

# Aberrant somatic hypermutation in primary mediastinal large B-cell lymphoma

*Leukemia* (2005) **19**, 2363–2366. doi:10.1038/sj.leu.2403982;  
published online 6 October 2005

TO THE EDITOR

Primary mediastinal large B-cell lymphoma (PMLBCL) is recognized as a subtype of diffuse large B-cell lymphoma (DLBCL) arising in the mediastinum.<sup>1</sup> However, the clinical, morphological and molecular peculiarities of PMLBCL have been taken to suggest that the disease may represent a distinct clinico-pathologic entity. Clinically, PMLBCL patients display younger median age (35 vs 60 years) and a higher female:male ratio when compared to DLBCL. Also, PMLBCL rarely has extra-thoracic disease at diagnosis, and at relapse preferentially involves extranodal sites.<sup>2</sup> Pathologically, PMLBCL is typically associated with various degrees of sclerosis and consists of a diffuse proliferation of large cells expressing B-lineage markers but lacking surface immunoglobulins.<sup>1,2</sup> At the molecular level, PMLBCL exhibits several genetic abnormalities, including gains of 9p and X that are otherwise absent in DLBCL, and lack genetic lesions, such as *BCL-2* and *BCL-6* rearrangements that are typically associated with DLBCL.<sup>2</sup>

An aberrant activity of the somatic hypermutation (SHM) mechanism, targeting the 5' sequences of *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* proto-oncogenes, has been advocated as a molecular feature distinctive of DLBCL. Aberrant SHM affects >50% of DLBCL of immunocompetent hosts, while it is rare or absent among other B-cell malignancies.<sup>3</sup> The involvement of aberrant SHM in PMLBCL has been suggested by a recent report based on a limited number of cases.<sup>4</sup> These observations prompted our comprehensive analysis aimed at exploring the involvement of aberrant SHM of *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* in a sizeable panel of PMLBCL and at comparing the mutational spectrum with that of systemic DLBCL.

This study was based on 19 primary samples of PMLBCL. The clinical characteristics of this series were consistent with PMLBCL diagnosis (median age of 37 years; male:female ratio of 0.8; Ann Arbor stage I–II). Diagnosis was pathologically confirmed by three expert hematopathologists (MP, AC and SAP) according to the World Health Organization classification of hematopoietic tumours.<sup>1</sup> Malignant cells expressed CD20 in all cases, CD30 in 16 cases and CD23 in 13 cases, but lacked CD15. For comparison, 19 systemic DLBCL, matched for stage, sex and age, were also analysed. Mutations of *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* genes were detected by DNA direct sequencing.<sup>3</sup> The presence of intraclonal heterogeneity, indicative of ongoing SHM, was assessed by sequencing cloned PCR products generated using the proof-reading Pfu Turbo polymerase.<sup>3</sup> The prevalence of aberrant SHM in PMLBCL and DLBCL was compared by the  $\chi^2$  test. Differences in the mutation frequency between PMLBCL and DLBCL were analysed by the *t*-test. Mutation frequencies were normalized based on the nucleotide composition of the sequences analysed. The

normalized mutation frequency of nucleotides occurring in the context of an RGYW/WRCY motif was compared to the expected mutation frequency by the goodness-of fit  $\chi^2$  test.

Mutation analysis of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* is summarized in Table 1. Overall, the prevalence of aberrant SHM did not significantly differ between PMLBCL and DLBCL. Mutations targeting at least one of the four proto-oncogenes were found in 14/19 (73.6%) PMLBCL and 13/19 (68.4%) DLBCL, while mutations targeting more than one gene were found in 7/19 (36.8%) PMLBCL and 9/19 (47.3%) DLBCL. Each of the four genes analysed was altered in a significant fraction of PMLBCL and DLBCL, since *PAX-5* was mutated in 9/19 (47.3%) PMLBCL and 7/19 (36.8%) DLBCL, *RhoH/TTF* in 6/19 (31.5%) PMLBCL and 8/19 (42.1%) DLBCL, *PIM-1* in 3/19 (15.7%) PMLBCL and 7/19 (36.8%) DLBCL, and *c-MYC* in 6/19 (31.5%) PMLBCL and 5/19 (26.3%) DLBCL.

The detailed characterization of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* mutations is reported in Tables 1 and 2. Overall, the molecular profile of mutations was similar between PMLBCL and DLBCL. A total of 74 mutational events were detected in PMLBCL. The overwhelming majority of the mutations included single base-pair substitutions ( $n=66$ ), whereas deletions of a short DNA stretch were observed in eight instances. Of the 66 single base-pair substitutions observed, 41 were transitions and 25 were transversions, with a transition/transversion ratio of 1.64 (expected 0.5;  $P=0.001$ ). When considering all the nucleotide substitutions observed, analysis of the nucleotide exchange pattern showed that G + C base pairs were targeted 3.33-fold more frequently than A + T (expected 1.28;  $P=0.009$ ). In all, 19 of 66 single base-pair substitutions detected in PMLBCL fell within RGYW/WRCY motifs. Considering all the genes together, the frequency of mutations targeting RGYW/WRCY motifs was significantly higher than the frequency of mutations occurring outside RGYW/WRCY motifs (2.3 vs 1.3%;  $P=0.03$ ). As mutations introduced by physiological SHM preferentially affect specific dinucleotides,<sup>5</sup> we also analysed the distribution of mutations within dinucleotide motifs. Considering together all the four proto-oncogenes, the mutation frequency was higher than expected in the following motifs: AA (4.3 vs 1.3%;  $P<0.0001$ ), AG (3.0 vs 1.3%;  $P=0.028$ ), GC (5.1 vs 1.1%;  $P<0.0001$ ), GG (4.2 vs 1.1%;  $P<0.0001$ ) and TA (4.6 vs 1.3%;  $P=0.003$ ).

Among DLBCL, a total of 87 mutational events were detected. Mutations were preferentially represented by single base-pair substitutions ( $n=81$ ), whereas only four deletions and two insertions of a short DNA stretch were observed. Of the 81 single base-pair substitutions, 42 were transitions and 39 were transversions, with a transition/transversion ratio of 1.07 (expected 0.5;  $P=0.02$ ). Analysis of the nucleotide exchange pattern showed that G + C base pairs were targeted 1.89-fold more frequently compared to A + T (expected 1.28;  $P=ns$ ). In all, 41 out of 81 single base-pair substitutions fell within RGYW/WRCY motifs. The frequency of mutations targeting RGYW/WRCY motifs was significantly higher than the frequency of mutations occurring outside RGYW/WRCY motifs (5.0 vs 1.7%;  $P<0.0001$ ). Similarly to PMLBCL, also in DLBCL the mutation frequency was higher than expected in specific dinucleotide motifs: AA (6.4 vs 1.5%;  $P<0.0001$ ), AC (5.4 vs 1.4%;  $P<0.0001$ ), AG (4.5 vs 1.6%;  $P<0.0001$ ), AT (4.2 vs 1.7%;  $P=0.033$ ), CT (4.4 vs 1.6%;

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Received 6 April 2005; accepted 6 September 2005; published online 6 October 2005

**Table 1** Mutation analysis of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* in PMLBCL and DLBCL

Case	Mutation type and position <sup>a,b</sup>				
	<i>PAX-5</i>	<i>RhoH/TTF</i>	<i>PIM-1</i>	<i>c-MYC</i> exon 1	<i>c-MYC</i> exon 2
<b>PMLBCL</b>					
1	—	—	—	—	—
2	G847A, C1108A, G1299A	—	—	—	—
3	C853G, $\Delta$ 1337–1358	—	—	—	—
4	—	—	—	—	—
5	T788C, C848G, C853A, A885T G971A, $\Delta$ 1046–1048, G1079C G1161A, G1193C, C1207T, C1210A G1286T, G1308A, C1334G, G1347A $\Delta$ 1434–1450	—	T1353A T1354C G1368C:Gly28Ala	C3203G	—
6	—	—	—	—	—
7	—	—	—	—	—
8	C917T, G943A, C1087G	—	—	—	—
9	—	C855G	—	—	—
10	$\Delta$ 886–953	A227T	—	G3019A	—
11	G1025A (+/+), G1175A	—	—	—	—
12	T1356C	G821A	—	—	—
13	C1112T	T987C, $\Delta$ 418–448 (+/+)	G1335A	—	—
14	—	—	—	C2879T, A2884T (+/+), T2905G G2946A (+/+), G3026T (+/+) G3027A (+/+), G3028T (+/+) A3079G (+/+), A3098G (+/+) C3119G (+/+), C3203T (+/+) A3248G (+/+)	—
15	—	G165A	—	$\Delta$ 3224	C4695T:Pro59Ser G4792A:Gly91Glu
16	$\Delta$ 885–894	—	—	—	—
17	—	—	C1071T	G3049A, A2772G	—
18	—	T756C	—	—	G4711A:Ser64Lys
19	—	—	—	—	—
<b>DLBCL</b>					
20	—	—	—	—	—
21	—	T614A, T747G	G1518C, C1531G	—	—
22	A1181T	—	—	—	—
23	—	G859T, A984C	—	C2438T, C2687G, G3321T	$\Delta$ 4976–4980 frameshift
24	G1435T A1428C G1287C $\Delta$ 806–821 +GGG 1269	A459C, T477C, G479A A619T, A693G, G694C A777G, A853C, C855T G881A, T901G, T916G G924A, T947C, A957C T976G, T987A, T992A T1043G, G1061T	C1756T	—	—
25	—	G1029C	—	—	C5045T
26	—	G799C	G1568T	—	—
27	G904A, C905G, T906C, C1046T C1423T, +G 1417	G597A G875A	C1201T: His11Tyr C1087T	—	—
28	G672A, G701C, C886T	—	—	—	—
29	G1325C, C1334G C1436G, A1428G	T417C, T472C A985G, $\Delta$ 413	G1623A, C1128T	—	—
30	—	—	C1689G G1774A: Gly99Ser G1857A	G3022A (+/+) G3028A (+/+)	—
31	—	—	—	—	—
32	—	—	—	—	—
33	—	—	—	—	—
34	—	—	—	—	—
35	G1435A, A1428C	—	—	C2734A	—
36	—	—	—	—	—
37	—	C544G, T710C T778C, G843A, C855G	—	—	—
38	$\Delta$ 829–830	—	G1368A (+/+) :Gly28Asp C1858T:Gln127Stop	G2540C, C2541T T2542A, A2543G	—

<sup>a</sup> $\Delta$ , deletion; —, wild-type sequence; (+/+), apparently homozygous mutations (apparently homozygous mutations may be a consequence of mutations affecting the primer binding sites, which may prevent amplification of both alleles or may be related to the deletion/translocation of one allele).

<sup>b</sup>Numbering according to GenBank accession Nos. AF386792 (*PIM-1*), AF386791 (*PAX-5*), AF386789 (*RhoH/TTF*) and X00364 (*c-MYC*).

$P < 0.0001$ ), GC (5.1 vs 1.1%;  $P < 0.0001$ ), GG (3.5 vs 1.6%;  $P < 0.0001$ ) and TA (8.6 vs 1.5%;  $P < 0.0001$ ).

In *PIM-1* and *c-MYC*, a number of mutations were located in coding exons and led to amino-acid substitutions, with potential functional consequences. In particular, among PMLBCL, one

missense mutation in case 5 affected *PIM-1* exon 2 leading to the substitution of Ala for Gly at position 28 of the amino-acidic sequence. Three missense mutations in cases 15 and 18 affected *c-MYC* exon 2, leading to the substitution of Ser for Pro at position 59, Glu for Gly at position 91 and Lys for Ser at position

**Table 2** Features of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* mutations in PMLBCL and DLBCL

	Mutation frequency (range) <sup>a,b</sup>	Transitions/transversions	RGYW-WRCY	G+C/A+T	Single bp substitutions	Deletions/insertions
<i>PIM-1</i>						
PMLBCL	0.09 (0.05–0.16) × 10 <sup>-2</sup>	3/2 (1.50)	3	3/2	5	0/0
DLBCL	0.11 (0.05–0.17) × 10 <sup>-2</sup>	10/4 (2.50)	9*	14/0	14	0/0
<i>PAX-5</i>						
PMLBCL	0.20 (0.06–0.93) × 10 <sup>-2</sup>	15/11 (1.36)	9	23/3	26	5/0
DLBCL	0.18 (0.06–0.35) × 10 <sup>-2</sup>	8/10 (0.80)	11*	14/4	18	2/2
<i>RhoH/TTF</i>						
PMLBCL	0.08 (0.06–0.18) × 10 <sup>-2</sup>	4/2 (2.0)	0	3/3	6	2/0
DLBCL	0.27 (0.06–1.18) × 10 <sup>-2</sup>	16/20 (0.80)	14	14/22	36	1/0
<i>c-MYC</i> exon 1						
PMLBCL	0.20 (0.04–0.84) × 10 <sup>-2</sup>	16/10 (1.60)	6	16/10	26	1/0
DLBCL	0.11 (0.04–0.15) × 10 <sup>-2</sup>	7/5 (1.40)	6	10/2	12	0/0
<i>c-MYC</i> exon 2						
PMLBCL	0.13 (0.08–0.17) × 10 <sup>-2</sup>	3/0	1	3/0	3	0/0
DLBCL	0.08 (0.08–0.08) × 10 <sup>-2</sup>	1/0	1	1/0	1	1/0
<i>All genes</i>						
PMLBCL	NA	41/25 (1.64)*	19*	48/18 (3.33)*	66	8/0
DLBCL	NA	42/39 (1.07)*	41*	53/28 (1.89)	81	4/2

<sup>a</sup>NA: not applicable.<sup>b</sup>Calculated on the entire region analyzed and on mutated cases only, considering two alleles/gene/case.\**P* < 0.05.

64 (Table 1). Among DLBCL, three missense mutations affected respectively *PIM-1* exon 1 (case 27), leading to the substitution of Tyr for His at position 11; *PIM-1* exon 2 (case 38), leading to the substitution of Asp for Gly at position 28; and *PIM-1* exon 4 (case 30), leading to the substitution of Ser for Gly at position 99. One non-sense mutation affected *PIM-1* exon 4 (case 38), truncating the amino-acidic sequence at position 127. With respect to *c-MYC*, a short DNA stretch deletion led to a frameshift mutation within exon 2 in case 23 (Table 1).

To investigate whether the aberrant mutational activity is ongoing in PMLBCL, we selected the gene that displayed the highest mutation frequency, namely *PAX-5*. Among PMLBCL carrying mutations of *PAX-5*, a subset of cases (*n* = 4), shown by direct sequencing to carry the highest mutation frequency, were analysed for the presence of intraclonal heterogeneity. The results showed that, in all PMLBCL analysed, one or two predominant alleles recapitulate the mutations observed by direct sequencing of the PCR product, confirming their presence in the tumor clone (not shown). Ongoing SHM was found in the *PAX-5* sequence of only 1/4 PMLBCL examined, namely case 5.

The implications of our results are two-fold. First, aberrant SHM is a frequent event in PMLBCL and may contribute to the pathogenesis of the disease. Second, because aberrant SHM targets both PMLBCL and DLBCL with similar prevalence, distribution and mutational pattern, our results disclose some degree of identity in the molecular pathogenesis of these two lymphoma types.

The molecular pathogenesis of PMLBCL is largely unknown.<sup>2</sup> Here, we document that 73% PMLBCL are targeted by aberrant SHM of at least one of four proto-oncogenes, namely *PAX-5*, *RhoH/TTF*, *PIM-1* or *c-MYC*. These genes encode proteins with different functions since they include both signal transducers (*PIM-1* and *RhoH/TTF*) and transcription factors (*PAX-5* and *c-MYC*) involved in B-cell development/differentiation, proliferation and apoptosis.<sup>3</sup> Activation of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* may be relevant for B-cell lymphomagenesis, since, as previously noted, these four genes represent known proto-

oncogenes that have been implicated in lymphoma-associated chromosomal translocations.<sup>3</sup>

In PMLBCL, aberrant SHM may alter the function of *PAX-5*, *RhoH/TTF*, *PIM-1*, and *c-MYC* by at least two modalities. As mutations cluster around the gene 5' untranslated region, it is conceivable that mutations may deregulate gene transcription by affecting specific regulatory regions, with a mechanism analogous to that observed for *BCL-6* and *c-MYC* in DLBCL and Burkitt's lymphoma, respectively.<sup>6,7</sup> A subset of mutations of *c-MYC* and *PIM-1* in PMLBCL lead to amino-acid substitutions, and, consequently, may alter the biochemical and/or structural properties of the protein. In particular, *in vitro* and *in vivo* studies have shown that mutations in the *c-MYC* transactivation domain can deregulate *c-MYC* function by interfering with its phosphorylation, protein stability or the repression of its transactivation activity by the Rb-related protein p107.<sup>8</sup> In two PMLBCL samples, three missense mutations were found within the sequences of *c-MYC* exon 2 that encode the transactivation domain (Table 1). Additionally, one missense mutation of *PIM-1* affected the protein ATP-binding site, and predicted a change in the structure and, potentially, in the function of this serine/threonine protein kinase (Table 1).

In our study, we demonstrate that aberrant SHM affects both PMLBCL and DLBCL with similar prevalence. Moreover, the molecular profile of aberrant SHM in PMLBCL is superimposable to that observed in DLBCL and is reminiscent of the mutational spectrum of immunoglobulin variable genes.<sup>3</sup> In fact, mutations in the *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* proto-oncogenes (i) are predominantly represented by single nucleotide substitutions, with occasional deletions and insertions; (ii) display a preference for transitions over transversions and an elevated ratio of G + C over A + T substitutions; and (iii) display a preferential distribution within the RGYW/WRCY motifs. Recent reports have suggested that the mutation profile of the physiological SHM process targeting the *BCL-6* gene may differ between PMLBCL and DLBCL.<sup>5</sup> With respect to aberrant SHM, though, our data do

not reveal any difference in the mutation profile of PMLBCL vs DLBCL, since in both neoplasms mutations of *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* preferentially affect dinucleotides that are included within RGYW/WRCY motifs.

## Acknowledgements

This study has been supported by Ricerca Sanitaria Finalizzata and Ricerca Scientifica Applicata, Regione Piemonte, Torino, Italy; PRIN 2005 – MIUR, Rome, Italy; Istituto Superiore di Sanità, Programma Nazionale di Ricerca sull'AIDS – Progetto Patologia, Clinica e Terapia dell'AIDS, Rome, Italy; AIRC, Milan, Italy; and Novara-AIL Onlus, Novara, Italy. EB has been supported by Fondazione 'Piera Pietro Giovanni Ferrero', Alba, Italy. LP is a special fellow of the Leukemia & Lymphoma Society.

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## A rare case of acute myeloid leukemia with a CHIC2-ETV6 fusion gene and multiple other molecular aberrations

*Leukemia* (2005) **19**, 2366–2368. doi:10.1038/sj.leu.2403957; published online 13 October 2005

### TO THE EDITOR

Acute myeloid leukemia (AML), like other human cancers, is the consequence of more than one mutation. Data from animal models of leukemia strongly support a multistep pathogenesis of the disease.<sup>1</sup> Chromosomal translocations most frequently target transcription factors involved in the regulation of normal hematopoietic differentiation, whereas molecular mutations often affect genes involved in signal transduction pathways associated with cell proliferation. Here, we present a case with multiple genetic and molecular defects targeting transcription factors as well as tyrosine kinases in a rare constellation.

A 39-year-old woman was referred to the hospital in July 2004 due to tonsillitis, hematomas and weakness. The laboratory data showed a white blood cell count of 39 000/ $\mu$ l with 85% blast cells, hemoglobin 7.1 g/dl and platelet count 70 000/ $\mu$ l. Bone

marrow examination revealed 95% myeloblasts with little differentiation. The diagnosis was AML M1 according to FAB classification. Cytogenetic analysis of bone marrow cells revealed a t(4;12)(q12;p13) as well as a dic(7;12)(q11.2;p13). On the molecular level, the molecular correlate of the t(4;12)(q12;p13) a *CHIC2-ETV6* fusion transcript could be demonstrated (Figure 1a, b). In addition a *FLT3* length mutation (*FLT3-LM*) (Figure 1c), a mutation of *NRAS* codon 12 (Figure 1d), *MLL-PTD* and a p53 mutation (*TP53*) (Figure 1e) were detected. After being treated according to the German AMLCG study with HAM/HAM induction and TAD consolidation, the patient received allogeneic bone marrow transplantation from her HLA matching sister in January 2005. The patient had a good initial response to chemotherapy as well as to transplantation and attained morphologic and cytogenetic remission until now. As target for minimal residual disease monitoring, the *MLL-PTD* was used, reflecting the clinical follow-up (Figure 1e).

*CHIC2* was initially described in the t(4;12)(q11;p13) translocation and is an in-frame fusion gene generated between *CHIC2* (previously BTL) (exons 1–3) and the *ETV6* gene (exons 2–8).<sup>2</sup> Probably due to its infrequent occurrence in AML no association with other mutations has been described yet. *FLT3* length mutations (*FLT3-LM*) are frequently found in AML and are associated with a potentially worse prognosis.<sup>3</sup> However, this seems to be restricted to cases with a high *FLT3-LM/WT* ratio.<sup>4</sup> In the present case only a low ratio

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Received 25 July 2005; accepted 12 August 2005; published online 13 October 2005