

Genotoxicity of Therapeutic Intervention in Children with Acute Lymphocytic Leukemia

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ABSTRACT

The survival rates of children treated for cancer have dramatically increased after the development of standardized multiple-modality treatment protocols. As a result, there is a rapidly growing population of pediatric cancer survivors in which the long-term genotoxic effects of chemotherapeutic intervention is unknown. To study the genotoxic effects of antineoplastic treatment in children, we performed a comparative analysis of the changes in the frequency of somatic mutations (Mfs) at the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*)-reporter gene in children treated for acute lymphocytic leukemia (ALL). We measured *HPRT* Mfs from 130 peripheral blood samples from 45 children with ALL (13, low risk; 22, standard risk; and 10, high risk) from the time of diagnosis, as well as during and after the completion of therapy. We observed a significant increase in mean *HPRT* Mfs during each phase of therapy (diagnosis, 1.4×10^{-6} ; consolidation, 52.1×10^{-6} ; maintenance, 93.2×10^{-6} ; and off-therapy, 271.7×10^{-6}) that were independent of the risk group treatment protocol used. This 200-fold increase in mean somatic Mf remained elevated years after the completion of therapy. We did not observe a significant difference in the genotoxicity of each risk group treatment modality despite differences in the compositional and clinical toxicity associated with these treatment protocols. These findings suggest that combination chemotherapy used to treat children with ALL is quite genotoxic, resulting in an increased somatic mutational load that may result in an elevated risk for the development of multi-factorial diseases, in particular second malignancies.

INTRODUCTION

Acute lymphocytic leukemia (ALL) is the most common pediatric malignancy and is highly responsive to chemotherapeutic intervention (1). Since the early 1960s, the remission rate for children with ALL has increased to an overall 5-year survival rate approaching 80%. This success is the result of the development of national standardized multiple-modality risk of relapse directed chemotherapeutic treatment protocols (2) in which between 73% (children age <20 years) and 85% (children age <15 years) of children with ALL participate (3, 4). Children with ALL are currently grouped into specific risk of relapse categories (low, standard, and high), which determines protocol modality, therapy intensity, and length of exposure. These relapse risk designations have been correlated to clinical and biological prognostic factors such as the patient's age at disease onset, WBC counts at diagnosis, sex, race, and the karyotype of leukemic cells (4). Treat-

ment regimens consist of three specific phases (induction, consolidation, and maintenance) that use antimetabolites, anthracyclines, topoisomerase inhibitors, and steroids over a 2–3 year period. These antineoplastic agents were selected for their broad genotoxic and cytotoxic properties, which target cell cycle mechanisms of malignant cells, thereby inhibiting proliferation and inducing cell death leading to senescence or apoptosis (5).

The current success in the treatment of children with ALL is leading to a growing population of adolescent and adult cancer survivors. As a result, there is an increasing need to examine the long-term genetic consequences of prolonged exposure to genotoxic antineoplastic agents in these children and young adults. An intrinsic complication of combination chemotherapy is that selected agents can also target normal somatic cells. The cellular and genetic alterations to nontumor somatic cells as a consequence of antineoplastic therapy contribute to late effect complications observed with treatment that include cardiac and pulmonary toxicity, nephrotoxicity, neurotoxicity, and neuropsychological abnormalities (6, 7). In addition, children treated for ALL have a 5–20 times greater risk for developing secondary malignant neoplasm's (SMN), especially with associated radiation (1, 6, 8–10). One specific question is whether chemotherapeutic treatment in children results in the accumulation of somatic mutations in nontumor somatic cell populations that could contribute to their increased risk of developing multi-factorial diseases and SMN later in life. This question can be studied by monitoring changes in the frequency of somatic mutations (Mfs) at the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*)-reporter gene in peripheral human T cells. The *HPRT* enzyme is ubiquitous and catalyzes reactions involved with purine nucleotide salvage in mammalian cells. It also binds and ribophosphorylates many toxic purine analogs including 6-thioguanine, which allows for the *in vitro* selection of mutant T cells that have acquired an *HPRT* mutation *in vivo* (11–13). In addition, the *HPRT* gene is an excellent biomarker of effect because mutations at the *HPRT* locus have no direct clinical consequences and have been shown to reflect genome-wide mutational events (13, 14). This approach for somatic mutation analysis in humans has been widely used to determine *in vivo* background as well as acquired somatic cell Mfs in pediatric and adult populations exposed to known and unknown environmental mutagens (15–21).

In this study, we performed a longitudinal assessment of the frequency of somatic mutations at the *HPRT* locus (*HPRT* Mf) in children with ALL enrolled in low-, standard-, and high-risk chemotherapeutic treatment protocols, with blood samples collected at the time of diagnosis before the start of therapy (diagnosis), during therapy (induction, consolidation, maintenance), and after therapy cessation (off-therapy). We observed a dramatic increase in *HPRT* Mf after the onset of therapy through completion in each of the risk groups. Of importance is the observation that *HPRT* Mf remains elevated after the completion of therapy and then continues to increase at a rate similar for normal age matched controls. These findings suggest that combination chemotherapy treatment protocols used to treat children with ALL are quite genotoxic, resulting in a sustained

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increase in somatic mutational load in exposed cell populations with a long *in vivo* life span that could result in an elevated risk for the development of SMN or other multi-factorial diseases later in life.

MATERIALS AND METHODS

Study Population and Sample Collection. A total of 130 heparinized peripheral blood samples from 45 children with ALL from the oncology units at the University of Vermont and other participating Pediatric Oncology Group/Children's Oncology Group institutions were collected and analyzed at diagnosis, induction, consolidation, maintenance, and off-therapy phases of treatment. Thirteen children were enrolled from low-risk protocols (9201, 9904, and 9004), 22 children were enrolled from standard-risk protocols (9405, 9605, 9005, and 9905), and 10 children were enrolled from high-risk ALL protocols (9406, 9906, 9006). Patients were classified as low-, standard-, and high-risk for ALL relapse based on their age at diagnosis, cytogenetic and molecular properties of WBC populations, and leukocyte cell counts as outlined by the cooperative group protocols (Fig. 1). Informed consent was obtained following procedures approved by the Committee on Human Research at the University of Vermont and participating Pediatric Oncology Group/Children's Oncology Group institutions. Some protocols/phases of treatment within each prospective risk group contained scheduling differences, which were taken into account in the analysis. All treatment schedules were finished in 130 weeks. A synopsis of chemotherapeutic agents used for risk directed treatment protocols for patients included in this study is summarized in Fig. 1 and included vincristine, cyclophosphamide, cytosine arabinoside, prednisone, hydrocortisone, and dexamethasone. The topoisomerase II inhibitor teniposide (VM-26), anthracycline daunomyacin and folic acid derivatives such as leucovorin were also included. All treatment protocols incorporated long-term use of purine analogs 6-mercaptopurine (6-MP)/6-thioguanine, as well as methotrexate during the consolidation and maintenance phases of therapy.

HPRT T-Cell Cloning Assay. Determination of somatic mutant frequency at the *HPRT* locus has been described previously in detail (17). Heparinized blood samples were diluted with basic RPMI 1640. Mononuclear cell fractions were isolated with ficoll hypaque (specific gravity 1.077) by density sedimentation and washed twice with saline G. The mononuclear cells were inoculated at 1, 2, and 5 cells/well in 96-well round-bottomed microtiter plates for determining unselected cloning efficiency (CE). To select *HPRT*-mutant isolates, 1–4 × 10⁴ cells/well were plated in the presence of 10 μM 6-thioguanine. Cells were grown in humidified incubators (10–21 days) in an atmosphere of 5–6% CO₂, at 37°C, and scored for positive colonies on days 12–14 and 17–21 using an inverted phase-contrast microscope. CEs were calculated using the Poisson distribution relationship, $P = e^{-x}$, where P is the fraction of wells without colony growth and x is the average number of clonable cells per well. $CE = -\ln P_0/N$, where N is the average number of cells inoculated per well by limited dilution. The mean CE was determined from three different concentrations of unselected cells (1, 2, and 5 cells/well). The Mf is the ratio of the mean CE in the presence of (selected) and absence (unselected) of 6-thioguanine. Because the distribution of Mf is skewed, statistical analyses are based on the natural logarithms of Mf × 10⁻⁶ (lnMf).

Statistical Analysis. Longitudinal analysis of somatic *HPRT* Mfs was based on data obtained from children at the different time points and phases of therapy (diagnosis, induction, consolidation, maintenance, and off therapy) for each risk group protocol used (low, standard, and high), Table 1 and Fig. 2. A two-stage hierarchical model was fitted to the data from all time points to estimate the effects of the three-treatment phases on Mf and to determine whether these effects differed in low-, standard-, and high-risk children (Fig. 3). For the first stage, a person's lnMf at a particular time point was modeled as a linear function of age at diagnosis, unselected CE, and the number of weeks of each treatment phase completed at the time of the measurement, as well as the number of weeks off therapy if the measurement was made after completion of treatment. No lnMf measurements were obtained during the induction phase; therefore, this treatment phase was included in the model as an indicator variable having the value of 0 if the

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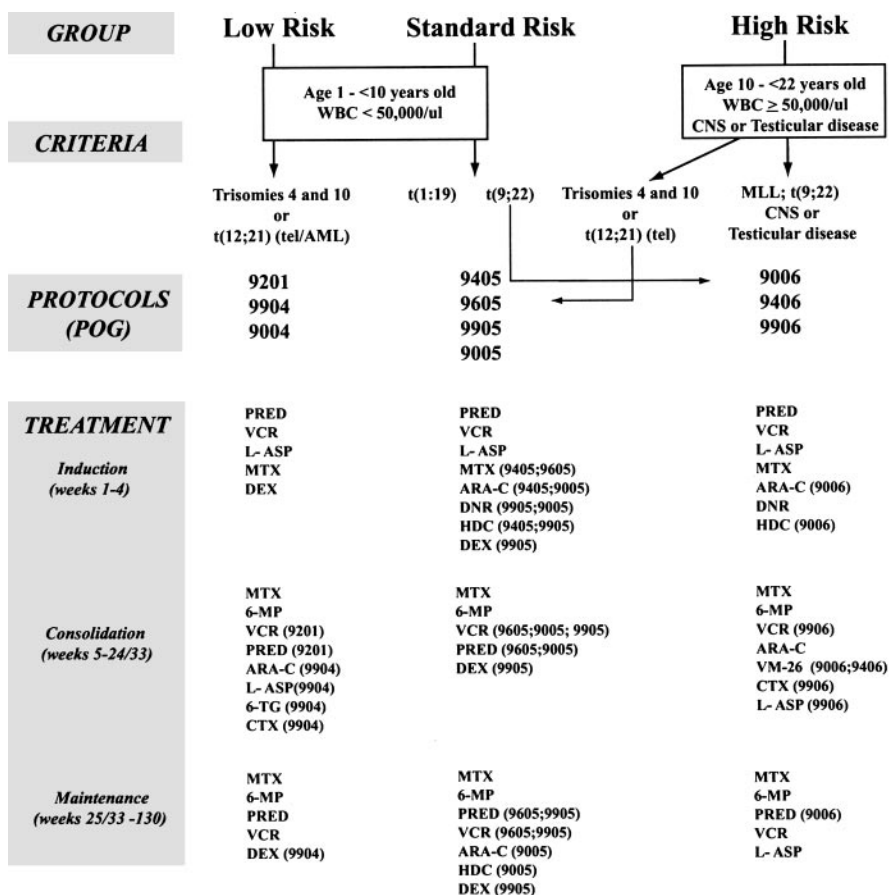


Fig. 1. Risk group designations, criteria, and Pediatric Oncology Group (POG) treatment protocols for children treated for acute lymphocytic leukemia. CNS, central nervous system; AML, acute myelogenous leukemia; MLL, mixed lineage leukemia; PRED, prednisone; DEX, dexamethasone; VCR, vincristine; L-ASP, L-asparaginase; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; MTX, methotrexate; HDC, hydrocortisone; ARA-C, cytosine arabinoside; DNR, daunomyacin; VM-26, teniposide; CTX, cyclophosphamide.

Table 1 Summary of age, sex, unselected cloning efficiency (CE), mutant frequency (Mf), lnMf, and phase of therapy for children treated for acute lymphocytic leukemia (ALL)

Subject/samples	Sex	Age	CE	Weeks ^a	Mf (×10 ⁻⁶)	lnMf (×10 ⁻⁶)	Phase of therapy
Low risk ALL							
CS-49	M	4.0	0.59	0	0.15	-1.897	Diagnosis
		4.2	0.35	11	1.00	0.000	Consolidation
		4.4	0.35	20	5.09	1.627	Consolidation
		4.5	0.31	26	7.84	2.059	Maintenance
		5.5	0.39	77	30.90	3.431	Maintenance
CS-218	M	9.8	0.54	303	70.10	4.250	Off-therapy
		1.8	0.59	0	0.30	-1.204	Diagnosis
		2.3	0.86	25	1.20	0.182	Maintenance
		3.6	0.24	96	67.30	4.209	Maintenance
		5.2	0.62	176	194.30	5.269	Off-therapy
CS-227	M	6.2	0.19	0	0.47	-0.755	Diagnosis
		7.4	0.25	60	3.67	1.300	Maintenance
		7.8	0.82	84	9.01	2.198	Maintenance
CS-275	M	8.3	0.30	108	9.70	2.272	Maintenance
		3.8	0.79	0	0.66	-0.416	Diagnosis
CS-265	M	3.5	1.22	0	0.30	-1.204	Diagnosis
		4.1	0.37	30	6.06	1.802	Consolidation
CS-029	M	3.1	0.22	26	1.06	0.058	Maintenance
CS-175	M	10.9	0.18	276	13.50	2.603	Off-therapy
CS-209	F	2.4	0.07	0	1.60	0.470	Diagnosis
		3.1	0.96	36	4.70	1.548	Maintenance
		4.2	0.36	96	184.70	5.219	Maintenance
		4.9	0.29	129	536.80	6.286	Maintenance
		5.8	0.33	177	215.00	5.371	Off-therapy
CS-250	F	10.1	0.67	53	37.60	3.627	Maintenance
		10.3	0.58	61	48.00	3.871	Maintenance
		10.6	0.96	75	146.60	4.988	Maintenance
		11.0	0.38	97	544.10	6.299	Maintenance
CS-254	F	2.6	0.23	24	7.45	2.008	Consolidation
		3.3	0.72	60	41.90	3.735	Maintenance
CS-272	F	6.4	0.92	0	0.29	-1.238	Diagnosis
		6.8	1.07	22	0.74	-0.301	Consolidation
CS-071	F	8.3	0.05	184	20.94	3.042	Off-therapy
CS-292	F	8.6	0.89	0	0.29	-1.238	Diagnosis
Standard risk ALL							
CS-36	M	6.6	0.14	59	15.37	2.732	Maintenance
		9.7	0.46	217	29.20	3.374	Off-therapy
CS-37	M	1.7	0.31	4	0.22	-1.514	Induction
		2.4	0.30	39	1.16	0.148	Maintenance
		2.7	0.35	54	5.10	1.629	Maintenance
		3.1	0.04	78	353.80	5.869	Maintenance
		3.7	0.04	106	79.40	4.374	Maintenance
		4.2	0.05	131	129.20	4.861	Off-therapy
		6.0	0.47	226	341.10	5.832	Off-therapy
		7.8	0.33	322	87.17	4.468	Off-therapy
CS-51	M	2.7	0.68	31	1.08	0.077	Consolidation
		5.2	0.11	162	177.30	5.178	Off-therapy
		7.7	0.19	292	214.70	5.369	Off-therapy
CS-69	M	2.9	0.55	0	0.64	-0.446	Diagnosis
		3.1	0.37	9	0.94	-0.062	Consolidation
CS-75	M	8.2	0.76	274	353.70	5.868	Off-therapy
		8.8	0.51	5	6.43	1.861	Consolidation
CS-82	M	11.5	0.07	144	443.40	6.094	Off-therapy
CS-82	M	4.3	0.40	0	0.87	-0.139	Diagnosis
		3.6	0.49	0	3.40	1.224	Diagnosis
		3.8	0.34	11	3.10	1.131	Consolidation
		4.5	0.17	51	6.20	1.825	Maintenance
CS-122	M	6.0	0.53	129	9.20	2.219	Maintenance
		8.1	0.82	237	15.50	2.741	Off-therapy
		5.0	0.76	0	0.63	-0.462	Diagnosis
CS-150	M	5.1	0.12	4	0.30	-1.204	Induction
		7.9	0.21	151	1100.00	7.004	Off-therapy
CS-211	M	11.5	0.10	127	84.98	4.442	Maintenance
CS-222	M	4.3	0.70	0	1.50	0.405	Diagnosis
		5.0	0.59	33	1.60	0.470	Consolidation
		5.1	2.11	39	30.10	3.405	Maintenance
CS-258	M	5.5	0.47	61	174.60	5.162	Maintenance
		3.2	0.20	0	1.16	0.148	Diagnosis
		3.8	0.50	33	1052.00	6.958	Consolidation
CS-268	M	5.2	0.32	102	575.20	6.355	Maintenance
		5.3	0.32	132	3.27	1.185	Off-therapy
		6.3	0.39	187	6.76	1.911	Off-therapy

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Table 1 Continued

Subject/samples	Sex	Age	CE	Weeks ^a	Mf (×10 ⁻⁶)	InMf (×10 ⁻⁶)	Phase of therapy
CS-284	M	14.0	0.42	252	545.50	6.302	Off-therapy
CS-285	M	9.5	0.09	0	3.00	1.099	Diagnosis
		9.6	0.30	4	1.70	0.531	Induction
CS-287	M	11.4	0.47	0	3.66	1.297	Diagnosis
CS-43	F	4.1	0.69	0	2.06	0.723	Diagnosis
		4.5	0.77	17	1.14	0.131	Consolidation
CS-23	F	3.2	0.13	0	2.30	0.833	Diagnosis
		3.4	0.21	12	0.47	-0.755	Consolidation
		3.7	0.26	26	2.02	0.703	Consolidation
		4.4	0.17	64	6.16	1.818	Maintenance
		6.0	0.07	144	149.70	5.009	Off-therapy
CS-62	F	10	0.22	304	13.70	2.617	Off-therapy
CS-74	F	3.5	0.41	0	0.21	-1.561	Diagnosis
		3.6	0.32	6	1.64	0.495	Consolidation
		4.4	0.36	106	7.40	2.001	Maintenance
		6.3	0.09	148	132.80	4.889	Off-therapy
		6.5	0.33	159	76.50	4.337	Off-therapy
		6.8	1.11	175	64.90	4.173	Off-therapy
		8.3	0.72	250	234.50	5.457	Off-therapy
		9.2	0.12	298	733.00	6.597	Off-therapy
CS-217	F	6.3	0.83	0	1.40	0.336	Diagnosis
		8.3	0.47	103	29.00	3.367	Maintenance
		8.8	0.22	129	232.80	5.450	Maintenance
CS-290	F	1.6	0.18	0	1.16	0.148	Diagnosis
CS-273	F	11.2	0.27	7	9.10	2.208	Consolidation
High Risk ALL							
CS-241	M	12.8	0.35	47	5.66	1.733	Maintenance
		13.3	0.13	71	11.10	2.407	Maintenance
		13.6	0.32	87	6.05	1.800	Maintenance
CS-269	M	17.6	0.15	124	49.20	3.896	Maintenance
		17.8	0.35	136	53.40	3.978	Off-therapy
CS-276	M	17.3	0.25	0	4.21	1.437	Diagnosis
		17.3	0.16	4	0.84	-0.174	Induction
CS-033	M	1.2	0.16	0	0.68	-0.386	Diagnosis
		1.8	0.30	33	1.16	0.148	Consolidation
		2.3	0.40	60	1.75	0.560	Maintenance
		3.8	0.23	137	131.20	4.877	Off-therapy
		4.7	0.46	185	190.10	5.248	Off-therapy
CS-005	F	6.0	0.08	0	1.63	0.489	Diagnosis
		8.3	0.66	120	1.59	0.464	Maintenance
		9.0	0.18	152	3.70	1.308	Off-therapy
		9.1	0.41	161	1.95	0.668	Off-therapy
		13.1	1.12	368	7.84	2.059	Off-therapy
		14.1	0.41	420	13.60	2.610	Off-therapy
CS-027	F	2.5	0.10	0	0.62	-0.478	Diagnosis
		6.1	0.46	188	21.40	3.063	Off-therapy
		9.4	0.16	362	18.00	2.890	Off-therapy
CS-044	F	4.3	0.07	124	120.00	4.787	Maintenance
		4.5	0.01	137	252.00	5.529	Off-therapy
		4.5	0.14	137	556.20	6.321	Off-therapy
		5.2	0.05	172	1848.00	7.522	Off-therapy
		5.2	0.15	172	607.70	6.410	Off-therapy
		5.3	0.20	181	1375.00	7.226	Off-therapy
		10.6	0.15	455	558.30	6.325	Off-therapy
CS-045	F	4.4	0.11	10	1.05	0.049	Consolidation
CS-223	F	12.7	0.14	0	2.10	0.742	Diagnosis
		12.8	0.09	5	5.80	1.758	Consolidation
		14.3	0.68	81	2.10	0.742	Maintenance
		14.8	0.36	105	2.18	0.779	Maintenance
CS-237	F	22	0.13	427	133.90	4.897	Off-therapy

^a Weeks since diagnosis.

measurement was obtained before treatment and a value of 1 if obtained after induction. The unselected CE associated with each Mf was included in the model as a time-varying covariate. For the second stage, each of these coefficients was in turn modeled as a function of risk group and a person's random deviation from the average value of the coefficient of his/her group. All models were fitted using restricted maximum likelihood methods, as implemented by SAS, PROC Mixed software (22).

RESULTS

A summary of subjects and samples, age, gender, CE, Mf, InMf, weeks on therapy, phase of therapy, risk group, and protocol is shown in Table 1. At the time of diagnosis, the mean age of the children was 5.5 years, and, as reported in a previous paper, their CEs and InMFs

did not differ significantly from those of healthy children of the same age (23). Longitudinal analysis of Mfs at the *HPRT* locus was performed on 130 peripheral blood samples from 45 children. Thirty-four observations from 13 low-risk children, 62 observations from 22 standard-risk children, and 34 observations from 10 high-risk children were included in this study (Table 1; Fig. 2). The mean age of children on low-risk protocols was 4.8 years, for children on standard-risk treatment protocols, 5.4 years, and children on high-risk treatment protocols, 8.8 years. Children on high-risk treatment protocols had significantly lower unselected CE at the time of diagnosis. There were no significant differences in unselected CE by phase of therapy (Table 2). The number of measurements obtained from each child ranged from 1 to 8. Previous studies have shown a relationship among

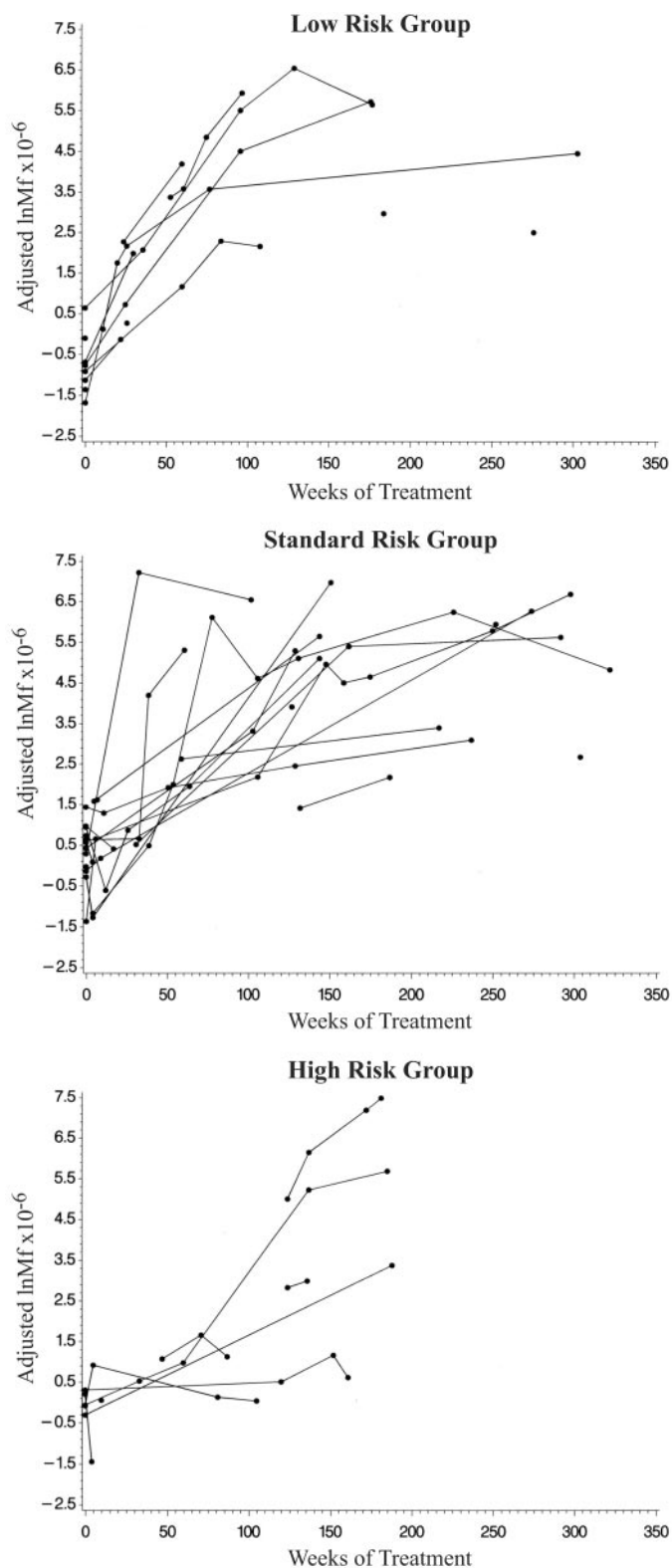


Fig. 2. Adjusted lnMf versus weeks of treatment for children on low-, standard-, and high-risk treatment protocols.

unselected CE, age, and lnMf (17, 24). We corrected for the effects of age and unselected CE on lnMf by including these variables in the regression analysis and adjusting the values in Fig. 2 to correspond to the lnMf of a 5.4-year-old child with a CE of 0.39 (averages for the entire study group). Several unselected CEs are >1 , which can occur

if cell growth is robust and the average number of clonable cells per well has been underestimated. Although CEs over 1 are not intuitively meaningful, they reflect the variability inherent in the assay and must be used in the computation of Mf, as well as statistical analysis of CE, to avoid bias.

Phase of Treatment and Mutant Frequency. Data showing the effect of phase of therapy on the frequency of somatic mutation at the *HPRT* locus in children from each risk group is given in Table 2. Samples were obtained from the time of diagnosis to the end of therapy (130 weeks) as well as up to 455 weeks after the start of therapy. All but five measurements were obtained within 350 weeks of diagnosis. Standard deviations within each phase of treatment are large, not only because of variability between individual subjects but also because Mf values were grouped together based on phase, without consideration for the cumulative effects of weeks on therapy. There is overlap in the timing of measurements at various phases of treatment because the length of these treatment phases differed slightly between protocols. All treatments ended at 130 weeks. During and after the completion of therapy, the nonselected CE remained relatively stable. There was a nonsignificant decrease in CE during the induction phase compared with the other phases of therapy that is probably indicative of the lympholytic aspect of the treatment during this phase. There was a steady and dramatic increase in the frequency of somatic mutations at the *HPRT* locus (Mf and lnMf) after induction in each of the groups (Table 2; Fig. 2). Specifically, there was an incremental 38- to 68-fold increase over Mf values at diagnosis from consolidation to the end of the maintenance-treatment phase. Of importance is that after the completion of therapy, there continued to be a striking increase in the mean Mf that was 200-fold higher than at the start of treatment.

Regression Analysis for the Longitudinal Effects of Treatment on Somatic Mutant Frequency. To rigorously assess the longitudinal genotoxic effects of chemotherapy and risk group, we fitted a hierarchical regression model to lnMf values obtained from children throughout each phase of therapy from each risk group protocol (Table 3; Fig. 3). The intercept of the model represents the lnMf before treatment whereas the remaining coefficients represent the change in lnMf after induction and the rate of change in lnMf per week during consolidation, maintenance, and off-therapy phases of treatment. This random coefficient regression analysis takes into account the correlations between repeated measurements on the same person and does not require an equal number of measurements or comparable time points for each person.

This analysis showed that standard-risk children had a significantly higher lnMf at diagnosis than low-risk children with ALL ($P < 0.05$) after adjustment for age and unselected CE. Regression estimates for the effects of the induction phase of treatment on lnMf demonstrated that children on low-risk protocols had significantly increased Mfs at the end of induction compared with the time of diagnosis. In addition, lnMf in children on high-risk protocols were significantly lower at the end of induction compared with children on low and standard-risk protocols (Table 3).

The analysis also provided estimates of the changes in lnMf per week for children during the consolidation and maintenance phases of treatment as well as after the completion of therapy (Table 3). The estimated longitudinal changes in lnMf over time shown graphically in Fig. 3 include the different durations for the consolidation and maintenance phases for low-, standard-, and high-risk treatment protocols. Fig. 3 also shows the age-related changes in normal children, as estimated previously from cross-sectional data (25). The regression analysis did not indicate significant differences between the three risk groups in the rate of lnMf change per week during each phase of therapy. There were significant increases in lnMf per week for chil-

Table 2 The effects of phase of treatment on cloning efficiency (CE) and frequency of mutation (Mf) at the HPRT locus

Treatment phase	Subjects (N)	Samples (N)	Weeks ^a	CE ^b	Mf (×10 ⁻⁶)	lnMf (×10 ⁻⁶)
Diagnosis	25	25	0	0.45 ± 0.33	1.38 ± 1.16	-0.055 ± 0.939
Induction	4	4	4-4	0.22 ± 0.10	0.76 ± 0.68	-0.596 ± 0.938
Consolidation	18	22	5-33	0.47 ± 0.43	52.04 ± 223.02	1.168 ± 1.657
Maintenance	22	37	25-130	0.40 ± 0.25	93.18 ± 158.32	3.078 ± 1.873
Off Therapy	23	41	131-455	0.33 ± 0.27	271.83 ± 393.44	4.506 ± 1.760

^a Weeks since diagnosis.

^b ± = SD.

dren on all three risk group treatment protocols during both the consolidation ($P = 0.027$) and maintenance phases of treatment ($P = 0.001$). In addition, the rate of increase in lnMf during the consolidation phase of treatment was significantly higher than during the maintenance phase ($P = 0.039$). The increase in lnMf per week in children after the completion of therapy ($P = 0.077$) was not statistically significant. This observation is not surprising because of the short time period and the large variability of the data. Of interest is that the estimated rate of change in lnMf after the completion of therapy is similar to the age-related changes observed in normal children although their Mfs are significantly higher.

This analysis further supports our observations of a rapid sustained stable increase in lnMf in children during and after the completion of antineoplastic therapy for ALL that is independent of risk group. Of significance is that this increased mutational load remained elevated in these children for years after the completion of therapy.

DISCUSSION

The success of pediatric cooperative cancer group treatment protocols using standardized multiple-modality risk-directed chemotherapeutic regimens for the treatment of ALL and other pediatric cancers has resulted in a rapidly growing population of children and young adult cancer survivors. An important aspect of the clinical follow-up of these patients involves screening for late effect cardiac, renal, endocrine, and neuro-toxicity associated with treatment. The current supposition is that the short- and long-term clinical toxicity of these protocols is greater in the higher risk-directed treatment regimens, which use higher or more frequent dosing of anthracyclines and alkylating agents than low- and standard-risk protocols.

In addition to these late effect risks, children treated successfully

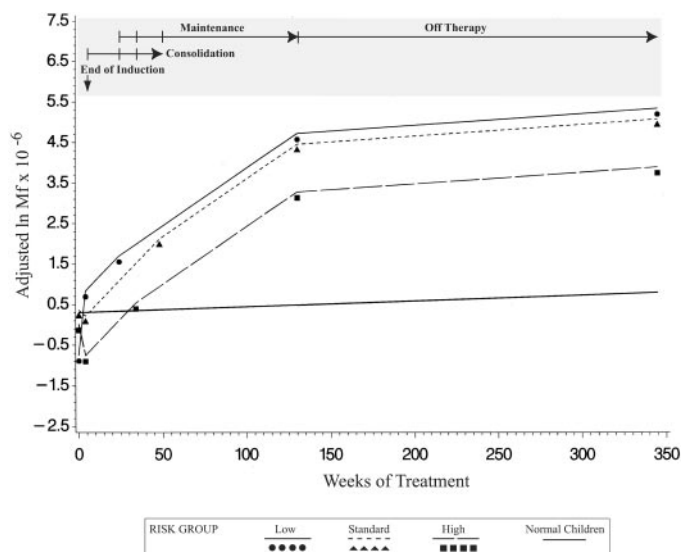


Fig. 3. Estimated longitudinal effects of antineoplastic therapy on somatic mutant frequency in children treated for acute lymphocytic leukemia (ALL).

Table 3 Regression analysis model for estimating the effect of treatment on somatic mutant frequency (lnMf)

Stage of treatment	Estimate	SE	P
At diagnosis ^a			
Low risk	-0.7542	0.2923	
Standard risk	0.4271 ^b	0.2290	
High risk	0.0037	0.3704	
Start of consolidation ^a			
Low risk	0.8331 ^b	0.3443	
Standard risk	0.2161	0.3435	
High risk	-0.7479 ^c	0.4369	
Change per week ^d			
Consolidation	0.0431	0.0172	0.027
Maintenance	0.0286	0.0077	0.001
Off therapy	0.0029	0.0016	0.077

^a Regression model lnMf estimates adjusted for age at diagnosis and cloning efficiency (CE), corresponding to the lnMf for a 5.4-year-old with a CE of 0.39 (the averages).

^b Significantly higher compared to low risk children at diagnosis prior to treatment.

^c Significantly lower compared to children treated with low and standard risk protocols after induction.

^d Rate of change in lnMf per week in all risk groups (low, standard, and high).

for ALL also have a 5-20 times greater risk for developing a SMN (1, 6, 8-10). The etiology of this increased cancer risk in children after treatment is unclear. One possibility is that children with cancer have a specific genetic predisposition to acquiring somatic mutations that lead to an overall increased cancer risk. Another possibility is that genotoxic chemotherapy results in an increase in somatic mutations in long-lived cell pools that contribute to the development a SMN. To date, there is no evidence to support a direct link between an increase in the frequency of somatic mutations after antineoplastic treatment and an increased risk of SMN.

In this study, the genotoxic effects of chemotherapeutic treatment of children with ALL were determined by measuring the frequency of somatic mutations at the HPRT locus before, during, and after the completion of therapy. Longitudinal analysis of somatic Mf after the start of therapeutic intervention demonstrated a dramatic and sustained increase in Mf over time in each ALL risk group. The rate of increase varied by phase of therapy but was highest during the consolidation phase of treatment and independent of the risk protocols used. During the induction phase, there was a significant increase in Mf in children from the low-risk treatment protocol whereas children from high-risk protocols had a significantly lower Mf compared with children from low- and standard-treatment protocols. The lower Mf at diagnosis in children on high-risk protocols may be the result of technical difficulties calculating mononuclear cell concentrations used to set up the T cell cloning assay, because these children present with markedly elevated WBC counts that make these initial determinations difficult.

Similar genotoxic biomonitoring studies have shown elevations in somatic cell Mfs after exposure to chemotherapy and radiation in multiple reporter gene model systems that were lower than those we observed (15, 26-31). Specifically, Hirota *et al.* (32) reported an increase in HPRT Mfs in children after treatment for ALL (7.8×10^{-6}) compared with healthy control values (1.1×10^{-6}). Similarly, somatic Mfs in adult cancer patients during and after combination chemotherapy were higher ($15.9 \pm 15.0 \times 10^{-6}$) com-

pared with pretreatment mean Mfs ($9.1 \pm 3.9 \times 10^{-6}$; Ref. 33). Unlike our observations in children, somatic Mf in adult cancer patients has been shown to eventually return to control levels after treatment cessation (34).

Our most significant finding is a dramatic increase in the accumulation of somatic mutations in children during exposure to various risk-directed therapeutic protocols that resulted in an extraordinary increase in overall mutational load that remained elevated even years after the completion of therapy. Of interest is that we observed no significant differences in the overall genotoxic effect in each risk group therapy used, despite considerable difference in the composition, length of exposure, and clinical toxicity associated with these therapies. Specifically, children treated on low-risk protocols have very little exposure to alkylating agents and anthracyclines whereas children on high-risk protocols have elevated exposure to these agents as well as epipodophyllotoxins. Exposure to these agents for children on the standard-risk protocols is somewhere in between. Our statistical power to detect a difference in genotoxicity was limited by the small number of subjects and time points per subject in each risk group, as well as by the considerable variability between children receiving the same treatment. It is therefore possible that the risk protocols do differ in their genotoxic effects, but our results suggest that any such differences are likely to be small.

To the best of our knowledge, the increased frequency of mutations observed in these children is the highest recorded in humans after genotoxic exposure. Previously, Russian liquidators exposed to high doses of radiation at the Chernobyl Nuclear Facility and atomic bomb survivors were shown to have a 2- to 24-fold increase in *HPRT*-somatic Mf compared with controls that persisted for 6–10 years (35, 36). A 10-fold increase in *HPRT*-somatic Mf was observed in patients who received total body irradiation after radioimmunoglobulin therapy (37). These observed increases are significantly less than the sustained mean 200-fold increase in somatic Mf observed in children after therapeutic intervention for ALL. An argument could be made that the long-term use of 6-MP during the consolidation and maintenance phases in these treatment protocols would lead to *in vivo* selection of *HPRT*-negative mutants and subsequent distortion of the mutant frequencies in a similar manner to that observed with chronic azathioprine treatment for type I diabetes, an autoimmune disease (38). With respect to our study, the foundation of the maintenance phase of therapy for children with ALL includes simultaneous chronic exposure to 6-MP as well as methotrexate. Purine analogs of hypoxanthine such as 6-MP are pro-drugs requiring intracellular activation by *HPRT* that results in the accumulation of cytotoxic nucleotides. Methotrexate blocks the folic acid cycle and results in inhibition of both purine and thymidine biosynthesis. The combination of the two drugs results in increased accumulation of 5-phosphoribosyl-1-pyrophosphate because of the inhibition of the reduced folate and purine biosynthetic pathways and leads to increased levels of accumulated MP nucleotides. *HPRT* mutants not only show the phenotype of resistance to 6-thioguanine but also an inability to grow in the presence of methotrexate *in vitro*, which has been confirmed by their inability to grow in hypoxanthine-aminopterin-thymidine media. As a result, there is the potential *in vivo* selection for or against *HPRT* mutants in subjects receiving this combination therapy. It has not yet been fully determined what the net effect of the simultaneous exposure to 6-MP and methotrexate will be. However, we do have the ability to address these questions by studying the relationship among *HPRT*-mutational spectra, cell proliferation, and selection through the use of *TCR β* gene analysis of each mutant isolate as an independent marker of clonality. We have previously used this relationship to demonstrate that in a subset of children treated for ALL, there is evidence of the emergence of both genomic instability and clonal

proliferation (39, 40). In the later study, we reported on the extent of clonal proliferation of *HPRT*-mutant isolates from 12 children treated for ALL who had Mfs > 30-fold higher than age-matched controls. We observed that the percent of unique mutations in these patients ranged from 31–100% and that even after correcting for clonality, the Mfs remained >30-fold higher than age-matched controls. This demonstrates that despite the extensive clonal proliferation observed in some of these patients, extremely high frequencies of somatic mutations still exist in these subjects independent of *in vivo* selection after exposure to purine analogs. Follow-up mutational spectra and clonality studies on the subjects included in this study will enable us to more fully test the effect of chronic 6-MP and methotrexate exposure on the potential selection for or against *HPRT* mutants.

This study further supports an increasing body of evidence demonstrating that children are more susceptible to the genetic and cellular consequences of genotoxic agents (41–43). The overall level of exposure relative to body surface area is markedly higher in children compared with adults during a time in human development when there is a decrease in the expression of phase I and phase II carcinogen-metabolizing enzyme systems. In addition, exposure to these agents during childhood and adolescence is occurring at a time when virtually all somatic cell populations are replicating and undergoing extensive expansion that results in the potential establishment of somatic mutations in a multitude of cell populations. With respect to children treated for ALL, they receive regimented doses of multiple antineoplastic agents over specific time periods using agents that historically have been collectively more genotoxic, especially in combination with radiation regimens. The use of multiple-modality treatment protocols makes investigating the *in vivo* genotoxicity of a specific single therapeutic agent difficult in humans. However, our findings indicate that antineoplastic therapeutic treatment protocols used for the treatment of children with ALL are quite genotoxic, in which there was a significant increase in somatic mutational load in peripheral T cells that have a long *in vivo* life span. Such events in this as well as other somatic cell populations following chemotherapy could lead to an elevated risk for the development of multi-factorial diseases in adulthood, in particular secondary tumors.

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