Analysis of Genetic Alterations and Clonal Proliferation in Children Treated for Acute Lymphocytic Leukemia

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Abstract

The development of risk-directed treatment protocols over the last 25 years has resulted in an increase in the survival rates of children treated for cancer. As a consequence, there is a growing population of pediatric cancer survivors in which the long-term genotoxic effects of chemotherapy is unknown. We previously reported that children treated for acute lymphocytic leukemia have significantly elevated somatic mutant frequencies at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene in their peripheral T cells. To understand the molecular etiology of the increase in mutant frequencies following chemotherapy, we investigated the HPRT mutation spectra and the extent of clonal proliferation in 562 HPRT T cell mutant isolates of 87 blood samples from 47 subjects at diagnosis, during chemotherapy, and postchemotherapy. We observed a significant increase in the proportion of CpG transitions following treatment (13.6±23.3%) compared with healthy controls (4.0%) and a significant decrease in V(D)J-mediated deletions following treatment (0-6.8%) compared with healthy controls (17.0%). There was also a significant change in the class type percentage of V(D)J-mediated HPRT deletions following treatment. In addition, there was a >5-fold increase in T cell receptor gene usage–defined mean clonal proliferation from diagnosis compared with the completion of chemotherapeutic intervention. These data indicate that unique genetic alterations and extensive clonal proliferation are occurring in children following treatment for acute lymphocytic leukemia that may influence long-term risks for multifactorial diseases, including secondary cancers. (Cancer Res 2006; 66(17): 8455-61)

Introduction

Acute lymphocytic leukemia (ALL) is the most common childhood cancer. Since the early 1960s, the overall 5-year survival rate for children with ALL has improved from ~4% to between 80% and 90% following the development of national cooperative standardized multiple modality, risk of relapse–directed chemotherapy protocols (1). Children with ALL are placed into specific risk of relapse categories—low, standard, or high—based on clinical and biological prognostic factors (2). Chemotherapeutic treatment is divided into three phases: induction, consolidation, and maintenance. The therapy consists of steroids, antimetabolites, topoisomerase inhibitors, antimicrotubules, and anthracyclines, and lasts for 2.5 years. The drugs used are designed to induce cell death via apoptosis through several different mechanisms (3). Treatment protocols vary with respect to subjects’ risk of relapse classification.

A consequence of the improved survival rate of ALL is that there is a rapidly growing population of pediatric and young adult cancer survivors in which the short-term and long-term genetic effects of the chemotherapy are unknown. A potential adverse effect of treatment is the acquisition of cellular and genetic alterations in nonmalignant somatic cells that may contribute to late-effect clinical complications including an increased risk for developing second malignant neoplasms (4, 5). As a result, it is important to investigate the long-term genetic consequences of prolonged exposure to the chemotherapy drugs in these children.

We previously reported that children treated for ALL have significantly elevated somatic mutant frequencies (MFs) at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) reporter gene in their nonmalignant peripheral T cells, compared to children at diagnosis and to healthy controls, and that the MFs remained elevated for years (6, 7). The precise genetic mechanisms associated with the dramatic increase in MFs following chemotherapy and the potential consequence of this increased mutational load is unknown. In this study, we investigated the specific genetic effects of chemotherapeutic treatment by determining the mutational spectra and extent of clonal proliferation of HPRT mutant isolates from children with ALL at multiple time points during therapy and posttherapy in comparison with healthy age-matched controls and subjects at the time of diagnosis. We observed unique HPRT mutational spectral changes in somatic peripheral T cells as a consequence of chemotherapy, as well as a significant increase in clonal proliferation over the course of chemotherapeutic treatment that contributes to, but does not fully account for, the previously determined increase in HPRT MF in these children.

Materials and Methods

Study population and sample collection. A total of 87 heparinized blood samples from 47 children with B cell ALL, from oncology units at the University of Vermont and other participating Pediatric Oncology Groups/Children’s Oncology Group institutions were collected at the times of diagnosis, consolidation, the beginning and end of maintenance, and following the completion of chemotherapy for primary ALL. Subjects with recurrent disease were excluded from this analysis. Blood samples were stratified by age to ensure that all age groups were represented in each of the treatment phases. Eleven children were enrolled in low risk of relapse protocols, 28 children were enrolled in standard-risk protocols, and 8 children were enrolled in high-risk protocols. Informed consent was obtained from all subjects following procedures approved by the Committee.
on Human Research at the University of Vermont and participating institutions of the cooperative Pediatric Oncology Group/Children’s Oncology Groups. Peripheral blood samples were placed into the following groups/phases: (a) diagnosis, samples obtained prior to the start of chemotherapy; (b) consolidation, samples obtained between 5 and 25 to 33 weeks from the time of diagnosis; (c) early maintenance, samples obtained between 25 and 65 weeks from the time of diagnosis; and (d) late maintenance, samples obtained between 95 and 130 weeks from the time of diagnosis. The posttherapy groups were also separated into two groups for analysis: (a) posttherapy group I, samples from subjects 157 to 390 weeks from the time of diagnosis (6 months to 5 years following the completion of treatment); and (b) posttherapy group II, samples from subjects 391 to 910 weeks from the time of diagnosis (3-15 years after the completion of treatment). A list of the chemotherapy drugs administered during each phase of therapy for each of the risk of relapse groups was previously described (7). Frequently, blood samples were obtained from subjects at more than one time point.

**HPRT T cell cloning assay.** Determination of HPRT MFs and the isolation of T cell mutant isolates from blood samples were done as previously described (6, 8). HPRT mutant isolates were expanded, pelleted at 1 × 10^6 cells, and then frozen at −80°C for molecular analysis.

**Molecular analysis of HPRT mutant isolates.** In contrast with our previous study (7), only peripheral blood samples that yielded viable and expandable HPRT mutants were included in this molecular analysis. A total of 562 HPRT mutant T cell isolates, originating from 87 blood samples from 47 children with ALL were characterized for both the type of HPRT mutation by sequence analysis of cDNA and/or genomic DNA, and clonality by characterizing the T cell receptor-β (TCRβ) mRNA sequence of each mutant isolate. For unique HPRT mutation spectra distribution analysis, five mutant isolates per subject blood sample were randomly selected to avoid potential bias. If a sample had fewer than five mutants, all available mutants were analyzed. To estimate the extent of clonal proliferation, we included 2 to 10 mutant isolates per blood sample.

For the determination of HPRT spectra, 1 × 10^6 cell pellets were used for reverse transcriptase (RT)-mediated production of cDNA (9). Reverse transcriptase products were amplified with specific HPRT primers in a nested PCR reaction and the PCR products were run on 1% to 1.5% agarose gels, extracted, purified via Qiagen’s QIAquick gel extraction kit, and then sequenced (9). Because HPRT is located on the X chromosome, males are screened for large deletions or insertions within the nine exons at the genomic level via a multiplex PCR (10). When exon exclusions or intron inclusions occurred in the cDNA, genomic regions were amplified using the genomic multiplex PCR primers for each of the exons, then sequenced to determine the type of mutation which resulted in the exclusion or inclusion observed in the cDNA. HPRT mutants that showed exclusion of exons 2 to 3 were screened for VDJ recombinase–mediated deletions of HPRT exons 2 to 3 with specific primers spanning that region (11). There are three classes of HPRT V(D)J-mediated deletions. Each class has the same intron 1 breakpoint at a cryptic recombination signal sequence (cRSS) at position 2198 to 2204, in combination with one of three 3′-cRSSs located in intron 3 (class I at 22244-22251, class II at 20149-20155, and class III at 22562-22568; ref. 12).

The rearrangement of the TCR genes in mutant isolates provides an independent measure of clonality and thus allows measurement of clonal proliferation that can be followed over time (13, 14). The extent of clonal proliferation was determined by sequence analysis of the TCRβ CDR3/variable regions. RT-PCR-generated cDNA was amplified using a primer to the TCRβ constant region and a mix of 26 TCRβ V region primers (15). The PCR products were then run on an agarose gel, excised, purified, and sequenced. The TCRβ CDR3 region sequence and the type of HPRT mutation(s) were used together to determine if mutant isolates were unique or of clonal origin, as previously described (13).

**Statistical analysis.** Of the 319 mutants from children with ALL that were analyzed for mutation spectra distribution, 51 (16.0%) were classified as being uncharacterized, which is consistent with other human spectra studies (16). The percentage of uncharacterized mutations in the phase of therapy groups ranged from 11.4% to 21.3%. They did not differ significantly between groups and were excluded from computations of the proportions of specific types of mutations. Thus, a total of 268 unique mutant T cell isolates from children with ALL were used for the final HPRT mutational spectra distribution analysis. These data were compared with 100 unique mutations previously reported for healthy children up to 16 years of age (17). Logistic regression was used to test the statistical significance of differences in the proportion of specific types of mutations between healthy children and children with ALL in differing phases of treatment, as well as between differing risk of relapse groups during the same phase of treatment. Because our previous analysis of data from healthy children showed that the mutation spectra distribution is age-dependent during human development, from birth through adolescence (17), age group (0–4, 6–11, or 12–18 years) was included as a covariate in the logistic regression analyses to control for age-related differences in mutation spectra distributions (17). For some risk of relapse groups, there were small sample sizes in some of the treatment phases and we were not able to control for the effects of age in the logistic regressions. In situations in which it was possible to adjust for age, the results were consistent with the unadjusted results. To compare differences in the proportions of specific types of mutations between children with ALL at diagnosis and in differing phases of treatment, a random effect was included in the logistic regression models to take into account correlations between samples from the same person at differing time points. Among V(D)J HPRT mutations, the proportion of class III mutations in children who had received chemotherapy was compared with the proportion in children at diagnosis and healthy children of comparable age using Fisher’s exact test. For all analyses, differences were considered statistically significant at P ≤ 0.05.

Clonal proliferation for each blood sample was estimated as one minus the ratio of unique to total mutations (1 − unique / total). For samples with ≤10 mutant isolates, all available mutant isolates were used to estimate clonal proliferation. For samples having >10 mutant isolates, 10 were randomly selected for our analysis. Regression analysis to examine changes in clonal proliferation over time since diagnosis was done using a linear random effects model to take into account correlations between samples that were obtained from the same individuals at different time points. Similar linear random effects models were used to test for differences in clonal proliferation between treatment phases and between risk of relapse groups by including these as categorical variables. To eliminate potential bias due to confounding between the number of available mutants and phase of therapy, we reanalyzed the extent of clonal proliferation using five randomly selected mutants as the total number of mutants for each subject. With this approach, the increase in clonal proliferation during therapy remained statistically significant, hence, only the original results are presented. To estimate the frequency of unique mutations, the MF for each sample was multiplied by the ratio of unique to total mutations to adjust for clonal proliferation. The frequencies of unique mutations in the differing treatment phases were compared using random effects models as described above for clonal proliferation, with the logarithms of the adjusted MFs as the dependent variable.

**Results**

**Mutation spectra distribution and phase of therapy.** The mutation spectra distribution analysis was done on 319 unique T cell HPRT mutant isolates. A summary of the subject samples in each phase of therapy group from diagnosis to posttherapy is summarized in Table 1. A summary of the unique HPRT mutations for each subject, including both cDNA and genomic DNA analysis by each phase of therapy, is provided as a Supplemental Table.

There were no statistically significant age-adjusted mutation spectra distribution differences between healthy children and children at the time of ALL diagnosis (Table 2). This suggests that an underlying genetic susceptibility or an extensive environmental component is not strongly affecting mutation spectra prior to diagnosis with ALL.
Mutational spectra distribution of small alterations and phase of therapy. We observed a significantly higher proportion of small alterations, or equivalently, a significantly lower proportion of large alterations, in children with ALL at each therapeutic phase during and following the completion of treatment when compared with healthy children. Of the mutants isolated during consolidation through posttherapy group II, 72.7% to 85.4% were small alterations, compared with 57.0% in healthy children (Table 2). There were significantly larger proportions of transitions in children following completion of chemotherapy compared with healthy children. This increase is primarily the result of an increase in transition mutations at CpG dinucleotides (13.6-23.3% versus 4.0%, respectively). The proportion of CpG transitions is significantly higher in children during each phase of therapy from early maintenance through posttherapy group II, compared with healthy children (Table 2; Fig. 1). Of the 37 total CpG transition mutations characterized from consolidation through posttherapy group II, 17 (46%) were at position c.151 (exon 3), 16 (43%) at c.508 (exon 7), and 4 (11%) at genomic g.1,714 (intron 1; GenBank accession no. M26434). There was also a significant increase in transversions observed in children during the late maintenance phase, 18 (41.9%), compared with 24 (24.0%) in age matched healthy children (Table 2). Of the 18 transversions, 5 (28%) were G→T’s, 4 (22%) were T→G’s, with the remaining being equally distributed (Supplemental Table).

Table 1. Summary of patient samples by phase of therapy

<table>
<thead>
<tr>
<th>Phase of therapy</th>
<th>Risk group</th>
<th>Samples (n)</th>
<th>Mean no. of weeks since diagnosis</th>
<th>Female (%)</th>
<th>Mean age at diagnosis</th>
<th>Mean age at testing</th>
<th>Mean MF x 10^-6</th>
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</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>Low</td>
<td>6</td>
<td>0</td>
<td>16.7</td>
<td>4.6</td>
<td>4.6</td>
<td>0.7</td>
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<tr>
<td></td>
<td>Standard</td>
<td>8</td>
<td>0</td>
<td>37.5</td>
<td>6.3</td>
<td>6.3</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>6</td>
<td>0</td>
<td>33.3</td>
<td>9.1</td>
<td>9.1</td>
<td>2.4</td>
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<tr>
<td>Consolidation</td>
<td>Low</td>
<td>4</td>
<td>23</td>
<td>50</td>
<td>4</td>
<td>4.5</td>
<td>4.9</td>
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<td></td>
<td>Standard</td>
<td>6</td>
<td>17</td>
<td>33.3</td>
<td>5.7</td>
<td>6.0</td>
<td>101.5</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1</td>
<td>5</td>
<td>100</td>
<td>12.7</td>
<td>12.8</td>
<td>5.8</td>
</tr>
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<td>Early Maintenance</td>
<td>Low</td>
<td>8</td>
<td>40</td>
<td>50</td>
<td>4.4</td>
<td>5.2</td>
<td>14.4</td>
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<tr>
<td></td>
<td>Standard</td>
<td>5</td>
<td>42.4</td>
<td>40</td>
<td>3.7</td>
<td>4.3</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2</td>
<td>54.5</td>
<td>0</td>
<td>10.9</td>
<td>12.0</td>
<td>26.7</td>
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<td>Late Maintenance</td>
<td>Low</td>
<td>6</td>
<td>109.2</td>
<td>66.7</td>
<td>4.7</td>
<td>6.8</td>
<td>291.9</td>
</tr>
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<td>122.5</td>
<td>50</td>
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<td>8.4</td>
<td>1,882.1</td>
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<td></td>
<td>High</td>
<td>2</td>
<td>112.5</td>
<td>100</td>
<td>9.4</td>
<td>11.6</td>
<td>1.9</td>
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<tr>
<td>Posttherapy Group I</td>
<td>Low</td>
<td>6</td>
<td>210</td>
<td>50</td>
<td>4.3</td>
<td>8.3</td>
<td>221.5</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>8</td>
<td>279.6</td>
<td>37.5</td>
<td>4.5</td>
<td>9.9</td>
<td>490.2</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2</td>
<td>277</td>
<td>50</td>
<td>3.6</td>
<td>8.9</td>
<td>98.9</td>
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<tr>
<td>Posttherapy Group II</td>
<td>Low</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>11</td>
<td>410</td>
<td>54.5</td>
<td>5.1</td>
<td>16.7</td>
<td>511.1</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2</td>
<td>605.5</td>
<td>50</td>
<td>3.6</td>
<td>11.5</td>
<td>162.6</td>
</tr>
</tbody>
</table>

Table 2. Distribution of unique HPRT mutations by phase of therapy

<table>
<thead>
<tr>
<th>Phase of therapy</th>
<th>Healthy, n (%)</th>
<th>Diagnosis, n (%)</th>
<th>Consolidation, n (%)</th>
<th>Early maintenance, n (%)</th>
<th>Late maintenance, n (%)</th>
<th>Posttherapy group I, n (%)</th>
<th>Posttherapy group II, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All mutations</td>
<td>100 (100)</td>
<td>38 (100)</td>
<td>25 (100)</td>
<td>44 (100)</td>
<td>43 (100)</td>
<td>70 (100)</td>
<td>48 (100)</td>
</tr>
<tr>
<td>Small alterations</td>
<td>57 (57.0)</td>
<td>24 (63.2)</td>
<td>20 (80.0)*</td>
<td>32 (72.7)*</td>
<td>34 (79.1)*</td>
<td>52 (74.3)*</td>
<td>41 (85.4)*</td>
</tr>
<tr>
<td>Transversions</td>
<td>24 (24.0)</td>
<td>10 (26.3)</td>
<td>5 (20.0)</td>
<td>12 (27.3)</td>
<td>18 (41.9)*</td>
<td>13 (18.6)</td>
<td>14 (29.2)</td>
</tr>
<tr>
<td>Non-CpG transitions</td>
<td>20 (20.0)</td>
<td>13 (34.2)</td>
<td>8 (32.0)</td>
<td>10 (22.7)</td>
<td>6 (14.0)</td>
<td>20 (28.6)</td>
<td>17 (35.4)</td>
</tr>
<tr>
<td>CpG transitions</td>
<td>4 (4.0)</td>
<td>0 (0.0)</td>
<td>2 (8.0)</td>
<td>5 (13.6)*</td>
<td>10 (23.3)*</td>
<td>12 (17.1)*</td>
<td>7 (14.6)*</td>
</tr>
<tr>
<td>≥2 bp insertions and deletions</td>
<td>9 (9.0)</td>
<td>1 (2.6)</td>
<td>5 (20.0)</td>
<td>4 (9.1)</td>
<td>0 (0.0)</td>
<td>7 (10.0)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>Large alterations</td>
<td>43 (43.0)</td>
<td>14 (36.8)</td>
<td>5 (20.0)*</td>
<td>12 (27.3)*</td>
<td>9 (20.9)*</td>
<td>18 (25.7)*</td>
<td>7 (14.6)*</td>
</tr>
<tr>
<td>V(D)J deletions</td>
<td>17 (17.0)</td>
<td>5 (13.2)</td>
<td>0 (0.0)</td>
<td>3 (6.8)*</td>
<td>2 (4.7)*</td>
<td>0 (0.0)*</td>
<td>0 (0.0)*</td>
</tr>
<tr>
<td>Non-V(D)J deletions</td>
<td>26 (26.0)</td>
<td>9 (23.7)</td>
<td>5 (20.0)</td>
<td>8 (18.2)</td>
<td>6 (14.0)</td>
<td>17 (24.3)</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>&gt;2 bp insertions and duplications</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (2.3)</td>
<td>1 (2.3)</td>
<td>1 (1.4)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>All mutations (including uncharacterized)</td>
<td>108 (100)</td>
<td>44 (100)</td>
<td>30 (100)</td>
<td>53 (100)</td>
<td>52 (100)</td>
<td>79 (100)</td>
<td>61 (100)</td>
</tr>
<tr>
<td>Uncharacterized mutations</td>
<td>8 (7.4)</td>
<td>6 (13.6)</td>
<td>5 (16.7)</td>
<td>9 (17.0)</td>
<td>9 (17.3)</td>
<td>9 (11.4)</td>
<td>13 (21.3)</td>
</tr>
</tbody>
</table>

NOTE: Statistical analysis adjusted for age.
*Indicates significantly lower or higher from healthy children (P < 0.0).
Due to limited power, it was not possible to perform statistical comparative analysis on the specific types of transversions.

When only single base substitutions were considered, the proportions that were CpG transitions ranged from 13.3% to 29.4% during and following therapy. The largest proportions were seen in the late maintenance phase and posttherapy group I (29.4% and 26.7%, respectively), and these were significantly higher than the proportions observed in healthy children (8.3%; Fig. 1).

Although the spectra of HPRT mutations in children at diagnosis with ALL were similar to healthy children, it did not differ significantly from the spectra in children during or following therapy. This is likely due to the small number of mutants available from children at the time of diagnosis, which limited the statistical power to detect differences in this group.

Of interest is that mutant isolate M30 from subject CS033B7 had two point mutations in cDNA (T49 → G and C69 → T; Supplemental Table). The transversion at position c.49 was recorded as part of the spectra data as it is predicted to cause an amino acid change (Tyr → Asp), whereas the transition at position c.69 was not included, as it is a conserved mutation. The acquisition of two independent allelic mutations at the HPRT locus further supports our previously reported observations of mutants with genomic instability following chemotherapy for ALL (6).

**Mutation spectra distribution and risk of relapse.** A comparative analysis was also done between HPRT mutation spectra distributions from subjects in low, standard, and high-risk groups. This analysis revealed no differences between these risk groups at diagnosis, during any phase of therapy, or following the completion of therapy.

**Figure 1.** Percentage of CpG transitions of total HPRT unique mutations and single base substitutions. +, significantly higher than healthy children (P < 0.05) after adjustment for age.

**Figure 2.** A, percentage of V(D)J-mediated deletions of unique HPRT mutations for each therapy phase. *, significantly lower than observed in healthy children (P < 0.05) after adjustment for age. B, percentage of HPRT V(D)J class I and class III deletions of healthy children compared with children who received chemotherapy for ALL (P < 0.001).
Table 3. Clonal proliferation analysis in children treated for ALL

<table>
<thead>
<tr>
<th>Phase of therapy</th>
<th>Diagnosis</th>
<th>Consolidation</th>
<th>Early maintenance</th>
<th>Late maintenance</th>
<th>Posttherapy group I</th>
<th>Posttherapy group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>20</td>
<td>11</td>
<td>15</td>
<td>12</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>No. of subjects included in analysis</td>
<td>11</td>
<td>7</td>
<td>13</td>
<td>11</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>Total no. of mutants</td>
<td>37</td>
<td>40</td>
<td>79</td>
<td>92</td>
<td>159</td>
<td>126</td>
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<td>No. of unique mutants</td>
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<td>34</td>
<td>55</td>
<td>66</td>
<td>115</td>
<td>81</td>
</tr>
<tr>
<td>Mean clonal proliferation*</td>
<td>0.07</td>
<td>0.18</td>
<td>0.24</td>
<td>0.24</td>
<td>0.28</td>
<td>0.36</td>
</tr>
<tr>
<td>Mean HPRT MF × 10^{-6}</td>
<td>2.1</td>
<td>88.5^1</td>
<td>19.4^1</td>
<td>843.8^1</td>
<td>340.5^1</td>
<td>457.4^1</td>
</tr>
<tr>
<td>Mean frequency of unique mutations × 10^{-6}^1</td>
<td>2.0</td>
<td>86.6</td>
<td>13.0^1</td>
<td>440.2^1</td>
<td>271.3^1</td>
<td>317.3^1</td>
</tr>
</tbody>
</table>

*Clonal proliferation = 1 – (unique / total mutant isolates).

^1Indicates significantly higher compared with children at diagnosis (P < 0.05).

^2Mean frequency of unique mutations = MF for each sample × unique / total mutations.

Clonal proliferation analysis. We investigated the number of unique mutations in each subject sample by determining both the sequence of the TCRβ CDR3 gene region as well as the specific HPRT mutation for each T cell HPRT mutant. This approach allows us to determine the extent of pre- or postthymic clonal proliferation, and allows calculation of the frequency of unique HPRT mutations (13). Analysis included 540 mutants (2-10 per sample) characterized from 71 blood samples from 42 subjects, in which some were represented in more than one phase of therapy. Sixteen samples with a single isolate were excluded because clonality could not be determined. For this analysis, clonal proliferation was defined as one minus the ratio of unique to total mutants (Table 3).

Clonal proliferation increased significantly with treatment, over all treatment phases (P = 0.004 for linear trend; Table 3; Fig. 3). Compared with the diagnosis group, there was significantly higher clonal proliferation in both posttherapy groups. Regression analysis showed that clonal proliferation increased linearly with the logarithm of weeks since diagnosis, reflecting a rapid increase throughout chemotherapeutic intervention that slowed at the cessation of treatment (Fig. 3). Of interest, there was no evidence of differences in the extent of clonal proliferation between the low, standard, and high-risk of relapse groups as well as age groups. In addition, although MFs during and after therapy were reduced after adjustment for clonal proliferation, they still increased significantly with treatment and were higher at each treatment phase than at diagnosis (Table 3).

Of the 24 subjects who were sampled at two or more time points, 7 (29.2%) of them had mutant T cell isolates that persisted between phases of therapy. We observed one instance of prethymic and two instances of postthymic clonal proliferation (13) from 3 of 20 subjects at the time of diagnosis. We observed 15 examples of postthymic proliferation and 3 examples of “proliferative” clonality (ref. 13; same TCR and different HPRT mutations) from 12 of 38 children during treatment. During therapy, there were also six instances of clonality from four children in which the particular type could not be determined. Posttherapy, we observed 2 instances of prethymic clonality (13) from 2 children, 25 instances of postthymic clonality from 20 children, and 3 examples of proliferative clonality from 2 of 29 children. The posttherapy groups had eight instances of undefined clonality from six subjects.

Discussion

The goal of this study was to determine the HPRT mutation spectra and the extent of clonal proliferation in mutant isolates following chemotherapy in order to investigate the genotoxicity of treatment for pediatric ALL.

Mutation spectra analysis showed that chemotherapeutic intervention resulted in a significant increase in small alterations, in particular, CpG transitions compared with that of healthy children (Table 2; Fig. 1). These CpG transitions were in the nontranscribed strand, consistent with previous findings of strand bias in HPRT at CpG sites (16, 18, 19). The mechanism associated with our observed increase in CpG transitions is unclear, and to date, no mutagen has been identified that could increase the mutation rate at these sites (18, 20, 21). Methylation of cytosine residues has been reported to increase its mutation potential by at least a factor of 10 in somatic tissues (20), which may be a contributing factor following treatment when lymphocyte populations are significantly decreased and need to be reconstituted (22). The reconstitution of immune cell populations following treatment...
is an analogous developmental stage as a developing fetus and newborn infant when there is also significant cell proliferation during the emergence of a mature immune system (17, 23). This comparison is supported by our observations that CpG transitions are most comparable to the percentages observed for pre-term and full-term infants (Fig. 1). In addition, the fact that the percentage of CpG transitions remain elevated following the completion of treatment implies that these T cell mutants are sustained, possibly from a mutation in a stem cell or a memory cell that occurred during lymphocyte reconstitution. Alternatively, because we observed primarily postthymic clonal proliferation of mutants following treatment, it is also possible that at least some of these mutants are from mature dividing cells, as a consequence of antigen stimulation and/or a lack of cell cycle control. This has clinical relevance because a similar accumulation of CpG transition mutations may also occur in other replicating tissues that need to be cyclically replenished as a consequence of chemotherapy, such as epithelial mucosa. Although CpG dinucleotides are underrepresented in the genome, they are common sites for mutations in disease genes associated with hemophilia, retinoblastoma, and multiple cancers, especially at the disease genes involved with cell cycle control, which may have long-term deleterious consequences in these individuals.

We also observed a significant increase in the proportion of transversions during the late maintenance phase compared with healthy children. The most prevalent were G → T’s, which are also the most common type of transversion seen among healthy children and adults (16, 17). G → T transversions have been shown to occur following the oxidation of guanine by endogenous reactive oxygen species (ROS; ref. 25). These transversions were seen in children from both low and standard risk groups during the maintenance phase of therapy when vincristine is used. In Jurkat ALL cells, ROS have been shown to participate in a mitochondrial controlled pathway of vincristine-induced apoptosis (26). Thus, ROS produced via vincristine-induced apoptosis or by another chemotherapeutic agent may cause an increase in the proportion of G → T transversions during the maintenance phase treatment for ALL.

The mutational spectra analysis revealed that 51 out of the 319 mutant isolates (16%) were “uncharacterized” and were excluded from our analysis. If we assume an extreme scenario in which all of the uncharacterized mutations are large alterations, the higher proportion of small alterations, or conversely, the lower proportion of large alterations, in children with ALL at each phase when compared with healthy controls would no longer be significant, whereas the proportion of CpG transitions would remain significantly higher in both the late maintenance phase and posttherapy group II, compared with healthy children. However, these results would not be accurate because a conservative interpretation of our uncharacterized mutants shows that 7 out of 20 (35%) male subjects do not have large structural alterations in the HPRT coding region because exons 1 to 9 are present according to genomic PCR (Supplemental Table). Our assumption is that this same distribution would hold true for uncharacterized mutants from female subjects as well. These uncharacterized mutations could be in the promoter, noncoding regions affecting mRNA stability or translation, or could be translocations with breakpoints within the HPRT locus. In addition, some mutants from females which show exon exclusions in cDNA, and no splice alterations in genomic DNA, are still considered uncharacterized because the mutation could not be confirmed.

In an earlier study, Hirota et al. observed an increase in undefined large structural HPRT alterations as determined by Southern blot analysis in children treated for ALL compared with healthy controls (27). The analysis was done on samples at various time points and small alterations were not characterized. In this study, we observed a significantly lower proportion of large alterations during and following the completion of treatment when compared with healthy children. V(D)J-mediated deletions were significantly lower from early maintenance through post-therapy, even after adjustment for age, compared with healthy children. V(D)J recombinase normally rearranges immunoglobulin and TCR loci at specific RSSs to generate gene diversity for antigen recognition (28). Cryptic RSSs occur outside of the immune loci throughout the genome, and four cRSS sites within HPRT can mediate V(D)J recombinase deletion of HPRT exons 2 and 3 (11, 12, 29). The frequency of V(D)J recombination events can be influenced by CpG methylation status at or near RSSs. Specifically, there is an increased frequency of V(D)J recombination events at hypomethylated sites, whereas hypermethylated sites are refractory to V(D)J recombination (30, 31). Several chemotherapy drugs used during consolidation, specifically, 6-thioguanine, cyclophosphamide, and teniposide, induce DNA hypomethylation, whereas methotrexate, 1-β-D-arabinofuranosylcytosine, and vincristine, given throughout treatment, induce hypermethylation (32, 33). We postulate that the reduction in the proportion of V(D)J-mediated HPRT deletions is a result of hypermethylation at the cRSS regions within the HPRT locus as a consequence of chemotherapy. Such therapy-induced changes could also affect the accessibility of other areas of the genome, thereby affecting gene expression, and potentially, secondary V(D)J recombinase-mediated genomic rearrangements. An important observation was the reversal in the percentage of distribution of class III versus class I type V(D)J-mediated HPRT deletions compared with the proportion in healthy children. This reversal was observed in children from 4 to 13 years of age, which is noteworthy because class III V(D)J HPRT deletions are rarely detected in children after birth (17, 29). This suggests that exposure to chemotherapeutic agents may induce changes in DNA accessibility in regions containing cRSSs that were previously not substrates for V(D)J recombination. An alternative explanation is that the use of class III cRSS sites is more prevalent during early immune system development, which may occur following each round of chemotherapy. Such a response to genotoxic exposures could potentially increase V(D)J-mediated events at disease loci with pathologic potential.

Our analysis showed that there were no significant differences in mutational spectra between the risk of relapse groups at any phase of therapy. Although this implies that the difference in the chemotherapy protocols between risk groups may not affect mutation spectra distribution, sample sizes in the risk groups provided limited statistical power to detect differences. We can infer that differences between the mutational spectra of healthy children and those undergoing treatment are a consequence of treatment, because there were no differences between healthy children and children at the time of diagnosis with ALL. It would be preferable to longitudinally assess spectrum changes in individual subjects. However, this would require not only obtaining samples from each patient before therapy and during each treatment phase, but also obtaining sufficient numbers of mutations from each
sample to adequately estimate a patient’s mutational spectrum at each time point. Although we included as many subjects with samples at multiple time points as possible, 34 of the 47 subjects in our study had available samples at only one or two time points, and none had samples at all six time points before, during, and after therapy. This precluded a longitudinal analysis of the data and reduced our statistical power to detect differences between mutational spectrum at diagnosis and during treatment.

Regression analysis showed that there is extensive clonal proliferation of the mutant isolates as therapy proceeds, but proliferation slows once therapy has ceased. However, when HPRT MF is corrected for clonal proliferation, the MFs are still significantly elevated. This indicates that other mechanisms, such as chemotherapy drug-induced genetic damage at HPRT, or alterations in genes affecting mutation rate, may also be responsible for the elevated MFs. We observed persistent clonal expansion of the same mutant isolates in several subjects. These clones survived between chemotherapy treatments, indicating that they may have acquired resistance to the therapy as a consequence of selective pressure. The cyclical rounds of chemotherapy may drive the selection of proliferating clones, and when therapy is complete, this selective pressure is no longer present, resulting in a decrease in proliferation.

This study shows that chemotherapy intervention for childhood ALL results in a unique HPRT mutation spectra distribution that persists for up to 15 years following the completion of treatment. Because genetic alterations at the HPRT biomarker are reflective of genome-wide somatic mutational events, the observed genetic changes at this locus could also have occurred in other replicating cell populations affected by the chemotherapy, in genes pivotal to cell growth control, development, or genome stability. As a result, these genetic changes may have long-term clinical consequences that may not manifest until decades following treatment for ALL. In addition, we observed a significant increase in clonal proliferation, a hallmark of tumors, as subjects proceeded with chemotherapy. Investigations into the genetic consequences of chemotherapy provide important insights into potential future genetic risks for secondary cancers and other multifactorial diseases, as well as providing information that may lead to successful treatment protocols with decreased genetic toxicity.

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