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

Ashley N. Mays,1 Neil Osheroff,2 Yuanyuan Xiao,3 Joseph L. Wiemels,3 Carolyn A. Felix,4 Jo Ann W. Byl,2 Kandeepan Saravanamuttu,5 Andrew Peniket,6 Robert Corser,7 Cherry Chang,8 Christine Hoyle,9 Anne N. Parker,10 Syed K. Hasan,11,12 Francesco Lo-Coco,11,12 Ellen Solomon,1 and David Grimwade1

1Department of Medical & Molecular Genetics, King’s College London School of Medicine, London, United Kingdom; 2Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN; 3Department of Epidemiology and Biostatistics, University of California San Francisco; 4Department of Pediatrics, University of Pennsylvania, Division of Oncology, The Children’s Hospital of Philadelphia, PA; 5Department of Haematology, Lincoln County Hospital, Lincoln, United Kingdom; 6Department of Haematology, John Radcliffe Hospital, Oxford, United Kingdom; 7Department of Haematology, Royal Derby Hospital, Derby, United Kingdom; 8Department of Haematology, Glan Clwyd Hospital, Rhyl, United Kingdom; 9The Beatson Institute, West of Scotland Cancer Centre, Glasgow, United Kingdom; 10Department of Biopathology, University of Tor Vergata, Rome, Italy; and 11Laboratorio di Neuro-Oncoematologia, Fondazione Santa Lucia, Rome, Italy

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, RUNXI 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 anthracerediones (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/m² 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
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at APL diagnosis, y</th>
<th>Primary malignancy</th>
<th>Treatment of primary malignancy</th>
<th>Cumulative dose of epirubicin, mg/m²</th>
<th>Latency, mo*</th>
<th>Cytogenetics</th>
<th>PML breakpoint</th>
<th>RARA breakpoint¶</th>
<th>APL therapy</th>
<th>Current status of APL</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN1</td>
<td>40</td>
<td>Breastcarcinoma</td>
<td>4 Cycles of epirubicin (165 mg), 6 cycles of CMF (cyclophosphamide 990 mg, methotrexate 65 mg, 5FU 990 mg)</td>
<td>400</td>
<td>48</td>
<td>t(15;17)(q22;q12-21) idem, del(5)(q?31q?35)</td>
<td>1186†</td>
<td>1185†</td>
<td>13463</td>
<td>13437</td>
</tr>
<tr>
<td>UPN2</td>
<td>47</td>
<td>Breastcarcinoma</td>
<td>4 Cycles of epirubicin (175 mg) + cyclophosphamide (1180 mg) DXT</td>
<td>400</td>
<td>28</td>
<td>t(7;14)(q32;q22), t(15;17)(q22;q21)</td>
<td>1270§</td>
<td>1267§</td>
<td>16192</td>
<td>16192</td>
</tr>
<tr>
<td>UPN3</td>
<td>41</td>
<td>Breastcarcinoma</td>
<td>6 Cycles of epirubicin (118 mg) + cyclophosphamide (940 mg) DXT</td>
<td>450</td>
<td>18</td>
<td>t(15;17)(q22;q21)</td>
<td>379-80§</td>
<td>375-76§</td>
<td>9291-92</td>
<td>9293-94</td>
</tr>
<tr>
<td>UPN4</td>
<td>55</td>
<td>Breastcarcinoma</td>
<td>4 Cycles of epirubicin (200 mg), 4 cycles of CMF (cyclophosphamide 1200 mg, methotrexate 80 mg, 5FU 1200 mg) DXT</td>
<td>400</td>
<td>18</td>
<td>der(12), (8;12)(q13;p13), t(15;17)(q22;q21)</td>
<td>1184-85†</td>
<td>1187-1191†</td>
<td>1332-33</td>
<td>13336-40</td>
</tr>
<tr>
<td>UPN5</td>
<td>60</td>
<td>Breastcarcinoma</td>
<td>6 Cycles of FEC (5FU 975 mg, epirubicin 98 mg, cyclophosphamide 980 mg), DXT</td>
<td>360</td>
<td>24</td>
<td>t(15;17)(q22;q21)</td>
<td>1968</td>
<td></td>
<td></td>
<td>1964-65</td>
</tr>
<tr>
<td>UPN6</td>
<td>55</td>
<td>Breastcarcinoma</td>
<td>6 Cycles of FEC (5FU 1100 mg, epirubicin 110 mg, cyclophosphamide 1110 mg × 3; 5FU 1000 mg, cyclophosphamide 1000 mg × 3) DXT</td>
<td>300</td>
<td>27</td>
<td>t(15;17)(q22;q21)</td>
<td>955-57†</td>
<td>955-59†</td>
<td>14882-84</td>
<td>14884-88</td>
</tr>
</tbody>
</table>

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

\(\text{t}(15;17)\) 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 topoi 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\(^{12,17}\) and included epirubicin, dissolved in 20 \(\mu\)L of DMSO used at a concentration of 160\(\mu\)M.

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 (\(P = 0.014\) using scan statistics for the 1056-bp intron 6 only with 3 patients; \(P = 0.009\) 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 (\(P = 0.017\) 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).

\(\text{t}(15;17)\) Translocation breakpoints are preferential sites for epirubicin-induced DNA cleavage by topoi

To investigate mechanisms by which the \(\text{t}(15;17)\) may have been formed after epirubicin exposure, we evaluated topoiα-mediated cleavage of the normal homologues of PML and RARA encompassing the respective breakpoints detected in 4 cases in the presence or absence of epirubicin. Differentiation between the cases 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 topoi-dependent manner (Figure 1B). Cleaveage 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 topoi 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 topoi cleavage sites in the vicinity of the breakpoints, and known mechanisms by which topoi induces double-strand breaks in DNA,\(^{3,18}\) it was possible to generate models as to how the \(\text{t}(15;17)\) 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 topII 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, 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. However, our studies involving the characterization of t-APL cases after mitoxantrone or epidurubicin provide very strong support for a third hypothesis whereby topII induces double-strand DNA breaks in susceptible regions of the genome which are aberrantly repaired to generate leukemia-associated chromosomal translocations.

This work was supported by a Leukaemia Research Gordon Piller Studentship (A.N.M., D.G., and E.S.), and by the National Institutes of Health (grant CA077683, C.A.F.; and grant GM33944, N.O.). S.K.H. and F.L.-C. were supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), the Progetto Integrato Oncologia of the Italian Ministry of Health. J.L.W. and Y.X. were supported by the Children with Leukaemia Fund UK.

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., 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: David Grimwade, Department of Medical & Molecular Genetics, King’s College London School of Medicine, 8th Fl, Tower Wing, Guy’s Hospital, London SE1 9RT, United Kingdom; e-mail: david.grimwade@genetics.kcl.ac.uk.

Acknowledgments

We thank Jelena Jovanovic for RT-PCR analyses to define PML-RARα isomorph type and Glynis Lewis for provision of clinical data.

References

17. Stanulla M, Wang J, Chernevinsky DS, Tandla S, Aplan PD. DNA cleavage within the MLL breakpoint cluster region is a specific event which occurs as part of higher-order chromatin fragmentation during the initial stages of apoptosis. Mol Cell Biol. 1997;17(7):4070-4079.
Evidence for direct involvement of epirubicin in the formation of chromosomal translocations in t(15;17) therapy-related acute promyelocytic leukemia

Ashley N. Mays, Neil Osheroff, Yuanyuan Xiao, Joseph L. Wiemels, Carolyn A. Felix, Jo Ann W. Byl, Kandeepan Saravanamuttu, Andrew Peniket, Robert Corser, Cherry Chang, Christine Hoyle, Anne N. Parker, Syed K. Hasan, Francesco Lo-Coco, Ellen Solomon and David Grimwade