejoinings occur at regions of microhomology that are 1–4 nucleotides in length. These are nucleotides that, by chance, are shared between the two ends of different chromosomes. We and others have demonstrated the presence of short homologies at t(15;17) translocation breakpoint junction suggesting that *PML* and *RARA* joining may have been mediated by the classical NHEJ pathway[10,19-20]

1.2 Therapy related acute myeloid leukemia with t(16;21) (q24;q22)

Hematopoietic malignancies are frequently characterized by recurrent chromosomal translocations involving genes that play an important role in the regulation of hematopoietic cell proliferation and differentiation[21]. The *AML1* (*RUNX1*) gene at cytogenetic band 21q22 is one of the most frequent targets of chromosomal translocations observed in both *de novo* acute leukemia and therapy-related myelodysplastic syndrome (t-MDS) and acute myeloid leukemia (t-AML). Translocations involving *AML1* have been reported in up to 15% of t-MDS/t-AML cases and the most common chromosome/gene rearrangements described in this clinical context are the t(8;21)(q22;q22), t(3;21)(q26;q22) and t(16;21)(q24;q22) translocations involving the *ETO-1 (MTG8)*, *EAP/MDS1/EVI1*, and *MTG16* (*ETO-2*) genes, respectively[22].

The t(16;21)(q24;q22) is a rare but non random chromosome abnormality associated mostly with t-AML[23-25]. It involves *MTG16* (myeloid translocation gene on chromosome 16) which encodes one of a family of novel transcriptional corepressors (MTG proteins) and shows a high degree of homology to the *MTG8* gene, the fusion partner in the t(8;21)[24] . The evolutionary conserved structural features between AML1-MTG8 and AML1-MTG16[24], suggests that the two chimeric proteins are both involved in hematopoiesis, as subsequently demonstrated in functional studies[26].

1.3 t-APL following multiple sclerosis and genetic variants of DNA double strand break repair genes

The occurrence of APL as a second tumor has been increasingly reported in patients with Multiple Sclerosis (MS). It is believed that the genetic contribution to leukemia susceptibility is a complex interplay of environmental exposures and many susceptibility alleles, each of which contributes only a small amount to overall risk. Multiple genetic variants, deriving from Single Nucleotide Polymorphisms (SNPs) within coding sequences, have been described for many genes involved in DNA repair processes. Such variants may be associated with functional differences of the encoded proteins, which may in turn be responsible for inefficient DNA repair mechanisms that become evident particularly after exposure to chemotherapeutic agents. Furthermore, specific SNP variants of apoptosis and DNA damage-regulatory genes have recently been described as risk factors for MS and may hence be associated with sAPL occurring in patients with this disease.

We, therefore, intend to investigate the possibility that specific genetic variants in DNA repair genes or genes that predispose to MS are significantly associated with t-APL.

1.4 Aims and objectives

We aim to investigate whether specific chromosomal regions are particularly susceptible to DNA damage (either spontaneously or induced by chemotherapeutic agents). In particular, we proposed:

a) To determine the mechanism of chromosomal translocations of t(15;17) and t(16,21) in therapy related acute leukemia through characterization of DNA breakpoint regions using patient samples.

b) To provide evidences of mitoxantrone and epirubicin induced DNA cleavage at translocation breakpoint loci using *in vitro* DNA cleavage assays.

c) To compare the translocation breakpoint distribution between *de novo* and t-APL.

d) To analyse genetic variability of genes involved in DNA double strand break repair in multiple sclerosis patients and t-APL developing after multiple sclerosis.

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Chapter 2

Mechanisms of the formation of t(15;17) in mitoxantrone related therapy related-APL following multiple sclerosis

blood

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Molecular analysis of t(15;17) genomic breakpoints in secondary acute promyelocytic leukemia arising after treatment of multiple sclerosis

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Molecular analysis of t(15;17) genomic breakpoints in secondary acute promyelocytic leukemia arising after treatment of multiple sclerosis

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Therapy-related acute promyelocytic leukemia (t-APL) with t(15;17) translocation is a well-recognized complication of cancer treatment with agents targeting topoisomerase II. However, cases are emerging after mitoxantrone therapy for multiple sclerosis (MS). Analysis of 12 cases of mitoxantrone-related t-APL in MS patients revealed an altered distribution of chromosome 15 breakpoints versus de novo APL, biased toward disruption within *PML* intron 6 (11 of 12, 92% vs 622 of 1022, 61%: *P* = .035). Despite this intron spanning approximately 1 kb, breakpoints in 5 mitoxantrone-treated patients fell within an 8-bp region (1482-9) corresponding to the "hotspot" previously reported in t-APL, complicating mitoxantrone-containing breast cancer therapy. Another shared breakpoint was identified within the approximately 17-kb *RARA* intron 2 involving 2 t-APL cases arising after mitoxantrone treatment for MS and breast cancer, respectively. Analysis of *PML* and *RARA* genomic breakpoints in functional assays in 4 cases, including the shared *RARA* intron 2 breakpoint at 14 446-49, confirmed each to be preferential sites of topoisomerase IIα-mediated DNA cleavage in the presence of mitoxantrone. This study further supports the presence of preferential sites of DNA damage induced by mitoxantrone in *PML* and *RARA* genes that may underlie the propensity to develop this subtype of leukemia after exposure to this agent. (Blood. 2008;112: 3383-3390)

Introduction

The occurrence of acute promyelocytic leukemia (APL) as a second tumor (sAPL) frequently has been reported as a late complication of chemotherapy and/or radiotherapy (therapy-related APL [t-APL]), although sAPL cases arising in patients whose primary tumors were treated by surgery alone have also been described.[1-3] The agents most often associated with development of t-APL induce DNA damage through targeting of topoisomerase II, with mitoxantrone, epirubicin, adriamycin, and etoposide being most commonly implicated.[3,4] The latency period between chemotherapy exposure and the onset of t-APL is relatively short (< 3 years) and typically occurs without a preceding myelodysplastic phase.[3,4]

In a study by Mistry et al[5] concerning molecular mechanisms underlying formation of the t(15;17) in t-APL, breakpoints in cases arising after mitoxantrone exposure for prior breast carcinoma were found to be clustered in an 8-bp region within *PML* intron 6; this corresponded in functional assays to a preferential site of mitoxantrone-induced topoisomerase II–dependent cleavage at position 1484. Although these findings highlighted the leukemogenic role of drug-induced DNA cleavage at specific sites in the genome, the precise mechanism by which secondary leukemias with balanced chromosomal translocations such as the t(15;17) in APL develop remains controversial.[6-9] This is compounded by the fact that many patients have been exposed to multiple cytotoxic drugs often accompanied by radiotherapy, making it difficult to categorically ascribe the etiology of therapy-related acute myeloid leukemia (t-AML) in any given case.

Previous studies on t-AML have focused on patient populations that feasibly could have been enriched for persons at particular risk of leukemia, having already developed one form of cancer. Therefore, to investigate whether particular chemotherapeutic agents have a propensity to induce specific molecular subtypes of t-AML, it is of interest to study patients exposed to topoisomerase II targeting drugs used in the treatment of nonmalignant conditions, such as mitoxantrone in the management of multiple sclerosis (MS). MS is a putative autoimmune disease affecting the central

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nervous system for which mitoxantrone represents the latest in a long list of general immunosuppressive agents used in the treatment of this condition.[10,11] In recent years, an increasing number of APL cases have been reported in MS patients treated with mitoxantrone.[3,5,12-20] However, to date, no attempts have been made to systematically characterize translocation breakpoints in APL cases that developed in this setting.

In the present study, we analyzed at the genomic level the *PML* and *RARA* breakpoints of 14 patients who developed APL on a background of MS, including 12 who received mitoxantrone for their primary disease. Furthermore, we used functional cleavage assays to better elucidate the mechanisms underlying the formation of the t(15;17) in this setting.

Methods

Patients and samples

The main patient characteristics, including demographic data, MS type, and treatments received for MS, are reported in Table 1. Seven patients were diagnosed in 5 Italian institutions, 3 in 2 Spanish institutions, 3 in the United Kingdom, and the remaining patient in Austria. Analyses were undertaken after informed patient consent was obtained in accordance with the Declaration of Helsinki with ethical approval of University Tor Vergata of Rome and St Thomas' Hospital of London. Bone marrow samples were obtained at the time of diagnosis of APL. Mononuclear cells were collected after centrifugation on a Ficoll-Hypaque gradient and stored at −70°C as dry pellets. In all cases, APL diagnosis was confirmed at the genetic level by reverse-transcriptase polymerase chain reaction (RT-PCR) amplification of the *PML-RARA* hybrid gene.

Amplification of DNA spanning possible break points (*PML-RARA*): long-range PCR and DNA sequencing

To determine the exact chromosomal breakpoint position in *PML* and *RARA* genes, genomic DNA extracted from APL blasts collected at diagnosis was amplified by a 2-step, long-range nested PCR method as reported elsewhere.[5,21] Two forward and 8 reverse primers were designed for each step to cover the *PML* breakpoint region (bcr1 or bcr3, as previously known based on diagnostic RT-PCR results available for all cases) and the 16.9-kb-long *RARA* intron 2. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Samples were loaded in 96-well plates and covered with mineral oil. The amplified products were separated with a capillary electrophoresis-based system (CEQ 8000 Genetic Analysis System; Beckman Coulter, Fullerton, CA) using the "LFR1 Test" default run method and sequenced using appropriate primers.[5,21] Rigorous procedures were used to reduce risk of PCR contamination,[22] and genomic breakpoints were in all cases confirmed by PCR analysis of a fresh aliquot of DNA. Moreover, in 3 cases, breakpoint analyses were performed independently in parallel in the Rome and London laboratories, yielding identical results.

Amplification and sequence analysis of the reciprocal *RARA-PML* genomic breakpoint junction

Genomic *RARA-PML* was amplified using patient specific primers (designed on the basis of *PML* and *RARA* breakpoints) and fresh aliquots of DNA. In 13 cases, the reciprocal *RARA-PML* genomic breakpoint junction was sequenced, providing further confirmation of the t(15;17) translocation breakpoints at the genomic level. In one case (unique patient number [UPN] 10), no DNA was available to carry out sequencing of the reciprocal *RARA-PML*.

Alignment of sequenced nucleotides using BLAST algorithm

The patients' genomic *PML-RARA* junction sequences were aligned against normal *PML* (GenBank accession number S57791 for bcr1 and S51489 for bcr3) and *RARA* intron 2 (GenBank accession number AJ297538) nucleotides as a reference text input in BLAST/alignment program. The purpose of alignment was to identify any microhomologies between *PML* and *RARA* in the vicinity of the breakpoint.[23]

In vitro DNA cleavage assays

Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae*[24] and purified as described previously.[25,26] Assays were performed as described previously.[5] Briefly, having identified genomic junction sequences, regions of the normal homologs encompassing the breakpoint sites were amplified by PCR and subcloned into the pBluescript SKII(+) vector. The optimal insert size for the assay was 200 to 500 bp, with the breakpoint site located approximately 50 to 100 bp from the 5′ end of the insert. Substrates containing 25 ng of the normal homologs of the translocation breakpoints were 5′end-labeled (30 000 cpm) and incubated with 147 nM of human DNA topoisomerase IIα, 1 mM of ATP in the presence or absence of 20 μM mitoxantrone.[5] In all cases, additional reactions were carried out to evaluate the heat stability of the covalent complexes formed. Cleavage complexes were irreversibly trapped by the addition of sodium dodecyl sulfate, and purified products were resolved in an 8% polyacrylamide-7.0 M of urea gel in parallel with dideoxy sequencing reactions primed at the same 5′-end, visualized by autoradiography, and quantified using PhosphoImager and IMAGEQUANT software (GE Healthcare, Little Chalfont, United Kingdom).

Results

Clinical features

As shown in Table 1, a total of 14 patients with APL developing in a background of MS were studied. The series included 12 cases exposed to a median total dose of 105 mg mitoxantrone (range, 30-234 mg), whereas 2 patients received other treatments for their primary disease (interferon-β in UPN 10 and corticosteroids in UPN 12). The median latency period between the first exposure to mitoxantrone and APL diagnosis was 28 months (range, 4-60 months). Patients were treated with all-*trans* retinoic acid and anthracycline-based chemotherapy, mostly using AIDA-like (all-*trans* retinoic acid + idarubicin) protocols[27] (Table 2); however, UPN 13 died of cerebral hemorrhage within 3 hours of APL diagnosis before antileukemic therapy was started. The remaining 13 patients achieved hematologic and molecular remission. Of these, 11 remain in first molecular remission at a median follow-up of 10 months, whereas UPN 7 relapsed at 28 months and achieved second molecular remission after salvage therapy with arsenic trioxide, and UPN 1 died of cerebral hemorrhage while in remission after 7 months.

Location of t(15;17) translocation breakpoints within the *PML* and *RARA loci*

RT-PCR showed the bcr1 *PML-RARA* isoform (*PML* intron 6 breakpoint) in 12 cases, whereas in the remaining 2 cases the *PML* breakpoint fell within intron 3 (bcr3; Figure 1). This breakpoint distribution appeared skewed in favor of the bcr1 isoform, which previously has been reported to account for approximately 55% of unselected APL cases.[28-30] Comparison of the breakpoint distribution in MS patients with mitoxantrone-related APL relative to a cohort of 1022 consecutive cases of newly diagnosed de novo APL from GIMEMA, PETHEMA, and United Kingdom MRC trials confirmed significant overrepresentation of involvement of *PML* intron 6 in the former group (11 of 12, 92% vs 622 of 1022, 61%: $P = .035$ by Fisher exact test). *PML* genomic breakpoints within intron 6 were found to fall between nucleotide positions 1482 and 1489 in 6 patients (UPNs 3, 6, 11, 12, 13, and 14; Figure 1A),

Table 1. Main clinical and molecular features of 14 sAPL patients

Patient no.	Age (y) at time of MS diagnosis	Sex	Primary disease	Therapy of MS			Latency between MS and APL, mo	Latency between mitoxantrone and APL, mo	Bcr subtype	*PML** breakpoint der(15)-der(17)	*RARA** breakpoint der(15)-der(17)
				Type of treatment	Mitoxantrone schedule	Total dose, mg					
UPN 1	59	F	SPMS	Mitoxantrone	10 mg/m^2 every 1 mo	30	13	10	1	1496-97	6104-05
										1496-97	6104-05
UPN 2	43	F	SPMS	Mitoxantrone	10 mg/m^2 every 2 mo	35	192	37	1	1657-60	14446-49
										1657-60	14446-49
UPN 3	56	F	PPMS	Mitoxantrone	10 mg/m^2 every 3 mo	70	30	4	1	1485-87	15266-68
										1485-87	15266-68
UPN 4	24	F	RRMS	Mitoxantrone	12 mg/m^2 every 2 mo	147	150	6	1	1922-23	12418-19
										1922-23	12418-19
UPN 5	21	F	RRMS	Mitoxantrone	12 mg/m^2 every 2 mo	170	66	18	1	1150-51	14082-83
										1169-70	14054-55
UPN 6	53	F	RRMS	Mitoxantrone	10 mg/m^2 (every 1 mo, 3 doses), 10 mg/m^2 (every 3 mo, 7 doses)	234	72	51	1	1483-84	12909-10
										1484-85	12908-09
UPN 7	25	M	RRMS	Mitoxantrone	12 mg/m^2 (every 1 mo, 3 doses), 6 mg/m^2 (every 3 mo, 5 doses)	110	37	27	1	1165	7974
										1161	7978
UPN 8	44	M	SPMS	Mitoxantrone	1.9 mg/m^2 every 1 mo	100	204	29	1	1409-10	718-19
										1429-30	675-76
UPN 9	33	M	PPMS	Mitoxantrone	10 mg/m^2 every 2 mo	176	144	30	3	1286-87	15386-87
										1286-87	15386-87
UPN 10	26	M	RRMS	INF beta	NA	NA	18	NA	3	1117-22†	14920-25
UPN 11	37	M	RRMS	Mitoxantrone	13 mg monthly (5 doses)	81	97	17	1	1483-86	11586-89
										1486-1506	11683-84
UPN 12	49	M	RRMS	Prednisone	NA	NA	216	NA	1	1489-91	14785-87
										1485	14117
UPN 13	45	F	PPMS	Mitoxantrone	8 mg/m^2 every 1 mo	64	120	60	1	1485-87	11569-71
										1486-88	11569-71
UPN 14	25	F	SPMS	Mitoxantrone	11.36 mg/m^2 every 1 mo	120	237	34	1	1488-89	12038-39
										1483-86	12035-38

UPN indicates unique patient number (some details regarding UPNs 1, 4 and 5, and 7 were reported in references 20, 17, and 5, respectively); RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis; and NA, not applicable.

*Breakpoint locations are numbered according to the following GenBank accession numbers: *PML* intron 6 (bcr 1), S57791; *PML* intron 3 (bcr 3), S51489; and *RARA* intron 2, AJ297538.

†No DNA was available to sequence the reciprocal *RARA-PML* in this case.

Table 2. Main clinical features and treatment outcome of sAPL patients

Patient no.	Age (y) at time of APL diagnosis	Sex	APL characteristics			Therapy of APL	Outcome
			WBC, $\times 10^9$/L	PLT, $\times 10^9$/L	DIC, Yes/No		
UPN 1	60	F	5.5	25	Yes	PETHEMA APL 2005	Died at 7 months due to cerebral hemorrhage
UPN 2	59	F	8.9	74	Yes	GIMEMA AIDA protocol	CCR 21 mo
UPN 3	58	F	0.5	37	No	PETHEMA APL 2005	CCR 16 mo
UPN 4	36	F	0.8	74	No	GIMEMA AIDA protocol	CCR 16 mo
UPN 5	26	F	1.1	30	Yes	GIMEMA AIDA protocol	CCR 24 mo
UPN 6	59	F	1.1	58	Yes	GIMEMA AIDA protocol	CCR 19 mo
UPN 7	28	M	0.7	5	No	UK MRC protocol	Relapse at 28 mo
UPN 8	61	M	3.3	31	No	PETHEMA APL 2005	CCR 4 mo
UPN 9	45	M	13	12	No	GIMEMA AIDA protocol	CCR 4 mo
UPN 10	27	M	33.7	11	Yes	GIMEMA AIDA protocol	CCR 10 mo
UPN 11	45	M	0.6	66	No	GIMEMA AIDA protocol	CCR 10 mo
UPN 12	67	M	1.1	9	No	PETHEMA APL 2005	CCR 9 mo
UPN 13	55	F	8.9	13	Yes	NA	Died 3 h after APL diagnosis
UPN 14	45	F	72.2	40	Yes	PETHEMA APL 2005	CCR 1 mo

DIC indicates disseminated intravascular coagulation; CCR, continuous complete remission; and NA, not applicable.

coinciding precisely with the "hotspot" previously identified in t-APL after mitoxantrone treatment for breast cancer.[5] Interestingly, one of these patients (UPN 12) had not received mitoxantrone therapy for MS. In the 2 patients (UPNs 9 and 10) with the bcr3 *PML-RARA* isoform, the breakpoints in *PML* intron 3 were detected between nucleotides 1286 and 1287 and 1117 through 1122, respectively (Figure 1A). The breakpoints within the *RARA* locus were distributed across intron 2 without particular clustering in any restricted small region (Figure 1B). However, one breakpoint (in UPN 2) mapped precisely to a breakpoint found in a case of t-APL arising after mitoxantrone therapy for breast cancer, studied previously by Mistry et al.[5]

Sequence analyses of the reciprocal *RARA-PML* fusion revealed a balanced translocation in 7 of 13 analyzed cases. Six patients showed size variable deletions and/or insertions at the breakpoint junction (Table 1). Microhomologies at the breakpoint junctions were indicative of DNA repair by the nonhomologous end-joining (NHEJ) pathway.[5]

t(15,17) translocation breakpoints are preferential sites for mitoxantrone-induced DNA cleavage by human topoisomerase IIα

To investigate the mechanisms by which the t(15;17) chromosomal translocation may have been formed in MS patients treated with

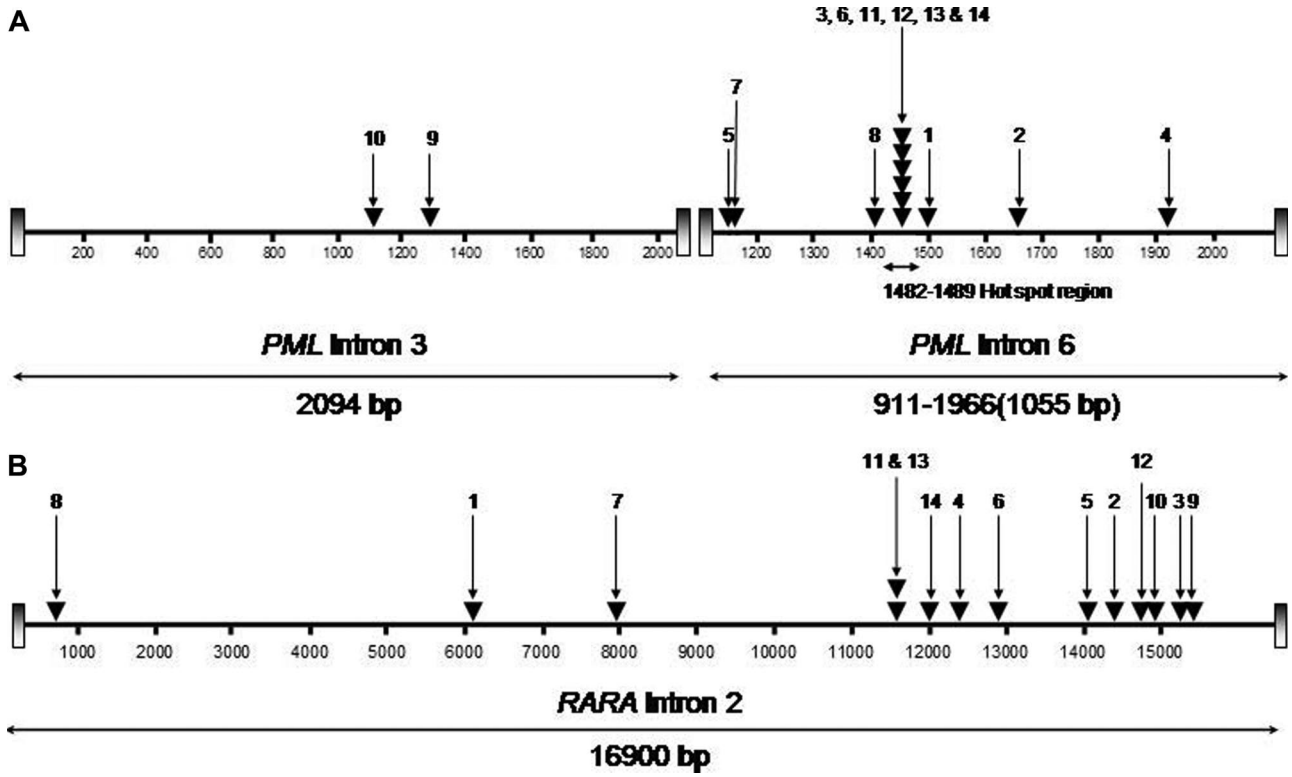

Figure 1. Characterization of t(15;17) breakpoints within the *PML* and *RARA* loci. The location of breakpoints indicated by ▼ in the 14 patients (numbers correspond with UPNs in Tables 1 and 2) within the *PML* gene on chromosome 15 (A; bcr3 region and bcr1/2 region) and intron 2 of *RARA* on chromosome 17 (B) are shown. Breakpoint locations are numbered according to the following GenBank accession numbers: *PML* intron 6 (bcr 1), S57791; *PML* intron 3 (bcr 3), S51489; and *RARA* intron 2, AJ297538.[23]

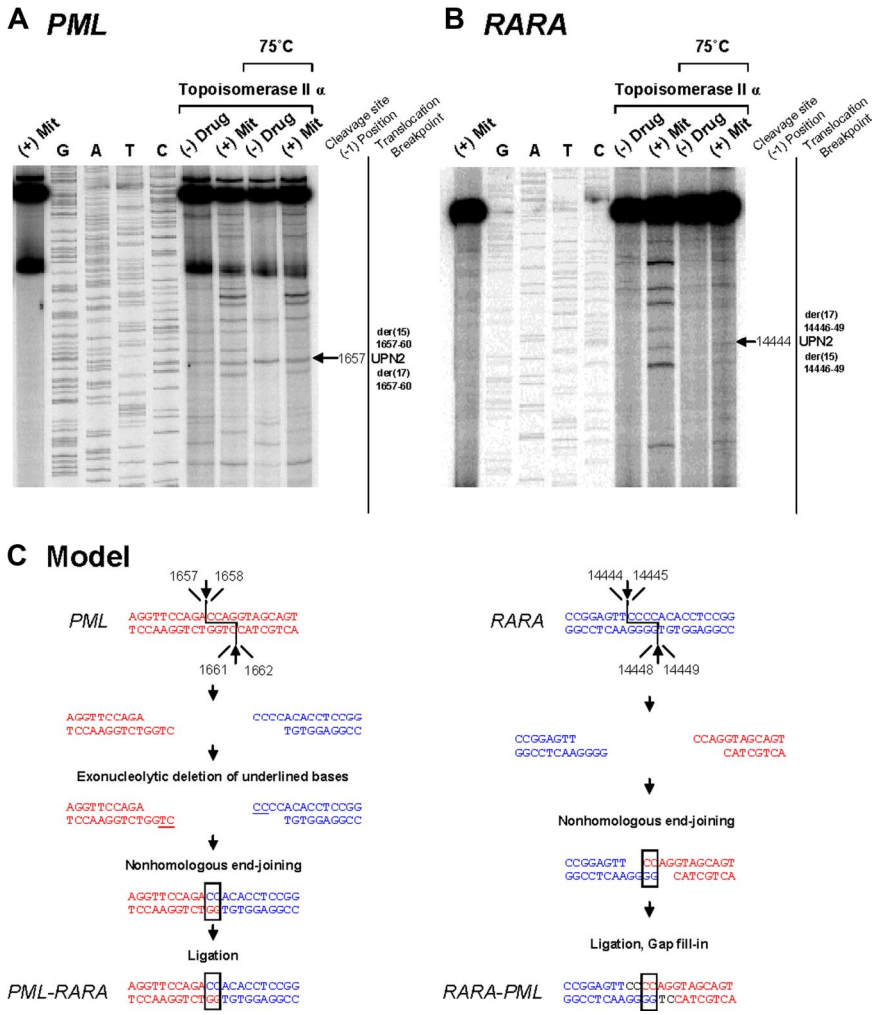

Figure 2. Investigation of t(15;17) translocation mechanism in UPN 2 by in vitro topoisomerase IIα DNA cleavage assay. Chromosomal breakpoint junctions were examined in an in vitro topoisomerase IIα cleavage assay using substrates containing *PML* (A) and *RARA* (B) translocation breakpoints in the APL case of UPN 2. Reactions in lane 1 were performed without DNA topoisomerase IIα and lanes 2 to 5 show dideoxy sequencing reactions. DNA cleavage reactions were performed in the presence of 147 nM of human DNA topoisomerase II alpha and in the absence (lanes 6 and 8) or presence of 20 μM mitoxantrone (lanes 7 and 9). Reactions in lanes 8 and 9 were incubated at 75°C to assess the heat stability of the cleavage products seen in lanes 6 and 7. In each case, the location of the relevant heat stable cleavage site is indicated by an arrow on the far right. (C) Native *PML* and *RARA* sequences are shown in red and blue, respectively. In the creation of the *PML-RARA* genomic fusion, processing includes exonucleolytic deletion to form a 2-base homologous overhang that facilitates repair via the error prone NHEJ pathway. In the creation of the reciprocal *RARA-PML* genomic fusion, 2-base homologies facilitate NHEJ repair, whereas in both instances polymerization of the relevant overhangs fills in any remaining gaps (shown black font).

mitoxantrone, we evaluated topoisomerase IIα–mediated cleavage of the normal homologs of *PML* and *RARA* encompassing the respective breakpoints detected in 4 cases (UPNs 2, 7, 8, and 14) in the presence or absence of this agent. These included cases (ie, UPN 2 and UPN 14) in which the genomic breakpoint in the *RARA* or *PML* locus coincided with those reported previously in cases of t-APL arising in breast cancer patients treated with multiple DNA-damaging agents, including mitoxantrone.[5] Few cleavage sites were observed in the absence of drug; however, bands of various sizes and intensities were observed in the presence of mitoxantrone in a topoisomerase IIα-dependent manner (Figures 2, 3 top panels). Cleavage bands that were significantly enhanced by mitoxantrone corresponding to the location of the observed genomic breakpoints in the *PML* and *RARA* loci were detected in each of the cases analyzed (Figures 2A,B, 3A,B; and data not shown). These bands remained detectable after heating, indicating stability of the cleavage complexes. In UPN 2, the case in which the *RARA* breakpoint was shared with a t-APL case that arose after mitoxantrone-containing breast cancer therapy,[5] a functional site of mitoxantrone-induced cleavage by topoisomerase II was identified at position 14 444 (Figure 2B).

To provide further evidence that the region between positions 1482 and 1489 within *PML* intron 6 (which was involved in almost half the cases) is also a preferential site of mitoxantrone-induced DNA cleavage mediated by topoisomerase IIα, the reverse complement of the described *PML* substrate[5] was used in the cleavage assay. A strong heat-stable cleavage band was detected in the presence of mitoxantrone at position 1488, which corresponds to the described functional cleavage at position 1484 on the upper strand[5] (Figure 3A,C). Given that the chromosome 15 breakpoint in UPN 12 (in which there was no history of mitoxantrone exposure) also fell within this "hotspot," it is interesting to note that a weak cleavage band was apparent in the presence of topoisomerase IIα in the absence of drug (Figure 3A lane 6). This finding suggests that the sequence may be a natural site of topoisomerase IIα–mediated cleavage that could be relevant to the etiology of APL in this case.

Based on sequence analysis of *PML-RARA* and reciprocal *RARA-PML* genomic breakpoints, the location of functional topoisomerase IIα cleavage sites in the vicinity of the breakpoints, and known mechanisms by which topoisomerase II induces double-strand breaks in DNA[31] and their subsequent repair,[6] it was possible to generate models as to how the t(15;17) chromosomal translocation could have been formed in the studied cases (Figure 2,3C). Type II topoisomerases introduce staggered nicks in DNA creating 5′-overhangs. In the models, repair of the overhangs in *PML* and *RARA* entails exonucleolytic digestion, pairing of complementary bases, and joining of DNA free ends by the NHEJ pathway, with template-directed polymerization to fill in any gaps.

Discussion

In this study on sAPL that developed after MS, we were able to identify a biased distribution of breakpoints in the *PML* gene that

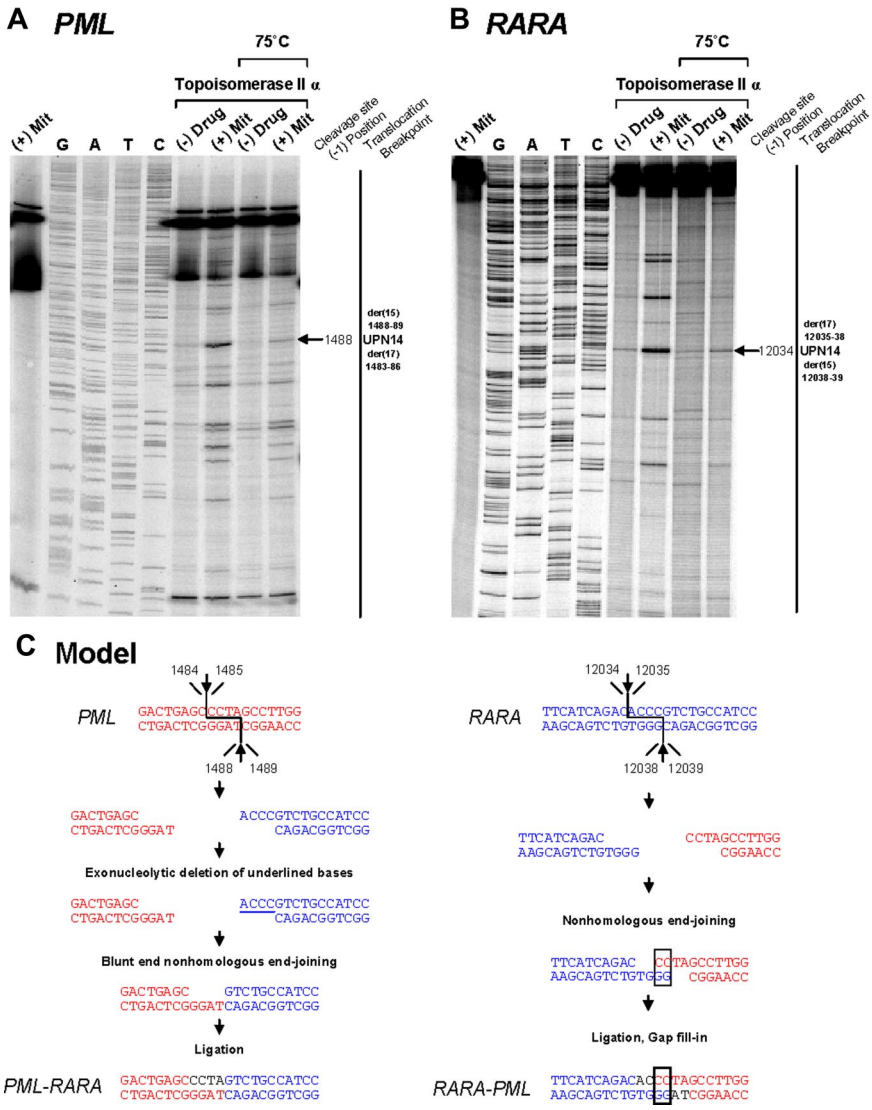

Figure 3. Investigation of t(15;17) translocation mechanism in UPN 14 by in vitro topoisomerase IIα DNA cleavage assay. DNA cleavage assays are shown for *PML* (A) and *RARA* (B) genomic breakpoint regions. For the *PML* assay, the reverse complement of the substrate containing the "hotspot" region between 1482 and 1489 described by Mistry et al[5] was used. Lanes 1 to 9 of each cleavage assay are described in the legend to Figure 2. (C) Native *PML* and *RARA* sequences are shown in red and blue, respectively. In the creation of *PML-RARA*, processing includes exonucleolytic deletion and repair via the NHEJ pathway. In the creation of *RARA-PML*, 2-base homologies facilitate repair via the NHEJ pathway, whereas in both instances polymerization of the relevant overhangs fills in any remaining gaps (shown in black font).

clustered in the same "hotspot" region previously identified in APL cases arising after treatment with mitoxantrone for breast cancer.[5] In addition, we established in one patient who the breakpoint in *RARA* intron 2 at position 14446-49 coincided with a breakpoint identified by Mistry et al in 1 of 5 t-APL cases arising in breast cancer patients treated with the same agent.[5] Given that intron 2 is almost 17 kb in length, such tight clustering of breakpoints between 2 different t-APL cases would be highly improbable to occur by chance. This observation strongly suggests that this is a preferential site of mitoxantrone-induced cleavage of DNA by topoisomerase IIα. The hypothesis is further supported by our functional in vitro data that show that this *RARA* site, together with the previously identified 8-bp "hotspot" region in *PML* intron 6, are preferential targets of mitoxantrone-induced DNA damage mediated by topoisomerase IIα.

Interestingly, of the 6 patients found to have *PML* breakpoints involving the "hotspot" region, one (UPN 12) did not receive mitoxantrone. Although mitoxantrone may significantly increase the chances of inducing DNA damage at this site, it is conceivable that this region represents a preferential site of cleavage by the native topoisomerase IIα and could in some instances act in concert with environmental or dietary agents that also target the enzyme.[32-36] Accordingly, in the other case of sAPL that arose in the absence of mitoxantrone exposure (UPN 10), the in vitro DNA cleavage assay also revealed sites of cleavage in the *PML* and *RARA* substrates with topoisomerase II alone, which corresponded to the observed breakpoints (data not shown).

In some cases, the occurrence of short homologies of 1 or 2 nucleotides at the breakpoint region between the *PML* and *RARA* genes precluded precise assignment of the breakpoint within each respective gene. However, further investigation using the in vitro functional assays enabled the location of preferential sites of mitoxantrone-induced topoisomerase IIα–mediated DNA cleavage to be mapped. Taking into account the mechanisms by which type II topoisomerases induce double-strand DNA breaks[31] and the processes mediating their repair, most probably involving the NHEJ pathway,[5,30] it was possible to model the generation of the t(15;17) chromosomal translocation underlying the development of APL in these cases. Previous studies have established the presence of functional topoisomerase II cleavage sites at translocation breakpoints in *MLL*-associated t-AML, indicating that direct DNA damage coupled with aberrant repair by the NHEJ pathway is probably relevant to the formation of translocations that disrupt other genes that are commonly involved in t-AML.[37-39]

To the best of our knowledge, 20 cases of sAPL occurring in patients with MS have been reported to date,[3,5,12-20] 4 of which are

included in the present series (UPNs 1, 4, 5, and 7) [5, 17, 20]. However, this is the first study to systematically analyze such cases at the genomic level. The incidence of sAPL arising in MS patients treated with mitoxantrone is not firmly established, as no systematic analysis has been undertaken to address this issue and only case reports have been published. On reviewing the records of 2336 MS patients treated with mitoxantrone, Voltz et al[40] described 5 cases of t-AML and 2 sAPL as a case report. Ghalie et al[41] assembled the records of 1378 patients treated with mitoxantrone in 3 MS studies and reported 2 patients who developed t-AML with an observed incidence proportion of 0.15% (95% confidence interval, 0.00%-0.40%). In addition to the reported 20 cases of sAPL, 8 cases of t-AML (non-M3) arising after mitoxantrone treatment for MS have been described, with the majority showing balanced translocations in their leukemic cells.[40,42-46] Therefore, although it appears that an excess of sAPL cases are observed in the MS setting, the reasons underlying this phenomenon remain unclear at present and warrant further basic and epidemiologic investigation. It is unknown, for example, whether factors other than mitoxantrone may play a role in sAPL development in the context of MS. Finally, it would be important to assess prospectively the true incidence of APL development in the MS setting (with or without mitoxantrone). Considering the risk of leukemia development and cardiac toxicity, the Therapeutics and Technology Subcommittee of the American Academy of Neurology recently has recommended that mitoxantrone be reserved for patients with progressive MS who have failed other therapies.[47]

In conclusion, this study lends further support to the presence of preferential sites of DNA damage induced by mitoxantrone within *PML* intron 6 and suggests the existence of a further "hotspot" at the distal end of *RARA* intron 2. The susceptibility of these regions of the *PML* and *RARA* loci to topoisomerase IIα–mediated cleavage by mitoxantrone may underlie the propensity to develop this particular subtype of AML after exposure to this agent. Further studies are warranted to investigate whether MS patients have a particular predisposition to the development of sAPL.

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Authorship

Contribution: S.K.H. and A.N.M. performed the experiments, analyzed the data, and contributed to the manuscript; T.O. and M.L. assisted in experimental design and performance of experiments; A. Ledda, G.L.N., C.S., C.C., E.B., L.M., E.M., J.C., G.S., A. Lennard, J.E., M.T.V., and W.R.S. contributed to the samples, clinical data, and interpretation of results; J.A.W.B. and N.O. supplied vital reagents; D.G., M.A.S., C.A.F., and S.A. analyzed the data, critically reviewed the manuscript, and amended the final report; and F.L.C. and D.G. designed the study, supervised the research, and wrote the manuscript.

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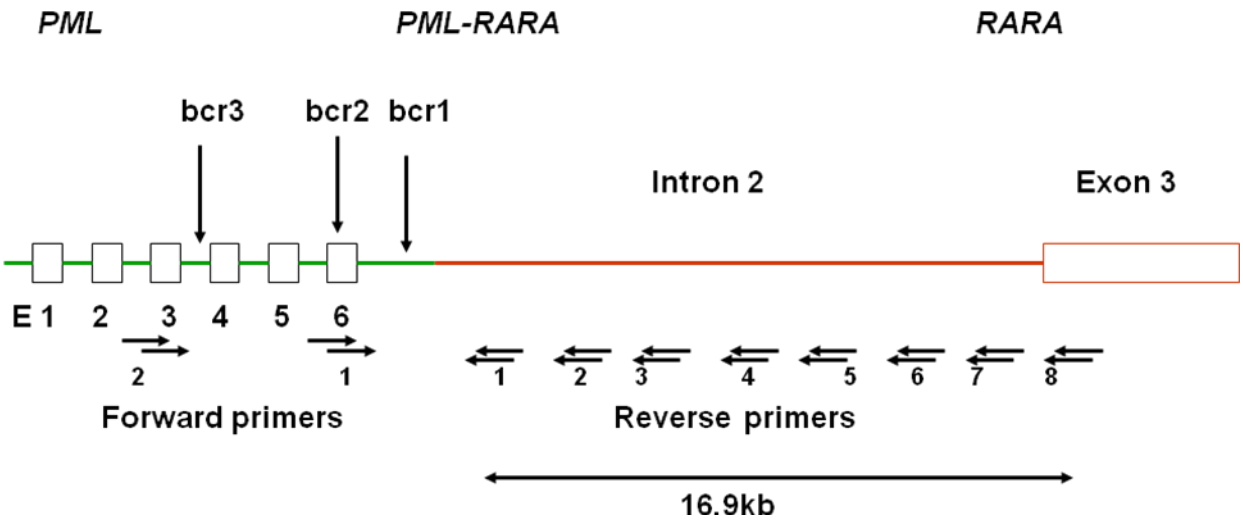
Note: Additional text and figures related to this chapter which are not available to online version of the manuscript.

Fig S1: Schematic diagram showing the strategy employed to identify t(15;17) genomic breakpoint junction locations. (A) *PML* is shown in green, *RARA* in red, and the locations of the nested primers used to perform long range nested PCR are indicated by the horizontal arrows. Vertical arrows indicate the regions in which breakpoints are most likely to occur. (B) An example of the chromatogram obtained revealing the breakpoint junction sequence.

A



B

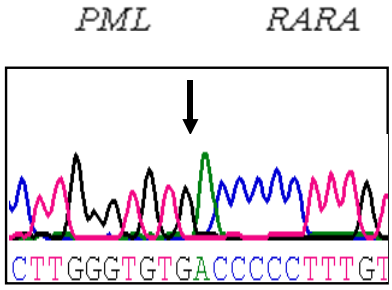
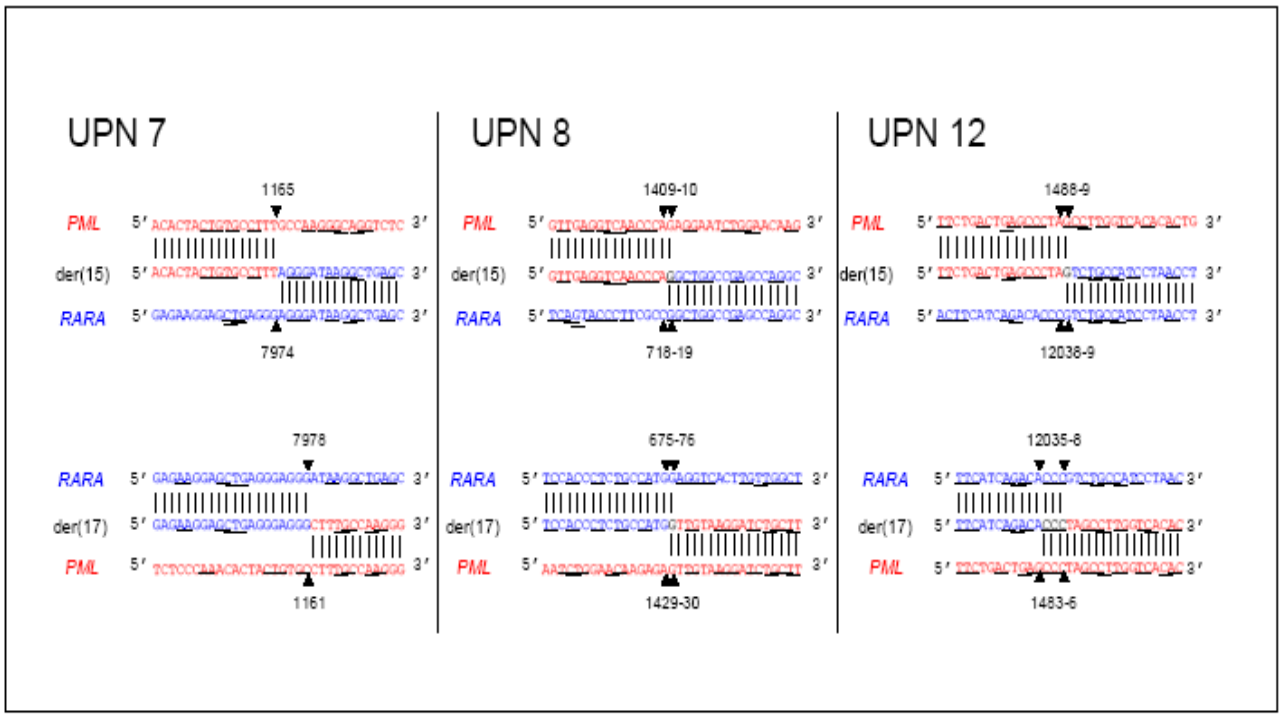


Figure S2. Locations of t-APL breakpoints in multiple sclerosis patients exposed to mitoxantrone. Der(15) and der(17) genomic breakpoint junctions in 3 cases. Native *PML* sequences are in red and *RARA* in blue. Vertical lines indicate sequences from the derivative chromosomes, and horizontal lines indicate regions of microhomologies consistent with DNA repair of chromosomal breaks by the non-homologous end joining pathway. Homologies prevented determining precise localization of breakpoint positions (black font).



TableS1: ***PML* and *RARA* homologues provided the substrate for *in vitro* topoisomerase II cleavage assays**

Patient Numbers	Gene and Accession Number	Substrate Positions	Length of Substrate (in bps)	Forward Primer (5'→3')	Reverse Primer (5'→3')	Sequence of Substrates
UPN 2	*PML* S57791	chr15:72113430 +72113710	281	ATGCGAATTCGATATTCCTT ACCCACAAATGCTATTGGT AATCG	AGAGAGGGATCCCTTTG CATTTGGATTTGAGTAAT TCACA	GATATTCCTTACCCACAAATGCTATTGGTAATCGatgggttttgattaacta aatgaagcgtcttttaactctagacatcccagctcatggagctgagaatggg gacagtgtgcttctttaggattgcagaaggttccagaccaggtagcagttgt caggcctcctgtacaggggggcaaattctgaattctgggcagataacttaatt gaatattcatagataaggcacagcaagaaattatggaaacccattTGTGAAT TACTCAAATCCAAATGCAAAG
UPN 2	*RARA* AJ297538	chr17:35755496 +35755758	263	ATGCGAATTCGAGGTTAGC AGGGATGGGCCAG	AGAGAGGGATCCGTCAC CCAGGTAAGCCAGCTCT G	GAGGTTAGCAGGGATGGGCCAGgcccgggcagtcctccccccgttggtgtcc ctcccactccacctgtgtgtgcagggagttatggccgtgtcctaactcttg cagaggctgtgaggattccggagttccccacacctccgggccttggtccttgt acctcacctccttggactgctggctggaggcctggggaggtggggcatcgag ctctgggttcaaagggcagagcagggaaacctCAGAGCTGGGTTACCTGGGT GAC
UPN 7	*PML* S57791	chr15:72112949 +72113240	292	ATGCGAATTCCACAGTGTG CGTGGGGGTGAG	AGAGAGGGATCCTAGGT GGCCTTTGCAGAAACACT TGG	CACAGTGTGCGTGGGGGTGAGagtggacacagtttaccatgtgttttgccat gattgaggtgtggggagcctgacaggctaaaatgcacagttctatgagtcct ttctcccaaacactactgtgcctttgcacagggcaggtctccacaagaagtt tgttggctgatgcctagcatactcccagagagggcttgggcataccataaca ggaaactggactcccctgacctttaactctgctctctcagtctacacccatc atttccCCAAGTGTTTCTGCAAAGGCCACCTA
UPN 7	*RARA* AJ297538	chr17:35749047 +35749337	291	ATGCGAATTCATGGGGTTG GGGACAGGAGGG	AGAGAGGGATCCCTAGG GCAAGAGGCCCGTTCTG	ATGGGGTTGGGGACAGGAGGGgcccctgcctggcacaagtgggggccagttt ggcctgcacctgctgagggcttactaaacgcaaagccctgagaaggagctga gggagggataaggctgagcaggatgggcccccaggcctgcagacagctgccg ctggaggcccccagcccccagctgcgggccagagcctttcttgtttcccact tctcccactgccccaggacactgtgttctgctctgacctgcccccatgacctt gctgttgccCAGAACGGGCCTCTTGCCCTAG
UPN 8	*PML* S57791	chr15:72113173 +72113462	290	ATGCGAATTCCCTTCCTTAA CTCTTCTCTCTCAGTCTACA CC	AGAGAGGGATCCATTAAC CAATAGCATTTGTGGGTA AGGAATATCAG	CCTTAACTCTGCTCTCTCAGTCTACACCcatcatttcccccaagtgtttctgc aaaggccacctacctaagtatctcatttgtcagagttatcttctatgaacac tgccaggcaggtagtcagttgggcaggagttgaggtcaaccccagaggaatct ggaacaagagagttctaaggatctgcttcacccagagtacagctttgttcct cattctgactgagccctagcctttgtgtcacacactgagcactgaCTGATATTC CTTACCCACAAATGCTATTGGT
UPN 8	*RARA* AJ297538	chr17:35741791 +35742054	264	ATGCGAATTCGCCTCGGAC GCATGCATGGTC	AGAGAGGGATCCAGGCC AGGCCCCGGCTC	GCCTCGGACGCATGCATGGTCtggcagggcctgtgttttccttgcctccac cctctgccatggaggtcacttgttggctgggcaggggtctcagtaccttcg ccggctggccgagccaggtagtggaggtggcggcagctctggccagctggc agagtccagggctggggcctgccaggccgcttacagtaactctggccccag ggcccagctgggcgccaggccagggctgggggaggaggGAGCCGGGGCCTG GCCT
UPN 12	*PML* S57791	chr15:72113268 -72113541	274	ATGCGAATTCACACTGTCC CCATTCTCAGC	AGAGAGGGATCCCTTCTA TGAACACTGCCAGG	ACACTGTCCCCATTCTCAGCtccatgagctgggatgtctagagttaaaagAc gcttcatttagttaatcaaaaccccatcgattaccaatagcatttgtgggtaa ggaatatcagtcagtgctcagtgtgtgaccaaggctagggctcagtcagaat gaggaacaaagctgtactctgggtgaagcagatccttagaactctcttgttc cagattcctctgggttgacctcaactcctgcccaactgactacctgCCTGGC AGTGTTCATAGAAG
UPN 12	*RARA* AJ297538	chr17:35753093 +35753395	303	ATGCGAATTCTCCTGTGCG ACAGCTGCTGCC	AGAGAGGGATCCGAACA AACCGGATTAGGATTAGG GGG	TCCTGTGCGACAGCTGCTGCCcccagcccttgctcttgcgtcattagagtgg gtgtcaggggagcactcagtgtcttttggtattgacagtggggtgtggcagaa aggggcaacttcatcagacaccccgtctgccatcctaaccttccatcttggca aggggcactgggtccttatggggttgttgtcctggccccagcacacttggctg tcatctttgaggctttcatccccaggagtggaggggagaagctgctctggtg agaagaatccctctCCCCCTAATCCTAATCCGGTTTGTTC

Figure S3**: Investigation of t(15;17) translocation mechanism in UPN 7 by *in vitro* topoisomerase IIα DNA cleavage assay.** Cleavage results of *PML* (A) and *RARA* (B) translocation breakpoints in the t-APL case of UPN 7. Lanes 1-9 of each cleavage assay are as previously described in the manuscript Figure 3. The location of the relevant heat stable cleavage sites are indicated by an arrow on the far right.

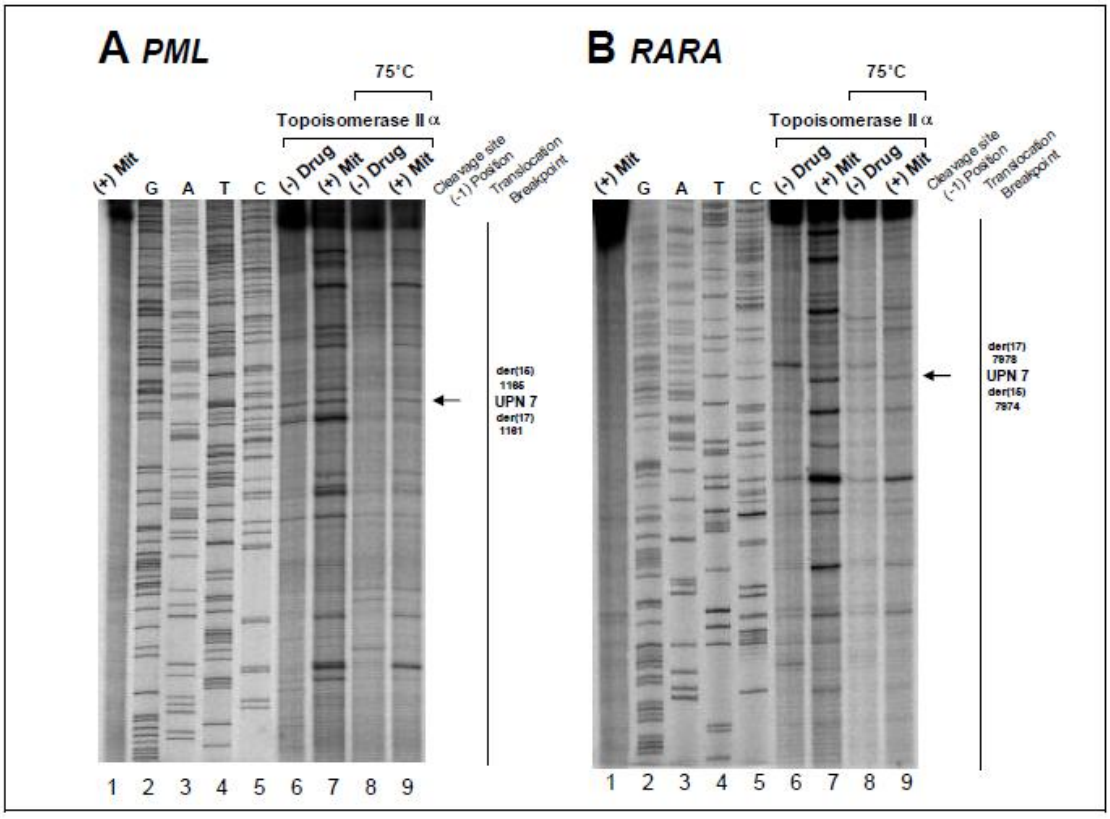


Figure S4. Investigation of translocation mechanism in UPN 8 by *in vitro* topoisomerase IIα DNA cleavage assay. Cleavage data using *PML* and *RARA* substrates corresponding to the locations of UPN 8 breakpoints previously identified. Lanes are as previously described, with relevant heat stable cleavage bands indicated by arrows on the far right.

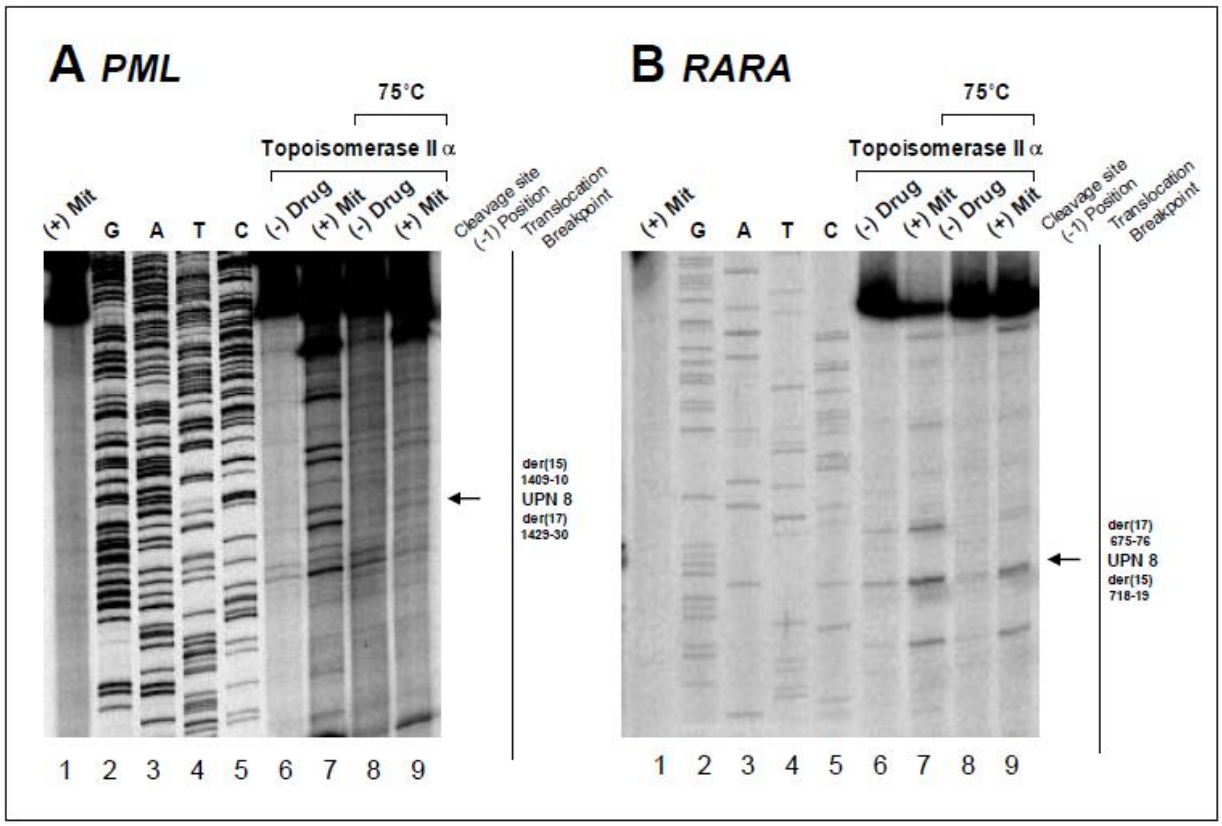
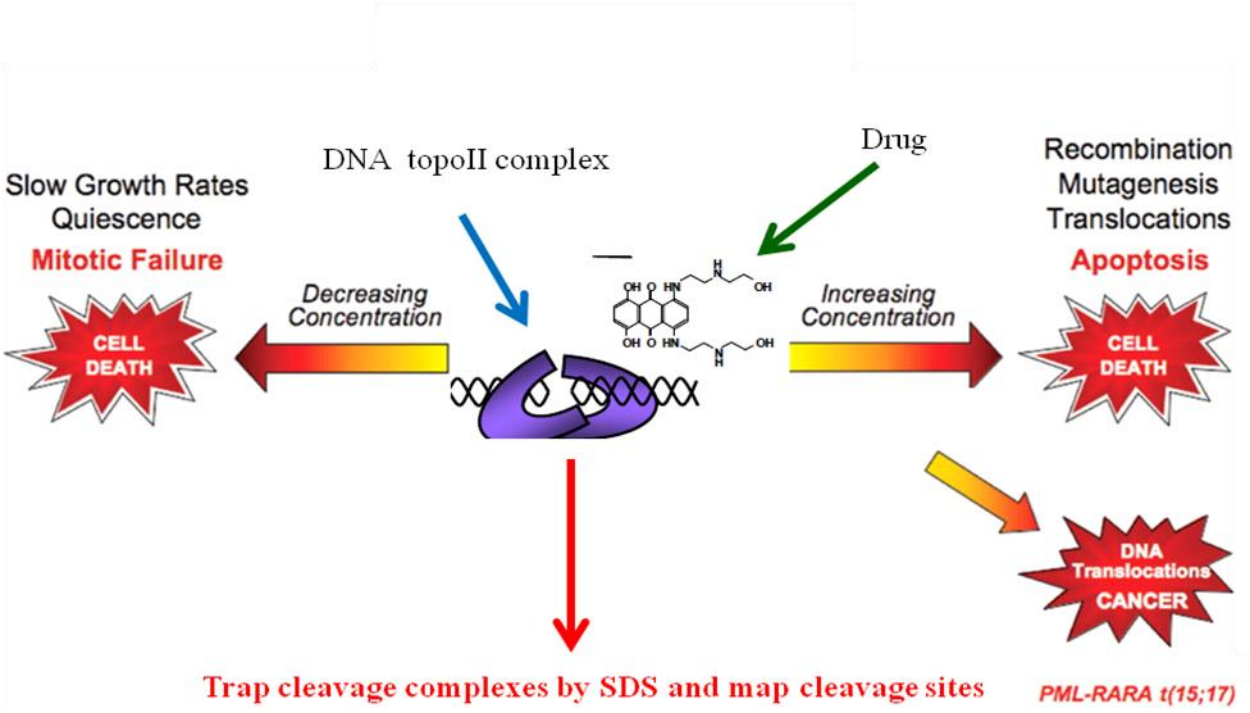


Figure S5**: The working model of DNA topoisomerase IIα cleavage assay.** Normal homologues encompassing translocation breakpoint regions were end labelled, incubated with human DNA topoisomerase II, ATP, and mitoxantrone. Cleavage complexes were irreversibly trapped upon the addition of SDS (sodium dodecyl sulfate), purified, and resolved in a polyacrylamide gel alongside sequencing to map the sites of cleavage precisely, allowing analysis of the position of the cleavage sites with respect to translocation breakpoint sites. Levels of cleavage complexes are maintained in a critical balance. When levels drop below threshold concentrations, daughter chromosomes remain entangled following replication. As a result, chromosomes cannot segregate properly during mitosis and cells die as a result of catastrophic mitotic failure. When levels of cleavage complexes rise too high, cells also die, but for different reasons. Accumulated topoisomerase II–DNA cleavage intermediates are converted to permanent strand breaks when replication forks, transcription complexes or DNA tracking enzymes such as helicases attempt to traverse the covalently bound protein 'roadblock' in the genetic material . The resulting collision disrupts cleavage complexes and ultimately converts transient topoisomerase II-associated DNA breaks to permanent double-stranded breaks that are no longer tethered by proteinaceous bridges. The resulting damage and induction of recombination/repair pathways can trigger mutations, chromosomal translocations and other aberrations.



Chapter 3

Molecular analysis of the t(15;17) genomic breakpoints in epirubicin associated therapy-related APL following breast carcinoma

Brief report

Evidence for direct involvement of epirubicin in the formation of chromosomal translocations in t(15;17) therapy-related acute promyelocytic leukemia

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Therapy-related acute promyelocytic leukemia (t-APL) with t(15;17)(q22;q21) involving the *PML* and *RARA* genes is associated with exposure to agents targeting topoisomerase II (topoII), particularly mitoxantrone and epirubicin. We previously have shown that mitoxantrone preferentially induces topoII-mediated DNA damage in a "hotspot region" within *PML* intron 6. To investigate mechanisms underlying epirubicin-associated t-APL, t(15;17) genomic breakpoints were characterized in 6 cases with prior breast cancer. Significant breakpoint clustering was observed in *PML* and *RARA* loci ($P = .009$ and $P = .017$, respectively), with *PML* breakpoints lying outside the mitoxantrone-associated hotspot region. Recurrent breakpoints identified in the *PML* and *RARA* loci in epirubicin-related t-APL were shown to be preferential sites of topoII-induced DNA damage, enhanced by epirubicin. Although site preferences for DNA damage differed between mitoxantrone and epirubicin, the observation that particular regions of the *PML* and *RARA* loci are susceptible to these agents may underlie their respective propensities to induce t-APL. (Blood. 2010;115: 326-330)

Introduction

For many years it has been appreciated that exposure to drugs targeting topoisomerase II (topoII) predisposes to the development of secondary leukemias characterized by balanced translocations, particularly involving *MLL* at 11q23, *NUP98* at 11p15, *RUNX1* at 21q22, and *RARA* at 17q21.[1-3] Indeed, therapy-related leukemias are becoming an increasing health care problem because more patients survive their primary tumors.[3,4] TopoII is a critical enzyme that relaxes supercoiled DNA by transiently cleaving and religating both strands of the double helix by the formation of a covalent cleavage intermediate.[5] Epipodophyllotoxins (eg, etoposide), anthracyclines (eg, epirubicin), and anthracenediones (eg, mitoxantrone) act as topoII poisons, inducing DNA damage by disrupting the cleavage-religation equilibrium and increasing the concentration of DNA topoII covalent complexes.[5]

The association between exposure to chemotherapeutic agents targeting topoII and development of leukemias with balanced chromosomal rearrangements has naturally implicated the enzyme in this process, but the mechanisms involved have remained subject to debate. Interestingly, the nature of the drug exposure has a bearing on the molecular phenotype of the resultant secondary leukemia, with translocations involving 11q23 being particularly associated with etoposide exposure,[6,7] and development of therapy-related acute promyelocytic leukemia (t-APL) with the t(15;17) being linked to mitoxantrone and epirubicin treatment.[8-11] Previously, we identified that t-APL cases arising in patients with breast cancer receiving mitoxantrone display tight clustering of chromosome 15 breakpoints within an 8 base pair (bp) "hotspot" region in *PML* intron 6.[12] Furthermore, these breakpoints were shown by functional assay to be a preferred site of mitoxantrone-induced DNA topoII cleavage.[12] Subsequent analysis of an independent cohort of t-APL cases arising after mitoxantrone therapy for multiple sclerosis confirmed chromosome 15 breakpoint clustering in the hotspot and identified recurrent breakpoints within *RARA* intron 2.[13] Once again, these breakpoints were preferential sites of mitoxantrone-induced cleavage in vitro.[13]

No studies to date have investigated epirubicin-induced leukemias. This agent is widely used in adjuvant breast cancer therapy, with cumulative doses of 720 mg/m^2 or less associated with a secondary leukemia risk of 0.37% at 8 years.[14] Several balanced rearrangements have been reported in this context, including translocations involving the *MLL* locus, core binding factor leukemias, and t-APL with the t(15;17).[14,15] To gain further

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The online version of this article contains a data supplement.

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Table 1. Patient characteristics

Patient	Age at APL diagnosis, y	Primary malignancy	Treatment of primary malignancy	Cumulative dose of epirubicin, mg/m^2	Latency, mo*	Cytogenetics	*PML* breakpoint		*RARA* breakpoint¶		APL therapy	Current status of APL
							der(15)	der(17)	der(15)	der(17)		
UPN1	40	Breastcarcinoma	4 Cycles of epirubicin (165 mg), 6 cycles of CMF (cyclophosphamide 990 mg, methotrexate 65 mg, 5FU 990 mg)	400	48	t(15;17)(q22;q12-21) idem, del(5)(q?31q?35)	1186†	1185†	13463	13437	PETHEMA‡	Alive in first CRm at 61 mo
UPN2	47	Breastcarcinoma	4 Cycles of epirubicin (175 mg) + cyclophosphamide (1180 mg) DXT	400	28	t(7;14)(q32;q22), t(15;17)(q22;q21)	1270§	1267§	16192	16192	MRC‡	Alive in first CRm at 43 mo; treatment complicated by anthracycline-related cardiomyopathy (ejection fraction 30%–40%)
UPN3	41	Breastcarcinoma	6 Cycles of epirubicin (118 mg) + cyclophosphamide (940 mg) DXT	450	18	t(15;17)(q22;q21)	379-80§	375-76§	9291-92	9293-94	MRC‡	Death in first CRm (fungal infection) at 5 mo
UPN4	55	Breastcarcinoma	4 Cycles of epirubicin (200 mg), 4 cycles of CMF (cyclophosphamide 1200 mg, methotrexate 80 mg, 5FU 1200 mg) DXT	400	18	der(12), t(8;12)(q13;p13), t(15;17)(q22;q21)	1184-85†	1187-1191†	13332-33	13336-40	PETHEMA‡	Alive in first CRm at 42 mo
UPN5	60	Breastcarcinoma	6 Cycles of FEC (5FU 975 mg, epirubicin 98 mg, cyclophosphamide 980 mg), DXT	360	24	t(15;17)(q22;q21)	1968‖	1964-65‖	16196	16192-93	MRC‡	Induction death (typhlitis) at 13 da
UPN6	55	Breastcarcinoma	6 Cycles of FEC (5FU 1100 mg, epirubicin 110 mg, cyclophosphamide 1100 mg × 3; 5FU 1000 mg, epirubicin 100 mg, cyclophosphamide 1000 mg × 3) DXT	300	27	t(15;17)(q22;q21)	955-57†	955-59†	14882-84	14884-88	PETHEMA‡ #1 ATO + ATRA consolidation	Alive in first CRm at 15 mo

APL indicates acute promyelocytic leukemia; CRm, molecular remission; DXT, radiotherapy; 5FU, fluorouracil; UPN: Unique patient number; and #1, course 1.

*Length of time between first epirubicin exposure and presentation with therapy-related APL.

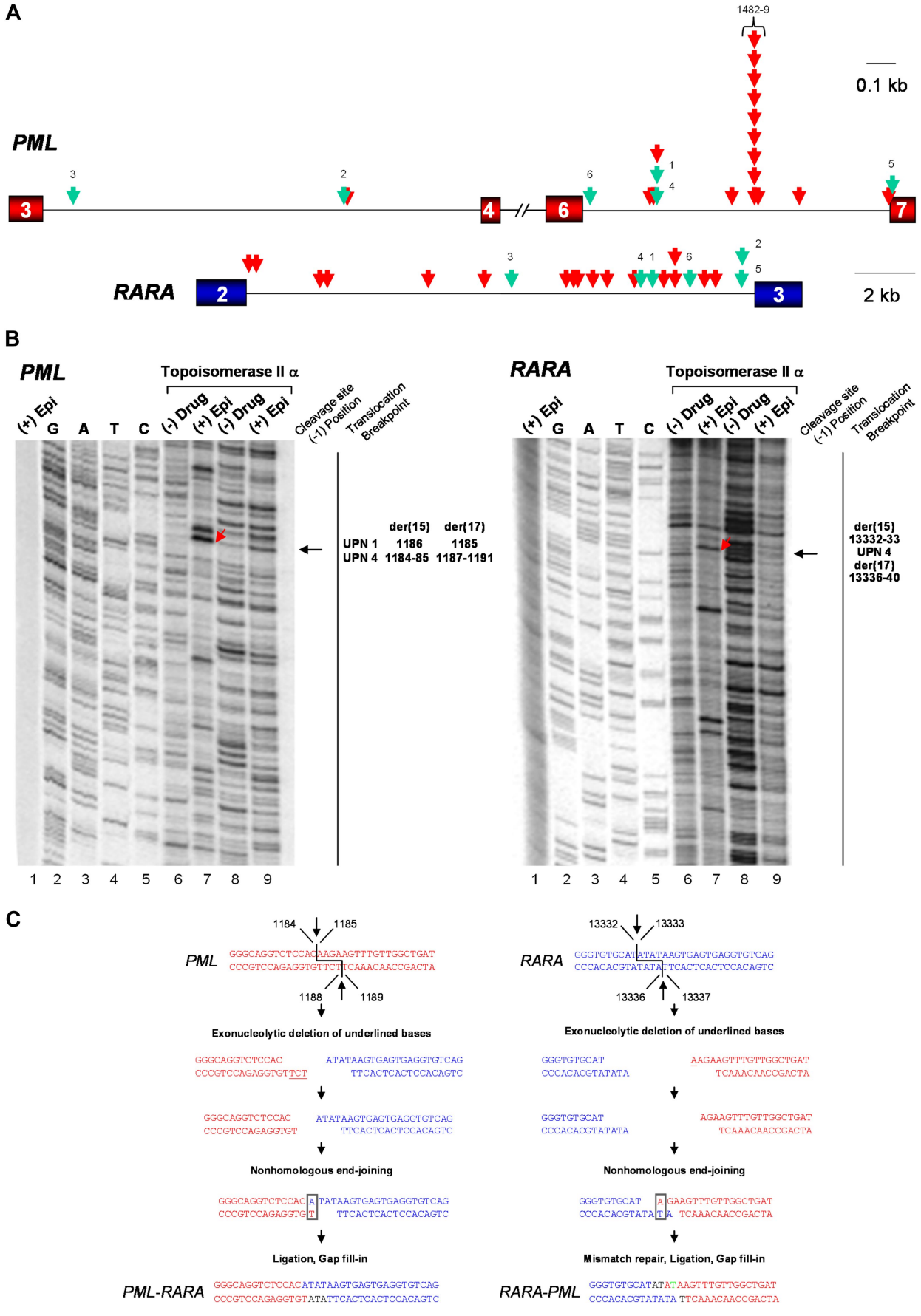
†Breakpoint locations for *PML* intron 6 are numbered according to the GenBank accession no. S57791.

‡Patients were treated with an extended course of *all-trans* retinoic acid (ATRA) given simultaneously with induction chemotherapy. Medical Research Council (MRC) and PETHEMA treatment schedules were given as described.[16] UPN6 received consolidation with arsenic trioxide (ATO) and ATRA according to the National Cancer Research Institute AML17 protocol (http://aml17.cardiff.ac.uk).

§Breakpoint locations for *PML* intron 3 are numbered according to the GenBank accession no. S51489.

‖Breakpoint locations for *PML* exon 7 are numbered according to the GenBank accession no. S57791.

¶Breakpoint locations for *RARA* intron 2 are numbered according to the GenBank accession no. AJ297538.


A
1482-9
0.1 kb
PML
RARA
2 kb
B
PML
Topoisomerase II α
(+) Epi
G A T C
(-) Drug
(+) Epi
(-) Drug
(+) Epi
Cleavage site (-1) Position
Translocation Breakpoint
der(15) der(17)
UPN 1 1186 1185
UPN 4 1184-85 1187-1191
RARA
der(15)
13332-33
UPN 4
der(17)
13336-40
1 2 3 4 5 6 7 8 9
C
PML
1184 1185
GGGCAGGTCTCCACAAGAAGTTTGTTGGCTGAT
CCCGTCCAGAGGTGTTCTTCAAACAACCGACTA
1188 1189
Exonucleolytic deletion of underlined bases
GGGCAGGTCTCCAC ATATAAGTGAGTGAGGTGTCAG
CCCGTCCAGAGGTGTTCT TTCACTCACTCCACAGTC
GGGCAGGTCTCCAC ATATAAGTGAGTGAGGTGTCAG
CCCGTCCAGAGGTGT TTCACTCACTCCACAGTC
Nonhomologous end-joining
GGGCAGGTCTCCAC A TATAAGTGAGTGAGGTGTCAG
CCCGTCCAGAGGTG T TTCACTCACTCCACAGTC
Ligation, Gap fill-in
PML-RARA
GGGCAGGTCTCCACATATAAGTGAGTGAGGTGTCAG
CCCGTCCAGAGGTGTATATTCACTCACTCCACAGTC
RARA
13332 13333
GGGTGTGCATATATAAGTGAGTGAGGTGTCAG
CCCACACGTATATATTCACTCACTCCACAGTC
13336 13337
Exonucleolytic deletion of underlined bases
GGGTGTGCAT AAGAAGTTTGTTGGCTGAT
CCCACACGTATATA TCAAACAACCGACTA
GGGTGTGCAT AGAAGTTTGTTGGCTGAT
CCCACACGTATATA TCAAACAACCGACTA
Nonhomologous end-joining
GGGTGTGCAT A AGAAGTTTGTTGGCTGAT
CCCACACGTATATT A TCAAACAACCGACTA
Mismatch repair, Ligation, Gap fill-in
RARA-PML
GGGTGTGCATATATAAGTTTGTTGGCTGAT
CCCACACGTATATA TTCAAACAACCGACTA

insights into molecular mechanisms underlying epirubicin-related leukemias, we characterized t(15;17) genomic breakpoint junction regions in t-APL after breast cancer therapy.

Methods

t(15;17) Genomic breakpoint characterization

Samples from 6 patients with t-APL (Table 1) were received by the APL Reference Laboratory, Guy's Hospital. The study including patient information sheets and consent forms was approved by St Thomas' Hospital London Research Ethics Committee (ref 06/Q0702/140), and performed with informed consent in accordance with the Declaration of Helsinki. Reverse transcriptase–polymerase chain reaction (PCR) was used to establish *PML* breakpoint region.[16] Genomic breakpoint junction regions were then amplified with appropriate primer sets by nested long-range PCR, followed by sequence analysis, as described.[17] *PML-RARA* breakpoint junctions were confirmed by PCR amplification and sequence analysis with the use of fresh aliquots of genomic DNA. Patient-specific primers were designed to PCR amplify and sequence the reciprocal *RARA-PML* genomic breakpoint junction regions. The distribution of genomic breakpoints was analyzed by scan statistics, as previously described.[12,17]

In vitro topoII DNA cleavage assays

Normal homologues of *PML* and *RARA* encompassing the location of the relevant breakpoint were cloned into the pBluescript SKII (+) vector. Cleavage assays were performed as reported previously[12,13] and included epirubicin, dissolved in 20 μL of DMSO used at a concentration of 160μM.

Figure 1. Molecular characterization of the t(15;17) in therapy-related APL arising after epirubicin therapy. (A) Distribution of translocation breakpoints within the *PML* and *RARA* loci in t-APL cases arising after epirubicin and mitoxantrone. *PML* exons are represented by red boxes, *RARA* exons are in blue, and introns are represented by black lines. Arrows indicate the location of *PML* and *RARA* translocation breakpoints identified in patients with t-APL arising after mitoxantrone (red arrows) or epirubicin (green arrows), and numbers of the epirubicin-related cases correspond to those presented in Table 1. Details of the mitoxantrone cases have been reported previously.[12,13] (B) *PML* and *RARA* breakpoints in epirubicin-related t-APL are preferred sites of epirubicin-induced topoII-mediated DNA cleavage. To identify epirubicin-enhanced cleavage by topoIIα, chromosomal breakpoint junctions were examined in an in vitro assay. DNA cleavage reactions were performed with 25 ng of 5′-labeled DNA (30 000 cpm), 1mM ATP, DMSO, and in the presence or absence of 147nM human DNA topoIIα and 160μM epirubicin. Cleavage complexes were trapped on the addition of SDS and were resolved in an 8% acrylamide–7.0M urea gel. In both panels, reactions in lane 1 were performed with epirubicin (Epi) but lacking DNA topoIIα and show little evidence of cleavage in the absence of the enzyme. Lanes 2 to 5 show dideoxy sequencing reactions primed at the same 5′ end, which allows high-resolution mapping of cleavage sites. Substrates were incubated with topoIIα and DMSO only (lanes 6 and 8) and also in the presence of epirubicin (lanes 7 and 9). Reactions in lanes 8 and 9 were further incubated at 75°C to assess the heat stability of the cleavage complexes. On the left, DNA topoIIα-dependent cleavage is shown within a *PML* substrate that encompassed the locations of the genomic breakpoints identified in UPN1 and UPN4. The location of the arrows indicate the epirubicin-enhanced heat-stable complexes at position 1184, corresponding precisely to these translocation breakpoints. On the right, cleavage within a substrate that contains the normal homologue of *RARA* encompassing the breakpoint junction identified in UPN4 is shown, whereby the arrows indicate the epirubicin-enhanced heat-stable complexes corresponding to the der(15) and der(17) translocation breakpoints. (C) Model for formation of the t(15;17) underlying epirubicin-induced t-APL in UPN4. Normal homologues of *PML* and *RARA* are indicated in red and blue fonts, respectively. Models show where topoIIα introduces 4-bp staggered nicks in the DNA (as indicated by in vitro experiments), followed by exonucleolytic processing to reveal microhomologies (indicated by gray boxes) that are probably repaired by the error-prone nonhomologous end joining repair pathway. Template-directed polymerization (indicated with black font), mismatch repair (represented by green font), and ligation fills in any remaining gaps to generate the *PML-RARA* and *RARA-PML* genomic breakpoint junctions that were identified in the t-APL arising in this patient.

Results and discussion

Clinical features

Demographic features and details of the treatment received by the 6 patients with t-APL for their original breast cancer are shown in Table 1. Median latency from time of first epirubicin exposure to t-APL diagnosis was 26 months (range, 18-48 months).

Identification of t(15;17) genomic translocation breakpoints

Chromosome 15 breakpoints were localized to *PML* intron 6 (UPN1, UPN4, UPN6), intron 3 (UPN2, UPN3), and exon 7 (UPN5), with breakpoints in 2 of the cases (UPN1, UPN4) found to fall within 1 to 2 bp of one another (Table 1). Given the size of *PML* intron 6 (~ 1 kb), the close apposition of these breakpoints was unlikely to have occurred by chance ($P = .014$ using scan statistics for the 1056-bp intron 6 only with 3 patients; $P = .009$ for the 3921-bp exon 5-7b region and 4 patients). The chromosome 17 breakpoints of the 6 cases were distributed within *RARA* intron 2, with breakpoints in 2 patients (UPN2, UPN5) falling within 4 nucleotides of one another between positions 16192 and 16196. Considering the length of this intron (~ 17 kb), the proximity of the breakpoints in these 2 patients was also unlikely to have occurred by chance ($P = .017$ for the 16913-bp intron).

The breakpoint locations within the *PML* locus of the epirubicin-related t-APL cases occurred outside the hotspot region in intron 6 (1482-9) previously mapped in cases occurring after mitoxantrone treatment for breast cancer[12] or multiple sclerosis[13] (Figure 1A).

t(15;17) Translocation breakpoints are preferential sites for epirubicin-induced DNA cleavage by topoII

To investigate mechanisms by which the t(15;17) may have been formed after epirubicin exposure, we evaluated topoIIα-mediated cleavage of the normal homologues of *PML* and *RARA* encompassing the respective breakpoints detected in 4 cases in the presence or absence of this agent, including those in which the *PML* (UPN1, UPN4) or *RARA* breakpoints (UPN2, UPN5) were closely apposed. Some DNA cleavage bands were observed in the absence of drug, but the addition of epirubicin increased DNA cleavage in a topoII-dependent manner (Figure 1B). Cleavage bands that were significantly enhanced by epirubicin corresponding to the location of the observed genomic breakpoints in the *PML* and *RARA* loci were detected in each of the cases analyzed (Figure 1B; supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). These bands remained detectable after heating at 75°C, indicating stability of the cleavage complexes. The shared breakpoints in *PML* and *RARA* related to functional sites of epirubicin-induced cleavage by topoII at positions 1184 (Figure 1B) and 16192 (supplemental Figure 1A), respectively.

On the basis of sequence analysis of *PML-RARA* and reciprocal *RARA-PML* genomic junction regions, the location of functional topoII cleavage sites in the vicinity of the breakpoints, and known mechanisms by which topoII induces double-strand breaks in DNA,[5,18] it was possible to generate models as to how the t(15;17) chromosomal translocation could have been formed in the studied cases (Figure 1C; supplemental Figure 1B). Type II topoisomerases introduce staggered nicks in DNA, creating 5′-overhangs. In the models, repair of the overhangs in *PML* and *RARA* entails

exonucleolytic digestion, pairing of complementary bases, and joining of DNA free ends by the nonhomologous end-joining pathway, with template-directed polymerization to fill in any gaps.

Although there is strong circumstantial evidence linking exposure to agents targeting topoII to the development of leukemias with balanced chromosomal translocations, the precise mechanisms remain uncertain. One hypothesis takes into account reports that leukemia-associated translocations can be detected in hematopoietic cells derived from healthy persons without overt leukemia,[19,20] suggesting that administration of chemotherapy provides a selective advantage to progenitors with preexisting translocations during regrowth of depopulated bone marrow. In this case, exposure to DNA-damaging agents is postulated to induce additional mutations that cooperate with the chimeric fusion protein to mediate leukemic transformation. A second hypothesis proposes that chromosomal translocations arise through an indirect mechanism involving induction of apoptotic nucleases.[21-24] However, our studies involving the characterization of t-APL cases after mitoxantrone[12,13] or epirubicin provide very strong support for a third hypothesis whereby topoII induces double-strand DNA breaks in susceptible regions of the genome which are aberrantly repaired to generate leukemia-associated chromosomal translocations.[25]

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Authorship

Contribution: A.N.M. performed the experiments, analyzed the data, and wrote the manuscript; N.O. supplied vital reagents, analyzed the data, critically reviewed the manuscript, and amended the final report; Y.X. undertook statistical analyses; J.L.W. undertook statistical analyses, analyzed the data, critically reviewed the manuscript, and amended the final report; C.A.F. analyzed the data, critically reviewed the manuscript, and amended the final report; J.A.W.B. supplied vital reagents; K.S., A.P., R.C., C.C., C.H., and A.N.P. provided samples and clinical data and contributed to interpreting the results; S.K.H. assisted in performing the experiments, F.L.-C. and E.S. analyzed the data, critically reviewed the manuscript, and amended the final report; and D.G. designed the study, supervised the research, and wrote the manuscript.

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Note: Additional text and figures related to this chapter which is not available online.

Table S1: **. Reported cases of t-leukemia after epirubicin exposure.** Abbreviations: ALL- acute lymphoblastic leukemia; AML- acute myeloid leukaemia

Reference	Size of Cohort/ % Developing t-AL	Number and Subtype of t-Leukemia	Cytogenetics	Latency
(Campone et al., 2005)	2603/ (0.3%)	2 ALL	t(9;22) Not specified	55 months 69 months
		2 AML (M2)	t(9;22) t(8;21)	49 months 125 months
		3 AML (M4)	t(8;16), del(17q21) del(16q) Not specified	8 months 81 months 58 months
		1 AML (M3)	t(15;17)	23 months
(van der Hage et al., 2001)	698	0	Not applicable	Not applicable
(Coombes et al., 1996)	380	0	Not applicable	Not applicable
(Bernard-Marty et al., 2003)	522/ (0.5%)	2 AML (M5)	t(1;9;11)(q31-q32;p21;q23) t(9;11)	21 months 32 months
		1 AML (M6)	del(7q)	57 months
(Wils et al., 1999)	303/ (0.6%)	2 AML (M4)	Not specified	18 months 58 months
(Levine et al., 1998)	351/ (1.4%)	1 ALL	Not specified	15-41 months
		4 AML (Not specified)	11q23 (n=1) Not specified (n=3)	
(Crump et al., 2003)	636/ (1.3%)	3 AML (M5)	Normal t(9;11) Not specified	14 months 15 months 15 months
		2 AML (M4)	11q23 Normal	27 months 18 months
		2 AML (Not specified)	Not specified complex	39 months 79 months
		2 pre-B ALL	Not specified t(9;11)	24 months 14 months
(Bergh et al., 2000)	525/ (1.1%)	6 AML	Not specified	9-33 months

Figure S1. Location of t-APL breakpoints in breast carcinoma patients exposed to epirubicin. Der(15) and der(17) genomic breakpoint locations are shown for six epirubicin exposed patients with *PML* sequences shown in red font, and *RARA* in blue. Vertical lines indicate sequence homology with derivative chromosomes while horizontal lines indicate microhomologies between the *PML* and *RARA* sequences.

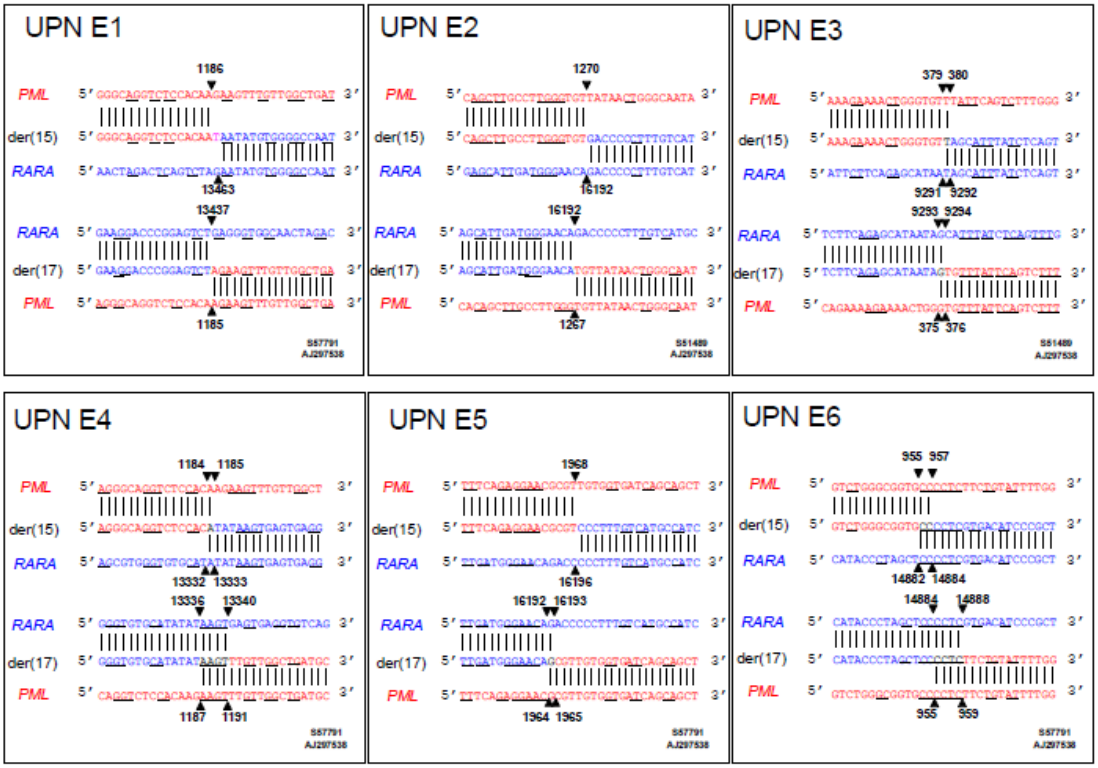

Table S2. *PML* and *RARA* homologues used as substrates for *in vitro* assays.

Patient Numbers	Gene and Accession Number	Substrate Positions	Length of Substrate (in bps)	Forward Primer (5'→3')	Reverse Primer (5'→3')	Sequence of Substrates
UPN E2	*PML* S51489	chr15:72103675 +72103895	221	ATGCGAATTC AGCTGGACATGG GTCATGAAGGATA ATC	AGAGAGGGATCC ATGATCTTCCTTATCT GCAGCCTGGT	AGCTGGACATGGGTCATGAAGGATAATCttagagggcctggacctgtgc cttctccacctaggtagtgaatgtcacccatattaaggaaatgacccca cagagtttacacagcttgccttgggtgttataactgggcaatagcagag agaagggttatttttattctgaagcataagagatttagatttaataaaaA CCAGGCTGCAGATAAGGAAGATCAT
UPN E2/ UPN E5	*RARA* AJ297538	chr17:35757240 +35757515	276	ATGCGAATTC GGCCAGTGAGGG CTGCCC	AGAGAGGGATCC CACAAATGCTGGGCA CAGTAGGAGG	GGCCAGTGAGGGCTGCCCctgctgtctgggtcacccctcctggctgccc tcttggagctgaataacagaaggggagggggttagtaacccggacatagt attgaggccagacagacagagcattgatgggaacagacccccttttgtca tgccatctctccccagatgggggggtacccagaataatgggcttttgggg ccctggggactcttctccctgtattcagggtatctcccccctatctcagg gagacaCCTCCTACTGTGCCCAGCATTTGTG
UPN E1/ UPN E4	*PML* S57791	chr15:72112973 +72113231	259	ATGCGAATTC GGACACAGTTTAC CATGTGTTTTGCC ATG	AGAGAGGGATCC TTTGCAGAAACACTTG GGGAAATGATGGG	GGACACAGTTTACCATGTGTTTTGCCATGattgaggtgtggggagcctg acaggctaaaatgcacagttctatgagtcctttctcccaaacactactg tgccttgcacagggcaggtctccacaagaagtttgttggctgatgcct agcatactcccagagagggcttgggcatacccataacaggaaactggact ccccatgaccttaactctgctctctcagtctacaCCCATCATTTCCCCA AGTGTTTCTGCAAA
UPN E4	*RARA* AJ297538	chr17:35754375 +35754643	269	ATGCGAATTC GCAGTGTATTGGT GAGCCCTTCCC	AGAGAGGGATCC CTAGACTGAGTCTAGT TGCCACCCTC	GCAGTGTATTGGTGAGCCCTTCCCctaaaccactggacatggggaagtg gagacctgtccccacatccattctgggggtgggtagtagacctagaggc ctgggtttccagttcccgtagtctgagcgtgggtgtgcatatataagtg agtgaggtgtcagtggactcgggtcctgaggctgtgaggttgggagtga tgggggtctgggggcttgccttgaggcacaggaaggacccggagtctGA GGGTGGCAACTAGACTCAGTCTAG
UPN E5	*PML* S57791	chr15:72113752 +72114008	257	ATGCGAATTC TGCAGGGCATCC GTTTCC	AGAGAGGGATCC GAGAGTCACCTGCTG TGGCTGTG	TGCAGGGCATCCGTTTCCcccagccgactggcctgcaaggattcccata ggtgcacacccacacccctcccagcatgcatcctaggcagttcataatg catctcccctttccccgtttcagaggaacgcgtgtggtgatcagcagct cggaagactcagatgccgaaaactcggtgagtggcccagaagttcagcc caggactcctgcctccccccatttcaggtcccaggggggCACAGCCACAG CAGGTGACTCTC
UPN E1	*RARA* AJ297538	chr17:35754501 +35754737	237	ATGCGAATTC GTGGGTGTGCATA TATAAGTGAGTGA GG	AGAGAGGGATCC CCCCAAGAGGGGGCT GGAG	GTGGGTGTGCATATATAAGTGAGTGAGGtgtcagtggactcgggtcctg aggctgtgaggttgggagtgatgggggtctgggggcttgccttgaggca caggaaggacccggagtctgagggtggcaactagactcagtctagaata tgtggggccaatgccaccaccttggaagggtcccctgggtgtgtggaa gtccgctggtgactggagctgcCTCCAGCCCCCTCTTGGGG

Chapter 4

Analysis of t(15;17) chromosomal breakpoint sequences in therapy-related versus *de novo* APL

RESEARCH ARTICLE

Analysis of t(15;17) Chromosomal Breakpoint Sequences in Therapy-Related Versus De Novo Acute Promyelocytic Leukemia: Association of DNA Breaks with Specific DNA Motifs at *PML* and *RARA* Loci

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We compared genomic breakpoints at the *PML* and *RARA* loci in 23 patients with therapy-related acute promyelocytic leukemia (t-APL) and 25 de novo APL cases. Eighteen of 23 t-APL cases received the topoisomerase II poison mitoxantrone for their primary disorder. DNA breaks were clustered in a previously reported 8 bp "hot spot" region of *PML* corresponding to a preferred site of mitoxantrone-induced DNA topoisomerase II-mediated cleavage in 39% of t-APL occurring in patients exposed to this agent and in none of the cases arising de novo ($P = 0.007$). As to *RARA* breakpoints, clustering in a 3′ region of intron 2 (region B) was found in 65% of t-APL and 28% of de novo APL patients, respectively. Scan statistics revealed significant clustering of *RARA* breakpoints in region B in t-APL cases ($P = 0.001$) as compared to de novo APL ($P = 1$). Furthermore, ~300 bp downstream of *RARA* region B contained a sequence highly homologous to a topoisomerase II consensus sequence. Biased distribution of DNA breakpoints at both *PML* and *RARA* loci suggest the existence of different pathogenetic mechanisms in t-APL as compared with de novo APL.

INTRODUCTION

In the acute promyelocytic leukemia (APL) specific t(15;17)(q22;q21) translocation, rearrangements between chromosome 15 and 17 are generated because of endogenous or exogenous DNA breaks at these loci. Chemotherapeutic drugs targeting topoisomerase II (topoII) produce exogenous DNA double strand breaks at the site of the enzyme-DNA complex and previous studies have implicated these agents in the pathogenesis of therapy-related APL (t-APL) (Beaumont et al., 2003; Mistry et al., 2005; Hasan et al., 2008).

Therapy-related leukemias provide an extraordinary opportunity to investigate key mechanisms of leukemogenesis by relating specific genetic abnormalities to the biological effects of chemotherapeutic agents. We and others (Mistry et al., 2005; Hasan et al., 2008) have shown that mitoxantrone-induced topoII mediated DNA damage at the *PML* and *RARA* breakpoint loci and their subsequent repair via the error prone nonhomologous end joining pathway lead to the formation of the t(15;17) in t-APL. Moreover, both studies

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(Mistry et al., 2005; Hasan et al., 2008) have confirmed a tight clustering of translocation breakpoints at the *PML* locus in mitoxantrone related t-APL.

The proposed mechanisms mediating chromosomal rearrangements in de novo leukemias are diverse. Several studies on cloned genomic breakpoints from many patients with various translocations revealed specific DNA sequence motifs in the vicinity of the breakpoint junctions (Tsujimoto et al., 1985; Haluska et al., 1986; van der Reijden et al., 1999; Kolomietz et al., 2002; Abeysinghe et al., 2003) while other reports did not bring convincing evidence for conserved sequences at DNA breaks (Yoshida et al., 1995; Reichel et al., 1998; Zhang et al., 2006). However, the role of V-D-J recombinase has been shown in several de novo translocations where genes involved in fusion transcripts are non-Ig-TCR loci but still contain recombination signal sequences (Tsujimoto et al., 1985; Haluska et al., 1986). A previous study on molecular analysis of t(15;17) in de novo APL (Yoshida et al., 1995) reported random DNA breaks at *PML* and *RARA* loci without any specific consensus sequence motif around them. By contrast, genomic analysis of *PML* and *RARA* loci from another study reported recombination prone sequences such as *ALU* elements and recombination signal sequences (RSS) at the 5′end of *RARA* intron 2 (Reiter et al., 2003).

Here, we investigated *PML* and *RARA* DNA breakpoints in t-APL with or without prior exposure to topoII targeting agents and in APL arising de novo. The current study is also an attempt to correlate the specific DNA motifs with observed translocation breakpoints in a series of de novo APL patients.

PATIENTS AND METHODS

Twenty-three patients with t-APL and 25 patients with de novo APL were included in the study. The t-APL cases were collected from several European Hematology Units as part of a collaborative study on t-APL arising after mitoxantrone treatment for multiple sclerosis (MS). Molecular characterization of DNA breakpoints in 14 (UPN 1 to 14) of 23 patients with t-APL has been reported elsewhere (Hasan et al., 2008). The de novo APL cases were consecutively diagnosed and treated at the Department of Biopathology, Policlinico Tor Vergata in Rome between 2001 and 2009. The main clinico–biological features of 9 unreported t-APL cases are described in Table 1. In six of these

TABLE I. Clinical and Molecular Characteristics of 9 t-APL Cases

Patient ID	Age/sex	Primary disease	Therapy	Mitoxantrone schedule	Mitoxantrone total dose (mg)	Interval between MS and APL (mos)	Bcr isoform	*PML* breakpoints	*RARA* breakpoints
GE205	61F	Breast cancer	Radiation and tamoxifen	NA	NA	14	1	1,169–1,170	2,146–2,147
GE477	65F	Corpus uteri carcinoma	Radiation	NA	NA	69	1	1,274–1,276	7,096–7,098
GEI	29F	Multiple sclerosis	Mitoxantrone	10 mg/m^2 every 2 month	95	29	1	1,075	8,375
PAV	31F	Multiple sclerosis	Mitoxantrone	13 mg/m^2/month	39	36	1	1,483–1,485	8,760–8,762
PAL	36F	Multiple sclerosis	Interferon and Mitoxantrone	24 mg/m^2 every 3 month	195	135	3	142–145	12,853–12,856
CTN	27M	Multiple sclerosis	Interferon and Mitoxantrone	14 mg/m^2/month	14	25	1	1,682–1,683	14,585–14,586
GE723	19F	Hodgkin lymphoma	ABVD and Radiation	NA	NA	33	1	1,941–1,942	15,462–15,463
MLN	33M	Multiple sclerosis	Mitoxantrone	10 mg/m^2/month	90	86	1	1,487–1,488	15,583–15,584
VLN	48M	Multiple sclerosis	Steroids and Mitoxantrone	10 mg/m^2 every 3 month	120	180	3	299–302	16,613–16,616

ABVD, adriamycin, bleomycin, vinblastine dacarbazine; NA, not applicable; MS, multiple sclerosis; APL, acute promyelocytic leukemia; mos, months.

nine cases, t-APL developed after mitoxantrone treatment for MS whereas in the remaining three patients (GE205, GE477, and GE723) t-APL followed treatment for breast cancer, corpus uteri carcinoma, and Hodgkin's lymphoma, respectively.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from leukemic blasts using Trizol and reverse transcribed according to the Roche diagnostics cDNA kit manual. *PML-RARA* isoforms were amplified by a reported RT-PCR based method (van Dongen et al., 1999).

Genomic *PML-RARA* Specific PCR and Direct Sequencing

DNA was extracted from frozen bone marrow pelleted cells collected at the time of APL diagnosis, using the salting out protocol. On the basis of isoform type determined by RT-PCR, a long-range nested PCR strategy based on appropriate intronic regions of *PML* (intron 6 for bcr1 and intron 3 for bcr 3) and *RARA* (intron 2) genes was adopted to amplify the genomic *PML-RARA* fusion transcripts. To identify the exact location of *PML* and *RARA* breakpoints, amplified PCR products were sequenced. In brief, all purified amplicons were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in conjunction with GeneAmp 9700 PCR Systems (Applied Biosystems). Each 10 μl sequencing reaction contained the following: 1 μl of BigDye v3.1(Applied Biosystems), 2 μl of BigDye® Terminator v1.1/3.1 Sequencing Buffer (5×), 1 μl of PCR primer (5 μM), ~3 ng/200 bp of purified PCR product, and enough Gibco distilled water (Invitrogen, Grand Island, New York) to bring the total volume to 10 μl. Thermocycling parameters for PCR product sequencing were as follows: 1′ at 96°C; 10′′ at 96°C, 5′′ at 50°C, and 4′ at 60°C for 40 cycles. Sequencing reaction products were purified using the Centri·Sep columns (Applied Biosystems) according to the manufacturer's recommendations. Samples were diluted with 16 μl of ABI HiDi Formamide (Applied Biosystems) and resolved on an ABI 3130 automated sequencer (Applied Biosystems).

Identification of Recombination Prone Sequences at *PML* and *RARA* Loci

ALU elements are rare within the human genome (Stenger et al., 2001) but are known to be hotspots for genomic instability (Gebow et al., 2000; Lobachev et al., 2000; Stenger et al., 2001). A web based tool (http://transpogene.tau.ac.il/) was used for the identification of *ALU* recombination sequences in the vicinity of the *PML* and *RARA* breakpoint regions. Computational analysis was further narrowed on the webpage by selecting all intronic transposable elements (TE) of human alone. The exclusion of other species in the TE selection criteria helps in unbiased interpretation of the data. V-D-J RSS in *PML* and *RARA* genes were searched for using the criteria described elsewhere (Gellert, 1992; Gu et al., 1992; van der Reijden et al., 1999).

Statistical Method

The significance of the cluster regions of *RARA* intron 2 in cases of t-APL or de novo APL was assessed with the use of scan statistics, which are based on the maximal number of events occurring in a prescribed interval. The statistic is defined as follows. Let $N(x, x + d)$ be the number of breakpoints contained in the interval $(x, x + d)$. Then the scan statistic for the prescribed interval length d is $N_d = \max N(x, x + d)$, where the maximum is taken overall positions x such that the interval $(x, x + d)$ is within the intron. This statistic is then referenced against a uniform (null) distribution reflecting the absence of clustering. In the case of translocation breakpoint clustering in *RARA* intron 2, the event is the occurrence of a breakpoint, the interval is the number of base pairs spanning the putative cluster, and the reference interval is the intron length. To compute significance of clustering, we used the accurate, end point-corrected, large deviation approximation to the one-dimensional scan statistic (Segal et al., 2002).

RESULTS AND DISCUSSION

According to breakpoint sequencing data, we identified two potential breakpoint cluster regions in *RARA* intron 2 (GenBank accession number AJ297538), i.e., one at the 5′ proximal end (Chr17: region A, 6,000–9,800; 3.8 kb) and the other one at the 3′distal end (region B, 11,500–15,600; 4.1 kb). *RARA* breakpoints were located in region A in 5 (21%) of 23 t-APL patients including one patient (GE477) who did not receive topoII targeting agents, and in 8 (32%) of 25 de novo APL patients (Fig. 1). Notably, in this region, we observed a 26 bp (Chr17:

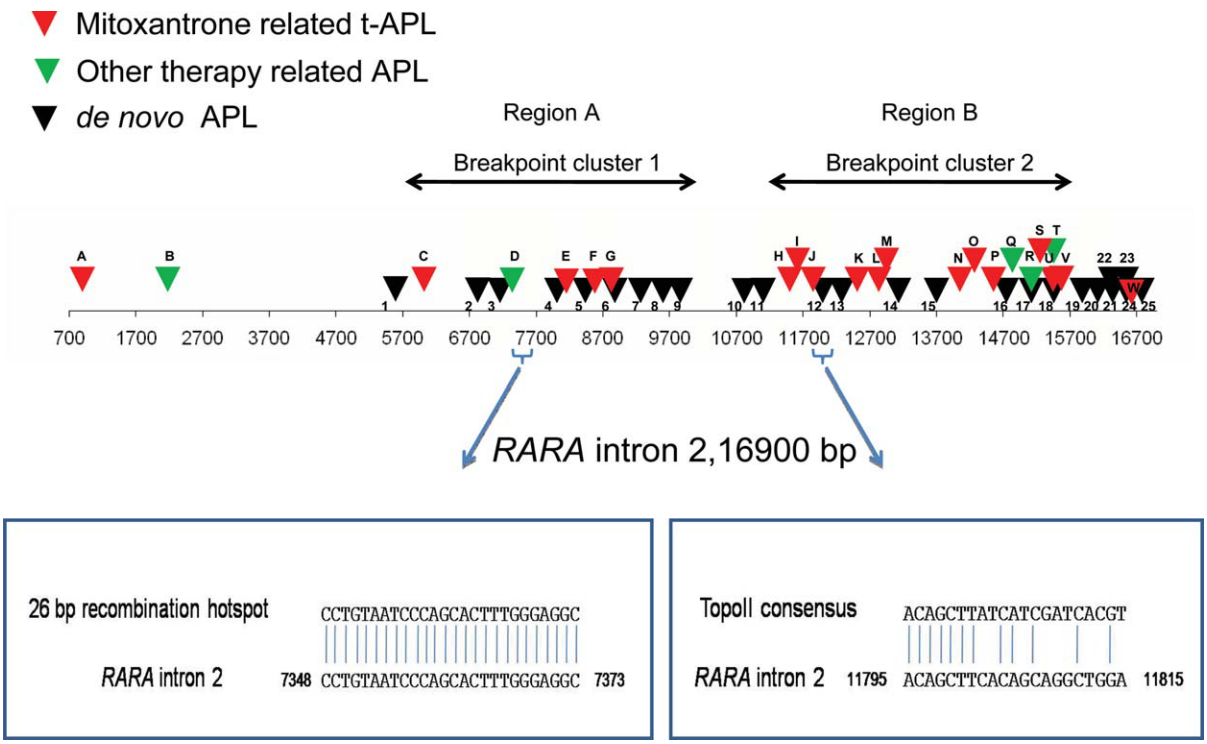

Figure 1. Genomic breakpoint distribution at *RARA* locus in de novo and t-APL patients. Region A include nucleotide location from 6,000 to 9,800 while region B consist of bases from 11,500 to 15,600 of *RARA* intron 2. Recombination prone sequences in region A and topoisomerase consensus in region B of *RARA* intron 2 have been shown. See text for details. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

7,348–7,373) recombination hotspot (RHS), a core sequence that is highly conserved in all reported *ALU* elements and reported to stimulate gene rearrangements (Rudiger et al., 1995) and recombination signal consensus sequences. By assuming that RHS distribute randomly/uniformly on the chromosome 17, we calculated the expected number of 26nt-sequences in a window of 16.9 kb and 3.8 kb region A of *RARA* intron 2. The 26 bp sequence was found only once in the 16.9 kb long region (P value = 0.17) based on Poisson probability distribution. Such distribution supports the hypothesis that occurrence of these nucleotide sequences in region A (3.8 kb) and not in region B (4.1 kb) of *RARA* intron 2 are not just by chance. Moreover, in one t-APL patient (PAV) the *RARA* breakpoint (8,760–8,762) was located within a recombination signal sequence between the last nucleotide of a 23 bp spacer and the first base of a nonamer (Chr17: 8,730–8,768). Matching sequences were 7 per nonamer and 4 per heptamer separated by a 23 bp spacer. *RARA* breakpoints in region B were detected in 15 (65%) of 23 t-APL patients, and 7 (28%) of 25 de novo APL patients (Fig. 1). To determine whether the breakpoints in regions A and B of *RARA* intron 2 differed significantly from a uniform pattern, we used scan statistics. In region A (16,913 bp), none of the breakpoint clusters in de novo APL (eight events), t-APL (five events) and mitoxantrone related t-APL (four events) was found to be significantly different from a random uniform pattern, with P values equaling 1, 0.91, and 0.97, respectively. In region B, breakpoints in t-APL (15 events) and mitoxantrone related t-APL (12 events) were identified to be significantly clustered (P = 0.001 and P = 0.004, respectively). However, no significant clusters were detected in de novo APL (seven events, $P = 1$).

Interestingly, nucleotide sequences of region B (Chr17: 11,795–11,815) showed ~60% homology with a recently identified 21bp long DNA sequence preferentially cleaved by topoII in response to its inhibition (Masliah et al., 2008). On screening the entire *RARA* locus (GenBank accession number NM_000964.2), such high nucleotide homology was not detected elsewhere.

With respect to *PML* breakpoints, in 8/19 (42%) t-APL cases DNA breaks in intron 6

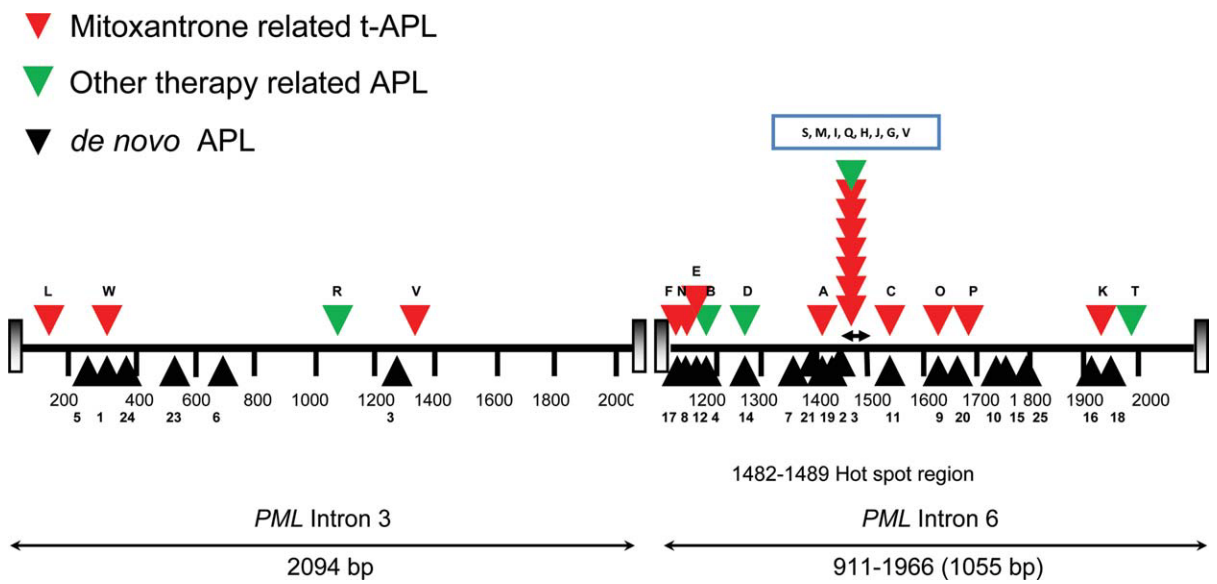

Figure 2. Genomic breakpoint distribution at *PML* locus in de novo and t-APL patients. "Hot spot" region is an 8 bp long nucleotide sequence (AGCCCTAG) from 1,482–1,489 in *PML* intron 6. In both the figures numerals corresponds to de novo APL as described in Table 2 while alphabets are patient identification numbers for therapy-related APL. A, UPN8; C, UPN1; E, UPN7; H, UPN13; I, UPN11; J, UPN14; K, UPN4; M, UPN6; N, UPN5; O, UPN2; Q, UPN12; R, UNP10; S, UPN3; U, UPN9 described elsewhere (Hasan et al., 2008), unreported t-APL patient identification codes are B, GE205; D, E477; F, GEI; G, PAV; P, CTN; T, GE723; V, MLN; W, VLN. GenBank accession numbers *PML* intron 6 (bcr1) S57791, *PML* intron 3 (bcr3) S51489 and native *RARA* intron 2 DNA sequence (GenBank accession number AJ297538) using the BLAST/alignment program of NCBI. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

(GenBank accession number S57791) were located in a previously reported 8 bp "hot spot" region (Fig. 2), corresponding to a preferred site of mitoxantrone-induced DNA topoII cleavage as determined by functional assay (Mistry et al., 2005; Hasan et al., 2008). All except one patient (UPN12) with breaks in this particular region had received topoII targeted treatment with mitoxantrone prior to developing t-APL. In three (GE205, GE477, and UPN10) of the four t-APL cases in which there was no history of exposure to drugs targeting topoII, *PML* intron 6 breaks were located outside this hot spot. *PML* breakpoints in all 18 bcr1 positive de novo APL cases were found to fall outside the 8 bp hotspot region where 7 of 18 mitoxantrone related bcr1 positive patients were clustered ($P = 0.007$) (Fig. 2, and Table 2). Genomic breakpoint junction sequences in 20 of 23 t-APL and 18 of 25 de novo APL cases showed 1–5 common nucleotides between *PML* and *RARA* loci at the breakpoint sites.

The proximal end of *RARA* intron 2 (region A) consists of several recombination prone sequences including a 26-bp *ALU* core sequence that was suggested to be a recombinogenic hot spot. Web-based tools allowed the identification of 9 *ALU* repeat elements in the region of 1,638–9,194 of *RARA* intron 2, whereas no such regions were detected on the *PML* gene. Heptamer and

TABLE 2. Genomic Breakpoint Locations in 25 De Novo APL

Patient number	Bcr isoform	*PML* breakpoints	*RARA* breakpoints
1	3	245–246	5,413–5,414
2	1	1,457	6,752
3	3	1,260–1,261	7,083–7,084
4	1	1,164–1,166	7,906–7,908
5	3	210–211	8,220–8,221
6	3	639–640	8,889–8,890
7	1	1,374	9,286
8	1	1,017	9,631
9	1	1,655–57	9,771–73
10	1	1,743–1,745	11,265–11,267
11	1	1,504–1,507	11,330–11,333
12	1	1,159–1,160	12,289–12,290
13	1	1,458–1,460	12,323–12,325
14	1	1,235	13,150
15	1	1,756	13,700
16	1	1,913–1,916	14,773–14,776
17	1	926–927	14,821–14,822
18	1	1,925–27	15,002–04
19	1	1,419–1,420	15,764–15,765
20	1	1,671	15,825
21	1	1,392–1,394	16,192–16,194
22	2	905–906	16,373–16,374
23	3	437	16,644
24	3	360–361	16,678–16,679
25	1	1,781–82	16,835–36

nonamer like V-D-J RSS were identified at nucleotide 5,296–8,769 of *RARA* intron 2 and interestingly in one patient *RARA* breakpoint was found within V-D-J RSS. The exact mechanisms that render these recombination regions prone to translocation remain unclear. However, Bassing et al. (2002) have proposed the mechanism of RAG-mediated DNA rearrangement through V-D-J RSS. RAG complexes can create DNA double strand breaks that may result in translocation (Leiber et al., 2006). However, although DNA breaks in region A between t-APL and de novo APL were not statistically significant, the presence of recombination prone sequences in this region indicates a recombination mechanism can generate t(15;17) translocation.

The topoII consensus sequences were identified at the distal end of *RARA*, ~300bp downstream of region B where most of the t-APL cases arising after topoII-targeted therapy were clustered. This clustering of genomic breakpoints in *RARA* intron 2 region B was confirmed by scan statistics. Furthermore, previously reported in vitro functional cleavage assays showed that the sequences within region B are indeed cleaved by topoII in response to topoII poisons such as mitoxantrone (Mistry et al., 2005; Hasan et al., 2008).

The occurrence of breakpoint clustering at both *PML* and *RARA* loci in t-APL suggests a different pathogenetic mechanism underlying the disease as compared with de novo APL. By studying genomic breakpoints in de novo APL, Reiter et al. (2003) identified a strong clustering of topoII binding sites at nucleotide position 13,124–13,669 of *RARA* intron 2 which fall inside region B of our study. In agreement with Reiter et al., (2003); no *RARA* breakpoint was found in our series to fall within the previously reported (Tashiro et al., 1994) 50 bp hotspot located at distal end of *RARA* intron 2 (16,539–16,589).

Mays et al. (2010) recently characterized genomic breakpoints in epirubicin related t-APL cases and showed site specific preferences of DNA damage at *PML* and *RARA* loci. The role of topoII poisons in the generation of the t(15;17) translocation was reinforced by our study analyzing genomic breakpoints of t-APL arising in multiple sclerosis patients who received single agent therapy with mitoxantrone (Hasan et al., 2008). In the study reported by Mays et al., (2010) breakpoint locations within the *PML* locus of the epirubicin-related t-APL cases were observed to occur outside the hotspot region in intron 6 (1,482–9) previously mapped in cases occurring after mitoxantrone treatment for breast cancer or multiple sclerosis (Mistry et al., 2005; Hasan et al., 2009). The precise mechanism of different drugs inducing preferential breakage at given genetic loci has remained subject to debate. Compounds that impact the catalytic activity of topoII can be separated into two categories. The first includes drugs such as anthracylines (epirubicin) that decrease the overall activity of the enzyme and are known as catalytic inhibitors (Fortune and Osheroff, 2000; McClendon et al., 2007). Drugs in the second category e.g., etoposide, mitoxantrone increase levels of topoII-DNA cleavage complexes are called topoII poisons (Fortune and Osheroff, 2000; McClendon et al., 2007; Bender et al., 2008).

Genomic breakpoint subcloning of nine patients with de novo AML provided evidence to suggest that the genomic rearrangement involved a recombination event between *ALU* sequences (Strout et al., 1998). In acute lymphoblastic leukemia with 11q23 translocations, the presence of heptamer–nonamer recombination signals in the vicinity of genomic breakpoints has suggested that VDJ recombinase activity is involved in illegitimate recombination events leading to these translocations (Thandla et al., 1997). A similar mechanism has not been identified in patients who develop therapy-related leukemia following treatment of the primary disorder with drugs that target topoII. Although several topoII DNA binding sites have been identified within the 11q23 breakpoint cluster region (bcr) (Gu et al., 1994), the mechanism of translocation associated with these drugs remains unclear.

In summary, this study suggests a different distribution of breakpoints in either *PML* or *RARA* loci in de novo versus t-APL implying differences in t(15;17) translocation mechanism according to disease context. Although there is evidence supporting a role for recombination mechanisms underlying de novo APL, cases arising following exposure to topoII targeted agents are consistent with drug-induced DNA damage followed by repair mediated by the nonhomologous end joining pathway.

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Chapter 5

Genomic characterization of t(16;21) translocation in therapy-related acute myeloid leukemia

GENES, CHROMOSOMES & CANCER 48:213–221 (2009)

RESEARCH ARTICLES

Identification of a Potential "Hotspot" DNA Region in the *RUNX1* Gene Targeted by Mitoxantrone in Therapy-Related Acute Myeloid Leukemia with t(16;21) Translocation

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The translocation t(16;21) involving *RUNX1* (*AML1*) and resulting in the *RUNX1-CBFA2T3* fusion is a rare but recurrent abnormality mostly found in therapy-related acute myeloid leukemia (t-AML) associated with agents targeting topoisomerase II (topo II). We characterized, at the genomic level, the t(16;21) translocation in a patient who developed t-AML after treatment of multiple sclerosis with mitoxantrone (MTZ). Long template nested PCR of genomic DNA followed by direct sequencing enabled the localization of *RUNX1* and *CBFA2T3* (*ETO2*) breakpoints in introns 5 and 3, respectively. Sequencing of the cDNA with specific primers showed the presence of the expected *RUNX1-CBFA2T3* fusion transcript in leukemic cells. The *RUNX1* intron 5 breakpoint was located at nucleotide position 24,785. This region contained an ATGCCCCAG nucleotide sequence showing ~90% homology to a "hotspot" DNA region ATGCCCTAG present in intron 6 of *PML* previously identified in therapy-related acute promyelocytic leukemia cases arising following treatment with MTZ. This study suggests a wider distribution in the human genome, and particularly at genes involved in chromosome translocations observed in t-AML, of DNA regions (hotspot) targeted by specific topo II drugs.

INTRODUCTION

Hematopoietic malignancies are frequently characterized by recurrent chromosomal translocations involving genes that play an important role in the regulation of hematopoietic cell proliferation and differentiation (Renneville et al., 2008). The *RUNX1* (*AML1*) gene at cytogenetic band 21q22 is one of the most frequent targets of chromosomal translocations observed in both de novo acute leukemia and therapy-related myelodysplastic syndrome (t-MDS) and acute myeloid leukemia (t-AML). Translocations involving *RUNX1* have been reported in 15% of t-MDS/t-AML cases, and the most common chromosome/gene rearrangements described in this clinical context are the t(8;21)(q22;q22), t(3;21)(q26;q22) and t(16;21)(q24;q22) translocations involving the *CBFA2T1 (ETO1)*, *EAP/MDS1/EVI1*, and *CBFA2T3* (*ETO2*) genes, respectively (Slovak et al., 2002).

The t(16;21)(q24;q22) is a rare but nonrandom chromosome abnormality associated mostly with t-AML (Nucifora and Rowley, 1995; Gamou et al., 1998; Roulston et al., 1998). It involves *CBFA2T3* (myeloid translocation gene on chromosome 16), which encodes one of the family of novel transcriptional corepressors (MTG proteins) and shows a high degree of homology to the *CBFA2T1* gene, the fusion partner in the t(8;21) (Gamou et al., 1998). The evolutionarily conserved structural features between RUNX1-CBFA2T1 and RUNX1-CBFA2T3 (Gamou et al., 1998) suggests that the two chimeric proteins are both involved in hematopoiesis, as subsequently demonstrated in functional studies (Rossetti et al., 2005).

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As observed in t(8;21), the *RUNX1* breakpoints in the t(16;21) are localized between exons 5 and 6 of *RUNX1*, immediately downstream of a phylogenetically conserved DNA-binding domain, whereas breakpoints in *CBFA2T3* are usually localized between exons 1 and 2 or exons 3 and 4 (Gamou et al., 1998; Zhang et al., 2002). However, to our knowledge, no studies have yet analyzed this aberration at the genomic level.

Genomic studies of translocations associated with t-AML are relevant to identify DNA regions targeted by cytotoxic agents, in particular drugs targeted by topoisomerase II (topo II) and may provide important clues in the understanding of t-AML pathogenesis. Recently, Mistry et al. (2005) demonstrated that drug-induced cleavage of DNA by topo II mediates the formation of chromosomal translocation breakpoints in mitoxantrone (MTZ)-related acute promyelocytic leukemia (APL) and in APL developing after therapy (t-APL) with other drugs targeting topo II.

Here, we report the characterization at the genomic level of the t(16;21) translocation in a 49-year-old man who developed t-AML after treatment with MTZ given for multiple sclerosis (MS). This investigation allowed the identification of a novel potential "hotspot" in the *RUNX1* gene, which is targeted by MTZ and shows a high degree of homology to the previously defined hotspot sequence present in *PML* intron 6 (Mistry et al., 2005; Hasan et al., 2008).

MATERIALS AND METHODS

Case History

A 49-year-old man with a 3-year history of MS was admitted at the Institute of Hematology, Hospital of S. Andrea, University "La Sapienza" of Rome, in February 2008, for severe pancytopenia. He had been treated for MS with MTZ (15 mg every 2 months for 1 year) receiving a total cumulative dose of 90 mg. On admission, laboratory evaluations revealed hemoglobin 8.8 g/dl, WBC 5,130/mm^3 and platelet count 56,000/mm^3. Bone marrow examination disclosed 35% infiltration by peroxidase positive granular blasts with some Auer bodies. Flow cytometric immunophenotyping performed on bone marrow cells demonstrated a predominance of leukemic blasts staining positive for CD33/HLA-DR/CD13/CD19/CD117 and negative for CD14/CD64/CD34/CD9. Based on the morphological and cytochemical findings, a diagnosis of AML-M2 was established according to the French-American-British criteria (Bennett et al., 1976). Patient received standard induction therapy with fludarabin, cytarabine and G-CSF (FLAG regimen), and achieved complete remission in March 2008. He therefore received consolidation chemotherapy according to the same protocol and presently persists in hematologic remission at 5 months from diagnosis.

Conventional Cytogenetic and FISH Analyses

Conventional karyotyping was performed on the bone marrow diagnostic aspirate after short-term culture and analyzed after G-banding. The description of the karyotype was according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

For FISH analysis, fresh slides were prepared from the cytogenetic pellets stored in fixative and allowed to dry for 20 min at 80°C on a hot plate (Hybrite, Vysis, Downers Grove, IL) followed by dehydration at room temperature (RT) in 70, 80, and 100% ethanol (3 min each). Codenaturation was carried out at 68°C for 5 min and hybridization at 37°C in a humid chamber over-night using whole chromosome painting probes of chromosome 16 and 21 (Vysis). Posthybridization washing was done at 72°C in 0.5× SSC for 2 min and for 5 min in 4T solution (SSC 2×, Tween 20). Slides were washed and counterstained with 4′,6′-diamine-2-phenylindole dihydrochloride (DAPI) (Vysis) and analyzed using an Olympus BX65 microscope equipped with a 100-W lamp and a complete set of filters.

Molecular Screening of Recurring AML Fusion Genes and Mutations

Total RNA was extracted from Ficoll-Hypaque-isolated bone marrow mononuclear cells using standard procedures (Chomczynsky and Sacchi, 1987) and reverse-transcribed using random hexamers as primers. According to our routine laboratory protocol for AML genetic diagnosis, cDNA was used to amplify the most common AML gene fusions, i.e., *RUNX1-CBFA2T1*, *PML-RARA*, *CBFB-MYH11*, and *DEK-CAN* as described in protocols standardized by the European BIOMED-I Concerted Action (van Dongen et al., 1999) as well as for the mutational analysis of *FLT3*, *NPM1*, and *JAK2* genes (Baxter et al., 2005; Noguera et al., 2005). High-molecular

TABLE 1. Primers Used in This Study

Number	Name	Sequence	Application[a]
F1	RUNX1-F1	ATCCACTTGGGGCTGGTACAC	LTP
F2	RUNX1-F2	CTTATAGACTCTTTGACCTGGCCTC	LTP
F3	RUNX1-F3	AATCGTATACCTTGCCCAAAGTC	LTP
F4	RUNX1-F4	GTCAGAAAGAAAAGTCACGTGTGG	LTP
F5	RUNX1-F5	TGCCTACTGCACAGGGTTCTTG	LTP
F6	RUNX1-F6	AATGAGGCTGTCATGACACAAAC	LTP
F7	RUNX1-F7	TTCATTCAGCCAACATTAGTGAGC	LTP
F8	RUNX1-F8	GTACTCCAACCTTGTGGTGTTGTC	LTP
F9	RUNX1-F8-1	TGAGTATCCAATTGACTGGCCAA	LTP
F10	RUNX1-F8-2	GCAGCTCGGTTATCAACGAGATA	LTP
F11	RUNX1-F8-3	TGTTCAGAGCTGCATCCTGGTT	LTP
F12	RUNX1-F8-4	CATTGTGAGCCTGAGGGTCAA	LTP
F13	RUNX1-F8-5	TGGCTGTAGACTCTACCACGTCAA	LTP
F14	RUNX1-F8-6	GAGTCACACCATGGCTGACCAA	LTP
F15	RUNX1-F8-7	TGAGACAATGTCAACTGTGCCAA	LTP
F16	RUNX1-F8-8	TATATAGACACTGAGGGGCCCAT	LTP
F17	RUNX1-F8-9	CCAACAATTAATGCGCCTCTT	LTP and nested PCR
R18	CBFA2T3-R1-6	CAACACAACAGAGGCAAT	LTP
R19	CBFA2T3-R1-5	CATTTTACAGGTGGGGAAAC	LTP
R20	CBFA2T3-R1-4	CACAGAATAATGGCTGTGAA	LTP and nested PCR
R21	CBFA2T3-R1-3	ACGACAGGTGTGTTCCTAA	LTP
R22	CBFA2T3-R1-2	GTCAGGACTGTGGACCTT	LTP
R23	CBFA2T3-R1-1	CCTGGCTTGAACGATCTTA	LTP
R24	CBFA2T3-R1	CCACACCTAGTGGAATTCTGGAA	LTP
R25	CBFA2T3-R2	GCTGAGTGTTGTGGCCTCTGT	LTP
R26	CBFA2T3-R3	GGTGACAACACAACCCAGACG	LTP
/	AML1-A[b]	CTACCGCAGCCATGAAGAACC	RT-PCR of *RUNX1/CBFA2T3*
/	CBFA2T3-rev	TGGGTGTGCACGGTGCACCATT	RT-PCR of *RUNX1/CBFA2T3*
/	CBFA2T3-forw	GAGGTCTTCACCGCAGCATC	PCR for genomic *CBFA2T3/RUNX1*
/	RUNX1-rev	CTATTGTGGGGAGCAGGGAG	PCR for genomic *CBFA2T3/RUNX1*
/	CBFA2T3-F1-ex2	CCCAGTGGACAGGAAAGCTAACG	RT-PCR of *CBFA2T3/RUNX1*
/	RUNX1-R1-ex7	GCACAGAAGGAGAGGCAATGGAT	RT-PCR of *CBFA2T3/RUNX1*
/	ABL-A2B-5	GCATCTGACTTTGAGCCTCAG	RT-PCR for *ABL*
/	ABL-A3E-3	TGACTGGCGTGATGTAGTTGCTT	RT-PCR for *ABL*

[a]LTP, long range template PCR.
[b]van Dongen et al., 1999.

weight DNA was isolated by proteinase K digestion and phenol/chloroform extraction (Sambrook et al., 1989) and used for long template PCR (LTP) to characterize the exact chromosome breakpoint position in *RUNX1* (genomic coordinates chr21:35081968-35343465) and *CBFA2T*3 (genomic coordinates chr16:87468768-87570902) genes. *RUNX1* and *CBFA2T*3 gene annotations were adapted using the University of California at Santa Cruz (UCSC) Genome Browser Reference Sequence (Refseq) gene track.

Long Template PCR to Amplify the DNA Spanning Possible Breakpoints in *RUNX1-CBFA2T3*

To characterize the *RUNX1-CBFA2T3* fusion at the genomic level, leukemic DNA was amplified by a two-step LTP method. Seventeen forward and nine reverse primers were designed to cover the 24.7-kb long *RUNX1* intron 5 and 5.5-kb long *CBFA2T3* intron 3 (Table 1 and Fig. 1). LTP containing an enzyme mix of thermostable Taq DNA polymerase and a proof-reading polymerase was performed following the manufacturer's instructions (TAKARA Biotechnology, Dalian Biotechnology, Dalian). In the first round of LTP, carried out using a Gene Amp PCR System 2400 (Perkin-Elmer, Emeryville, CA), 400 ng of genomic DNA, 500 nM of each primer, 400 μM dNTPs, and 2.5 U of TAKARA long Taq polymerase (Biotechnology, Dalian) was used in a total reaction volume of 50 μl. After an initial melting step of 1 min at 94°C, first and second rounds of the reaction consisted of 30 cycles of 98°C for 10 sec and 15 min at 68°C (annealing/extension) followed by a final extension at 72°C for 10 min. For sequencing purposes, nested PCR products were generated using 0.5 μl of the

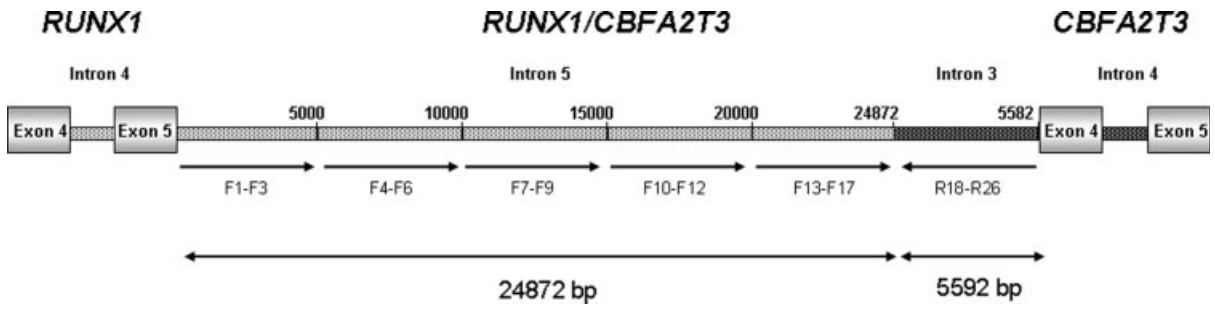

Figure 1. Schematic representation of position of primers for long template PCR. F1-17 are forward (*RUNX1*) primers and R18-26 are (*CBFA2T3*) reverse primers.

second-round LTP reaction and a pair of primers RUNX1-F8-9 and CBFA2T3-R1-4 (Table 1) located in close proximity to the *RUNX1* and *CBFA2T3* breakpoints. Preheating of the mixture at 95°C for 7 min was followed by 30 cycles of 45 sec at 95°C, 45 sec at 55°C, 2 min at 72°C, and final extension of 7 min at 72°C. An aliquot (5 μl) of nested PCR product was subjected to electrophoresis on a 2% (w/v) agarose gel to check the size of the PCR product, and then the remaining PCR product was purified using a QIAquick PCR extraction kit (Qiagen, Chatsworth, CA) for sequencing analysis. Samples were loaded in 96-well plates and covered with mineral oil. The amplified products were separated with a capillary electrophoresis-based system (CEQ 8000 Genetic Analysis system, Beckman Coulter, USA) using the "LFR1 test" default run method.

Based on the *RUNX1-CBFA2T3* fusion and to confirm the genomic breakpoint, 1 μl of cDNA was analyzed using the primers AML1-A and CBFA2T3-rev (Table 1). Preheating of the mixture at 95°C for 7 min was followed by 35 cycles of 30 sec at 95°C, 45 sec at 67°C, 45 sec at 72°C, and final extension of 7 min at 72°C. PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel.

Amplification of the Reciprocal *CBFA2T3-RUNX1* Genomic Breakpoint Junction

On the basis of *RUNX1* and *CBFA2T3* breakpoints, genomic *CBFA2T3-RUNX1* was amplified following the design of specific primers, CBFA2T3-forw and RUNX1-rev (Table 1) using 100 ng of fresh DNA. Preheating of the mixture at 95°C for 7 min was followed by 35 cycles of 30 sec at 95°C, 45 sec at 64°C, 45 sec at 72°C, and final extension of 7 min at 72°C. PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel.

To determine if the reciprocal *CBFA2T3-RUNX1* chimera was expressed, 1 μl of cDNA was amplified using the CBFA2T3-F1-ex2 and RUNX1-R1-ex7 primers (Table 1). Preheating of the mixture at 95°C for 7 min was followed by 35 cycles of 30 sec at 95°C, 45 sec at 58°C, 45 sec at 72°C, and final extension of 7 min at 72°C. An aliquot (5 μl) of PCR product was visualized by electrophoresis on 2% (w/v) agarose gel, and the remaining PCR product was purified with a QIAquick PCR extraction kit (Qiagen) and used for sequencing analysis.

To confirm the integrity of RNA and to ensure adequate cDNA synthesis, for each RT-PCR reaction, the housekeeping *ABL* gene was amplified. A 258-bp fragment was obtained from 1 μl of cDNA using the ABL-A2B-5 and ABL-A3E-3 primers (Table 1). Preheating of the mixture at 95°C for 7 min was followed by 35 cycles of 30 sec at 95°C, 45 sec at 65°C, 45 sec at 72°C, and final extension of 7 min at 72°C. PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel.

All PCR experiments were performed on a Gene Amp PCR system 2400 (Perkin-Elmer) in a 25-μl final volume containing 0.7 U of Taq Gold DNA Polymerase (Applied Biosystems), 10× PCR buffer, 0.2 mmol/l deoxynucleoside-5-triphosphates, 2.5 mM $MgCl_2$, and 10 pmol of each primer.

Alignment of Sequenced Nucleotides Using BLAST Algorithm

Patient's genomic *RUNX1-CBFA2T3* junction sequences were aligned against normal *RUNX1* intron 5 and *CBFA2T3* intron 3 nucleotides as a reference text input in the BLAST/alignment program to detect the microhomologies in the vicinity of breakpoint location.

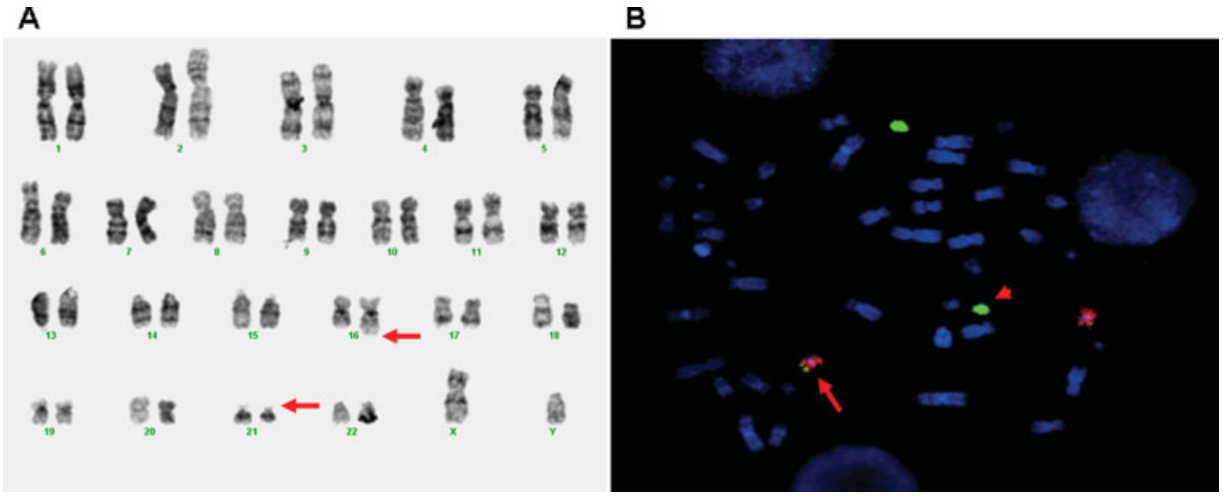

Figure 2. (A) A bone marrow G-banded metaphase showing a 46,XY, t(16;21)(q24;q22) karyotype. The arrows show the rearranged chromosomes. (B) FISH with painting chromosome 16 (red) and chromosome 21 (green). The normal chromosome 16 is red, whereas the der(16) (arrow) has both red and green signals. Both normal 21 and der(21) (arrowhead) show only green signals. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

Conventional Cytogenetic and FISH Analyses

Cytogenetic analysis of bone marrow cells revealed an abnormal karyotype with a translocation involving chromosome 16 and 21 in 5 of 10 metaphases analyzed resulting in the karyotype 46,XY,t(16;21)(q24;q22)[5]/46,XY[5] (Fig. 2A). Dual-color painting FISH analysis showed the translocation of the distal 21q (Fig. 2B) to the 16q derivative, but failed to reveal the reciprocal translocation, suggesting that the 16q segment was too small to be detected with painting FISH analysis or, alternatively, its deletion. However, sequencing results (see later) were in agreement with the former assumption.

Molecular Screening of Recurring AML Fusion Genes and Mutations

Screening for *RUNX1-CBFA2T1*, *PML-RARA*, *CBFB-MYH11*, and *DEK-CAN* fusion genes was negative. Mutational analysis showed no alterations in the *FLT3* and *NPM1* genes. The study of mutational status of the *JAK2* gene showed the presence of the V617F mutation in leukemic cells.

Location of t(16;21) Translocation Breakpoints Within the *RUNX1* and *CBFA2T3* Loci

A LTP strategy followed by direct sequencing was adopted to identify *RUNX1* and *CBFA2T3* breakpoints (Figs. 3A and 3B). After a series of PCRs with different combinations of forward and reverse primers, we amplified the genomic *RUNX1-CBFA2T3* junction region using RUNX1-F8-9 and CBFA2T3-R1-4 primers (Table 1). DNA breakpoints in the *RUNX1* and *CBFA2T3* genes were localized in intron 5 (chr21:35128769-35153640) and intron 3 (chr16:87486395-87491986), respectively. The RUNX1 genomic breakpoint in intron 5 was localized at nucleotide position chr21:35128855-35128856, whereas the breakpoint in *CBFA2T3* intron 3 was detected at nucleotide position chr16:87491463-87491464 with one base microhomology at the breakpoint junction precluding the precise assignment of the breakpoint (Fig. 3D). The *RUNX1* breakpoint region contained an ATGCCCCAG nucleotide sequence showing ~90% homology to a hotspot DNA region ATGCCCTAG contained in *PML* intron 6 gene previously identified in t-APL cases arising following treatment with MTZ (Mistry et al., 2005). Microhomologies at the breakpoint junctions in t(16;21) were indicative of DNA repair by the nonhomologous end-joining (NHEJ) pathway (Lovett et al., 2001a).

Based on the *RUNX1* and *CBFA2T3* breakpoints, the reciprocal *CBFA2T3-RUNX1* genomic translocation was amplified by PCR with specific primers. A fragment of the expected size corresponding to a reciprocal translocation *CBFA2T3-RUNX1* was visualized by agarose gel electrophoresis. Sequencing analysis of the PCR product confirmed a balanced chromosome translocation with no insertions/deletions at the genomic level (Fig. 4).

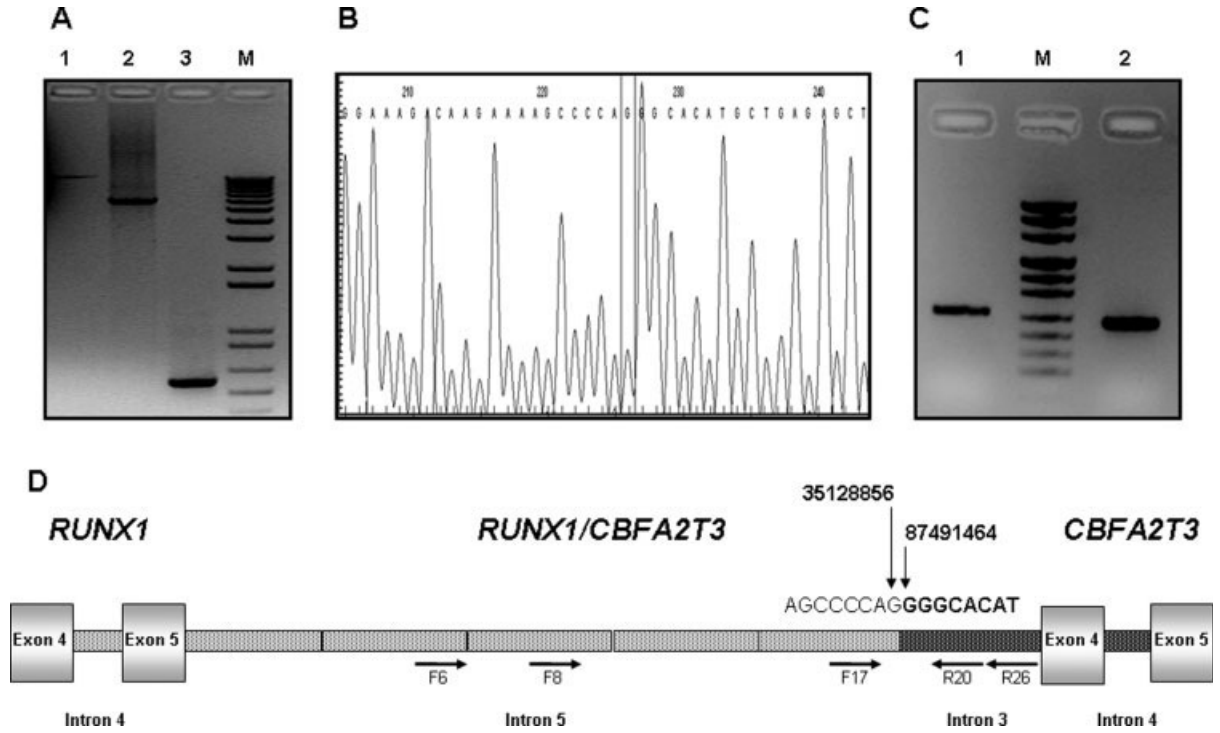

Figure 3. (A) Long template PCR (LTP) for genomic *RUNX1-CBFA2T3* analysis. Lane 1, amplification product of first round of LTP using F6 and R26 primers (10993 bp). Lane 2, second round of LTP using F8 and R26 primers (4991 bp). Lane 3, nested PCR reaction using F17 and R20 primers (570 bp); M molecular weight marker. (B) Sequence trace showing the genomic breakpoint position in *RUNX1-CBFA2T3* fusion genes. (C) RT-PCR analysis of the *RUNX1-CBFA2T3* chimeric transcript. Lane 1, *ABL* amplification used as internal PCR control (258 bp); M, molecular weight marker. Lane 2, *RUNX1-CBFA2T3* amplification product (231 bp). (D) Schematic representation of relevant primers and nucleotide sequences in the vicinity of the breakpoint. *RUNX1* and *CBFA2T3* gene annotations were adapted using the University of California at Santa Cruz (UCSC) Genome Browser Reference Sequence (Refseq) gene track.

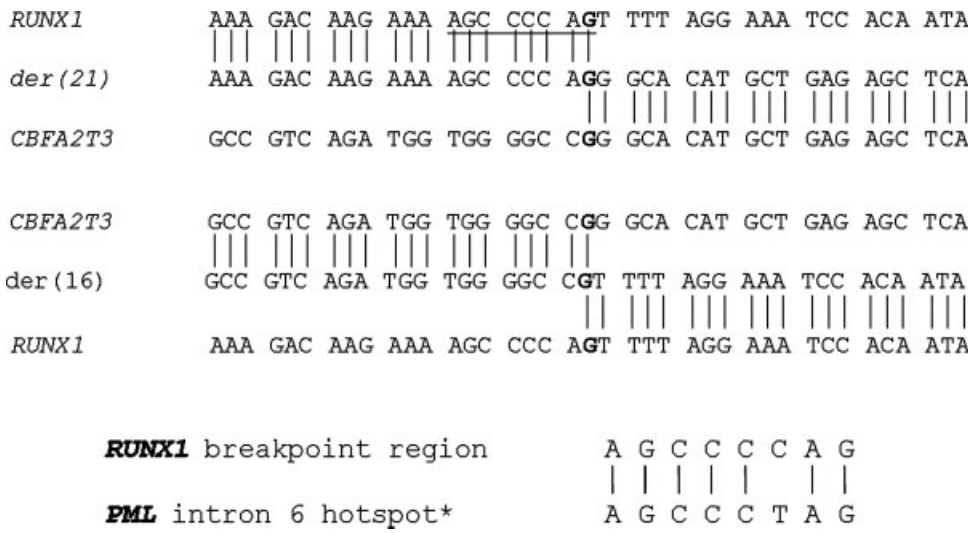

Figure 4. The der(21) and der(16) genomic breakpoint junctions. The nucleotide sequence underlined in *RUNX1* gene shows ~90% homology with a "hotspot" DNA region contained in *PML* intron 6 gene (*Mistry et al., 2005).

The results of RNA studies showed the expression of the *RUNX1-CBFA2T3* chimeric gene corresponding to an expected product size of 231 bp in the diagnostic sample (Fig. 3C). Sequence analysis of the PCR product confirmed this finding and was consistent with fusion

between exon 5 of *RUNX1* and exon 4 of *CBFA2T3* genes in accordance with the genomic breakpoints identified by LTP PCR. The expression of the reciprocal *CBFA2T3-RUNX1* chimera was examined using specific primers; the *CBFA2T3-RUNX1* fusion was not amplified indicating lack of expression of the reciprocal chimeric gene (data not shown).

DISCUSSION

In this report, which represents the first description at the genomic level of breakpoints involved in the t(16;21) translocation in t-AML, we describe a novel potential hotspot DNA region targeted by MTZ. This region shows striking homology to a previously identified region present in the *PML* gene, also targeted by the same agent and described as a preferential DNA break site in t-APL developing after treatment of breast cancer (Mistry et al., 2005). Following the report by Mistry et al. (2005), we have recently analyzed a series of t-APL cases occurring after MTZ treatment for MS at the genomic level. Interestingly, in 5 of 12 cases breakpoints were localized in the same 8-bp *PML* hotspot region identified by Mistry et al. (Hasan et al., 2008).

Twenty-one cases of acute leukemia with the t(16;21)(q24;q22) translocation have been described so far in the literature, including 17 patients who were affected by t-AML developing after chemotherapy and/or radiotherapy (Berger et al., 1996; Shimada et al., 1997; Takeda et al., 1998; Mitelman et al., 2008; Zatkova et al., 2007; Boils and Mohamed, 2008).

Although the type of chemotherapy agents used for the primary tumor is not always detailed in the published series, most patients with this aberration had received agents targeting topo II including MTZ and etoposide. Of the 17 t-AML cases, 13 were analyzed by FISH and/or RT-PCR with demonstration in all instances of the *RUNX1-CBFA2T3* fusion (Boils and Mohamed, 2008), while in none of them a genomic investigation of t(16;21) breakpoints was carried out.

It is well established that t-AML developing after previous therapy with topo II targeting drugs is associated with balanced chromosome translocations such as those involving *MLL* at band 11q23 (Bloomfield et al., 2002; Olney et al., 2002; Rowley and Olney, 2002; Schoch et al., 2003), *RUNX1* at 21q22 (Slovak et al., 2002) and *PML* at 15q22 (Mistry et al., 2005). The resulting gene fusions are likely due to illegitimate recombination that follows DNA damage induced by the drugs. Previous studies have established the presence of functional topo II cleavage sites at translocation breakpoints in *MLL*-associated t-AML (Lovett et al., 2001a,b; Whitmarsh et al., 2003) as well as in the *PML* gene in t-APL (Mistry et al., 2005; Hasan et al., 2008). Combined with the identification of nucleotide microhomologies at the translocation breakpoints, these data suggest that direct DNA damage at hotspot DNA regions coupled with aberrant repair by the NHEJ pathway is likely to be relevant to the formation of these translocations and ultimately to t-AML pathogenesis. By studying cell lines treated with various chemotoxic agents, Stanulla et al. (1997) identified another region within the *RUNX1* locus, located only 360 bp upstream of the hereby described breakpoint, which is highly sensitive to double-strand DNA cleavage induced by various drugs targeting topo II.

Although in vitro cleavage assays are the most reproducible experiments to establish a relationship between topo II targeting agents and translocation breakpoint sites, it should be considered that chromatin structure will limit access to these sites and may therefore change the apparent cleavage preference in vivo. In this context, a study by Mirault et al. (2006) showed that in vivo DNA cleavage by topoisomerase poisoning and apoptotic nuclease contribute synergistically to the generation of translocation breakpoints. Although in this report we did not perform functional in vitro studies to demonstrate the preferential MTZ targeting at the identified breakpoint, the considerably high homology of the detected sequence in our case with the hotspot described in *PML* by Mistry et al. (2005) and Hasan et al. (2008) strongly suggests that the ATGCCCCAG *RUNX1* sequence also represents a preferential target of topo II induced cleavage in the presence of this agent.

In a study by Zhang et al. (2002) focusing on de novo t(8;21), the authors found a strong correlation of genomic breakpoints in *RUNX1* and *CBFA2T1* gene with in vivo topo II cleavage and DNAase I hypersensitive sites. Unfortunately, to the best of our knowledge, no studies which analyze MTZ-associated *RUNX1* breakpoints at the DNA sequence level in either t(8;21) or t(16;21) t-AML patients have been carried out so far. It is likely that these genomic investigations will lead to the identification of other identical or highly homologous hotspots targeted by topo II drugs,

giving rise to other recurrent translocations associated with t-AML.

Although site-specific DNA cleavage induced by drugs targeting topo II at specific genes most likely represent the initial step leading to chromosomal translocations and resultant leukemia induced by these agents, additional events may be required for the development of the full leukemic phenotype. As to the coexistence in this case of the *JAK2* V617F mutation with the t(16;21) translocation, this has not been reported to date. Our finding is in keeping with previous reports of cases of t-AML with both t(8;21) and *JAK2* V617F (Döhner et al., 2006; Lee et al., 2006; Schnittger et al., 2007) suggesting that this alteration may represent a cooperating event in therapy-related leukemogenesis.

In conclusion, our findings highlight the relevance of genomic analysis to characterize DNA breakpoints in therapy-related leukemias and should foster similar investigations in other types of translocations associated with both de novo and secondary leukemia. In particular, comparison of the various aberrations among primary and secondary cases may provide relevant insights into leukemia pathogenesis and should lead to potential identification of novel hotspot regions at involved gene breakpoint sites. These studies may in fact allow the generation of models as to how these chromosomal translocations could have been formed and ultimately improve our understanding of leukemia pathogenesis.

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Chapter 6

To study the genetic markers of susceptibility to t-APL and their association with multiple sclerosis

Introduction

Therapy related acute leukemia (TRAL) has been increasingly reported in patients with Multiple Sclerosis (MS). In contrast to the low incidence reported earlier (0.29%) (Ghalie et al., 2002), recent reports have documented rates of TRAL between 2% and 3% (Pascual et al., 2009). The occurrence of therapy related acute promyelocytic leukemia (t-APL) was surprisingly high (65.6%) amongst all TRAL cases arising after MS (Pascual et al., 2009).

Multiple sclerosis is a heterogeneous neurological disease with different degrees of severity. In 2000 mitoxantrone (MTZ) has been approved by American Academy of Neurology with the aim to prevent the progression of the disease (Marriott et al., 2010). Since no single golden standard for disease severity exists, it is therefore crucial to take into account the reliable clinical predictors of the evolution of the disease. We considered two outcomes for the patients: the Bayesian Risk Estimate for Multiple Sclerosis (BREMS) score (Bergamaschi et al., 2007) which is calculated based on the clinical events of the first year of disease for every patient and progression index (PI). PI corresponds to the ratio between EDSS and disease duration in years.

The data from previous studies have concluded that MTZ reduces clinical attack rates, MRI activity, and disease progression. MTZ exerts anti-proliferative effects on lymphocytes through several mechanisms of action, including inhibition of topoisomerase II enzyme (Fox, 2006; Komori et al., 2009; Marriott et al., 2010; Ory et al., 2008). Although several reports have implicated MTZ in inducing TRAL but the dose dependent effect of MTZ has remained controversial. Of particular note we have previously reported two MS patients who received only 30 and 35 mg cumulative dose of MTZ (Hasan et al., 2008). Besides, there were 2 MS patients who developed leukemia even without MTZ treatment (Hasan et al., 2008). In addition, one case has recently been published of a MS patient developing chronic myeloid leukemia (CML) 16 months after MTZ therapy (Sadiq et

al., 2008). CML is not a recognized TRAL in either the cancer or MS population and therefore it is unclear whether this leukemia resulted from the MTZ therapy.

Given the fact that only a subset of all MS patients treated with MTZ develops t-APL suggests that these individuals may be genetically predisposed toward TRAL. There are data to suggest that genetic factors contribute to t-APL risk. In particular, variants of genes involved in DNA repair pathway are associated with increased t-APL susceptibility (Casorelli et al., 2006; Seedhouse and Russell, 2007). This led us to investigate the genetic variants of DNA repair or factors involved in genomic stability might contribute to t-APL risk in MS population.

Furthermore, specific SNP variants of apoptosis and DNA damage-regulatory genes have recently been described as risk factors for MS (Satoh et al., 2005) and may hence be associated with APL developed as a second tumor (sAPL) occurring in patients with this disease. We, therefore, intend to investigate the possibility that specific genetic variants in DNA repair genes or genes that predispose to MS are significantly associated with sAPL.

Materials and methods

Patients

All patients were initially divided into 2 groups. The sAPL group was composed of 20 patients in whom, primary disease was MS. The median latency between MS and sAPL was 24 months (range 4-60 months). All except 2 patients received MTZ before developing secondary leukemia. The median cumulative dose of MTZ was 105 mg (range 30-234 mg). The detailed clinical and biological features of these patients have been described elsewhere (Hasan et al., 2008) (Hasan et al 2010). The control group was defined as patients with MS (N=271) who 24 months or more after treatment did not develop a secondary malignancy. However, control group was further divided in to 2 subgroups based on the clinical status of disease that is stable vs progressive MS. Type of MS, BREMS score and progression index values were taken into account before classifying

them into stability and progression (Table 1). All RRMS patients with at least 24 months of follow-up (N=152) were considered stable while SPMS (N=34) and PPMS (N=19) were taken as a progressive form of the disease. Patients with incomplete clinical data and/or insufficient follow up were excluded from the study. We have also analyzed a group of 89 healthy individuals.

Selection criteria for genes and SNPs

The selection was based on the fact that the risk of therapy related leukemia may also, in part, be due to inherited genetic factors. These factors include polymorphisms within genes encoding DNA reapir enzymes (Casorelli et al., 2006; Ruttan and Glickman, 2002; Seedhouse and Russell, 2007) (Seedhouse et al BJH 2007, casorelli et al 2003). However, there is also risk -- theoretical, at least -- that disruption in DNA repair may increase the side effects of the drugs that functions through inhibiting DNA repair processess. Apart from DNA repair genes other coding variants of genes were selected prospectively based on their association either with multiple sclerosis or therapy related leukemia as demonstrated previously (Ellis et al., 2008; Felix et al., 1998; Hafler et al., 2007). We have studied 180 SNPs of 26 genes mostly relating to DNA repair pathway (ATM, BRCA1 and 2; CHK1 and 2; LIG 3 and 4; MRE11A; NBS1; PRKDC; RAD 21, 50, 51 and 52; XRCC1, 2, 3, 4, 5, and 6), apoptosis (p53; MDM2), drug detoxification pathways (CYP3A4) and genes that are associated with increased MS susceptibility (HLA-DR; IL2RA; IL7R) (Table 2).

Genotyping and quality control

Genotyping was performed using MassARRAY high-throughput DNA analysis with Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, Inc., San Diego, CA). SNPs were genotyped using iPLEX Gold technology following manufacturer protocol (Sequenom). One hundred and eighty SNPs (Table 4) were subdivided in 9 multiplex assays, designed by MassARRAY Assay Design software (version 3.1). SNPs were

genotyped using iPLEX Gold technology (Sequenom). We performed multiplex PCR in a total volume of 5 µL containing: 0.5 µL of 10xBuffer (2mM MgCl2), 0.4 µL of MgCl2 (2 mM), 0.1 µL dNTPs (500 µM, 1.0 µL of primer mix (500nM each), 0.1 µL of PCR Enzyme (0.5 U/rxn) (Sequenom) and 1 µL DNA (10ng/µL), all concentrations are related to 5 µL. The cycling conditions (DNA Engine® Petier Thermal Cycler Operations Manual (BIO RAD) were 94°C for 4 minutes followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds and 72°C for 1 minutes, and a final extension at 72°C for 3 minutes. PCR primers and dNTPs were removed by incubation with 2 µL SAP Enzyme solution (Sequenom) at 37°C for 40 minutes, followed by 5 minutes at 85°C. The primer extension reaction were performed adding 2 µL of iPLEX Cocktail (Sequenom) containing: 0.2 µL of 10x iPLEX Buffer Plus, 0.1 µL of iPLEX Termination mix 0.94 µL of Primer mix (from 7 µM to 14 µM) and 0.041 iPLEX enzyme. The cycling conditions (DNA Engine® Petier Thermal Cycler Operations Manual (BIO RAD) were 94°C for 30 seconds, followed by 40 cycles at 94°C for 5 seconds, 52°C for 5 seconds and 80°C for 5 seconds for 5 cycles, and a final extension at 72°C for 3 minutes. Then the iPLEX Gold reaction products were desalted with Clean Resin (Sequenom); this cleanup step was important to optimize mass spectrometry analysis of the iPLEX Gold reaction products. Twenty five nL of products were spotting to the SpectroCHIP (Sequenom) using Nanodispenser (Samsung); subsequently the SpectroCHIP was analyzed in the MALDI-TOF MS (Bruker).

Statistical analysis

Allele and genotype frequencies (Pearson X statistics), odd ratio (OR), 95% confidence intervals (CI) and p values, as well as dominant and recessive genetic models, were analyzed using deFinetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Hardy-Weinberg equilibrium of tested groups and Armitage's trend test (ATT) were also calculated using deFinetti. ATT assumes additive (or codominant) disease model where all disease allele are independent and have the same contribution to the disease risk.

For each statistically significant association found (P<0,05), we estimated the false positive report probability (FPRP) using methods described previously (Wacholder et al., 2004). The FPRP method calculates the probability that a single SNP association is a false positive report for a range of prior probabilities. We chose a prior probability of 0,10 and a FPRP cut-off value of 0,50 to identify which findings should be considered "noteworthy". The FPRP values of a range of prior probability from 0,10 to 0,0001 were also reported. FPRP values and the statistical power to detect an OR of 1,5 were computed by the excel spreadsheet provided by (Wacholder et al., 2004). Additional P values were calculated using the omnibus chi-square test, included in this spreadsheet.

A multiple correspondence analysis (CA) was also performed for the associated SNPs. This test is a multivariate exploratory technique suitable for complex tables of categorical data (Greenacre, 1992, 2010). The aim was to graphically display different SNP genotypes (categorical variables) and different patient subgroups (supplementary variables). To be introduced into the analysis, genotypes were categorized into 1, 2, 3 according to the major allele homozygous, minor allele homozygous and heterozygous respectively. Patients sub grouping were submitted to the analysis based on their disease status and were compared with healthy controls and MS patients who developed APL.

CA is of great help to globally understand data and to explore associations between different categories. Categories of data are represented by coordinates which are analogous to principal components of continuous data, with the exception the CA coordinates which are derived from a partition of 2 rather than total variance. Coordinates originate points whose position in a two-dimensional graphical display indicates certain levels of similarity or association of categories. CA is one of the few multivariate techniques available for categorical data without distributional assumptions (Greenacre, 1992, 2010). The analyses were computed using the software CA (BMDP PC-90 Statistical Software ,Los Angeles, CA, USA).

Results

We have successfully genotyped 180 SNPs across 26 candidate genes in total 374 cases. Sixty-two SNPs resulted polymorphic whereas 118 SNPs resulted homozygous for the major allele frequency. These 118 SNPs were excluded prior to the analysis. Allele and genotype frequencies of 62 polymorphic SNPs were compared in different subgroups of patients. Eight case-control comparisons have been performed, grouping the patients in five different categories based on MS, MS stable, MS progressive, MS-APL and healthy controls. Case-control comparisons, patient categories and their numbers are reported in Table 3. Many SNPs showed a statistically significant association in several comparisons, using deFinetti program and standard 'p' value criterion of 0,05 (data no shown). However, when the FPRP method was then applied to reduce the numbers of false-positive findings, only seven SNPs (p53: rs1042522, BRCA1: rs16940, BRCA2: rs1801406, LIG4: rs1805386, XRCC5: rs207906, NBS1: rs1063045 and CYP3A4: rs2740574) in different comparisons remain "noteworthy" (Table 4).

Analysis involving all MS patients versus healthy controls for the 7 significant SNPs revealed only p53 variant allele as a risk factor for MS, while only BRCA2 variant allele is protective, both in univariate as well as in multivariate logistic regression analyses (Table 4 Group A and data not shown).

Restricitng the analysis to MS patients with at least 2 years of follow-up and remained clinically stable versus MS patients who either diagnosed with PPMS at disease onset or progressed to SPMS, we found that the Lig4 variant allele was a risk factor and the NBS1 allele is protective (Table 4 Group B).

In univariate analysis, BRCA2 and CYP3A4 variant were risk factors for MS-APL in RRMS patients who have been followed for more than 2 year after diagnosis of MS. However, including all 7 SNPs into multivariate analysis did not confirm the two alleles to be risk factors (Table 4 Group C and data not shown).

On comparing, all MS patients as single group irrespective of the disease severity with MS-APL, the risk of MS-APL increased by rs1801406 in BRCA2 gene and rs207906 in XRCC5 gene. The GG homozygosity for these SNPs yielded the highest risks. In addition the G allele positivity of rs2740574 in CYP3A4 gene resulted associated MS-APL (Table 4 Group D). On analysing MTZ treated patients with MS-APL, BRCA1 variant allele found to be protective (Table 4 Group E). BRCA2, CYP3A4 and XRCC5 variant allele were significantly associated when compared with MS patients without MTZ treatment (Table 4 Group F). In a group of MS patients based on mitoxantrone treatment analysis revealed that homozygosity of BRCA1 variant allele (CC) was predominant in patients who received mitoxantrone. All the association patterns found by multiple CA were in agreement with the SNP associations obtained by standard case control association tests.

Conclusion

The TRAL results from endogenous or exogenous DNA damage which engages DNA damage response (DDR) pathways in hematopoietic stem and progenitor cells, leading to DNA repair or cell death. Cells that survive with genetic variation in pathways that mediate cellular responses to DNA damage can affect the risk of developing TRAL because of non- or misrepair of damaged DNA (Knight et al., 2009; Nickoloff et al., 2008; Roca, 2009).

Although genetic variation in pathways that mediate cellular responses to DNA damage can affect the risk of developing secondary leukemia but in majority of cases it is not a simple issue, primarily because of the heterogeneity of chemotherapeutic treatments which makes it difficult to analyze the genetic predisposition associated to each drug or radiation. In this context leukemia arising after non malignant disorder such as MS acts as a model system. It provides an opportunity to understand the role of genetic variants in predisposing leukemia development. This study show genetic variants of BRCA2 (rs rs1801406), XRCC5 (rs207906), and CYP3A4 (rs2740574) may

predispose MS patients at higher risk to develop leukemia. Besides, given germ line samples from patients with MS-APL are extremely scarce, the authors are cautious about drawing firm conclusions.

Table 1: Clinical characteristics of multiple sclerosis patients

Type of MS	Number of patients	Age in yrs median (range)	Number of females	BREMS score median (range)	Progression index median (range)	Follow-up in yrs, median (range)	
RRMS	152	30 (13-57)	115	0.21 (-0.01-2.76)	0.2 (0-2)	4 (2.4-23)	
SPMS	34	32 (14-48)	18	0.93 (-0.5-3.42)	0.3 (0-1)	6 (0.8-35)	
PPMS	19	41 (22-52)	11	2.3 (0.64-3.79)	0.44 (0.2-1.33)	7 (0.08-23)	

Table 2: Genes and their respective coding variants included in the study

Gene	SNP ID	Gene	SNP ID
ATM	rs3218690	BRCA1	rs1800062
ATM	rs2234997	BRCA1	rs28897672
ATM	rs2235003	BRCA1	rs55851803
ATM	rs3218706	BRCA1	rs56187033
ATM	rs3218674	BRCA1	rs55688530
ATM	rs1800727	BRCA1	34(1)
ATM	rs4987943	BRCA1	rs1799965
ATM	rs2235000	BRCA1	35(1)
ATM	rs4986761	BRCA1	rs1800063
ATM	rs2229019	BRCA1	rs1799950
ATM	rs3218695	BRCA1	rs55906931
ATM	rs1800056	BRCA1	rs56012641
ATM	rs3218673	BRCA1	41(1)
ATM	rs3218687	BRCA1	rs4986850
ATM	rs3218708	BRCA1	rs1799949
ATM	rs3218688	BRCA1	rs16940
ATM	rs3092857	BRCA1	rs56082113
ATM	rs1800057	BRCA1	rs1800709
ATM	rs3092856	BRCA1	rs41286300
ATM	rs4988008	BRCA1	rs799917
ATM	rs1800889	BRCA1	rs1800740
ATM	rs3218672	BRCA1	50(1)
ATM	rs3218670	BRCA1	rs16941
ATM	rs3218699	BRCA1	rs4986852
ATM	rs3218675	BRCA1	rs2227945
ATM	rs4988125	BRCA1	rs16942
ATM	rs3218680	BRCA1	56(1)

Table 2 (continued)

Gene	SNP ID	Gene	SNP ID
BRCA1	rs28897686	IL2RA	s2104286
BRCA1	58[(1)]	IL7R	rs6897932
BRCA1	rs28897689	LIG3	rs313598
BRCA1	60[(1)]	LIG3	rs1802880
BRCA1	61[(1)]	LIG3	rs3136022
BRCA1	rs1060915	LIG3	rs3136025
BRCA1	rs41293455	LIG4	rs1805389
BRCA1	rs1800744	LIG4	rs1805388
BRCA1	rs55815649	LIG4	rs3093764
BRCA1	66[(1)]	LIG4	rs3093765
BRCA1	rs28897693	LIG4	rs2232638
BRCA1	rs1799966	LIG4	rs2232639
BRCA1	70[(1)]	LIG4	rs2232640
BRCA1	rs41293463	LIG4	rs1805386
BRCA1	rs41293465	LIG4	rs2232641
CHK1	rs3731410	LIG4	rs3093766
CHK2	rs1805129	LIG4	rs2232642
CHK2	rs17883862	MRE11A	rs1061945
CHK2	rs17879961	MRE11A	rs1805364
CHK2	rs17880867	MRE11A	110[(1)]
CHK2	rs17881473	MRE11A	111[(1)]
CHK2	rs17886163	MRE11A	112[(1)]
CHK2	rs17881378	MRE11A	rs1805367
CHK2	rs17882942	MRE11A	114[(1)]
CYP3A4	rs2740574	MRE11A	115[(1)]
DMC1	rs2227914	MRE11A	116[(1)]
IL2RA	rs12722489	MRE11A	rs1805362

Table 2 (continued)

Gene	SNP ID	Gene	SNP ID
NBS1	rs1063045	BRCA2	rs1799952
NBS1	119(1)	BRCA2	rs543304
NBS1	120(1)	BRCA2	147(1)
NBS1	rs1061302	BRCA2	rs4987117
RAD21	rs1050838	BRCA2	rs1799954
RAD50	rs1047380	BRCA2	150(1)
RAD50	rs1047382	BRCA2	rs45574331
RAD50	125(1)	BRCA2	rs1799955
RAD50	126(1)	BRCA2	rs4986860
RAD50	rs1047386	BRCA2	154(1)
RAD50	rs1047387	BRCA2	rs11571833
RAD50	rs1047388	BRCA2	156(1)
RAD50	rs35861031	RAD52	rs4987207
RAD50	rs1804670	RAD52	rs4987208
RAD51	132(1)	XRCC2	rs3218536
RAD51	rs1804269	XRCC5	rs41296400
RAD51	rs1056742	XRCC5	rs41257924
XRCC4	rs28360135	XRCC1	rs1799782
XRCC4	rs1056503	XRCC1	rs915927
BRCA2	137(1)	XRCC1	rs25489
BRCA2	138(1)	XRCC1	rs25491
BRCA2	139(1)	XRCC1	167(1)
BRCA2	rs1801439	TOP2A	rs34300454
BRCA2	rs1801499	TOP2A	rs11656816
BRCA2	rs1799944	TOP2A	rs11540720
BRCA2	rs1801406	TOP2A	rs28969502
BRCA2	rs1799951	TOP2A	rs1804537

Table 2 (continued)

Gene	SNP ID
TOP2A	rs1804539
TOP2A	rs1804538
XRCC3	176[(1)]
RAD54	177[(1)]
RAD54	178[(1)]
RAD54	179[(1)]
XRCC6	rs11557348
XRCC6	rs5758399
XRCC6	rs11557356
XRCC6	rs1803107
PRKDC	rs8178017
PRKDC	rs8178032
PRKDC	rs8178040
PRKDC	rs8178070
PRKDC	rs8178087
PRKDC	rs8178088
PRKDC	rs8178090
PRKDC	rs8178104
PRKDC	rs8178106
PRKDC	rs8178141
PRKDC	rs8178147
PRKDC	rs8178225
PRKDC	rs8178228
PRKDC	rs8178235
PRKDC	rs8178236
PRKDC	rs8178247
PRKDC	rs8178248
PRKDC	rs8178249
P53	rs1042522
MDM2	rs2279744

Note: [(1)] indicates the corresponding reference paper by Ruttan et al, 2002 (included in the reference list below) from which we have selected these SNPs and have given a unique ID instead of rs number.

Table 3: Number of patients and characteristics of case-control analysis for each group.

Comparison	Controls	N	Cases	N
Group				
A	Healthy controls (CTR)	89	All MS patients	271
B	RRMS with >2 years of follow-up	152	SPMS+PPMS	53
C	RRMS with >2 years of follow-up	152	MS-APL	20
D	All MS patients	271	MS-APL	20
E	MS patients treated with mitoxantrone	18	MS-APL	20
F	MS patients without mitoxantrone treatment	253	MS-APL	20
G	MS patients without mitoxantrone treatment	253	MS patients treated with mitoxantrone	18
H	Healthy controls (CTR)	89	All MS patients	271
No significant association	Healthy Controls (CTR)	89	MS-APL	20

Table 4: SNP markers associated with risk of development of MS and/or MS-APL

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group A	rs1042522	p53	GG	52 (58)	126 (47)						
Healthy controls vs All MS patients			GC	35 (39)	119 (45)						
			CC	2 (2)	20 (7)						
						C	1,52 (1,02-2,2)	0,037	0,030	0,026	Allele frequency difference
	rs1801406	BRCA2	AA	42 (47)	141 (54)						
			AG	32 (36)	102 (39)						
			GG	15 (17)	15 (6)						
						G	3,284 (1,53-7,03)	0,0014	0,0201	0,0022	Allele positivity (AA+AG versus GG)

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group B	rs1805386	LIG4	TT	55 (61)							
RRMS with >2yrs of follow up vs			TC	30 (34)							
SPMS+PPMS			CC	4 (4)		T	1,82 (1,08-3,05	0,021	0,025	0,023	Allele frequency difference
	rs1063045	NBS1	GG	68 (46)	29 (54)						
			GA	61 (41)	23 (43)						
			AA	20 (13)	2 (3)						
						A	0,234 (0,051-1,06)	0,04	0,09	0,05	Homozygous (GG versus AA)

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group C	rs1801406	BRCA2	AA	120 (55)	8 (42)						
RRMS with >2yrs of follow up vs			AG	84 (39)	6 (32)						
MS-APL			GG	12 (6)	5 (26)						
						A	6,25 (1,76-22,6)	0,0016	0,022	0,005	Homozygous (AA versus GG)
	rs2740574	CYP3A4	AA	186 (95)	15 (75,0)						
			AG	9 (4,5)	5 (25)						
			GG	1 (0,5)	0						
						G	6,88 (2,04-23,1)	0,00043	0,0030	0,001	Heterozygous (AA versus AG)

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group D	rs207906	XRCC5	GG	166 (64,1)	14 (70,0)						
All MS patients vs			GA	87 (33,6)	3 (15,0)						
MS-APL			AA	6 (2,3)	3 (15,0)						
						A	5,9 (1,3-26,3)	0.0089	>0,05	0.019	Homozygous (GG versus AA)
Group D	rs2740574	CYP3A4	AA	251 (95,1)	15 (75,0)						
All MS patients vs			AG	12 (4,5)	5 (25)						
MS-APL			GG	1 (0,4)	0						
						G	6,4 (2,0-20,4)	0.00038	0.0013	0.0016	Allele positivity (AA versus AG+GG)

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group D	rs1801406	BRCA2	AA	141 (54,7)	8 (42,1)						
All MS patients vs			AG	102 39,5)	6 (31,6)						
MS-APL			GG	15 (5,8)	5 (26,3)						
						G	5,9 (1,79-20,2)	0.0020	0.027	0.0050	Homozygous (AA versus GG)
Group E	rs16940	BRCA1	TT	2 (11,1)	13 (65,0)						
MS patients treated with mitoxantrone vs			TC	12 (66,7)	5 (25,0)						
MS-APL			CC	4 (22,2)	2 (10,0)						
						C*	0,067 (0,012-0,38)	0.00069	0.0039	0.0022	Allele positivity (TT versus TC+CC)
						protective					

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group F	rs1801406	BRCA2	AA	131 (54,6)	8 (42,1)						
MS patients without mitoxantrone vs			AG	94 (39,2)	6 (31,6)						
MS-APL			GG	15 (6,3)	5 (26,3)						
						G	5,46 (1,58-18,83)	0.0033	0.031	0.0720	Homozygous (AA versus GG)
Group F	rs2740574	CYP3A4	AA	234 (95,1)	15 (75,0)						
MS patients without mitoxantrone vs			AG	11 (4,5)	5 (25)						
MS-APL			GG	1 (0,4)	0						
						G	7,09 (2,18-23,05)	0.00021	0.0014	0.0011	Heterozygous (AA versus AG)
Group F	rs207906	XRCC5	GG	152 (63,1)	14 (70,0)						
MS patients without mitoxantrone vs			GA	83 (34,4)	3 (15,0)						
MS-APL			AA	6 (2,5)	3 (15,0)						
						G*	0,072 (0,012-0,44)	0.00046	>0,05	0.0430	Heterozygous (AA versus GA)
						protective					

Table 4 (continued)

Comparison	SNP	gene	Genotype	Controls	Cases	risk allele	OR (95% CI)	P value Pearson's goodness-of-fit chi-square	P value Armitage's trend test	P value Omnibus	The most significant association (test)
Group G	rs16940	BRCA1	TT	105 (44,4)	2 (11,1)						
MS patients without mitoxantrone vs			TC	106 (45,0)	12 (66,7)						
MS patients treated with mitoxantrone			CC	25 (10,6)	4 (22,2)						
						C	8,4 (1,46-48,46)	0.005	0.005	0.0170	Homozygous (TT versus CC)

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Chapter 7

Conclusions and future directions

The work described throughout this thesis has provided credible evidences to support the notion that topoisomerase II plays a central role in the generation of chromosomal translocations. In the current study this mechanism of chromosomal rearrangements mediating via topoisomerase II has largely been demonstrated either in patients who had received topoisomerase II inhibitor before developing leukemic chromosomal translocation or by utilizing information derived from patient's DNA samples in *in vitro* DNA cleavage assays. To this end we have convincingly showed that specific genomic loci within the genome, for example *PML* intron 6, *RUNX1* intron 5 and *RARA* intron 2 at specific locations, are preferential sites of topoisomerase II mediated DNA damage which was significantly enhanced in the presence of chemotherapeutics agents targeting this enzyme. Prof Neil Osheroff rightly describes this enzyme (*Nucleic Acids Res*, 2009) as having Dr. Jekyll/Mr. Hyde character; on one hand proliferating cells cannot exist without topoisomerase II, thereofore it is absolutely essential to cell viability, on the other hand this enzyme has enormous capacity to fragment the genome.

Considering the fact that therapy related leukemia when arises after treatment of primary cancer which involves a number of chemotherapeutics agents and many of these agents have leukemogenic potential which makes it difficult to ascribe a particular role to a specific agent in inducing leukemia. In this context leukemia arising after non malignant disorder such as multiple sclerosis acts as a model system. It provides an opportunity to understand the role of a specific agent that is mitoxantrone, which is used as a single agent chemotherapy with the aim to prevent the disease progression, in inducing chromosomal translocation. During this study we have also noticed that only a subset of all multiple sclerosis patients treated with mitoxantrone develops leukemia which suggests that these individuals may be genetically predisposed toward therapy related leukemia. To address this issue we undertook a study based on genetic variations of DNA repair genes. . This study has shown that the genetic variants of BRCA2 (rs rs1801406), XRCC5 (rs207906), and CYP3A4 (rs2740574) may predispose multiple sclerosis patients at higher risk to

develop leukemia. Currently we are in the process to confirm and validate these results. Therefore we are cautious about drawing any firm conclusion at this point of time.

In spite of putting all the efforts in order to understand the mechanism of chromosomal translocations, there are still some questions that needs to be addressed in future studies. For example during this study we hypothesize the potential role of non-homologous end joining pathway (NHEJ) in the generation of translocations during cleavage-religation process in the presence of topoisomerase II at broken ends of DNA but one has to demonstrate precisely *in vivo* why NHEJ pathway at some time functions erroneously in the presence of DNA damage resulting either from endogenous or exogenous stimulation? However one would not expect topoisomerase II cleavage to depend upon DNA sequence only considering that enzyme alter the topology of supercoiled DNA throughout the entire genome.

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