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Deficient Innate Immunity, Thymopoiesis, and Gene Expression Response to Radiation in Survivors of Childhood Acute Lymphoblastic Leukemia

Wing Leung^{1,4}, Geoffrey Neale³, Fred Behm², Rekha Iyengar¹, David Finkelstein³, Michael B. Kastan^{1,4}, and Ching-Hon Pui^{1,4}

¹Department of Oncology, University of Tennessee, Memphis, TN.

²Department of Pathology, University of Tennessee, Memphis, TN.

³Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital, University of Tennessee, Memphis, TN.

⁴Department of Pediatrics, University of Tennessee, Memphis, TN.

Abstract

Background—Survivors of childhood acute lymphoblastic leukemia (ALL) are at an increased risk of developing secondary malignant neoplasms. Radiation and chemotherapy can cause mutations and cytogenetic abnormalities and induce genomic instability. Host immunity and appropriate DNA damage responses are critical inhibitors of carcinogenesis. Therefore, we sought to determine the long-term effects of ALL treatment on immune function and response to DNA damage.

Methods—Comparative studies on 14 survivors in first complete remission and 16 siblings were conducted.

Results—In comparison to siblings on the cells that were involved in adaptive immunity, the patients had either higher numbers (CD19+ B cells and CD4+CD25+ T regulatory cells) or similar numbers ($\alpha\beta$ T cells and CD45RO+/RA- memory T cells) in the blood. In contrast, patients had lower numbers of all lymphocyte subsets involved in innate immunity ($\gamma\delta$ T cells and all NK subsets, including KIR2DL1+ cells, KIR2DL2/L3+ cells, and CD16+ cells), and lower natural cytotoxicity against K562 leukemia cells. Thymopoiesis was lower in patients, as demonstrated by less CD45RO-/RA+ Naïve T cell and less S β TREC levels in the blood, whereas the V β spectratype complexity score was similar. Array of gene expression response to low-dose radiation showed that about 70% of the probesets had a reduced response in patients. One of these genes, SCHIP-1, was also among the top-ranked single nucleotide polymorphisms (SNPs) during the whole genome scanning by SNP microarray analysis.

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Correspondence: Wing Leung, MD, PhD, Mail Stop 260, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-2794. Telephone: 901-595-2166, Fax: 901-521-9005, wing.leung@stjude.org.

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CONFLICT OF INTEREST

All authors do not have any conflicts to declare.

Conclusion—ALL survivors were deficient in innate immunity, thymopoiesis, and DNA damage responses to radiation. These defects may contribute to their increased likelihood of second malignancy.

Keywords

children; lymphoblastic leukemia; second malignancy; immunity; DNA damage

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy (1). Optimal use of anti-leukemic agents, together with a stringent application of prognostic factors for risk-directive therapy, has resulted in an improvement in overall cure rate to approximately 80 percent (2,3). Each year in the United States, approximately 2,000 patients become five-year survivors of childhood ALL. As survivors increase in number, the delayed effects of their leukemia treatment become more apparent. For instance, survivors of childhood ALL, especially those who have received craniospinal irradiation, are known to be at risk of many late sequelae such as endocrinopathies, neurocognitive dysfunction, cataract, skeletal abnormalities, obesity and metabolic syndrome (4–13). Extended follow-up studies also demonstrated a higher standardized mortality ratio (1.8) and a higher standardized incidence ratio (6.07) of developing a new malignancy when compared with the general population (14).

The pathogenesis and etiology for the increased risk in the development of a second malignant neoplasm (SMN) are likely to be multi-step and multi-factorial (11–13). The chemotherapy and radiation that the patients receive are known to be genotoxic, resulting in genomic instability, clonal proliferation, and high frequency of somatic mutations and cytogenetic abnormalities (15–19). The risk of progression of a pre-malignant condition to a SMN may then depend on both the host immunity and DNA damage response (20,21). Currently, little is known about the long-term immune function and DNA damage response capacity in survivors of childhood ALL. Therefore, we conducted a comparative study on 16 pairs of survivors and their siblings. We found that ALL survivors were deficient in innate immunity, thymopoiesis, and DNA damage response to radiation, thereby potentially putting them at risk of second cancer and late mortality.

PATIENTS AND METHODS

Blood samples were obtained prospectively from 16 consecutive healthy siblings when they came to the St. Jude Children's Research Hospital (SJCRH) coincidentally on the same day when the patients returned for routine follow-up visits after completion of chemotherapy. Twelve patients came in with one sibling and two patients with two siblings. Patients who were eligible for this study must have completed therapy for at least 18 months because previous studies have demonstrated that the acute effects of leukemia therapy on lymphocyte populations usually recovered in 6 to 12 months (22–24). At the time of blood collection, all patients were in first completion remission after treatment with one of the institutional protocols: Total XI (n = 5), Total XII (n = 1), Total XIII (n = 6), Total XIV (n = 2). The median time off chemotherapy was 8.7 years (range 1.9 – 15.1 years). The age at the time of participation was similar between the patients and siblings (patient: median 17 years, range 9 – 35; sibling: median 19 years, range 7 – 35).

Informed consent was obtained from all participants and the study was approved by the institutional review board. After the paired blood samples were obtained, they were processed simultaneously on the same day for the following tests: complete blood counts,

flow cytometry quantification of lymphocyte subsets, functional assays for natural cytotoxicity, KIR genotyping, quantitative PCR of sjTREC for thymopoiesis, V spectratyping for TCR repertoire, gene expression microarray for radiation damage response, and whole genome scanning by SNP microarray analysis.

Quantification of lymphocyte subsets

Lymphocytes subsets were quantified by flow cytometry analyses. The monoclonal antibodies used included CD45-PCP, IgG1-FITC, IgG2a-PE, IgG1-APC, CD4-FITC, CD8-PE, CD3 APC, CD3FITC, CD19-APC, $\alpha\beta$ TCR-FITC, $\gamma\delta$ TCR-PE, CD25-PE, CD15-FITC, CD14-PE, CD57-FITC, CD56-PE, CD45RA-FITC, NKB1-PE, CD5-FITC, CD10-PE, CD16-PE (Beckton Dickinson, Franklin Lakes, NJ, USA), CD45RO-PE, CD158a-PE, CD158b-PE, NKG2A-PE, NKP30-PE, NKP44-PE, NKP46-PE (Beckman Coulter, Miami, FL, USA) and CD-94-PE (PharMingen, San Diego, CA, USA).²⁵

KIR genotyping and Natural cytotoxicity assay

KIR genotyping was performed by PCR using SSP as described previously (25). The natural cytotoxicity of NK cells against K562 leukemia cell line was determined by using a standard europium release assay with an effector to target (E: T) ratio of 40:1, 10:1, and 2.5:1 as reported earlier (26).

Quantitative PCR for sjTREC

Real-time PCR was used to quantify sjTREC as described previously (27) using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster city CA). Standards for sjTREC and α were made by cloning sjTREC and α fragment into pCR2.1-TOPO Vector (Invitrogen, Carlsbad, CA). TREC copies were reported as copies per milliliter of blood.

V spectratyping for TCR repertoire

RT-PCR to determine the size distribution of TCR β CDR3 was performed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) as reported earlier (28). The data was analyzed by using the Genescan Analysis Software version 3.1.2 (Applied Biosystems, Foster City, CA). The overall complexity of TCR β subfamilies was presented as the spectratype complexity score (28). Spectratype complexity score is calculated as the summation of number of subfamilies (i.e. 27) per score with a maximum possible score of 216 (8 peaks for each family and 27 family type; $8 \times 27 = 216$).

Gene expression microarray for radiation damage response

RNA was extracted from aliquots of peripheral blood mononuclear cells immediately before or two hours after 1 Gy of irradiation and was used to synthesize cDNA. Gene expression analysis was performed using the Affymetrix HG-U133A GeneChip microarray containing 22,215 probesets that interrogate the expression of 14,500 well-characterized human genes. An expression profile for each individual's response to radiation was generated by pair-wise comparison of the radiated sample to the non-radiated sample using the Affymetrix GCOS software.

Principal components analysis and hierarchical clustering were performed using GeneMaths XT software version 1.6 (Applied Maths, Belgium). For clustering, the similarity among profiles was calculated using the Pearson correlation coefficient while linkage was calculated using the Unweighted Pair Group Method with Arithmetic mean (UPGMA).

SNP microarray analysis

SNP analysis was performed using the Affymetrix 10K_Xba131 GeneChip array as described previously (29,30). This assay provides parallel genotyping of 11,560 SNPs that span the human genome with a median inter-marker distance of 105 kb. Genotype calls were determined using the Affymetrix Gene DNA Analysis Software (GDAS) version 2.0. DNA copy number estimates were determined using the Affymetrix Copy Number Analysis Tool (31).

Statistical analysis

Comparisons of continuous variable between patients and their siblings were performed using Wilcoxon matched-pairs signed-ranks tests. Natural cytotoxicity with different effector : target cell ratio was compared using the approach described by Liang and Zeger (32). Gene expression responses to radiation were analyzed using a two-factor ANOVA model. The log₂-ratio of radiated versus untreated cells was the response variable, while family status and patient status were the two factors applied. For SNP analyses, the association of an allele with a phenotype (patient versus sibling) was computed using an additive model. Genotype calls at each SNP position (AA, AB, BB) were transformed to ordered categories (0, 1, 2) and then tested for association with phenotype using the Pearson's correlation metric. Prior to analysis, SNPs with call rates <90% or with variance <0.05 were filtered from the data set (10,524 of 11,560 SNPs retained). To improve sensitivity, data from the twin siblings were excluded from the association analysis.

Statistical analyses were performed using Stata 9.2/SE (College Station, TX) and S-Plus (Insightful Inc., WA). The Benjamini-Hochberg procedure was used to estimate the false discovery rate at a given significance threshold (33).

RESULTS

Deficiency of innate lymphocyte subsets in patients

There was no significant difference in complete blood counts between the patients and their siblings (Table 1). Lymphocyte subsets analyses, however, revealed a divergent pattern in adaptive versus innate immunity. For the cells that were involved in adaptive immunity, the patients had either higher numbers (CD19+ B cells and CD4+CD25+ T regulatory cells) or similar numbers ($\alpha\beta$ T cells and CD45RO+/RA- memory T cells) in the blood (Figure 1). In contrast, patients had lower numbers of all lymphocyte subsets involved in innate immunity ($\gamma\delta$ T cells and all NK subsets, including KIR2DL1+ cells, KIR2DL2/L3+ cells, and CD16+ cells). We confirmed that the difference in the abundance of KIR+ NK cell subsets was not due to difference in KIR gene content (as expected because of sibling relationship). The genomic numbers of inhibitory KIRs (patients: 7.3 ± 0.4 ; siblings: 6.8 ± 0.4 ; $p=0.17$) and activating KIRs (patients: 3.4 ± 0.5 ; siblings: 2.5 ± 0.4 ; $p=0.11$) were similar.

Lower natural cytotoxicity in patients

To further investigate the function of their innate immunity, we performed cytotoxicity assays against K562 leukemia cell lines. Natural cytotoxicity was found to be higher using blood NK cells obtained from the siblings than those from the patients (Figure 2), especially at low effector to target cell ratio (31% higher specific lysis in siblings at 2.5:1 E:T ratio).

Thymopoiesis is impaired in ALL survivors

Flow cytometry analyses revealed that the number of CD45RO-/RA+ Naïve T cells was also lower in the patients (Figure 1). We then directly investigate thymic output by using QT-PCR for SjTREC. The SjTREC levels were significantly lower in patients as compared

to those of the siblings (Figure 3A). The TCR repertoire, however, was preserved in the patients as indicated by a similar V β spectratype complexity score (patient, 201 $\pm$ 11; sibling, 206 $\pm$ 6; p = 0.29; Figure 3B). Among the patients, the duration since completion of chemotherapy was inversely correlated with the number of CD45RO $^{+}$ /RA $^{+}$ naïve T cells (decreased by an average of 11.7/ul per year, p =0.003; Figure 3C) and the SjTREC levels in the blood (decreased by an average of 2.3/ul per year, p =0.02; Figure 3D). Neither the number of naïve T cells or SjTREC levels correlated with the patient's age (p =0.69 and 0.97, respectively).

Differential gene expression response to radiation

Because cell response to stress is critical for genomic integrity, we studied the gene expression response to low-dose radiation (1Gy). At a significance threshold of p <0.005, we identified 203 probesets with differential expression between patients and siblings with an estimated false discovery rate of <0.53 (Figure 4A). Of note, 141/203 (69.5%) probesets showed reduced response to radiation within the patient group as compared to the sibling group. Gene ontology analysis of the differentially expressed transcripts indicated the reduction in responses were primarily related to genes associated with mitotic spindle checkpoint, purine nucleoside metabolism, and RNA splicing and metabolism (Supplemental Table 1).

Of the 203 probesets, 69 had a greater than two-fold mean difference in expression between patients and siblings (Figure 4B). A pronounced bias between patient and sibling response was observed; 52/69 probesets (76.5%) had reduced response in patients as compared to siblings. Again, gene ontology analysis indicated significant enrichment of genes associated with mitotic spindle checkpoint function and purine base metabolism (e.g., PAICS and PPAT). Of note, the mitotic spindle checkpoint genes Bub1 and TTK, as well as the radiation response gene RAD52, showed a reduced response to radiation treatment in patient samples as compared to those from siblings.

Whole genome scanning by SNP microarray analysis

To further investigate single nucleotide polymorphism and genome-wide alterations (gene deletion, amplification, and loss of heterozygosity), we performed high-resolution whole-genome scan using the Affymetrix 10K_Xba131 GeneChip array and the sibling samples as controls. As expected with matched sibling-control analyses, the allele frequencies and genomic copy number profiles between the patients and their siblings at each SNP position did not differ significantly in general (data not shown). Remarkably, when we examined the top-ranked SNPs with p -values less than 0.05, the most significant SNP (p =0.0029) located approximately midway between two genes on chr3p25.2. Its physical position was 188 kb downstream of the nucleoporin NUP210 (which is a nuclear pore membrane protein involved in transports across the nuclear membrane), and 186 kb upstream of the IQSEC1 gene (which is an activator of ADP-ribosylation factor 6 for regulation of cell adhesion, actin, apoptosis, and purine metabolism). Analysis of the remaining top-ranked 85 SNPs (Ingenuity Pathways) showed 46 SNPs mapped nearby to genes (Supplemental Table 2). Of note, one of these top-ranked SNPs SCHIP-1, which interacts with the tumor suppressor schwannomin or Neurofibromatosis 2 (NF2), had a significant p -value by differential expression testing (ANOVA p =0.0054) during the experiments for gene expression response to radiation.

DISCUSSION

It is generally believed that abnormalities of immune function after completion of ALL therapy are short term (22). Antigen-specific T-cell and B-cell memory was found to be

preserved in children treated for ALL (23,24). However, we demonstrated herein for the first time that although their numbers of T cells and T-cell receptor repertoire appeared normal, ALL survivors were deficient in naïve T cell generation, as measured by TREC and CD45RO⁺/RA⁺ cells enumeration when compared with their age-equivalent sibling controls. Furthermore, the numbers of naïve T cells and TREC in the patient's blood progressively decreased overtime after cessation of chemotherapy and were independent of the patient's age. Together, these data suggest that thymopoiesis in ALL survivors is significantly compromised and, more notably, not sustainable.

Other novel findings are that ALL survivors were significantly deficient in natural cytotoxicity and lymphocyte subset involved in innate immunity, including $\gamma\delta$ T cells and all NK cell subsets. Deficiency in innate immunity with NK cells, rather than adaptive response, has been demonstrated by large cohort studies to be associated with high risk of cancer development in general (34,35). Deficiency of natural killer cell triggering receptors (NCRs) has been associated with the development of AML in particular (36). The 30% reduction in cytotoxicity of NK cells obtained from our patients as compared to those from the siblings is clinically significant, as previous study has demonstrated an ~60% increase in relative risk of cancer incidence in individuals with similar magnitude of decrease in natural cytotoxicity (34). Of note is the global deficiency in all KIR⁺ NK cell subsets, which may reduce the patient's ability to recognize MHC class-I downregulation (a "missing-self" recognition essential for NK cell tumor surveillance). We confirmed that the lack of KIR⁺ cells were acquired and not based on inherited KIR gene content, as there was no difference in KIR typing between the patients and their siblings. Certain hematopoietic and cutaneous tumor cells have been shown to be specifically recognized and lysed by $\gamma\delta$ T cells (37–39). Thus, the deficiency of lymphocytes involved in innate immunity may be a fundamental mechanism that impairs the capability of tumor surveillance for second malignancy in survivors of ALL.

To our knowledge, this is also the first study that investigates gene expression responses to radiation in survivors of childhood ALL. We saw a significant difference between patients and siblings, in particularly a consistent pattern of reduced induction of selected genes in the patients. About 70% of the probesets had a reduced response in patients, in contrast to only 30% of the probesets that had a lower response in siblings. Gene ontology analysis indicated the reduction in response were primarily related to genes involved in radiation response (e.g., RAD52), mitotic spindle checkpoint (e.g., Bub1 and TTK), or purine metabolism (e.g., PAICS and PPAT). SCHIP-1, which interacts with tumor suppressors schwannonin and NF2, was both a top-ranked SNPs in our high-resolution whole-genome scan and a significant differentially expressed gene in our radiation study. The most significant SNP was located between NUP210 and IQSEC1 gene. NUP210 controls transport such as mRNA across the nuclear membrane (40), and IQSEC1 is involved in TNF-alpha-induced apoptosis in monocytes (41), invasive activities of primary breast carcinomas (42), and purine nucleotide metabolism (43). Collectively, both our gene expression and SNP data suggest that DNA damage responses and purine metabolism may be abnormal in ALL survivors. In this regard, a high incidence of secondary brain tumor and myeloid leukemia has been observed in our institution in ALL survivors who had radiotherapy and a genetic defect in thioguanine nucleotide metabolism (15,46).

Previous study has demonstrated that most or all of the ALL survivors have already acquired during leukemia treatment some cytogenetically abnormal cells that may persist for years after completion of therapy (16–18). These chromosomal aberrations alone, however, are unlikely to be sufficient to induce cancer without additional cooperating oncogenic lesions. After completion of therapy, the genome of ALL survivors will continue to expose to exogenous DNA damaging events similar to those in the general population in the form of

background radiation, viral infections, and chemical exposure. Endogenous processes, such as DNA replication and free radical formation, also threaten the integrity of the genome. Facing these genomic “stresses”, a normal cell is equipped with DNA damage responses which can cause cell cycle arrest and induction of DNA repair functions, or, in certain settings, induction of apoptosis. DNA damage responses may also alert the immune system by inducing cell surface expression of ligands for activating immune receptors of innate immunity, such as NKG2D which is expressed by natural killer cells, as well as triggering the development of adaptive immunity (45–46). Thus, both DNA damage responses and immunosurveillance work in concert for cancer prevention (47–48). The deficiency in our ALL survivors in both innate immunity and naïve T cell generation, as well as in radiation damage response, may collectively contribute to their risk of subsequent cancer development.

Because ALL survivors were shown to be deficient in both immunity and DNA damage response in this study, it will be important to test thoroughly in future studies the specific immune response of ALL survivors to DNA damage (45). Also of interest is whether other components of the immune system, in addition to NK cells and NKG2D recognition, are regulated by DNA damage response. Finally, a better understanding of the role of different immune cells and DNA damage response in natural or chemotherapy-accelerated cell senescence may be helpful in defining safer regimens to treat leukemia and for the surveillance and prevention of late complications during aging (49,50). Because thymopoiesis is limited in ALL survivors, studies are ongoing at our institution to test comprehensively the immune response of our patients to various vaccine-preventable diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differences in lymphocyte subsets

When compared with their sibling, patients had higher numbers of CD19+ B cells ($p=0.003$), CD4+CD25^{bright} T regulatory cells ($p=0.01$), and similar numbers of $\alpha\beta$ T cells ($p=0.72$) and CD45RO+/RA- memory T cells ($p=0.82$). In contrast, patients had lower numbers of all lymphocyte subsets involved in innate immunity: $\gamma\delta$ T cells ($p=0.001$) and all NK subsets, including KIR2DL1+ cells ($p=0.03$), KIR2DL2/L3+ cells ($p=0.05$), and CD16+ cells ($p=0.05$). In addition, CD45RO-/RA+ naïve T cells ($p=0.02$) was also lower in the patients.

Figure 2. Natural cytotoxicity

Natural cytotoxicity against K562 cells was higher in siblings than in patients with all effector to target (E:T) cell ratio between 2.5:1 to 40:1 (all $p < 0.05$).

Figure 3. Thymopoiesis

(A) Number of copies of TREC in peripheral blood was significantly higher in siblings than in patient. (B) Representative v-beta spectratyping from patient and sibling demonstrating a diverse pattern of T-cell receptor usage. (C) Inverse correlation between the number of CD45RO-RA+ naïve T-cells, or (D) the number of TREC, and the duration after cessation of chemotherapy.

Figure 4. Differential gene expression response to radiation

Gene expression profiles that differ between patient and sibling samples, (A) based on 203 expression profiles with $p < 0.005$ by ANOVA, or (B) based on 69 expression profiles with an average difference of at least two-fold and with $p < 0.005$ by ANOVA. Left panels: principal components analysis of patient and sibling samples based on the response to radiation. Each sample is represented by a sphere plotted in three-dimensional space and color-coded for sibling versus patient. Right panels: hierarchical cluster analysis of patient and sibling samples based on the response to radiation. X axis, samples; Y axis, gene expression profiles. Scale bars indicate the relative expression of transcripts (irradiated / non-irradiated) using a $\log_2$ -transformed scale.

Table 1

Complete blood counts

	Patients Median (range)	Siblings Median (range)	P value
WBC count ($\times 10^3/\text{ul}$)	6.6 (3.7 – 16.3)	6.7 (2.8 – 9.1)	0.92
Hemoglobin (g/dl)	14.1 (12.4 – 15.2)	14.3 (12.9 – 15.3)	0.67
Platelet count ($\times 10^3/\text{ul}$)	248 (140 – 378)	266 (169 – 376)	0.43
ANC (/ul)	3507 (1400 – 7800)	3793 (1000 – 6600)	0.63
Lymphocyte count (/ul)	1952 (999 – 2793)	2116 (1092 – 2856)	0.45
Monocyte count (/ul)	289 (62 – 481)	438 (84 – 819)	0.14