Prospective Study of Mutant Frequencies at the hprt and T-Cell Receptor Gene Loci in Pediatric Cancer Patients during Chemotherapy

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Abstract
Mutant frequencies (MFs) at the hypoxanthine phosphoribosyl transferase gene and the T-cell receptor (TCR) gene loci were evaluated in nine pediatric cancer patients before and during anticancer chemotherapy. The study population consisted of three patients with Hodgkin’s disease, four patients with neuroblastoma, and two patients with Wilms’ tumor. Except for one patient with neuroblastoma and one patient with Wilms’ tumor, MFs at the hypoxanthine phosphoribosyl transferase locus tended to increase during the early cycles of treatment. The elevation was most striking and persistent in patients with Hodgkin’s disease. The clonal relationship was determined in mutant cells derived from Hodgkin’s disease patients by TCR-γ gene rearrangement pattern and showed that the elevation of MFs resulted from increased mutational events rather than from clonal expansion of mutants. An increase in TCR MF was also found during chemotherapy in most patients, but the time of TCR MF elevation was variable among patients. Among the chemotherapeutic agents used in this study, cyclophosphamide was considered to be the most mutagenic. Our present study clearly demonstrates that anticancer chemotherapy can induce mutagenesis in vivo in various pediatric cancer patients.

Introduction
Recently, the survival rate of pediatric cancer patients has dramatically improved. On the other hand, long-term survivors are at risk of developing second malignancies as late complications (1). Pui et al. (2) have reported the high occurrence of secondary myeloid leukemia in pediatric ALL patients successfully treated by intensive chemotherapy. They have nominated VP-16, an inhibitor of topoisomerase II, as a causative agent. Other investigators have demonstrated that patients with Hodgkin’s disease tended to have an increased risk of secondary malignancy or breast cancer (3, 4). Treatment with alkylating agents is considered to be associated with an elevated risk for a second malignancy.

Many anticancer drugs can cause DNA damage, and such an effect is considered to induce mutations. To evaluate such mutagenic effects in vivo, assays of somatic cell mutations at several genetic loci, including the hprt, TCR, and GPA genes and the human leukocyte antigen-A gene, are widely used (5). Elevated MF at the hprt locus has been reported in atomic bomb survivors (6), in patients with cancer-prone syndromes such as ataxia-telangiectasia (7) and xeroderma pigmentosum (8), and in patients with hepatoma receiving radioimmunoglobulin therapy (9). TCR MF has also been reported to be increased in patients with ataxia-telangiectasia (10) or with thyroid disease treated with 131I (11). Furthermore, several investigators have described elevated MFs at the hprt (12, 13) and GPA (14) loci in adult patients after cancer chemotherapy and/or radiotherapy. In pediatric patients, Hirota et al. (15, 16) reported an elevation of MFs at the hprt, TCR, and GPA loci in patients after chemotherapy.

These studies usually measured MFs after the completion of chemotherapy and compared them with values obtained from healthy controls. To demonstrate the in vivo mutagenic effect, however, it is necessary to monitor the changes of MFs during anticancer chemotherapy. Such studies are quite limited and, as far as we know, have been reported only on adult patients (17–19). This fact prompted us to examine the changes of hprt MF and TCR MF during therapy in nine pediatric patients with various solid tumors treated with different chemotherapeutic regimens.

Materials and Methods

Patients’ Profiles. Nine newly diagnosed pediatric cancer patients, three patients with Hodgkin’s disease, four patients with neuroblastoma, and two patients with Wilms’ tumor were enrolled in this study. The details of their profiles and therapeutic schedules are summarized in Table 1. Informed consent for blood sampling was obtained from either the patient or the patient’s parent. Peripheral blood was drawn before starting the first cycle of anticancer therapy and just before each course of chemotherapy. The intervals of treatment were 6 weeks for regimens 1 and 2, 4 weeks for regimens 3–5, and 12 weeks for regimens 6 and 7.

GPA, glycoporphin A; PBMC, peripheral blood mononuclear cell; 6-TG, 6-thioguanine; CE, cloning efficiency; CI, confidence interval; VP-16, etoposide; CPM, cyclophosphamide; MLL, mixed-lineage leukemia.
regimens 6–9. As the control, the MF data previously evaluated in 26 healthy children (mean age ± SD: 11.6 ± 4.0 years; range: 4.4–22.2 years) were used (20).

Cloning Assay for the hprt Mutation. A T-lymphocyte cloning assay was performed as described previously (21–23), with minor modifications. The PBMCs obtained by Ficoll-Hypaque were plated in 96-well U-bottomed microtiter plates at a density of 1 cell/well (nonselection) or 2 × 10^4 cells/well (6-TG selection). They were cultured in RPMI supplemented with 20% FCS, 1% human serum, 50 IU/ml recombinant human interleukin 2 (Takeda, Tokyo, Japan), 5 μg/ml phytohemagglutinin P (Difco, Detroit, MI), and 10^5 cells/well of irradiated (100 Gy) Raji cells. Irradiated (50 Gy) autologous PBMCs (2 × 10^5 cells/well) were also added to nonselection plates. Selection plates contained 30 μM 6-TG. After 14 days of culture, colony formation was observed under an inverted microscope. CE was calculated for each plate from the proportion of negative wells, assuming a Poisson distribution. MF was determined as the ratio of CE in the presence of 6-TG:CE in the absence of 6-TG. The clonal relationship between mutants was determined by RFLP of the PCR products (24). Cells obtained from each mutant clone were frozen and used for nested PCR targeting the TCR-γ gene. Restriction digestion of the PCR product was performed separately with BstNI and RsaI followed by electrophoresis on a 5% NuSieve agarose gel.

Flow Cytometric Assay for the TCR Mutation. PBMCs (1 × 10^6 cells) were stained with fluorescein-labeled microbead-labeled anti-Leu3a (CD4) and phycoerythrin-labeled anti-Leu4 (CD3) antibodies (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) as described previously (10). The lymphocyte fraction was gated by forward and right-angle light scatter, and a window for mutants was set in the region where the surface CD3 level was below one-twenty fifth of that for normal CD4⁺ cells. The frequency of mutant cells showing the CD3⁺ CD4⁻ phenotype was calculated as the number of cells in the window for mutants divided by the total number of CD4⁺ T cells in the flow distribution.

Statistics. Because the distribution of MF is skewed, the MF values were transformed to natural logarithms and calculated. The 95% CIs for each MF value were calculated based on the CE and the number of cells plated (25).

Table 1: Patients' profiles and therapeutic regimens

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Gender</th>
<th>Regimen*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hodgkin's disease</td>
<td>7 yr. 7 mo</td>
<td>Female</td>
<td>(1)+(2)+(3)+(4)+(5)</td>
</tr>
<tr>
<td>2</td>
<td>Hodgkin's disease</td>
<td>12 yr. 9 mo</td>
<td>Male</td>
<td>(1)+(2)+(3)+(4)+(5)</td>
</tr>
<tr>
<td>3</td>
<td>Hodgkin's disease</td>
<td>13 yr. 11 mo</td>
<td>Female</td>
<td>(1)+(2)+(3)+(4)+(5)</td>
</tr>
<tr>
<td>4</td>
<td>Neuroblastoma</td>
<td>0 yr. 2 mo</td>
<td>Male</td>
<td>(3)+(4)+(5)</td>
</tr>
<tr>
<td>5</td>
<td>Neuroblastoma</td>
<td>0 yr. 7 mo</td>
<td>Male</td>
<td>(3)+(4)+(5)</td>
</tr>
<tr>
<td>6</td>
<td>Neuroblastoma</td>
<td>1 yr. 7 mo</td>
<td>Female</td>
<td>(4)+(5)+(6)+(7)</td>
</tr>
<tr>
<td>7</td>
<td>Neuroblastoma</td>
<td>1 yr. 11 mo</td>
<td>Female</td>
<td>(5)+(6)+(7)+(8)</td>
</tr>
<tr>
<td>8</td>
<td>Wilms’ tumor</td>
<td>0 yr. 8 mo</td>
<td>Female</td>
<td>(6)+(7)+(8)+(9)</td>
</tr>
<tr>
<td>9</td>
<td>Wilms’ tumor (CCSK*)</td>
<td>5 yr. 0 mo</td>
<td>Male</td>
<td>(6)+(7)+(8)+(9)</td>
</tr>
</tbody>
</table>

* 1, 1.5 mg/m² vincristine (days 1–3), 45 mg/m² pirarubicin (day 1), 1200 mg/m² CPM (days 1–3), 60 mg/m² prednisalone (days 1–7, p.o.), and 100 mg/m² procarbazine (days 15–28, p.o.): 2, 25 mg/m² pirarubicin (days 1 and 15), 10 units/m² bleomycin (days 1 and 15), 6 mg/m² vinblastine (days 1 and 15), 400 mg/m² dacarbazine (days 1 and 5), 1.5 mg/m² vincristine (days 43, 50, 57, and 64), and 20 mg/m² Adriamycin (days 43–45); and 9, 1.5 mg/m² vincristine (days 1 and 8), 0.35 mg/m² actinomycin D (days 1 and 5), 0.45 mg/m² actinomycin D (days 1–5), and 300 mg/m² CPM (days 1–3); 0.5 mg/m² vincristine (days 15, 22, and 29), 0.35 mg/m² actinomycin D (days 1–5) → 1.5 mg/m² vincristine (days 43, 50, 57, and 64), and 20 mg/m² Adriamycin (days 43–45).

Results

MF at the hprt Locus. The changes in MFs at the hprt locus before and during therapy are summarized in Fig. 1. Each hprt MF value with the 95% CI is also shown in Table 2. hprt MFs before treatment were within the 95% confidence limit of the healthy control (20), except for patient 1, whose MF was above it. In patients 1–3 with Hodgkin’s disease, hprt MFs began to increase quickly after the first or second cycle of chemotherapy. The maximum increase was more than 10-fold (13.3–67.3 × 10⁻⁶) as compared with the initial MFs; such levels were then maintained until the end of therapy (Fig. 1a). Hereafter, a net increase in hprt MF, i.e., an observed hprt MF – hprt MF before treatment, is shown in parentheses. In neuroblastoma patients 4 and 5 treated with CPM and vincristine, a 5–10-fold elevation (3.1–4.6 × 10⁻⁶) was observed (Fig. 1b). Patients 6 and 7 had neuroblastoma at an advanced stage; therefore, they were treated with more intensive chemotherapy, including pirarubicin, VP-16, and cisplatin. As shown in Fig. 1c, the responses varied between the two patients. Patient 6 demonstrated a 5-fold increase (3.5 × 10⁻⁶) after the first cycle, but patient 7 showed only a 2-fold increase (1.6 × 10⁻⁷). In patients 8 with Wilms’ tumor, mutation induction occurred after the second cycle of treatment that contained CPM (6.3 × 10⁻⁶). Patient 9 (clear cell sarcoma of the kidney) received Adriamycin instead of CPM in addition to the drugs used for patient 8, but MFs were stable throughout the treatment (Fig. 1d).

MF at the TCR Locus. Fig. 2 and Table 2 demonstrate the serial measurement of TCR MF values before and during therapy. Before treatment, the TCR MFs were all within the 95% confidence limit of the healthy control (20). The TCR MFs in patients 1 and 2 with Hodgkin’s disease exhibited a marginal increase (3.6–4.4 × 10⁻⁶). Patient 3 had an approximately 5-fold TCR MF elevation (5.7 × 10⁻⁴) after the third cycle, and the levels plateaued thereafter (Fig. 2a). In patients 4 and 5, transient increases in TCR MF (7.1 and 2.1 × 10⁻⁴) during the early course of chemotherapy were observed (Fig. 2b). Patients 6 and 7 showed a TCR MF elevation of about 5- and 8-fold (6.9 and 5.8 × 10⁻⁴), respectively, at later cycles of treatment (Fig. 2c). In patients 8 and 9, the TCR MF had elevated severalfold (5.8–6.7 × 10⁻⁴) during the first or second cycle and sustained the level until the end of therapy (Fig. 2d).
**Clonal Relationships among hprt Mutants in Hodgkin’s Disease Patients after Chemotherapy.** Because striking elevations of hprt MFs were observed in patients with Hodgkin’s disease, the clonal relationships of mutants were determined in mutant clones of their last hprt MF assays. The numbers of analyzed mutant clones were 11, 9, and 11, respectively. TCR-γ gene rearrangement patterns were all different in patients 1 and 2. In patient 3, 2 of 11 clones had an identical rearrangement pattern (Table 3).

**Correlation between hprt MF and the Cumulative Dose of CPM.** Because our present investigation postulated the possibility that CPM is most mutagenic in hprt mutation, we examined the correlation between hprt MF and the administered dose of CPM. As indicated in Fig. 3, an increase in hprt MF seems to be correlated with the cumulative dose of CPM \( r = 0.79; P < 0.001 \). The ability of CPM to induce mutation at the hprt locus is especially evident during an early phase of treatment. The correlations between hprt MFs and the cumulative dose of pirarubicin (or Adriamycin) and VP-16 are not significant (data not shown).

**Discussion**

Second malignancy is the most serious late complication among pediatric patients who survive their first cancer (1-3).

Several factors are considered to be related to the occurrence of a second malignancy: (a) genetic predisposition to cancer, such as the germ-line mutation of the p53 tumor suppressor gene. Malkin et al. (26) reported four patients with second malignancy who had a p53 mutation in their germ line without family histories indicative of Li-Fraumeni syndrome (26); (b) patients with primary malignancies such as childhood ALL (2) and Hodgkin’s disease (3) are known to be at high risk for second malignancy; and (c) an epidemiological study has shown the possibility that chemotherapeutic agents can induce a second malignancy. Among them, the association of alkylating agents with myelodysplastic syndrome with abnormality in chromosome 5 or 7 and of topoisomerase II inhibitor with acute monocytic leukemia with rearrangement of the MLL gene at chromosome 11q23 have been well documented (2, 27). However, there is currently no exact method to evaluate the risk for second malignancy at an individual level.

At present, cancer is considered to be the outcome of several mutational events (28, 29). A variety of chemotherapeutic agents have been demonstrated to cause somatic cell mutation both in vitro and in vivo (30). Therefore, MF assays used in this study, at least in part, may be useful as a tool for estimating the risk for second malignancy, although the target loci of these assays are neither the oncogene nor the oncosup-
pressor gene. In adults, hprt or GPA MF has been observed to be elevated in cancer patients after chemotherapy and/or radiotherapy, but they returned to normal levels during a follow-up of several months (13). Hirota et al. (16) have also shown significantly higher hprt, TCR, and GPA MFs in pediatric ALL patients after chemotherapy compared with those of age-matched controls. Notably, we reported that an increase in hprt MF persisted for several years after the completion of chemotherapy (15). From these studies, however, it cannot be known whether cancer chemotherapy really induces somatic cell mutation in vivo, because the researchers usually measured MFs only after chemotherapy. In fact, Caggana et al. (31) have demonstrated that some adult patients with Hodgkin’s disease already had a high hprt MF before therapy. We recently examined hprt and TCR MFs in pediatric cancer patients before starting therapy and found that patients with Hodgkin’s disease or Askin tumor had significantly higher hprt MF than do age-matched controls (20).

Perera et al. (17) have investigated the changes of multiple biomarkers in 36 germ cell tumor patients receiving platinum-

### Table 2: Results of the hprt and TCR MF in patients before and during chemotherapy

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sample no.</th>
<th>Nonselective Negative wells</th>
<th>Total wells</th>
<th>Selective Negative wells</th>
<th>Total wells</th>
<th>CE</th>
<th>hprt MF (× 10⁻⁶)</th>
<th>95% CI (× 10⁻⁶)</th>
<th>TCR MF (× 10⁻⁶)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>72</td>
<td>96</td>
<td>175</td>
<td>180</td>
<td>0.29</td>
<td>4.90</td>
<td>12.8 – 1.87</td>
<td>2.32</td>
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<tr>
<td>2</td>
<td>1</td>
<td>70</td>
<td>96</td>
<td>108</td>
<td>117</td>
<td>0.32</td>
<td>12.7</td>
<td>27.1 – 5.93</td>
<td>4.50</td>
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<tr>
<td>3</td>
<td>5</td>
<td>83</td>
<td>96</td>
<td>124</td>
<td>139</td>
<td>0.15</td>
<td>39.2</td>
<td>82.5 – 18.7</td>
<td>5.43</td>
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<td>6</td>
<td>84</td>
<td>94</td>
<td>244</td>
<td>287</td>
<td>0.11</td>
<td>72.2</td>
<td>144 – 36.2</td>
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<tr>
<td>5</td>
<td>0</td>
<td>45</td>
<td>96</td>
<td>416</td>
<td>429</td>
<td>0.76</td>
<td>2.03</td>
<td>3.74 – 1.10</td>
<td>4.96</td>
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<tr>
<td>1</td>
<td>2</td>
<td>37</td>
<td>75</td>
<td>84</td>
<td>96</td>
<td>0.71</td>
<td>9.45</td>
<td>18.1 – 4.92</td>
<td>3.03</td>
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<tr>
<td>2</td>
<td>3</td>
<td>36</td>
<td>96</td>
<td>99</td>
<td>123</td>
<td>0.98</td>
<td>11.1</td>
<td>17.9 – 6.85</td>
<td>4.50</td>
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<tr>
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<td>95</td>
<td>323</td>
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<td>11.4</td>
<td>18.0 – 7.23</td>
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<td>2.85</td>
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<td>46</td>
<td>96</td>
<td>214</td>
<td>218</td>
<td>0.74</td>
<td>1.26</td>
<td>3.49 – 0.45</td>
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<td>96</td>
<td>259</td>
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<td>0.58</td>
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<td>73</td>
<td>91</td>
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<td>3.97</td>
<td>10.7 – 1.47</td>
<td>2.03</td>
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Lower CI

Upper CI

Note: CE indicates the chi-square value.

Data are given as median (range).
As a cohort, hppt MF increased several-fold during the initial several cycles of treatment and then plateaued at a reduced level. Consequently, the posttreatment value of hppt MF revealed only a marginal increase. In contrast, GPA MF increased gradually throughout therapy. The GPA MF demonstrated a significant elevation 3-6 months after the end of chemotherapy. Other investigators have reported the frequencies of hppt mutation and micronuclei of adult patients with various cancers during chemotherapy (18). Results vary widely among patients, but alkylating agents such as ifosfamide and CPM turned out to be the most mutagenic. These studies, which were carried out on adult patients, clearly indicate the mutagenic effects of cancer chemotherapy. As far as we know, however, no reports exist of such an investigation of pediatric cancer patients. This led us to examine the changes of MFs at two gene loci, hppt and TCR, in nine pediatric cancer patients from diagnosis until the end of therapy. Major findings in our study are as follows: (a) in most patients, MFs at the hppt locus tended to increase during the early cycles of treatment, irrespective of the chemotherapeutic regimens. Whether or not such an increase is statistically significant is difficult to determine because of interpatient variation. This led us to calculate the 95% CIs for MF based on the CE and the plating efficiency (25). During chemotherapy, patients with Hodgkin’s disease exhibited CIs that did not overlap with the CIs before treatment; (b) the clonality analysis of hppt mutant clones derived from Hodgkin’s disease patients revealed that an increased hppt MF was the result of independent mutational events; (c) an increase in TCR MF was also found during chemotherapy in most patients, ranging from 2.11-7.08 × 10^{-4}. Although the time of TCR MF elevation was variable among patients, a fold increase during chemotherapy was not so prominent as that of hppt MF; and (d) among the chemotherapeutic agents used in this study, CPM was considered to be the most mutagenic. However, the mutagenic effect of other drugs, including VP-16, seemed to be equivocal.

Alkylating agents, such as CPM, chlorambucil, dacarbazine, mechlorethamine, and melphalan, are well known to have
mutagenic effects (32, 33). CPM was used in all patients except for patient 9, but the administered dose varied from patient to patient. In our study, the in vivo mutagenic effect of CPM is quite evident, because a substantial increase in hprt MF was noted in patients with neuroblastoma who were treated with only CPM and vincristine. It is noteworthy that no mutagenic effect has ever been reported for vincristine. Although CPM is not the only agent used for chemotherapy, an elevation of hprt MF seems to be dependent on the cumulative dose of CPM. The hprt MF responses were most striking in patients with Hodgkin’s disease at a cumulative dose of 2000–4000 mg/m² CPM, but the fact that they were also treated with other alkylating agents, i.e., dacarbazine and procarbazine, should be taken into consideration. Our data may suggest the possibility that patients with Hodgkin’s disease are more susceptible to drug (alkylating agents)-induced mutagenesis. The finding that the addition of VP-16 to the chemotherapeutic regimen failed to further increase the induction of hprt mutation by CPM in patients with neuroblastoma is intriguing. Although the number of samples in this study is limited, this fact seems to be in accordance with our previous report demonstrating that patients with acute myeloid leukemia treated with a VP-16-containing regimen did not show increased hprt MF (15). Another study of hprt MF in small cell lung cancer patients receiving VP-16 also showed no significant increase (19). These data are in accordance with an in vitro study demonstrating that the mutagenic potency of VP-16 was much less than that of N-methyl-N′-nitro-N-nitrosoguanidine at approximately the same survival rate (34). As one possible explanation for this, VP-16 may cause a large DNA deletion in lymphocytes, leading to cell death before expressing the hprt mutation (19, 35). In fact, VP-16 has been shown to induce apoptosis in a variety of hematopoietic cells (36–38). Another possibility is that in patients treated with VP-16, the induction of chromosomal aberrations may be more common than that of hprt or TCR mutation (39).

In summary, we first presented the in vivo mutagenic effects of cancer chemotherapy at the hprt and the TCR gene loci in nine pediatric cancer patients by a sequential determination of MFs. Because the two biomarkers have different responses in the same individuals, it would be advisable to follow-up more biomarkers simultaneously (17). In fact, several investigators have reported the usefulness of the GPA assay to screen individuals known to be at high risk for secondary leukemia (40, 41). Finally, the molecular analysis of mutational spectra is considered to be potentially important, and such analysis is now in progress in our laboratory.

References
Prospective study of mutant frequencies at the hprt and T-cell receptor gene loci in pediatric cancer patients during chemotherapy.


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