Molecular characterization of identical, novel MLL-EPS15 translocation and individual genomic copy number alterations in monozygotic infant twins with acute lymphoblastic leukemia

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Haematologica 2012 [Epub ahead of print]

doi:10.3324/haematol.2012.065730

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Molecular characterization of identical, novel MLL-EPS15 translocation and individual genomic copy number alterations in monozygotic infant twins with acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) occurring in the first year of life is rare, accounting for 2-5% of pediatric ALL cases. Infant ALL is distinguished by unique clinical and biological characteristics, with an aggressive course following a short latency period. The mixed lineage leukemia (MLL) gene, located on chromosome 11q23, is involved in 80% of cases. Over 70 different MLL-fusion partner genes have been molecularly characterized,1 with t(4;11), t(9;11) and t(11;19) occurring most frequently in infant ALL.

The outcome of MLL-rearranged infant ALL remains poor with up to 50% five-year survival. The uncommon nature of ALL in infancy limits the rate of accrual for clinical trials and evidence for the best therapeutic approach has been conflicting, particularly regarding the benefit of hematopoietic stem cell transplantation. The clinical heterogeneity exhibited by ALL in infancy, such as poorer outcome under 90 days of age,2 underlies the need for stratification on these trials. The age-related difference in outcome may be a reflection of underlying molecular characteristics with differences in gene expression profiles according to age.3

Risk stratification according to MLL translocation partners has not been undertaken due to small patient numbers and as the same biological process was thought to occur irrespective of the partner gene, for MLL-mediated leukemogenesis. However, recent identification of distinct molecular differences among the MLL subtypes, including variations in epigenetic4 and gene expression profiles,3 has given rise to the notion that the many fusion partners of
MLL should be considered as distinct entities. The complex interplay between the MLL translocation and these additional molecular differences is not fully understood but further characterization of cases will contribute to the current knowledge in understanding the clinical heterogeneity of the disease.

The t(1;11) (MLL-EPS15) translocation is rare with a small number of clinical cases published and three reports of molecular characterization at the transcriptional level. The EPS15 (epidermal growth factor receptor pathway substrate 15) gene encodes a protein that is involved in receptor-mediated endocytosis of epidermal growth factor. We present a MLL-EPS15 rearrangement with novel breakpoints at the transcriptional and DNA level in monozygotic infant twins with ALL and characterization of the molecular changes in their leukemic cells. The methods are described in the Online Supplementary Appendix. Both twins were diagnosed at seven weeks of age, with peripheral blood blast populations of 99.96x10⁹/L in Twin One and 154.87x10⁹/L in Twin Two. CD19+, CD24+, CD10–, B-precursor ALL was confirmed on immunophenotyping. Banded chromosomal analysis revealed a 46,XX,t(1;11)(p32;q23)[13]/46,XX[7] karyotype in Twin One and a 46,XX,t(1;11)(p32;q23)[8]/ibid+X[4]/46,XX[8] karyotype in Twin Two, with fluorescent in situ hybridization confirming MLL involvement.

The MLL-fusion transcripts were sequenced and novel, identical breakpoints were identified in both twins, as shown in Figure 1a. For the MLL-EPS15 transcript, the breakpoint was located at the end of exon 8 of MLL, which was fused to exon 10 of EPS15. For the EPS15-MLL transcript, the breakpoint was located at the end of exon 9 of EPS15, which was fused to exon 10 of MLL. Skipping of MLL exon 9 was therefore evident at the transcriptional level. A novel, identical breakpoint was also identified at the DNA level, as shown in Figure 1b. The
genomic breakpoint occurred within exon 9 of *MLL* and intron 9 of *EPS15*. A deletion of 10bp from exon 9 of *MLL* coincided with a duplicated 10bp segment from intron 9 of *EPS15*. These findings are consistent with and provide the first known evidence for the non-homologous end joining mechanism (NHEJ) of DNA repair occurring in the leukemic cells of infants with the t(1;11) translocation. NHEJ has previously been shown in infants with the t(4;11) translocation.9

In order to detect molecular aberrations that may be unique to each twin, the Affymetrix Cytogenetics Whole-Genome 2.7M Array technology was applied. Each twin had two copy-number alterations (CNAs), shown in Table 1, that were considered tumor-associated genomic aberrations. One CNA was common in both twins, namely amplification at Xp21.1, which is a gene poor region. This region has previously been identified with a 50kbp deletion in one infant with t(4;11) *MLL*-rearranged ALL.10 The other CNAs were different for each twin, namely a 47kbp amplification at 6p12.1, which contains the *GFRAL* (*GDNF family receptor alpha like*) gene, for Twin One and a 28kbp deletion at 9q31.3, containing the *MUSK* (*muscle, skeletal, receptor tyrosine kinase*) gene, for Twin Two. Each twin had three regions of tumor-associated copy-number neutral loss of heterozygosity, shown in Table 1, two of which were common between the twins. In addition, Twin Two had Trisomy X occurring in a subpopulation of leukemic cells, evident from both the banded chromosomal and cytogenetic array analyses.

Studies using high-resolution SNP and cytogenetic arrays in singletons with infant t(4;11) *MLL*-rearranged ALL,10,11 revealed an exceptionally low frequency of CNAs. Our results corroborate these findings in a different cytogenetic subtype of *MLL*-rearranged infant ALL. Together with the concordance shown at the molecular level for the t(1;11) translocation, the
identification of additional common aberrations provides further support for the concept of *in utero* development of a pre-leukemic clone in one twin with transfer to the other by means of the shared placental circulation.\textsuperscript{12} The low frequency of additional aberrations points towards the t(1;11) translocation as the major driver for leukemogenesis, however, further work is required to ascertain as to whether these additional aberrations have a co-operating role.

CNAs of the Xp21.1 site have now been demonstrated in three infants with *MLL*-rearranged ALL and so this region merits particular attention in future research. Importantly, this study has shown that several of the additional aberrations present in the leukemic cells of the twins are unique and individualized to each patient providing evidence for molecular heterogeneity for cells that have originated from a common cell of origin. The distinct molecular differences between the twins can be plausibly explained by clonal evolution of the leukemic cells from a common precursor or by selection of pre-existing clones. Further elucidation of the molecular differences among different *MLL*-subgroups and indeed among patients of the same subgroup will contribute to explaining the clinical heterogeneity of the disease.

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Key words: Infant, ALL, MLL, EPS15, t(1;11), heterogeneity.

Authorship and Disclosures

RSK designed and performed experiments, interpreted the results of the cytogenetic arrays and wrote the manuscript. JF performed experiments. DA was responsible for the biostatistical analysis. AHB, CHC and URK were responsible for project design and writing the report. RSK, JF, AHB, DA, CHC and URK approved the manuscript as submitted. The authors declare no conflict of interest.

Acknowledgments

We thank Dr. Silvia Bungaro for provision of data to enable comparison with their previously published MLL-AF4-positive infant ALL patients. We acknowledge Gillian Northcott for her assistance with illustrations.
References


Table 1 List of tumor-associated genomic aberrations identified using whole-genome cytogenetic arrays in monozygotic twins with t(1;11)(p32;q23) infant acute lymphoblastic leukemia.

<table>
<thead>
<tr>
<th>Twin</th>
<th>Region</th>
<th>Type* (CN state)</th>
<th>Start</th>
<th>End</th>
<th>Size (Kb)</th>
<th>Confidence (%)</th>
<th>Relevant genes</th>
<th>sno/micro RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>(6)(p12.1)</td>
<td>Gain (3)</td>
<td>55,308,049</td>
<td>55,355,423</td>
<td>47.4</td>
<td>87.5</td>
<td>GFRAL</td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>(X)(p21.1)</td>
<td>Gain (3)</td>
<td>36,640,213</td>
<td>36,706,268</td>
<td>66.1</td>
<td>93.1</td>
<td>No genes</td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>(11)(p11.2p11.12)</td>
<td>LOH</td>
<td>47,847,646</td>
<td>49,645,413</td>
<td>1797.8</td>
<td>99.9</td>
<td>PTTPRJ, FOLH1</td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>(20)(q11.22q11.23)</td>
<td>LOH</td>
<td>32,377,194</td>
<td>34,166,072</td>
<td>1788.9</td>
<td>99.9</td>
<td>ITCCH, DYNLRB1, PHF20, TP53INP2, GGT7, MMP24, ROMO1, RBM39, mir-644, mir-499, mir-1289-1</td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>(X)(q28)</td>
<td>LOH</td>
<td>152,270,145</td>
<td>154,849,094</td>
<td>2578.9</td>
<td>99.9</td>
<td>BRCC3, H2AFB3, AVPR2, LICAM, MTCP1NB, LCAP, CTAG family, DUSP9, mir-1184, ACA36, ACA56</td>
<td></td>
</tr>
<tr>
<td>Two</td>
<td>(9)(q31.3)</td>
<td>Loss (1)</td>
<td>112,486,139</td>
<td>112,514,070</td>
<td>27.9</td>
<td>86.8</td>
<td>MUSK</td>
<td></td>
</tr>
<tr>
<td>Two</td>
<td>(X)(p21.1)</td>
<td>Gain (3)</td>
<td>36,627,235</td>
<td>36,736,354</td>
<td>109.1</td>
<td>92.5</td>
<td>No genes</td>
<td></td>
</tr>
<tr>
<td>Two</td>
<td>(16)(q12.2q21)</td>
<td>LOH</td>
<td>54,430,930</td>
<td>56,753,662</td>
<td>2322.7</td>
<td>99.9</td>
<td>GNAO1, AMFR, CCL17, CCL22, DOK4, GPR56, CIAPIN1, MMP15, mir-138-2</td>
<td></td>
</tr>
<tr>
<td>Two</td>
<td>(20)(q11.22q11.23)</td>
<td>LOH</td>
<td>32,377,194</td>
<td>34,434,692</td>
<td>2057.5</td>
<td>99.9</td>
<td>ITCCH, DYNLRB1, PHF20, TP53INP2, GGT7, MMP24, ROMO1, RBM39, mir-644, mir-499, mir-1289-1</td>
<td></td>
</tr>
<tr>
<td>Two</td>
<td>(X)(q28)</td>
<td>LOH</td>
<td>152,263,301</td>
<td>154,849,094</td>
<td>2585.8</td>
<td>77.4</td>
<td>BRCC3, H2AFB3, AVPR2, LICAM, MTCP1NB, LCAP, CTAG family, DUSP9, mir-1184, ACA36, ACA56</td>
<td></td>
</tr>
</tbody>
</table>

* Gain = gain of one copy number (CN); Loss = loss of one CN
Figure 1 Molecular analysis of monozygotic twins with cytogenetically confirmed t(1;11)(p32;q23) infant acute lymphoblastic leukemia. (a) Sequence analysis showing the breakpoints at the transcriptional level (b) Sequence analysis showing the breakpoints at the genomic level.
**Design and Methods**

**Patient material**

To search for novel and rare *mixed lineage leukemia (MLL)* translocation partners, we performed banded chromosomal analysis and fluorescent *in situ* hybridization on diagnostic bone marrow samples of all infants with acute lymphoblastic leukemia (ALL) presenting to our institution, Princess Margaret Hospital for Children, between January 1983 and December 2011. Of sixteen patients, two patients were unique being monozygotic twins bearing a t(1;11) translocation. We undertook further molecular analysis of the leukemic cells of the monozygotic infant twins.

Peripheral blood and bone marrow samples were collected at diagnosis and remission from both twins under Princess Margaret Hospital for Children’s Institutional Review Board approved cell procurement protocols after informed consent was obtained in accordance with the Declaration of Helsinki. Total cellular DNA and RNA were extracted from the primary samples of both twins at diagnosis and remission using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA), as per the manufacturer’s instructions. All samples and intermediate products from the two twins were stored in separate locations and were manipulated at different times and in different areas of the laboratory, to avoid cross-contamination.

**Polymerase chain reaction and direct sequencing**

Total cellular RNA was reverse transcribed, using the Omniscript RT Kit (Qiagen) as per the manufacturer’s instructions, to generate cDNA. The cDNA was amplified by polymerase chain reaction (PCR) using the following primers:
• 5’-GAAACCTACCCCATCAGCAA and 5’-AACGTGAGGAGGATCAATGC were used to amplify the **MLL-EPS15** transcript
• 5’-GATTTGGGATTTAGCCGACA and 5’-TTTCGGCACTTATTACACTC were used to amplify the **EPS15-MLL** transcript

Total cellular DNA was amplified by PCR with the following primers:
• 5’-TGCAGTGAGCTGTGACTGTG and 5’-TTAGCTTTTTCTGCAGGGGA were used to amplify the **MLL-EPS15** genomic fusion
• 5’-CAGCCTGCCTCTTGTCAGAT and 5’-CCAGTTGGTGCTGATTTCCT were used to amplify the **EPS15-MLL** genomic fusion

Each PCR was performed using an optimized GoTaq Flexi DNA Polymerase protocol (Promega, Madison, WI, USA) with cDNA/DNA template at a concentration between 100-200ng. The PCR conditions used to amplify the samples consisted of initial denaturation at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 4 minutes. A final extension at 72°C was performed for 10 minutes following the 35 cycles. The positive control comprised of amplification with beta actin primers.

Direct sequencing was performed according to the manufacturer’s instructions using the BigDye Terminator v3.1 Cycle Sequencing Kit and an automated sequencer – the ABI 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The same internal forward and reverse primers as specified in the PCR amplification reactions were used at a concentration of 1mM.

Whole-genome cytogenetic arrays
The Affymetrix Cytogenetics Whole-Genome 2.7M Array (Affymetrix, Santa Clara, CA, USA) technology was applied for detection of submicroscopic aberrations and copy-number neutral loss of heterozygosity in each twin at diagnosis and remission. The arrays provide unbiased, whole-genome coverage with 2.7 million markers, including 400,000 single nucleotide polymorphisms and 2.3 million non-polymorphic copy-number markers. For each sample, 100ng of genomic DNA was amplified by whole-genome amplification reaction, purified by magnetic beads and fragmented to generate small (<300bp) products, which were subsequently labeled and loaded onto a single array. After hybridization, the chip was washed, stained and scanned. Raw data were analyzed with Affymetrix Chromosome Analysis Suite 1.01 software (Affymetrix) with comparison to the manufacturers recommended normalized reference. Filters that were applied included 45kbp for gains, 25kbp for losses and 1500kbp for copy-number neutral loss of heterozygosity. A 20 marker filter and 85% confidence was applied throughout. The filters selected were based on the manufacturer’s recommendations and previous analysis of t(4;11) infant ALL using the same platform (Bardini et al, Leukemia 2011). Visual software inspection of all reported lesions was undertaken and for each identified region of interest, a further in-depth analysis was undertaken below the filter thresholds in the other twin to ensure that no relevant results were missed. A tumor-associated copy-number alteration was defined as a copy-number alteration present in the tumor but not the remission DNA of each patient and for which there was no overlap with the Database of Genomic Variants (The Centre for Applied Genomics, Toronto, ON, Canada).