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Etoposide Sensitivity Does Not Predict *MLL* Rearrangements or Risk of Therapy-related Acute Myeloid Leukemia

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Abstract

Therapy-related acute myeloid leukemia (t-AML) can arise from topoisomerase II agents (e.g. etoposide and teniposide), via drug-induced *MLL* gene fusions. However, whether *MLL* rearrangements (r*MLL*) and subsequent leukemogenesis are inextricably linked to the cytotoxic effects of the drug remains controversial. To this end, we compared 1) r*MLL* in blood of children with acute lymphoblastic leukemia (ALL) who did and did not develop t-AML, 2) epipodophyllotoxin-related toxicity in t-AML cases and controls, and 3) the level of r*MLL* in cells that were sensitive and resistant to etoposide. In children with ALL, r*MLL* during etoposide treatment appeared independent of the cumulative dose ($P = 0.5$), although slightly more frequent in children who developed t-AML than those who did not (7 case-control pairs, $P = 0.04$). Similarly, the frequency of etoposide- or teniposide-related acute toxicities did not differ between t-AML cases and controls (26 case-control pairs, $P > 0.17$). Finally, in 25 human lymphoblastoid cell lines, *MLL* fusions were common after equitoxic etoposide treatment vs. controls ($P < 0.0001$) but did not differ in etoposide-sensitive versus resistant cell lines ($P = 0.91$). Together, these results indicate that epipodophyllotoxin-mediated leukemogenesis is not directly linked to the cytotoxicity of the drug.

Introduction

Therapy-related acute myeloid leukemia (t-AML) is a formidable consequence of some efficacious anti-cancer therapies, such as topoisomerase II-directed agents (also known as topoisomerase II “inhibitors”), DNA alkylating agents, and radiotherapy. Among children with acute lymphoblastic leukemia (ALL), topoisomerase II agent-induced t-AML has been the most common type of secondary tumor(1;2). Unlike other types of t-AML, topoisomerase II-related cases frequently feature balanced translocations involving the *MLL* gene at 11q23 and occur with a relatively brief latency following the treatment for primary cancer(3–7).

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Nucleotide-resolution mapping of t-AML translocation breakpoints within the *MLL* breakpoint cluster region (bcr) showed that breakpoints cluster in a relatively short stretch of DNA and are sometimes flanked by topoisomerase II cleavage sites and at minor apoptotic cleavage regions(8), suggesting involvement of topoisomerase II as well as apoptosis-related cleavage.

By stabilizing DNA/topoisomerase II cleavage complexes, epipodophyllotoxins convert the enzyme into a genotoxin that introduces DNA double strand breaks (DSB) in the genome(9; 10). Subsequent illegitimate DNA repair gives rise to abnormal fusion products, such as the leukemogenic *MLL* fusion genes(11–13). However, even chemotherapeutic agents that do not target topoisomerase II (e.g. 5-fluorouracil) can induce cleavage in the *MLL* bcr, and etoposide-induced DSBs can be diminished by caspase inhibitors, implying that a topoisomerase II-independent but apoptosis-related mechanism may also contribute to *MLL* rearrangements (14;15).

A clinically relevant question is whether the pro-apoptotic cytotoxic effect of etoposide is separable from its potential to induce *MLL* rearrangements and whether cellular sensitivity to etoposide is linked to the incidence of t-AML. Within some clinical studies, the relative risk of t-AML appeared to be related to cumulative dose of epipodophyllotoxins(1). However, the risk varies substantially among clinical trials, and is not always related to cumulative dose (16–20). Thus, clinical observations are somewhat conflicting on whether there is a dissociation between cytotoxic and leukemogenic properties of epipodophyllotoxins.

To address this question, we quantified and compared rearrangements of the *MLL* gene in normal circulating leukocytes during treatment for primary ALL between children who developed t-AML and those who did not. Secondly, epipodophyllotoxin-related host toxicity was compared between identically treated patients who did vs. did not eventually develop t-AML. Finally, we directly examined whether the frequency of etoposide-induced *MLL* rearrangements differed among human lymphoblastoid cell lines that exhibit differential susceptibility to the cytotoxic effects of etoposide. The results from these analyses demonstrate that predisposition to *MLL* rearrangements and secondary leukemogenesis is not directly linked to sensitivity to drug-induced cytotoxicity.

Results

MLL rearrangements following etoposide treatment in t-AML cases and controls

Most topoisomerase II agent-induced t-AML cases feature *MLL* fusion genes, many of which have exhibited strong leukemogenic potential in *in vitro* cellular and animal models (27;28). This observation prompted us to test the hypothesis that an elevated level of *MLL* rearrangements during treatment with etoposide for primary malignancy is associated with a higher incidence of subsequent t-AML. To this end, we evaluated *MLL* gene rearrangements in peripheral blood samples obtained at fixed time points in therapy following various doses of etoposide, for 7 t-AML cases and 7 matched controls from St. Jude Total Therapy Study XIIIIB (Table 1). Quantified using long distance inverse PCR (LDiPCR) (Fig. 1), *MLL* rearrangements were detectable in both cases and controls but at a slightly greater frequency in the former group ($P=0.04$, Wilcoxon signed-rank test; Fig. 2, inset). In addition, across 14 patients analyzed, there was no correlation between the cumulative etoposide dose and the degree of rearrangements at the time point of interest ($P=0.5$, Spearman rank correlation; Fig. 2). These results suggest that 1) *MLL* rearrangements are detectable following etoposide treatment, 2) the level of translocations may be slightly higher in those who developed therapy-related secondary leukemia as compared to controls, but 3) the frequency of *rMLL* is not related to cumulative dose of etoposide, even in patients who developed t-AML.

Etoposide sensitivity and drug-related toxicities in t-AML cases and controls

Using epipodophyllotoxin-induced acute toxicity to host tissues as a marker for drug sensitivity, we asked if drug-related clinical toxicities are related to the occurrence of t-AML in 26 t-AML case and matched control pairs (Supplemental Table 2S). Neutrophil and white blood cell counts generally decreased after receiving etoposide (Fig. 3), but the reduction was not greater among t-AML cases than the controls ($P=0.17$ and 0.42 , respectively, Wilcoxon signed-rank test). Furthermore, we compared the incidence of infection and gastrointestinal toxicity possibly related to epipodophyllotoxin for those patients with toxicity data available (18 pairs of cases and controls). It appeared that, if anything, toxicity was somewhat more common in the control group, but the difference did not reach statistical significance. Thus, during induction therapy, a total of 6 infections were identified and 5 occurred in controls ($P=0.22$, McNemar's test); 2 gastrointestinal toxicity events were identified and both occurred in controls ($P=0.5$). During continuation therapy, 7 infection events were related to epipodophyllotoxins with 4 in control group ($P=1$); two gastrointestinal events occurred and both were in controls ($P=0.5$). It should be noted, however, that the total number of adverse events is relatively small and therefore carries limited statistical power. Nevertheless, these data suggest that topoisomerase II agent-mediated leukemogenesis is largely independent of the agents' toxic effects to host tissues, and argue against the notion that acute drug toxicity can be utilized to identify patients at risk of t-AML.

Cytotoxicity of etoposide and *MLL* rearrangements in the CEPH cells

The lack of correlation between *rMLL* and cumulative dose and the finding that patients who developed t-AML did not experience greater hematopoietic or non-hematopoietic toxicity than identically treated controls implied that etoposide cytotoxic effects and drug-induced leukemogenic *MLL* rearrangements are not related. To further address this possibility, we screened 144 CEPH lymphoid cell lines derived from individuals with Northern and Western European ancestry (<http://ccr.coriell.org/nigms/ceph/ceph.html>) to identify lines with differing intrinsic sensitivity to etoposide. Although immortalized by the Epstein-Barr virus transformation, these lymphocytic cell lines largely retain inherited germline variants of the donors. (29–31) The calculated etoposide IC_{50} values of CEPH cell lines ranged from 0.08 to 4 μM (Fig. 4A), confirming significant differences in inherent drug sensitivity among individuals. Utilizing the most sensitive ($n=15$) and the most resistant cell lines ($n=10$) (Supplemental Table 1S), we explored whether there was an association between the cytotoxic effects of etoposide and its potential to induce *MLL* rearrangements. To adjust for inherent sensitivity, each line was treated with etoposide at equitoxic concentrations of drug. After etoposide treatment, the frequency of rearranged *MLL* increased dramatically relative to control levels ($P<0.0001$, Paired t test), but did not differ significantly between the 15 most sensitive and the 10 most resistant cell lines ($P = 0.91$, Mann–Whitney U test; Fig. 4B), suggesting that susceptibility to *MLL* rearrangements is not directly related to inherent drug sensitivity.

Discussion

The aim of the present study was to evaluate the relationship between t-AML and epipodophyllotoxin-induced cytotoxicity. In children treated with etoposide or teniposide for ALL, drug-related toxicity did not differ between patients who eventually developed t-AML vs. those who did not. Likewise, in cultured human lymphoblastoid cells, the frequency of *MLL* gene rearrangements was unrelated to the inherent sensitivity to etoposide. Nor was the level of *MLL* rearrangements correlated with the cumulative dose of etoposide in children with ALL. Thus, we conclude that leukemogenic effects of topoisomerase II agents are not dependent on their cytotoxic effects.

Dissociation of etoposide sensitivity and secondary leukemia is of particular clinical relevance in that it suggests the possibility of developing regimens containing this class of agents that maintain their anti-cancer efficacy while minimizing leukemogenesis (32–34). It has been hypothesized that the risk of t-AML is inextricably linked to etoposide cytotoxicity because drug-induced stabilization of the topoisomerase II-DNA complex results in both apoptosis and the leukemogenic rearrangements of the *MLL* gene (35;36). However, there is a growing body of evidence that the pro-apoptotic DNA cleavage induced by topoisomerase II agents is not necessarily related to their leukemogenic properties. Recently, Azarova et al.³⁸ showed that etoposide-induced melanoma in carcinogen-challenged mice is largely attributed to the beta isoform of topoisomerase II while its cytotoxicity was executed through interaction with the alpha isoform, providing a plausible mechanism of the separation of cytotoxicity and leukemogenic effects.

Although many chemotherapeutic agents are as effective as topoisomerase II agents in inducing apoptosis, and even specific *MLL* cleavage, most are not leukemogenic, and do not cause the characteristic *MLL* translocation-bearing secondary leukemia associated with topoisomerase II agents. Pharmacological studies of etoposide demonstrated that plasma pharmacokinetics were not different between patients who did vs. did not develop secondary leukemia^{(37)–(38)}. In a trial in which cumulative doses of all cancer drugs were identical between two treatment arms, which differed only in the schedule, one arm had a much higher incidence of t-AML than the other, indicating a clear dissociation between cumulative etoposide dose and secondary leukemia. ⁽³⁷⁾ Related to this, we found that, despite comparable cytotoxicity, etoposide-induced gene rearrangements were more common following a short, intensive course than after a prolonged low-dose exposure,⁽³⁹⁾ again consistent with a dissociation of leukemogenic vs. cytotoxic effects.

It has not been clear whether the long-term adverse effect of t-AML occurs at a different frequency in patients who suffered short-term adverse effects from etoposide compared to those who did not. It has been speculated that bone marrow myelosuppression might indicate a more leukemogenic regimen. For instance, the cumulative incidence of t-AML was significantly higher in children and in adults who received G-CSF for myelosuppressive anticancer drug regimens.^{(24),(40)} Also, the apparent association between G-CSF with t-AML, in the setting of G-CSF treatment of chronic neutropenia,⁽⁴¹⁾ argues that patients experiencing severe neutropenia are more likely to need G-CSF, and that the myelosuppression might itself somehow predispose to t-AML. Similarly, it has also been suggested that more frequent infections in patients sensitive to etoposide could facilitate leukemogenesis via inflammation-related effects ⁽⁴²⁾. However, many intensive etoposide-containing regimens have a low incidence of t-AML despite a high rate of acute toxicity,⁽¹⁾ consistent with the notion that leukemic transformation is most likely to manifest itself with mildly-toxic therapy, in which sublethal DNA damage can persist and provide the cellular substrate for future leukemogenic processes. In line with this notion, our results demonstrate that etoposide-related toxicity during treatment of the primary ALL is not more frequent in t-AML cases than their matched controls, arguing that leukemogenesis is not related to acute cytotoxicity.

There is evidence that some of the predisposition to t-AML is attributable to inherited genetic variability. Germline polymorphisms in genes involved in the disposition of and response to etoposide show modest inherited predisposition to t-AML (reviewed in ⁽⁴³⁾). Genome-wide investigations of genetic variation indicate that germline variations in genes involved in focal adhesion and Wnt signaling pathways are predictors of both *MLL* rearrangements and t-AML ⁽⁴⁴⁾. Herein, we found that at any cumulative dose, there was a modest trend ($p = 0.043$) for greater levels of acquired gene rearrangements in patients destined to develop t-AML than in those who did not, a finding consistent with the idea that intrinsic susceptibility (possibly due to inherited genetic variability) is involved in the risk of t-AML.

Our results, interpreted in the context of other studies, support the contentions that distinct mechanisms are responsible for etoposide cytotoxicity compared to genesis of t-AML, and that factors other than etoposide dose and related cytotoxicity are involved in the development of t-AML.

Methods

Cytotoxicity

CEPH Cell lines were obtained from the Coriell Institute (Camden, NJ, USA, <http://ccr.coriell.org/nigms/ceph/ceph.html>) and maintained in RPMI 1640 medium containing 2 mM L-glutamine and 15% heat-inactivated FBS at 37°C, 5% CO₂ and 95% humidity. IC₅₀ was determined as previously described, using a 72 hour exposure to etoposide (21;22).

Drug Treatment to induce *MLL* Fusions

Of 144 cell lines evaluated for etoposide-induced cytotoxicity, 15 sensitive and 10 resistant cell lines were selected to assess etoposide-induced *MLL* rearrangements (Table 1S of the Supplementary Materials). Three flasks were prepared for each cell line by seeding at 5×10^5 cells/ml and culturing for 24h. The cells were treated with DMSO or etoposide (at a concentration ten fold higher than each line's respective 72-hr IC₅₀ values) and collected at 8 hours, at which time DNA was extracted, and *MLL* rearrangements (*rMLL*) were quantified by long distance inverse PCR (LDiPCR) as described below and normalized to control levels (i.e. the numbers of *MLL* rearrangements detected in non-treated cells). Differences in the number of drug-induced *MLL* rearrangements between the sensitive vs. resistant cell lines were assessed by Mann-Whitney U test.

Quantification of *MLL* rearrangements

MLL rearrangements were quantified as previously described with slight modifications (12) (Fig. 1). Briefly, DNA was digested with BamH I and circularized by T4 ligase, followed by purification using QIAGEN Genomic-tip 100. The number of circularized wild-type *MLL* molecules present in the samples was determined by quantitative real-time PCR (qRT-PCR) to adjust for efficiency of ligation. A pCR 2.1-Topo (Invitrogen, Carlsbad, CA, USA) construct that encompasses ~200bp sequence flanking the BamH I site was employed as an internal control to standardize the qRT-PCR assay (Fig. 1S). The primers used for qRT-PCR were: B1 (CTAGATCTGTACCAAGTGTGTTTC) and B2 (TGGAGTGGTGGCCTGTTTGGAT). qRT-PCR amplification was performed using Cybergreen PCR master mix (QIAGEN, Valencia, CA, USA) and an ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems).

The LDiPCR consisted of a 1st round with primers C1(GCTCCCCGCC CAAGTATCCCTGTAAAC), and C2 (TGCTGGCTGGGAGACCTGCTTGCTT GA), followed by a 2nd round PCR (primers D1(GAACATCCTCAGCACTCTCT CCAATGGC), and D2 (GAGACCTGCTTGCTTGACTTCCTGGAAG). The LDiPCR primers bind to sequence within the breakpoint cluster region (bcr) of *MLL*, and were designed to amplify any 5' to 3' fusion products of *MLL* from the der(11) chromosome and the wild-type *MLL* fragments from the normal chr 11. However, in the presence of the "blocking primers" (E1: GCTCTTACAGCGAA CACACTTGGTACAGATCT, and E2:AGCTGCTGGAGTGTAATAAGTGCCGA AAC), amplification of the latter (i.e. wild-type *MLL*) is inhibited. That is, modified at the 3'end by a 3 carbon chain (23), these blocking primers prohibit the extension of amplification primers (C1/C2, and D1/D2) in the presence of wild-type *MLL* and thus allowed for maximal amplification of fusion products and minimized the interference by wild-type circularized *MLL*. For the 1st round LDiPCR, the reaction included of a volume of DNA corresponding to 40,000 copies of wild-type circular

MLL and 600 nM of each blocker. 1 μ l of the 1st round LDiPCR product was the template for a 2nd round PCR. Each analysis also included a positive control prepared by diluting 5 copies of circularized DNA from MV(4;11) cells in 40,000 copies of circularized wild-type *MLL*. This quantity of positive control was detected in 40 of 40 replicates, confirming satisfactory sensitivity of the PCR assay. All the PCR reactions were performed on PCT200 thermal cycler (MJ Research, Ramsey, Minnesota, USA) and 8 LDiPCR replicates were assayed for each sample. PCR products were separated on 1% agarose gels and the number of bands corresponding to non wild-type *MLL* amplifications was counted using Total Lab v2003.03 software following visualization with 0.005% ethidium bromide. A subset of non wild-type *MLL* bands was excised, purified and sequenced. The number of non-wild-type *MLL* amplifications in the etoposide treated-cell lines was normalized by the number observed in the paired untreated control cell lines. Assuming that each cell retains 1 copy of wild type *MLL* gene after etoposide treatment and 320,000 copies of circular wild type *MLL* was included in the PCR assay (i.e. 8 replicates), the number of *MLL* fusion products identified in this assay thereby represented the total number of rearrangements in approximately 320,000 cells (12).

Patients

The St. Jude Institutional Review Board approved the studies, and informed written consent was obtained from parents/guardians or patients. In St. Jude Total XIIIIB protocol for childhood ALL, peripheral blood cells were prospectively collected for the purpose of assessing gene rearrangements. Therapy included the administration of topoisomerase II agents and alkylating agents (24). We compared the level of *MLL* gene rearrangements in peripheral blood from 7 patients (cases) who developed therapy-related leukemia and in 7 identically treated children (controls) who did not develop secondary leukemia (Table 1). Controls were matched to cases for G-CSF therapy, ALL risk group, treatment arm, immunophenotype, ALL molecular subtype, age, race, and sex, in that order of priority. Genomic DNA was isolated from peripheral blood mononuclear cells obtained after patients achieved remission of ALL at specific time points in therapy, thereby controlling for etoposide exposure at the time of evaluation (Table 1).

We screened St. Jude ALL protocols beyond Total XIIIIB to identify additional cases of t-AML for the purpose of evaluating acute toxicity following epipodophyllotoxins. Twenty six cases were identified who developed secondary leukemia within 5 years following primary ALL and the secondary leukemia blasts were characterized by balanced translocations at 11q23. Among them, 8 cases were from St. Jude Total XI(25), 3 were from Total XII(26), 11 were from Total XIII A, and 4 were from Total XIII B protocols (these 4 were the subset of the 7 t-AML cases evaluated for *in vivo MLL* rearrangements in blood whose t-AML harbored an 11q23 translocation). Controls were matched to each case for protocol, treatment arm, cranial irradiation, G-CSF treatment, race, age, and immunophenotype. For each case and control, absolute neutrophil counts (ANC) and white blood cell (WBC) counts obtained within 1–2 days prior to the first dose of etoposide or teniposide during continuation therapy were compared with those measured 6–8 days thereafter to assess myelosuppressive effects. The decrease of ANC or WBC between cases and controls was compared using the Wilcoxon signed-rank tests. Etoposide or teniposide-related toxic events were defined as infections and gastrointestinal toxicity. Adverse events were graded according to NCI toxicity criteria and were evaluated for two time frames that were most uniform with regard to actual delivery of therapy: two weeks following the last dose of epipodophyllotoxins during remission induction, and another 2-week period following the first dose of these drugs during continuation therapy. The difference in proportions of adverse events between case and controls was assessed by exact McNemar's tests.

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Figure 1.

Schematic representation of LDiPCR-based *MLL* rearrangement detection, illustrated for germline wild-type genomic *MLL* (chr 11) and for DNA isolated from a cell with chimeric *MLL* genomic rearrangements (Der (11)). Following digestion with BamHI (BH), the resultant fragments contain either wild-type (Chr 11) or rearranged *MLL* (Der 11). The breakpoint cluster region (bcr) is then ligated to form a circular DNA molecule. Subsequent inverse PCR consists of two rounds: first amplification with C1 and C2, followed by D1 and D2. Rearranged *MLL* fragment amplicons differ in size from those of wild-type *MLL* (~11 kb). Blocking primers E1 and E2 are chemically modified to block amplification of the wild-type *MLL* gene, and thereby facilitate amplification of chimeric *MLL* products.

Figure 2. Level of *MLL* rearrangements in peripheral blood cells of secondary leukemia (t-AML) cases and controls, indicating lack of correlation with cumulative etoposide dose during treatment for primary ALL ($P=0.5$, Spearman rank correlation). The inset (upper right) indicates the pooled level of *MLL* rearrangements in t-AML cases ($n=7$) and controls ($n=7$) ($P=0.043$, Wilcoxon signed-rank test). Open box and circles: t-AML cases; Hatched box and circles: matched controls. *MLL* rearrangements represent the number of *MLL* fusions identified in 320,000 cells.

Figure 3. Toxicity after epipodophyllotoxins and t-AML

The ratio of absolute neutrophil counts (ANC) (A) and white blood counts (WBC) (B) (post-etoposide/pre-etoposide) was compared between 26 matched t-AML case-control pairs. The degree of reduction in ANC and WBC did not differ significantly between case and control groups ($P=0.17$ and 0.42 , respectively, Wilcoxon signed-rank sum test).

Figure 4. *MLL* rearrangements and etoposide-sensitivity *in vitro*

A, Variability in etoposide sensitivity among CEPH cell lines (n=144). B, *MLL* rearrangements were determined by LDiPCR of circularized DNA fragments containing *MLL* BCR as described in Methods and Materials. The number of *MLL* gene rearrangements detected was comparable for etoposide sensitive (green, n=15) and resistant CEPH cell lines (red, n=10), selected from the most sensitive and the most resistant cell lines (highlighted in green and red, respectively in the panel A). *MLL* rearrangements represent the number of *MLL* fusions in 320,000 cells.

Table 1

Characteristics of ALL patients who were studied for *MLL* gene rearrangements in vivo in blood during etoposide treatment. M, male; F, female; W, white; B, black; B-lin, B lineage ALL; T-lin, T lineage ALL; Hyper, hyperdiploid ALL; GCSF, granulocyte-colony stimulating factor; HR, high risk.

Patient number	Patients	Primary ALL	G-CSF use, ALL risk arm	Total number of etoposide doses received by the time blood sample	Secondary leukemia cytogenetics
1	secondary leukemia, M, W, B-lin	Early Pre-B, TEL/AML1	GCSF, HR	7	AML, 46 XY, t(11;19) (q23;p13.1)
2	Control, F, W, B-lin	Early Pre-B, TEL/AML1	GCSF, HR	9	N/A
3	secondary leukemia, M, W, B-lin	Pre-B, TEL/AML1	GCSF, LR	4	AML, 46 XY, der (7) del (7) (p11.1) del (7) (q11.1)
4	Control, F, W, B-lin	Pre-B, TEL/AML1	GCSF, LR	4	N/A
5	secondary leukemia, F, W, B-lin	Pre-B, B-other	No GCSF, HR	9	MDS/AML, 46 XX, inv (11) (p15.5; q22.2)
6	Control, M, W, B-lin	Pre-B, B-other	No GCSF, HR	8	N/A
7	secondary leukemia, M, W, B-lin	Early Pre-B, Hyperdiploid 51+	GCSF, LR	3	AML, 46 XY t(11;19) (q23; p13.1)
8	Control, F, W, B-lin	Early Pre-B, Hyper 47-50	GCSF, LR	4	N/A
9	secondary leukemia, M, B, T-lin	T lineage	GCSF, HR	9	AML, 46 XY t(10;11)(p13-14;q21)
10	Control, M, B, T-lin	T lineage	GCSF, HR	9	N/A
11	secondary leukemia, F, W, B-lin	Early Pre-B, Hypodiploid	GCSF, HR	15	MDS, 44 XX, -5, -9, t(6;16)(q23;q13), +17(p11.2)
12	Control, M, W, B-lin	Pre-B, B-other	GCSF, HR	15	N/A
13	secondary leukemia, F, W, B-lin	Early Pre-B, Hyperdiploid 51+	GCSF, HR	10	AML, 46 XX, t(2;11)(p23;q23), +21(q33)
14	Control M, W, B-lin	Early Pre-B, Hyper 47-50	GCSF, HR	9	N/A