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Estrogen treatment induces *MLL* aberrations in human lymphoblastoid cells

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Abstract

Epidemiological data indicates increased risk of infant acute leukemia involving *MLL* gene aberrations with use of oral contraceptives. To determine whether estrogens might be implicated, we examined the effect of estradiol (E2) or 4-OH-E2 in an *in vitro* model of translocation susceptibility. Genomic DNA from the TK6 human lymphoblastoid cell line was screened by ligation mediated PCR and inverse PCR at a rearrangement hot spot within the *MLL* breakpoint cluster region to detect DNA aberrations. An increase in DNA double strand breaks was observed within this region after exposure to either E2 or 4-OH-E2. An increase in the frequency of *MLL* translocations was only found after exposure to E2. Induction of cleavage due to increased activation of apoptotic nucleases was excluded by pre-treatment with the pancaspase inhibitor, zVAD.fmk. We conclude that concentrations of E2 and 4-OH-E2 that may occur during pregnancy, or during use of oral contraceptives, can cause aberrations of the *MLL* gene and could thus be a factor in the early events of leukemogenesis occurring *in utero*.

Keywords

Infant acute leukemia; Estrogen; Estrogen metabolites; *MLL* translocations; 11q23; Breakpoint cluster region (BCR)

1. Introduction

Infant acute leukemia (IAL) is defined as acute leukemia in children less than 12 months old. Albeit rare—it accounts for 2.4–5% of childhood ALL and 6–14% of childhood AML cases [1,2]. IAL exhibits very distinct characteristics, most notably, 75–80% of patients with IAL carry an aberration of the *MLL* (mixed lineage leukemia) gene located on 11q23, including

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Conflict of interest statement

The authors have no conflicts to report.

Contributions. Sabine Schnyder was the instigator of the possible role between estrogen and leukaemia—she oversaw many of the experiments. Nga Du was involved in refining and then executing the studies using iPCR. Hongan Le was involved in verifying the studies using iPCR and optimizing Western and Southern blots. Sheetal Singh was involved in experimental oversight and project direction. Grace Loreda was involved in oversight of iPCR analysis. Andrew Vaughan had overall charge of project direction and analysis.

translocations with over 50 partner genes, deletions, inversions and partial tandem duplications. Leukemogenesis in IAL has a very short latency and is thought to occur *in utero*; the prognosis is however still poor (reviewed by Felix and Lange [3]). Translocations of the *MLL* gene are also found in adult leukemia, especially in t-AML (therapy-related AML) patients who received topoisomerase II targeted therapy of a primary tumor [4]. Topoisomerase II poisoning has therefore been implicated by many as the underlying mechanism creating *MLL* aberrations in t-AML [5–7]. Translocation breakpoints within tAML and IAL are both biased towards the 3' end of the *MLL* breakpoint cluster region that includes exons 8 and 14 [8]. Some effort has thus been made to investigate whether naturally occurring topoisomerase II inhibitors such as flavonoids might contribute to, or be the cause of, IAL and childhood leukemia through exposure in the uterus. Although one preliminary study suggested that dietary topoisomerase-II inhibitors may be associated with infant AML harboring *MLL* aberrations [9], a later study could not confirm these findings to be statistically significant [10]. *In vitro*, however, high concentrations of flavonoids induce DNA double strand breaks and translocations in CD34+ cells obtained from chord blood [11].

Other epidemiological studies have looked at exposures to infectious agents [12,13], medications, drugs and environmental toxins [14,15] as well as birth characteristics [16] which might be associated with IAL, but results tend to be inconsistent, possibly due to the rarity of the disease. A recent epidemiological study from Brazil addressed IAL and its association with various maternal exposures during pregnancy. This study found a significant increase in IAL risk in the offspring following maternal use of oral contraceptives, either before, or during, the first trimester of pregnancy [17]. Notably, the association was stronger for cases with *MLL* aberrations, and the authors raise the question whether estrogens could play a role in the etiology of IAL.

Estrogens have been studied extensively in connection with breast, endometrial and ovarian cancer as well as other solid tumors, and it is generally accepted that they play an important role in the development of these tumors [18,19]. The exact mechanism by which estrogens promote carcinogenesis is thought to be a combination of a proliferative effect in hormone-sensitive tissues, mediated mainly via estrogen receptor (ER) alpha, and the direct genotoxicity of metabolites of endogenous and/or exogenous estrogens, reviewed in Liehr [18]. Estradiol (E2) and estrone (E1) as well as the catechol estrogens 4-OH-E2 and 4-OH-E1 have been shown to cause double strand breaks and chromosomal aberrations in embryonic hamster cells and animal models [19].

Here, we investigated whether concentrations of E2 corresponding to levels found in maternal serum and cord blood during pregnancy, and similar levels of 4-OH-E2, could experimentally induce aberrations of the *MLL* gene in TK6 cells, an untransformed human lymphoblastoid cell line.

2. Materials and methods

2.1. Cell culture

TK6 cells were purchased from ATCC and cultured in RPMI-1640 without phenolred (Invitrogen, Carlsbad, CA), supplemented with 10% charcoal-dextran stripped fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), HEPES buffer and sodium pyruvate (Invitrogen, Carlsbad, CA), at 37°C and 5% CO₂ under standard tissue culture conditions.

2.2. Reagents

E2 and 4-OH-E2 were purchased from Sigma-Aldrich (St. Louis, MO), dissolved in 100% EtOH at 10 mM and stored at –80°C. For each treatment, fresh 1000× stock solutions were

made for each group by serial dilution and filtered under sterile conditions before addition to cells. The maximum final concentration of EtOH was <0.05%.

2.3. Immunoblotting

Whole cell lysates (5×10^6 per group) were obtained from treated TK6 cells using RIPA lysis buffer. Protein samples were then separated on a 10% SDS-Page gel, transferred to a nitrocellulose membrane by wet transfer and probed for ER α , ER β and cytochrome P4501B1 (CYP1B1) over night. Antibodies for ER alpha and CYP1B1 were purchased from Santa Cruz (Santa Cruz, CA) (cat.# sc-543 and sc-32882, respectively) and for ER beta from Sigma (cat.# E1276). Membranes were stripped with Re-blot Plus Strong Solution (Chemicon Intl, Temecula, CA) and reprobed for β -actin as a loading control.

2.4. Inverse PCR

2.4.1. Treatment and DNA harvest—Cells were split at 2.5×10^4 /ml in T25 flasks and either left untreated (controls) or treated with EtOH at 0.05% (vehicle control) or E2/4-OH-E2 respectively at 10 nM, 100 nM and 1000 nM final concentration for 24 h. After 24 h, $4-5 \times 10^6$ cells per group were harvested for DNA (genomic DNA) extraction by DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The remaining cells were supplied with fresh media, treated and cultured for an additional 48 h, and again $4-5 \times 10^6$ cells per group harvested for DNA extraction (72 h time point).

2.4.2. Digest and ligation of genomic DNA—5 μ g of DNA per group were incubated at 37°C for 3–4 h with the restriction enzyme *Sau3A1* (Promega, Madison, WI) and purified by Quickspin kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Digested DNA was eluted in 50 μ l of ddH₂O. Eluates were self-ligated with T4 DNA Ligase (Promega Madison, WI) at 4°C overnight, and the reaction was terminated by incubating at 70°C for 15 min.

In one of three experiments, an aliquot of 20 μ l of each ligated sample was further digested with *PvuII* (Promega, Madison, WI) and the reaction was inactivated at 70°C for 15 min. This step re-linearizes the native *MLL* fragment and therefore removes it as a template for the inverse PCR reaction that follows, increasing the detection efficiency of *MLL* rearrangements [20].

2.4.3. Inverse PCR conditions—A first round of semi-nested PCR was performed with 100 ng of ligated DNA as well as with *PvuII*-redigested DNA as a template. For inverse PCR (iPCR), the following cycles were used: denaturing for 10 min at 95°C, 30 cycles of 94°C (1 min), 58°C (1 min) and 72°C (1 min 30s), and final extension at 72°C for 10 min. The second round of iPCR was performed under the same conditions except with an annealing temperature of 60°C and round 2 forward primer. Sequences of primers used for iPCR were as follows:

Round 1 forward primer: 5'-CACTCTTAGGTCACCTAGCATGTT-3'

Round 2 forward primer: 5'-TACTCTGAATCTCCCGCAGTGTCC-3'

Reverse primer: 5'-ACAGTTGTAAGGTCTGGTTTGTCC-3'.

All reactions were performed with GoTaq DNA polymerase (Promega, Madison, WI) and run on a 1.2% agarose gel with ethidium bromide at 0.5 μ g/ml.

2.4.4. TOPO-TA cloning and sequencing—Aberrant bands were extracted (Quickspin gel extraction kit, Qiagen, Valencia, CA) and cloned into TOPO-TA vector pCR2.1 (Invitrogen, Carlsbad, CA) according to the protocols provided by the manufacturers. Plasmids extracted from positive clones were sent for sequencing to Seqwright (Fisher Scientific, Pittsburgh, PA). Sequences were analyzed by comparison to the BLAST database of the NCBI.

2.5. Annexin V staining

Apoptosis was assessed by flow cytometry. The cells were incubated with Annexin-V antibody conjugated to PE. Cells were also simultaneously stained with 7-AAD (Biosciences, San Jose, CA) for viability. Cells treated with Fas-ligand (eBioscience, San Diego, CA, cat # 16-0958-81) for 18 h were used as a positive control. Data were gathered from 3 separate experiments.

2.6. Cell cycle analysis

Cells were fixed in 70% EtOH at -20°C overnight, incubated with Propidium Iodide/RNase solution (Invitrogen Carlsbad, CA) for 15 min and analyzed by flow cytometry on FACScalibur (BD Biosciences, San Jose, CA). The analysis was performed on fixed cells from three different experiments.

2.7. Ligation mediated PCR (LM-PCR) and Southern blot

Single stranded linkers 11 and 25mers were annealed in the presence of T4 DNA ligase buffer (Promega, Madison, WI) by incubation in a thermocycler under the following conditions: 95°C for 5 min, 70°C for 1 sec, ramp temperature from 70°C to 25°C over 60 min, hold at 25°C for 60 min, ramp to 4°C over 60 min. Cells were pre-treated with zVAD.fmk (Promega, Madison, WI) for 2 h to suppress spontaneous apoptosis and apoptosis-induced cleavage of the *MLL* gene [21], prior to treatment with E2/4-OH-E2. Total genomic DNA was ligated by T4 DNA ligase (Promega, Madison, WI) overnight at 15°C with the double-stranded linker as previously described [22]. Two rounds of PCR were performed with the linker-specific forward primer and two *MLL* BCR (breakpoint cluster region) specific semi-nested reverse primers. PCR products were run on a 1.5% agarose gel and used for specific probing by Southern blot to detect amplified breaks in the *MLL* BCR region.

LM-PCR Primer Sequences:

Linker-11: 5'-GAATTCAGATC-3'

Linker-25 (and forward primer): 5'-GCGGTGACCCGGGAGATCTGAATTC-3'

Round 1 reverse primer: 5'-ATGCCCAAGTCCCTAGACAAAATGGTG-3'

Round 2 reverse primer: 5'-GTCTGTTACATAGAGTACAGAGGCAACTA-3'

The PCR products from the LM-PCR were transferred from a 1.5% agarose gel to a nylon membrane overnight at room temperature, and fixed by baking in a microwave oven. A biotinylated probe was generated using NEBlot Phototope kit, cat.# N7550S (New England Biolabs, Ipswich, MA) specific to the *MLL* BCR region hotspot in intron 11 [23] and hybridized to the immobilized DNA using a standard protocol [20]. Probe was detected using the Phototope-Star detection Kit (New England Biolabs), cat.# N7020S.

3. Results

3.1. Expression of estrogen receptors and CYP1B1

Western blot data showed that TK6 cells express both ER α and ER β at levels comparable to MCF-7 (breast cancer derived) cells, and the levels of expression were not reproducibly affected by any treatment (Fig. 1). TK6 cells also express relatively high levels of CYP1B1, which was not significantly altered by treatment with the same concentration of E2 and 4-OH-E2 when assayed by Western blot (data not shown).

3.2. *MLL* aberrations detected by inverse PCR

DNA was harvested 24 and 72 h after treatment with E2 or 4-OH-E2 as well as from non-treated and vehicle-treated controls, and processed for analysis by iPCR (inverse PCR). Each

potential rearrangement (Fig. 2) was screened to exclude PCR products generated by presumed false priming or those generated by ligation of several *Sau3A1*-site-flanked pieces of genomic DNA. After TOPOTATA-cloning and sequencing of approximately 30 aberrant bands per experiment (90 bands total), 9 translocations of the *MLL* gene with genomic DNA of another chromosome were identified, as well as one deletion of several Mbp (Table 1).

Consistently, the 10 nM E2 treatment group demonstrated the most *MLL* aberrations of any treatment, and this finding was statistically significant by *t*-test compared to controls for the 24 h time point. In addition to the translocations, sequences were found which contained a break in the *MLL* BCR without any foreign chromosomal material inserted. These breaks appeared more often after 72 h and at the highest rate in the 4-OH-E2 treated cells, but also under E2 treatment. One such aberration occurred in a control group at 72 h in one of three experiments. An increase in break frequency was statistically significant by *t*-test for both treatments when compared to controls (Table 2).

3.3. Assessment of apoptosis and cell cycle distribution

TK6 cells are rapidly dividing cells that exhibit a relatively high level of spontaneous apoptosis under normal growth conditions [24]. It has previously been shown that under apoptotic conditions the *MLL* BCR is cleaved in TK6 cells stimulated with Fas-ligand (anti-CD95 antibody) [20]. To determine whether induction of cleavage by E2 and 4-OH-E2 treatment was due to increased proliferation and/or increased levels of apoptosis, the effect of treatment on cell cycle distribution and apoptosis was assessed by flow cytometry. As expected from previous studies 5–10% of normal TK6 cells were undergoing spontaneous apoptosis, but there was no detectable alteration of this level in any of the treatment groups when compared to controls (Fig. 3). Similarly, no alteration in cell cycle distribution was observed after any treatment (data not shown). Thus the increase in *MLL* rearrangements is unlikely to be generated from alterations in cell proliferation or access to apoptosis.

3.4. *MLL* cleavage by LM-PCR

To confirm if the treatment regimes fragmented DNA through an apoptotic mechanism, cells were incubated with the pan-caspase inhibitor, zVAD.fmk for 2 h before the addition of E2 and 4-OH-E2 [21]. After 24 h of treatment, DNA from treated and control cells was analyzed by LM-PCR to detect DNA double strand breaks. While zVAD.fmk significantly suppressed background cleavage in the control group, likely due to the expected suppression of basal levels of apoptosis, it had little demonstrable effect on *MLL* BCR cleavage by E2 and no effect on cleavage by 4-OH-E2 (Fig. 4). This further suggests that E2 and especially 4-OH-E2 introduce breaks within the *MLL* gene through a mechanism that is independent of apoptotic activation.

4. Discussion

It is known that treatment of normal peripheral mononuclear cells from healthy donors, with *ortho*-quinones of benzene and estrogens, cause the formation of depurinating DNA adducts with an increase in proliferation [25]. Consistent with these results, we show an increase in *MLL* translocations following exposure to E2 at a level (0.01 μ M), comparable to that found in serum during the second trimester of pregnancy [26]. Also, both E2 and 4-OH-E2 induce DNA double strand breaks at this clinically relevant concentration. We estimate the frequency of translocations in the lowest E2 concentration group (0.01 μ M) to be about 1 in 2×10^4 cells, or 50 in 1×10^6 cells, which is approximately one third of that reported by Libura et al. after exposure of CD34+ primary cells to etoposide (150 in 1×10^6 cells) [6], and substantially lower than a report of *MLL* aberrations following exposure to flavonoids (500 in 1×10^6 cells) [11]. However, both of these groups used iPCR to interrogate a longer *MLL* fragment (1.5 kb) than that reported here (0.75 kb), likely increasing the frequency of detection [20]. In addition, the

levels of E2 and 4-OH-E2 used in our experiments corresponded to physiological levels during pregnancy, while the other two groups used higher concentrations of the respective treatments (1–50 μ M range).

A number of groups, including our own, have correlated the induction of *MLL* rearrangements with the process of apoptosis [5,22,27]. In these studies the apoptotic nucleases are thought to specifically target the *MLL* gene that is subsequently mis-repaired. In the present study, we examined the possibility that either E2 or 4-OH-E2 induced *MLL* rearrangements by such a mechanism. No such effect on apoptosis was detected either by Annexin V staining of apoptotic cells (Fig. 3) or using the more sensitive LM-PCR technique (Fig. 4). In the case of LM-PCR it was determined that *MLL* fragmentation occurred in response to 4-OH-E2 exposure that was not suppressed by the pan-caspase apoptotic inhibitor zVAD.fmk. These data strongly suggest that the DNA damage, and by extension the *MLL* rearrangements observed here, are not related to apoptotic cleavage.

Our data suggest that under certain circumstances, E2 and 4-OH-E2 can promote breakage within the *MLL* BCR (and probably other regions of the genome) and therefore increase the chances of recombination events. *MLL* gene aberrations-, are known to be crucial in the development of infant acute leukemia and therapy related acute leukemias [1–4]. Surprisingly, it was noted that while 4-OH-E2 treatment promotes a higher rate of *MLL* BCR fragmentation, determined from iPCR and LM-PCR techniques, the frequency of *MLL* translocations was only elevated after E2 exposure. This confirms the predicted genotoxicity of 4-OH-E2 but implies an additional role for E2 in mediating aberrant repair. As a possible explanation, there is evidence that E2 interacts with histone acetylases and other chromatin modifiers through the estrogen receptors, changing chromatin structure [27]. Also, ER α may interact with cofactors of DNA repair pathways affecting correct repair [28]. In the current study cells were grown in E2-free media, thus the 4-OH-E2 treated group would not have been exposed to any significant levels of E2, supporting the role of E2 in the creation of *MLL* rearrangements.

Two main pathways may be involved in generating E2 associated *MLL* rearrangements. First, an effect may be mediated through the estrogen receptors, which are both expressed in hematopoietic cells and which participate in the regulation of normal hematopoiesis. Though no change in the levels of either receptor was observed by Western blot in this study (Fig. 1), ER α knockouts develop autoimmune disease, while ER β null mice develop a CML-like condition over time that can turn into a blast crisis [29]. Estrogen receptors are also involved in shaping chromatin structure, epigenetic phenomena and possibly DNA damage repair [27, 28]. Of note, a cryptic promoter site was recently found at the *MLL* location studied here indicating that this region may be a site of active chromatin remodeling [30]. Secondly, catechol estrogens such as 4-OH-E2 are either eliminated by phase-II enzymes, or further metabolized by peroxidases to semiquinones and quinones, forming DNA adducts while also generating genotoxic reactive oxygen species by redox-cycling *in vivo* [32]. These quinones are also strong candidates for topoisomerase II poisoning, analogous to both benzene-metabolites and the flavonoids genistein and quercetin that have all been implicated in the induction of leukemia [27,33–35].

In regard to estrogen metabolism, special attention has been given to the cytochrome P450 variant CYP1B1, which is normally expressed at very low levels in most tissues, but is highly inducible by dioxin [36] and other environmental toxins [37]. Similarly to the finding with ER receptors, no change in CYP1B1 was observed after treatment in this study though it was robustly expressed (data not shown). CYP1B1 has been shown to metabolize estradiol (E2) and estrone (E1) to their respective 4-catechol estrogens. Studies in CYP1B1 null mice showed evidence of a reduction of pre-leukemic cells in the bone marrow after exposure to the classic polycyclic aromatic hydrocarbon DMBA, indicating a possible role for CYP1B1 in creation

of leukemogenic metabolites [38]. Further, dietary compounds containing flavonoids may affect estrogen metabolism by inhibition of phase II enzymes and lead to higher levels of genotoxic estrogen metabolites, in addition to acting as topoisomerase II poisons, increasing *MLL* translocation frequency. This would be especially disturbing since flavonoids are part of many over-the-counter dietary supplements, which are also consumed by pregnant women. We suggest these data warrant further investigations into the role of estrogens and estrogen metabolites in the etiology of infant acute leukemia.

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Fig. 1.

Expression of estrogen receptors in TK6 cells. TK6 express both estrogen receptors alpha (ERα) and beta (ERβ) at levels comparable to MCF-7 (breast cancer); levels were not affected by treatments. C = no treatment; E ethanol (vehicle) control. TK6 also express relatively high levels of CYP1B1, and treatment with either E2 or 4-OH E2 did not cause any significant changes in protein levels (data not shown). β-actin was used as loading control.

Fig. 2.

Rearrangements determined by iPCR. (a) Schematic of *MLL* BCR fragment analyzed by inverse PCR (iPCR) S = *Sau3A1* site used to excise fragment and create circular template. S¹ is a methylated *Sau3A1* site that is not excised in genomic samples. P_V = *PvuII* site, (b) iPCR result from *Sau3A1*-digested DNA. Plasmid DNA (P) produces a smaller iPCR product as the S¹ *Sau3A1* is preferentially cleaved, this also provides a reference product size to compare panels b) and c). Main PCR product (*) is derived from native *MLL* amplification is visible in all groups, plus aberrant bands under treatment. P = plasmid control, a vector containing the full 8.3 kb *MLL* bcr, C = Control sample, E = Ethanol (vehicle control). (c) iPCR result from *Sau3A1/PvuII* digested DNA. *PvuII* digest of *Sau3A1*-digested and religated DNA removes native *MLL* band revealing more potential aberrations (arrows). Such bands that represent translocations or breaks in the *MLL* gene were analyzed by gel extraction of product and sequencing. From experience, intense PCR products usually proved to be artifacts.

Fig. 3.

Assessment of apoptosis in TK6 cells by Annexin-V staining and flowcytometry. Results show averages from three independent experiments, error bars indicate standard error of the mean. Black bars, 24 h treatment; Gray bars, 72 h. Positive controls were TK6 cells treated with anti-CD95 antibody (Fas-ligand) for 18 h. C1 = control, C2 Ethanol (vehicle) control, E2 = estrogen treated, 4OH = 4-OH E2 treated. Levels of spontaneous apoptosis in controls are between 5– and 10% as previously reported. There is no detectable drug-induced induction of apoptosis in any of the treatment groups.

Fig. 4.

Southern blot for detection of *MLL* bcr cleavage in LM-PCR products. Cells were pre-treated with the pan-caspase inhibitor zVAD-fmk for 2 h where indicated. C = control E = ethanol (vehicle) control. In controls, zVAD.fmk treatment suppressed background *MLL* cleavage significantly, while under E2 and especially 4-OH-E2 treatment, levels of cleavage products remained elevated despite inhibition of apoptosis.

Table 1

Partner chromosomes and breakpoint sequences of translocations.

Treatment (μM)		Time (h)	Partner chromosome	Breakpoint sequence ^a
E2	0.01	24	Chr.6	GCCTCTGTATCAGTGGGTCTGA AATCTCCTAGACT <u>TCATAAGAGGATT</u>
	-		Chr.3	TGTATTGCCAAGTCTGTTG TGAGC <u>CAAATGTGAGTGACCATGGCCCATG</u>
	-		Chr.5	TGAATCTCCCGCAGTGTCCGATACTGTA ATAGCACTCTGCTT <u>GAAAGTT</u>
	-	72	Chr.11, del 3Mbp	TTTGTGTATTGCCAAGTCTGTTGTGAGC CTAGGAACAGACATAGGCTTC
E2	0.1	72	Chr.3	AAGGGTATGATTGATTATGTTTT CTACA <u>AGTACAAAAATTAGCCGGGC</u>
	-		Chr.11	TGTGTATTGCCAAGTCTGTTG TGAGCACTCTAGCCTGGGCAATAAAGCA
E2	1.0	24	Chr.2 ^b	ATTGCCAAGTCTGTTGT GAGCC <u>AGTGTGTCCCTGGGGGCCGGTTCTTT</u>
4-OH-E2	0.01	72	Chr.4	TACATTGTATTAGGTATGATAAG TAATCCAGAGATCCAGATCCCTAAGA
4-OH-E2	0.1	72	Chr.1	TTTGTGTATTGCCAAGTCTGTTGT GAGCTTCCATTTCTCTTCTGAAAA
Control		72	Chr.4	AGCGCCCTCTGGGG GACCCTAAAGAAATGGAGATCTTTACTGCATGAC

^aNormal text: *MLL* sequence. Boldface: microhomology. Underlined: partner sequence.^bbeta-Galactoside alpha-2,6-sialyltransferase II, NP_115917.

Table 2Summary of *MLL* aberrations.

Treatment	Time (h)	Breaks	Translocations
Controls	24	0	0
	72	1	1
E2	24	7 ^a	4 ^a
	72	7 ^a	3 ^{a,b}
4-OH-E2	24	6 ^a	0
	72	11 ^a	2

^a Statistical analysis by *t*-test found a significant increase in frequency when compared with controls ($p < 0.001$).

^b Includes 1 Mbp deletion.