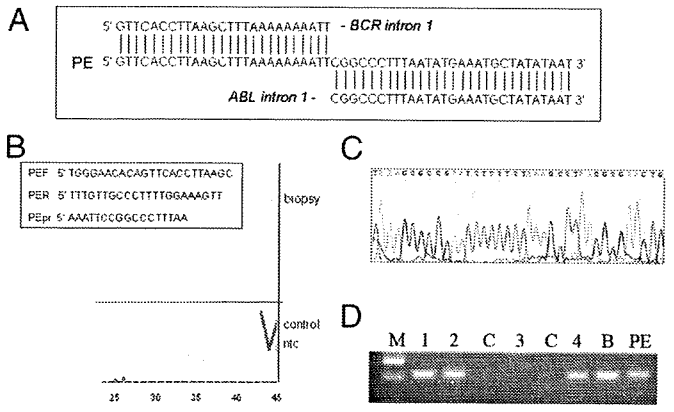


**Fig. 1.** Characterization of the *BCR-ABL1* fusion gene. (*A*) Comparison of the pleural effusion (PE) *BCR-ABL1* breakpoint DNA sequence with *BCR* intron 1 (NM_004327) and *ABL1* intron 1 (NM_007313) sequences. (*B*) Q-PCR amplification of the *BCR-ABL1* breakpoint in WGA DNA from mother's bone marrow biopsy. The primers and probe shown were chosen to span the PE fusion sequence obtained from the child's PE DNA. (*C*) Reverse strand DNA sequence of DNA from the mother's biopsy showing the same *BCR-ABL1* fusion sequence as found in the child. (*D*) PCR of the *BCR-ABL1* breakpoint in DNA from the neonatal blood spot confirming presence of the *BCR-ABL1* fusion gene. Lanes 1–4: slices from the child's card (1, 2, and 4 positive), C: DNA from control neonatal blood spot, B: mother's marrow biopsy DNA. PE: child's pleural effusion DNA. M: marker.

***BCR-ABL1* Genomic Fusion Sequencing.** We first cloned the *BCR-ABL1* genomic breakpoint region from the infant's pleural effusion (PE) (see *Materials and Methods*). DNA was Whole Genome Amplified (GenomiPhi, GE Healthcare), according to the manufacturer's instructions. The breakpoint was designated as a fusion between *BCR* intron 1 (46110 bp from ATG: NM_004327) and *ABL1* intron 1 (118930 bp from ATG: NM_007313) (Fig. 1*C*).

DNA from the mother's bone marrow was isolated by scraping cells from a formalin fixed paraffin embedded (FFPE) biopsy slide (the only sample available) using Recoverall (Ambion) as suggested by the manufacturer. Fragmented FFPE DNA was then subjected to whole genome amplification (WGA), and 2 $\mu$L amplified DNA subjected to 45 cycles Q-PCR with primers designed by Primer 3 software (5) and described in Fig. 1*B* and a FAM-labeled probe that spanned the specific *BCR-ABL1* breakpoint sequence. After successful Q-PCR (Fig. 1*B*), the product was purified and sequenced using the reverse *ABL1* primer. The fusion sequence in the mother's biopsy was verified as identical to that obtained from the pleural effusion of the child (Fig. 1*C*).

The archived neonatal blood spot (Guthrie card) of the infant was screened for the clonotypic *BCR-ABL1* genomic sequence using specific primers and as previously described for other fusion genes (6). Three out of four blood spot slices were positive (Fig. 1*D*), indicating that the cancer clone was present in the blood at birth.

**Microsatellite Markers.** Short tandem repeat (STR) microsatellite analysis of the DNA extracted from the jaw biopsy showed one predominant population (>95% of alleles) that did not correspond to the DNA profile obtained from the paternal sample (Fig. 2). The STR profile shows that it shared one allele with the patient's germline DNA for all of the 15 STR markers studied which was different from the paternally-inherited alleles, demonstrating that the jaw tumor sample was of maternal origin

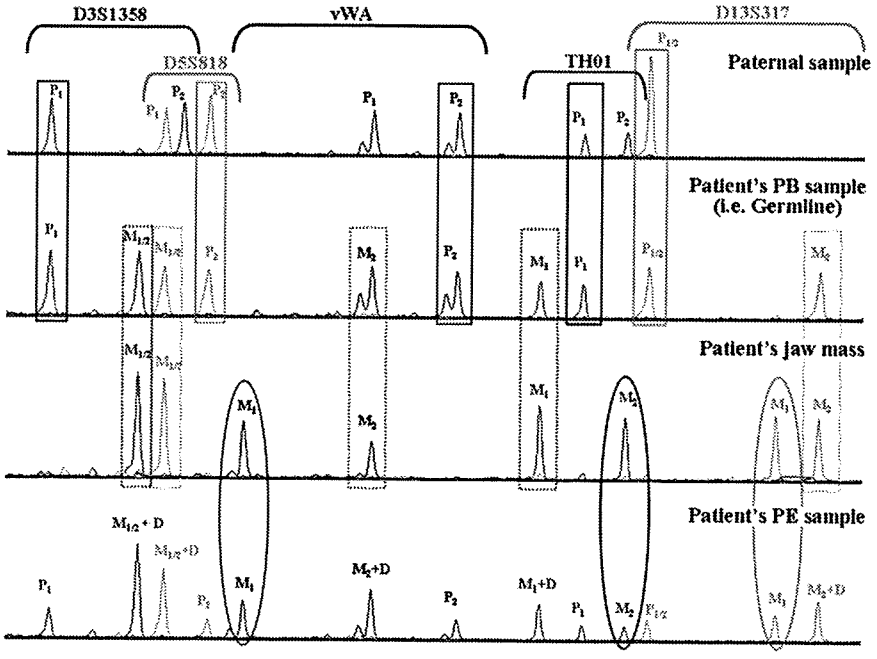


**Fig. 2.** STR typing for the paternal sample and patient's peripheral blood (PB), lymphoma (from the jaw mass) and pleural effusion samples. Figure shows typing for five out of the 15 STR markers analyzed. The STR profile of patient's jaw mass shows the sharing of one allele with the patient's germline DNA for all markers while the other allele is not present in the paternal DNA, demonstrating that the lymphoma DNA obtained from the jaw mass contained >95% of maternal cells. For each STR marker the Paternal (P1, P2) and Maternal (M1, M2) alleles are indicated. P and M alleles contributing to the patient's germline DNA are contained within solid and dotted rectangles, respectively. The PE sample shows a mixture of different alleles, with some markers showing up to three different alleles (vWA, TH01, and D13S317, contained in ovals), indicating heterozygosity for the maternal genotype on these markers. The remaining maternal alleles contributing to the daughter's genotype in this sample are indicated as M + D. These former markers were used for calculation of the percentage of maternal cells present in the PE sample (≈50–60%) by comparing the areas of the maternal-only peaks (M) versus the paternal peaks (corresponding to the patient's cells). This proportion of maternal cells approximates with the percentage tumor infiltration identified by immunophenotype (48.2%).

**Table 1. Loss of non-inherited maternal HLA allele expression in infants 'maternal' cancer cells**

| | HLA-A | HLA-B | DRB1 |
|---|---|---|---|
| Father | A2/A33 | B61/B58 | DR8/DR13 |
| Mother | A24/**A11** | B60/**B67** | DR9/**DR15** |
| Patient | A24/A33 2402/3303 | B60/B58 4001/5801 | DR9/DR13 0901/1302 |
| Patient's jaw mass | A24/-2402/- | B60/-4001/- | DR9/-0901/- |

Alleles were serotyped or genotyped (2402/3303, 4001/5801, 0901/1302) (see *SI Text*). Insufficient material was available to genotype parental samples. Alleles in bold are maternal HLA alleles lost from maternal cancer cells transmitted to infant.

(>95% maternal cells). STR analysis of the PE sample showed a mixture of alleles for most STR markers analyzed. These mixtures were identified either because of the presence of three different alleles in some markers, or because those markers with only two alleles presented an imbalance in the ratio of the peak areas (Fig. 2). These findings were consistent with the presence of two genetically different cell populations in the PE specimen–one maternal, one infant offspring (see Fig. 2 legend).

**HLA Analysis.** Survival of maternal cells in the offspring presumably requires some form of immunological acceptance or tolerance of cells expressing foreign, non-inherited maternal MHC antigens. We serotyped samples from both parents for HLA-A, HLA-B, and DRB1, and both serotyped and genotyped the infant's normal blood and lymphoma cells. The result was that the *BCR-ABL1*-positive jaw tumor had selectively deleted or lost the HLA alleles that were not inherited by the daughter (Table 1). The nature of this genetic lesion in the infant tumor cancer cells was further explored by high resolution SNP arrays. In the absence of normal germline maternal and infant DNA and only small quantities of degraded DNA from the maternal leukemia biopsy, we elected to analyze the infant lymphoma (jaw) sample for genome-wide lack of heterozygosity (LOH) in comparison with pooled normal control DNA. Table 2 summarizes the LOH analysis of the infant lymphoma. Recurrent deletions of *IKZF1* and *CDKN2A/B* have previously been described in *BCR-ABL1* ALL (7), as have deletions of *EBF1* and *RAG1/2* in B lineage childhood ALL (8). In addition to these anticipated oncogenic or 'driver' deletions, we observed a large region of homozygosity on the short (p) arm of chromosome 6 including the whole HLA locus. This loss was accompanied by duplication of the other parental 6p region resulting in uniparental disomy. A large genomic deletion, including the HLA loci, therefore accounts for the loss of maternal HLA alleles.

## Discussion

These data unambiguously mark the infant cancer as of maternal origin. Some 17 cases of probable metastasis to the fetus have now been recorded (1, 9, and current report), the first being in 1866, most being either melanoma (#6), a cancer with a notoriously metastatic proclivity, or leukemia/lymphoma (#8). Given the phenotypic features described in these cases, it is likely that they were all, as presumed, of maternal origin rather than coincidental cancers. Genetic markers can unambiguously resolve cellular origins in this context. In three of the reported leukemia/lymphoma cases, the male infant bone marrow contained cells with an XX karyotype (1). Whilst these most probably do reflect maternal leukemia/lymphoma cells, it cannot be excluded that they were non-malignant, normal maternal cells or infant male cells in which the Y chromosome was lost and X was duplicated. These are, individually, not rare events in leukemia (10, 11), although they seldom occur together in one clone. Other prior evidence for a maternal origin was the identification in a case of NK cell lymphoma of a specific chromosome translocation t(X;1)(q22;q12) in the maternal lymphoma and in three metaphases of the infant tumor (12).

The rarity of materno-fetal transmission of cancer is a testimony to the efficacy of the placental barrier and perhaps to immunosurveillance. In the present case, an additional feature was the selective loss in the infant maternally-derived tumor cells of maternal HLA alleles that were not inherited by the infant (Tables 1 and 2). Loss of HLA would be expected to render the transmitted cancer cells immunologically inert (13). HLA loci encoded cell surface proteins provide the major antigenic targets for allograft recognition and rejection, so it is likely that HLA deletion in this case contributed to successful transmission of the maternal leukemic cells. However, given the large size of the 6p deletion, it is possible that other gene losses could have contributed to the apparent lack of immuno-surveillance. Other unusual situations where cancer cell transmission occurs all appear to involve immunological invisibility (14): inter-monozygotic twin transmission in utero (4), immunosuppressed recipients of cancer-infiltrated donor organs (15), down-regulated MHC antigen expression in venereal sarcoma in dogs (2), and lack of MHC diversity in the Tasmanian devil (*Sarcophilus harrisii*) with transmissible facial tumors (16). In a recent report (17), loss of allorecognition of leukemic cell HLA by T cells, in a transplant context, also occurred by acquired uniparental disomy of chromosome 6p in the leukemic cells, as in the present study. It is possible that materno-feto transfer of cancer cells is more common than is reflected in the frequency of clinically diagnosed cases and that immuno-surveillance is the principal constraint.

**Table 2. Loss of heterozygosity analysis of infant tumor lymphoma**

| Region | LOH | Gene(s) |
|---|---|---|
| 5p33.3 | Loss | *EBF1* |
| 6p25.3–21.1 | UPD | *HLA*, and many other genes |
| 7p14.1 | Loss | *TCRG* |
| 7p12.2 | Loss | *IKZF1* |
| 9p21.3–12 | Loss | *MTAP, CDKN2A/B, PAX5* |
| 11p12 | Loss | *RAG1/2* |
| 14q11.2 | Loss | *TCRA* |
| 14q32.33 | Loss | *IGH* |
| 15q22.33 | Loss | *SMAD3* |
| 22q11.22 | Loss | *IGL* |

Loss of heterozygosity analysis of the infant tumor lymphoma in comparison with unpaired control DNA. UPD, uniparental disomy (see *Materials and Methods* for details).

## Materials and Methods

**Detection and Amplification of *BCR-ABL1* Genomic Breakpoints.** For detection and amplification of DNA breakpoints, ranging from 300 bp to 12 kbp the Expand Long Template PCR kit (Roche) with System 2 was used, with an annealing temperature of 64 °C. To cover the *BCR* and *ABL1* regions, within which breakpoints can occur, 21 *BCR* forward primers and 20 *ABL1* reverse primers were used in multiplex, combining each *BCR* forward primer with 4 mixes of 5 *ABL1* reverse primers.

The child's genomic breakpoint was initially amplified using *BCR* 3C F (GGGCTCATTTTCACTGGATGGAC) and the *ABL1* D reverse primer mix, and

upon split out PCR, a band was amplified with *BCR* 3C F and *ABL1* 1D R (AGC CAT AAC CAT TCT CCC AAG CA). The breakpoint was confirmed by re-amplification and sequencing of the breakpoint in both the original and WGA amplified patient sample with *BCR* 3C F (GGGCTCATTTTCACTGGATGGAC), and a breakpoint specific *ABL1* reverse primer (TTC AGG GGC CTT GGA TCA GAC TA) determined from sequencing the original cloned product. Forward and reverse primers for blood spot PCR were respectively (GATCCTTTTAAAT-AGGCAAG) and (GTAATGCCAAAAATAACACT).

Fifteen polymorphic STR markers were amplified in the paternal blood DNA and patient's PE and PB DNA samples using the Powerplex-16 system (Promega).

**Genome Mapping Analysis.** Mapping analysis was performed using 500 ng of tumor DNA from the infant lymphoma. DNA was prepared according to manufacturer's instructions using the GeneChip mapping 500K assay protocol for hybridization to GeneChip Mapping 250K Nsp and Sty arrays (Affymetrix). Briefly, genomic DNA was digested in parallel with restriction endonucleases *Nsp*I and StyI, ligated to an adaptor, and subjected to PCR amplification with adaptor-specific primers. The PCR products were digested with *DNase*I and labeled with a biotinylated nucleotide analog. The labeled DNA fragments were hybridized to the microarray, stained by streptavidin–phycoerythrin conjugates, and washed using the Affymetrix Fluidics Station 450 then scanned with a GeneChip scanner 3000 7G.

**Copy Number and LOH Analysis.** SNP genotypes were obtained using Affymetrix GCOS software (version 1.4) to obtain raw feature intensity and Affymetrix GTYPE software (version 4.0) using the Dynamic Model algorithm with a call threshold of 0.33 to derive SNP genotypes. The sample was analyzed using CNAG 3.0 (http://plaza.umin.ac.jp/genome), comparing tumor sample with unpaired control DNA to determine copy number and LOH caused by imbalance (18).

**HLA Typing.** Serotyping was by microdroplet lymphocyte cytotoxicity (19). Genotyping was carried out using a reversed SSO HLA DNA typing method using fluorescent microspheres and a flow analyzer (20).

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# Alteration of Enhancer of Polycomb 1 at 10p11.2 Is One of the Genetic Events Leading to Development of Adult T-Cell Leukemia/Lymphoma

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Adult T-cell leukemia/lymphoma (ATLL) is a malignant tumor caused by latent human T-lymphotropic virus I (HTLV-I) infection. We previously identified a common breakpoint cluster region at 10p11.2 in acute-type ATLL by spectral karyotyping. Single nucleotide polymorphism array comparative genomic hybridization analysis of the breakpoint region in three ATLL-related cell lines and four patient samples revealed that the chromosomal breakpoints are localized within the enhancer of polycomb 1 (*EPC1*) gene locus in an ATLL-derived cell line (SO4) and in one patient with acute-type ATLL. *EPC1* is a human homologue of the E(Pc) enhancer of polycomb gene of *Drosophila*. Inappropriate expression of the polycomb group gene family has been linked to the loss of normal gene silencing pathways, which can contribute to the loss of cell identity and malignant transformation in many kinds of cancers. In the case of the SO4 cell line, which carried a der(10)t(2;10)(p23;p11.2) translocation, *EPC1* was fused with the additional sex combs-like 2 (*ASXL2*) gene at 2p23.3 (*EPC1/ASXL2*). In the case with an acute-type ATLL, who carried a der(10)del(10)(p11.2)del(10)(q22q24) translocation, a putative truncated *EPC1* gene (*EPC1tr*) was identified. Overexpression of EPC1/ASXL2 enhanced cell growth in T-leukemia cells, and a GAL4-EPC1/ASXL2 fusion protein showed high transcriptional activity. Although a GAL4-EPC1tr fusion protein did not activate transcription, overexpression of EPC1tr accelerated cell growth in leukemia cells, suggesting that the *EPC1* structural abnormalities in the SO4 cell line and in the patient with acute-type ATLL may contribute to leukemogenesis. 

## INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is one of the peripheral $CD4^+$ T-cell malignant neoplasms associated with human T-lymphotropic virus 1 (HTLV-1). ATLL develops in 2–5% of HTLV-1-infected individuals with a latency of 20–40 years. The long latency period and the low frequency of clinical progression to ATLL suggest that complex viral and cellular events are involved in the development of ATLL. It has been proposed that after HTLV-1 infection, at least five independent genetic changes are needed before the onset of ATLL (Okamoto et al., 1989). Recently, we reported the existence of recurrent chromosomal rearrangements in acute-type ATLL (Hidaka et al., 2008). We precisely mapped 605 chromosomal breakpoints in 61 ATLL cases by spectral karyotyping (SKY) and identified frequent breakpoints at chromosome bands 10p11 (35%), 14q11 (31%), and 14q32 (30%). Using fluorescence in situ hybridization analysis, the breakpoints at 10p11.2 were localized within a common 1 Mb region with heterozygous deletions in all cases. Additionally, *ZEB1/TCF8* was isolated from the deletion region as a possible tumor suppressor for ATLL (Hidaka et al., 2008).

In this article, we report the molecular cloning of rearrangements in the enhancer of polycomb 1 (*EPC1*) locus from the 10p11.2 region of ATLL cells. During mapping of the chromosomal

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breakpoints at 10p11.2 by single nucleotide polymorphism (SNP) array-comparative genomic hybridization (CGH) analysis, the chromosomal breakpoints were found to localize within the *EPC1* locus in an ATLL-derived cell line (SO4) and in one acute-type ATLL patient (ATL071) (out of three ATLL-related cell lines and four patient samples that were examined). *EPC1* is a member of the polycomb group gene family and has a broader role in chromatin formation and gene regulation compared with other polycomb group genes (Stankunas et al., 1998; Doyon et al., 2004; Attwooll et al., 2005). In the SO4 cell line, which carried a der(10)t(2;10)(p23;p11.2) translocation, *EPC1* was fused with the additional sex combs like 2 (*ASXL2*) gene at 2p23.3. *ASXL2* is also a member of the polycomb group (Katoh and Katoh, 2003). In the case of patient ATL071, who carried a der(10)del(10)(p11.2)del(10)(q22q24) translocation, a truncated *EPC1* gene was isolated by inverse genomic PCR. Introduction of the *EPC1/ASXL2*-fusion protein or truncated *EPC1* into leukemia cells accelerated cellular proliferation, suggesting that structural alteration of *EPC1* may be one of the causes of ATLL development.

## MATERIALS AND METHODS

### Patient Samples

ATLL cells were collected from patients at the time of admission to the hospital and before chemotherapy (Sasaki et al., 2005). The diagnosis of ATLL was based on clinical features, hematologic characteristics, the presence of serum antibodies to HTLV-1 antigens, and the presence of the HTLV-1 proviral genome in DNA from leukemic cells. Peripheral blood mononuclear cells were isolated by Histopaque (Sigma, St. Louis, Missouri) by density gradient centrifugation. Each patient had more than 90% leukemic cells in the blood at the time of analysis. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, University of Miyazaki. Informed consent was obtained from all blood and tissue donors according to the Helsinki Declaration.

### $CD4^+$ T Cells

$CD4^+$ T cells were obtained from five healthy volunteers. $CD4^+$ T cells were isolated by negative selection by AutoMACS using the $CD4^+$-T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). After separation, $CD4^+$-T-cell enrichment of more than 90% was confirmed by flow cytometric analysis. Samples were quickly frozen within 3 hr and cryopreserved at −80°C.

### Cell Lines

Jurkat and MOLT4 are HTLV-1-negative human T-cell acute lymphoblastic leukemia (T-ALL) cell lines (Minowada et al., 1972; Schneider et al., 1977). MT2 and Hut102 are human T-cell lines infected with HTLV-1 (Miyoshi et al., 1981). KOB, SO4, and KK1 are interleukin 2 (IL-2)-dependent ATLL cell lines (Yamada et al., 1996). ED, Su9T, and S1T are IL2-independent ATLL cell lines (Okada et al., 1985). CTLL2 is an IL-2-dependent murine cytotoxic T cell line (Gillis and Smith, 1977). All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS (FCS), either with or without IL-2.

### High Density SNP Array-CGH Analysis

GeneChip Mapping 250K array set (Affymetrix, Santa Clara, California) analyses were performed according to the manufacturer's instructions. Briefly, genomic DNA was digested with *Sty*I, ligated to an adaptor, and amplified by polymerase chain reaction (PCR). Amplified products were fragmented, labeled by biotinylation, and hybridized to microarrays. Hybridization was detected by incubation with a streptavidin–phycoerythrin conjugate followed by scanning; analysis was performed as described previously (Hidaka et al., 2008).

## RESULTS

### Identification of a Chromosomal Translocation in the *EPC1* Gene Locus at 10p11.2

To identify gene(s) involved in the leukemogenesis of ATLL, we analyzed chromosomal rearrangements of 61 acute-type ATLL cases by SKY and reported that breakpoints were localized most frequently at 10p11 (21 cases) (Hidaka et al., 2008). Moreover, in four ATLL cases carrying 10p11.2 abnormalities, the breakpoints were found to localize within a common 1 Mb region containing heterozygous deletions. Further breakpoint mapping using SNP array-CGH analysis showed that the DNA copy number changed from two copies to one copy within the *EPC1* gene in one patient sample (ATL071) (Fig. 1),

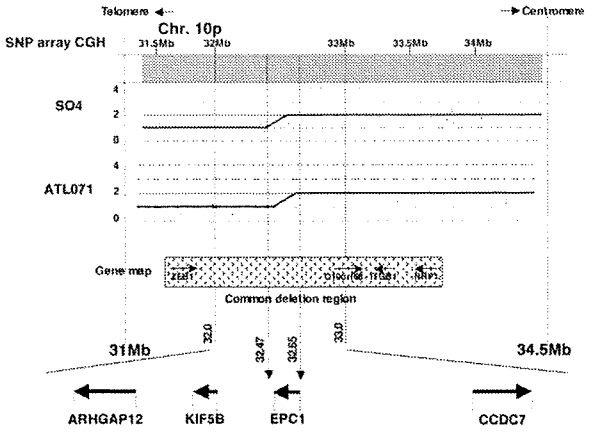


Figure 1. Array-CGH image of samples from the ATLL cell line SO4 and an acute-type ATLL patient (ATL071). The DNA copy numbers indicated as digits were changed at the same point on the *EPC1* gene in SO4 cells and ATL071. *ZEB1* through *NRP1* represent the names of the genes within the region from 31.6 Mb to 33.7 Mb of chromosome 10p11.2-p12 in the human genome map. Horizontal arrows show the direction of the gene. A common region of heterozygous deletions at 10p is also shown (hatched box).

while the other three cases had only one copy of the *EPC1* gene. We also detected a copy number decrease in the *EPC1* locus in the ATLL-derived cell line SO4. The 3′ region of the *EPC1* gene locus was included within the alteration point at 32.47–32.65 Mb on chromosome arm 10p (Fig. 1). Interestingly, the *ZEB1/TCF8* gene is located toward the telomere with respect to the *EPC1* gene and lies within the common region of heterozygous deletions in the SO4 cell line and patient ATL071. On the basis of the SKY analysis, the SO4 cell line and case ATL071 exhibited unbalanced chromosome 10 translocations, der(10)t(2;10)(p23;p11.2) and der(10)del(10)(p11.2)del(10)(q22q24), respectively (Hidaka et al., 2008).

### Identification of *EPC1/ASXL2*-Fusion mRNA in the ATLL-Derived SO4 Cell Line

To identify the 3′ end of *EPC1* mRNA in the SO4 cell line, we first performed reverse-transcription PCR (RT-PCR) analysis using primer sets to amplify several different DNA fragments within the region from exon 2 to exon 11 (Supporting Information Fig. 1). A PCR product covering exon 4 through exon 11 (4–11 in Supporting Information Fig. 1) was not amplified in the SO4 cell line, although three other *EPC1* cDNA products covering the region from exon 2 to exon 9 were amplified. These results indicated that the translocation might be localized within the region including exon 9 through exon 11 of the *EPC1* locus. We next performed 3′ rapid amplification of cDNA ends (RACE) experiments using primers from *EPC1* exon 7 (Supporting Information Materials and Methods). Several DNA bands, 0.5–2 kb in size, were amplified using 3′-RACE (Fig. 2A) and all of the cDNA bands were sequenced. The sequences of the cDNA fragments showed that a 1.9 kb band, which was detected in both the MT2 cell line carrying no rearrangement of the *EPC1* gene and SO4 cell lines, was derived from the *EPC1* gene; however, a 0.65 kb band was derived from a part of *EPC1* fused with the additional sex combs-like 2 (*ASXL2*) gene at the 3′ region of *EPC1* (Supporting Information Fig. 2). *ASXL2* belongs to the same polycomb repressive complex 2 (PRC2) at chromosome band 2p23 (Raaphorst, 2005). On the basis of the nucleotide sequencing of the cDNAs, the breakpoint in SO4 cells was located in intron 9 of the *EPC1* gene at 10p11.2 and intron 2 of the *ASXL2* gene at 2p23 (Fig. 2B, upper panel). As products of the truncated *EPC1* gene were 464 amino acid residues in length, and products of the *ASXL2* gene were 1,388 amino acid residues long, the entire EPC1/ASXL2 fusion protein should be composed of 1,852 amino acid residues (EPC1/ASXL2b in Fig. 2B). However, a short form of the EPC1/ASXL2 fusion product should also be produced with 555 amino acid residues of ASXL2, since the poly(A) tails of the *EPC1/ASXL2* fusion genes isolated by 3′-RACE were added after the intron 5 sequence of *ASXL2* (EPC1/ASXL2a in Fig. 2B).

To identify the fusion transcripts of *EPC1/ASXL2* or the reciprocal *ASXL2/EPC1* in the SO4 cell line, four sets of specific primers (Supporting Information Materials and Methods) were used for detecting four kinds of transcripts, *EPC1*, *ASXL2*, *EPC1/ASXL2*, or *ASXL2/EPC1*. As shown in Figure 2C, a 1,532 bp band of *EPC1*, 1,117 bp band of *ASXL2*, 606 bp band of *EPC1/ASXL2a*, and a 951 bp band of the *EPC1/ASXL2b*-fusion cDNA fragment was amplified; however, an expected 1,368 bp fragment of the *ASXL2/EPC1*-fusion cDNA fragment was not amplified, suggesting that the reciprocal fusion mRNA of *ASXL2/EPC1* was not expressed in the SO4 cell line, most likely because of the unbalanced translocation.

To confirm the expression of two *EPC1/ASXL2* fusion transcripts in the SO4 cell line, Northern blot was performed using an *EPC1* or *ASXL2* cDNA probe (Fig. 2D). Ten T-cell leukemia cell lines were used for this study, including two HTLV-1-uninfected T-ALL cell lines (Jurkat

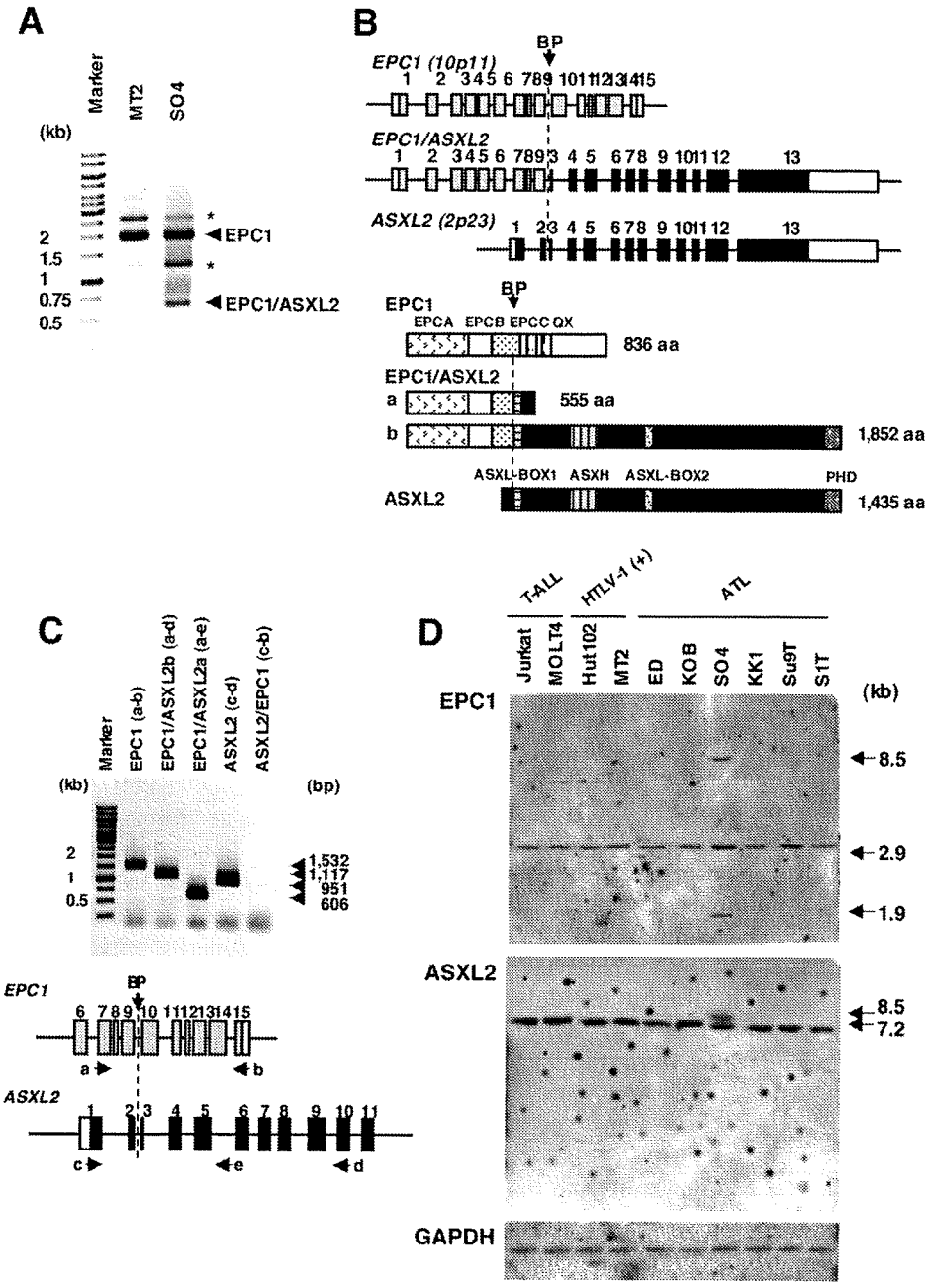


Figure 2. Identification of *EPC1/ASXL2*-fusion transcripts in the SO4 cell line. (A) 3′-RACE analysis of *EPC1* transcripts in MT2 and SO4 cell lines. The first round of PCR was performed by the primer in exon 7 (Supporting Information Fig. 1), and the second round of PCR was performed with the nested primers (Supplementary Materials and Methods). All of the cDNA bands amplified were subcloned and sequenced. An asterisk denotes nonspecific bands. (B) Schematic representation of the genomic structure and the protein domain structure of *EPC1/ASXL2*. (Upper panel) Genomic structure of *EPC1*, *ASXL2*, and *EPC1/ASXL2*-fusion. Coding exons are represented by gray in *EPC1* and black in *ASXL2*. A vertical arrow represents the chromosomal breakpoint (BP). (Lower panel) Schematic representation of the domain structure of EPC1/ASXL2-fusion proteins. Conserved EPCA, EPCB, EPCC domains, the glutamine-rich (QX) region in EPC1 proteins, conserved ASXL-BOX regions, ASX homology domain (ASXH), and COOH-terminal plant homeodomain (PHD) in ASXL proteins are indicated. (C) Detection of various types of mRNA by RT-PCR in the SO4 cell line. A fragment of *ASXL2/EPC1* cDNA was amplified by primers (c) of *ASXL2* exon 1 and (b) of *EPC1* exon 15, a fragment of *EPC1* was amplified by primers (a) and (b) of *EPC1*, a fragment of *ASXL2* was amplified by primers (c) and (d) of *ASXL2*, a fragment of *EPC1/ASXL2a* was amplified by primers (a) of *EPC1* and (e) of *ASXL2*, and a fragment of *EPC1/ASXL2b* was amplified by primers (a) of *EPC1* and (d) of *ASXL2*. SO4 cDNA was used as a template for PCR. Arrows under the gene structure indicate the position and the direction of the primers used. A vertical arrow represents the chromosomal breakpoint of t(2;10) (BP). Note that primer (e) is derived from intron 5. (D) Northern hybridization analysis for detecting *EPC1* or *ASXL2* mRNA in various types of T-cell leukemia cell lines. Jurkat and MOLT4 are T-lymphoid leukemia cell lines (T-ALL), Hut102 and MT2 are HTLV-1-infected cell lines (HTLV-1 (+)), and ED, KOB, SO4, KK1, Su9T, and S1T are ATLL-derived cell lines (ATL).

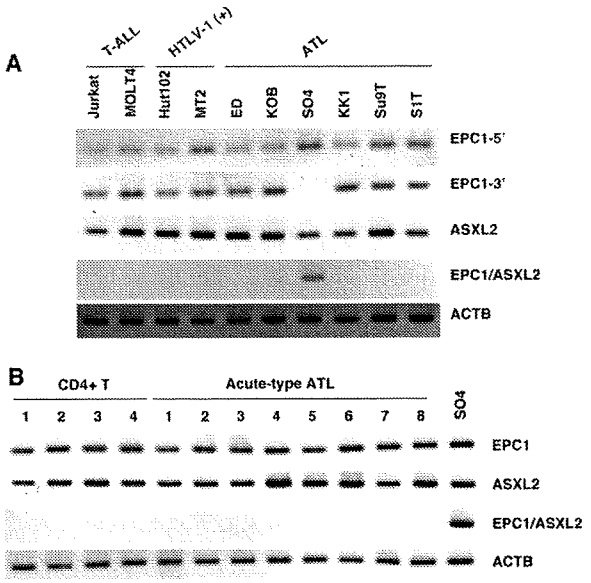


Figure 3. Semiquantitative RT-PCR of the *EPC1*, *ASXL2*, and *EPC1/ASXL2* genes. (A) Expression of *EPC1*, *ASXL2*, and *EPC1/ASXL2* in 2 HTLV-I-uninfected T-ALL cell lines and 2 HTLV-I-infected T cell lines or 6 ATLL cell lines was determined by semiquantitative RT-PCR. The expression of β-actin (*ACTB*) is shown at the bottom as a control. (B) Expression of *EPC1*, *ASXL2*, and *EPC1/ASXL2* in 8 primary ATLL cells and 4 $CD4^+$ T cells analyzed under the same conditions used for the cell lines (A). A SO4 cell line was used as a control.

and MOLT4), two HTLV-1-infected T cell lines (MT2 and Hut102), and six ATLL-derived cell lines (ED, KOB, KK1, SO4, S1T, and Su9T). A 2.9 kb band of the *EPC1* transcript was detected in all cell lines, and two additional bands of 8.5 and 1.9 kb were detected in the SO4 cell line using an *EPC1* cDNA probe. Additionally, a 7.2 kb band of *ASXL2* transcript was detected in all cell lines, and an additional 8.5 kb band was detected in the SO4 cell line using an *ASXL2* probe. Therefore, an 8.5 kb band of fusion mRNA was derived from *EPC1* fused with the longer form of *ASXL2* mRNA (EPC1/ASXL2b in Fig. 2B, lower panel), and a 1.9 kb band of fusion mRNA was suggested to be derived from *EPC1* fused with the shorter form of *ASXL2* mRNA (EPC1/ASXL2a in Fig. 2B). The *EPC1/ASXL2a* transcripts were not detected using the *ASXL2* cDNA probe, suggesting that the part of *ASXL2* in the *EPC1/ASXL2a* fusion mRNA is too short to detect the transcript.

### Expression of *EPC1/ASXL2*-Fusion Products

To determine the expression of *EPC1*, *ASXL2*, and *EPC1/ASXL2*, semiquantitative RT-PCR was performed using mRNA from 10 T-cell leukemia cell lines and eight patient samples with acute-type ATLL (Fig. 3). A 147 bp band of an *EPC1* cDNA fragment covering exons 1 and 2 (EPC1-5′) was comparably amplified in the mRNA from all leukemia cell lines (Fig. 3A). On the other hand, a 135 bp fragment of *EPC1* cDNA covering exons 14 and 15 (EPC1-3′) was not amplified in the SO4 cell line, although the same fragment was comparably amplified in all other cell lines. A 180 bp cDNA fragment covering exons 1 through 4 of *ASXL2* was also comparably amplified in all leukemia cell lines. A 355 bp band of *EPC1/ASXL2* fusion cDNA, which was amplified from both *EPC1/ASXL2a* and *EPC1/ASXL2b*, was only amplified in the SO4 cell line. Using the same primer sets, expression levels of these genes in eight leukemia cells from ATLL patients and 4 $CD4^+$ T lymphocytes from healthy volunteers were determined (Fig. 3B). The expression levels of *EPC1* and *ASXL2* cDNA in all leukemia cell samples were comparable with those in all $CD4^+$ T lymphocytes; however, *EPC1/ASXL2* fusion cDNA was not amplified in any patient samples.

### Identification of a Genetic Alteration within the *EPC1* Gene in the Case of ATL071

In the case of ATL071, inverse-genomic PCR analysis was performed to analyze the chromosomal breakpoint in the *EPC1* gene, since only genomic DNA from ATL071 remained. *Bam*HI-digested genomic DNA (fragments A to C in Fig. 4A) was subjected to inverse-PCR amplification using three sets of primers (horizontal arrows in Fig. 4A), which covered a part of intron 1 through a part of intron 9. A 4.9 kb germline band derived from fragment A and a 5.8 kb area from fragment C were amplified as a single band, but an additional 0.9 kb band was amplified with a 3.3 kb germline band from fragment B (Fig. 4B). As confirmed by DNA sequencing analysis, the 847 bp band (rearranged) contained only the 5′ part of the fragment B sequence from intron 2 of *EPC1* (Supporting Information Fig. 3). We also performed inverse PCR experiments with the same primer pairs using *Nde*I- or *Bgl*II-digested genomic DNA from ATL071 and obtained single bands derived from each germline allele (data not shown). To confirm this result, Southern blot analysis of *Bam*HI-digested genomic DNA from the leukemia cells from ATL071 and lymphocytes from a healthy volunteer was performed using a genomic probe corresponding to the 0.6 kb 5′ portion of fragment B depicted in Figure 4A. The blot showed that a 3.5 kb germline band of fragment B hybridized to both samples from

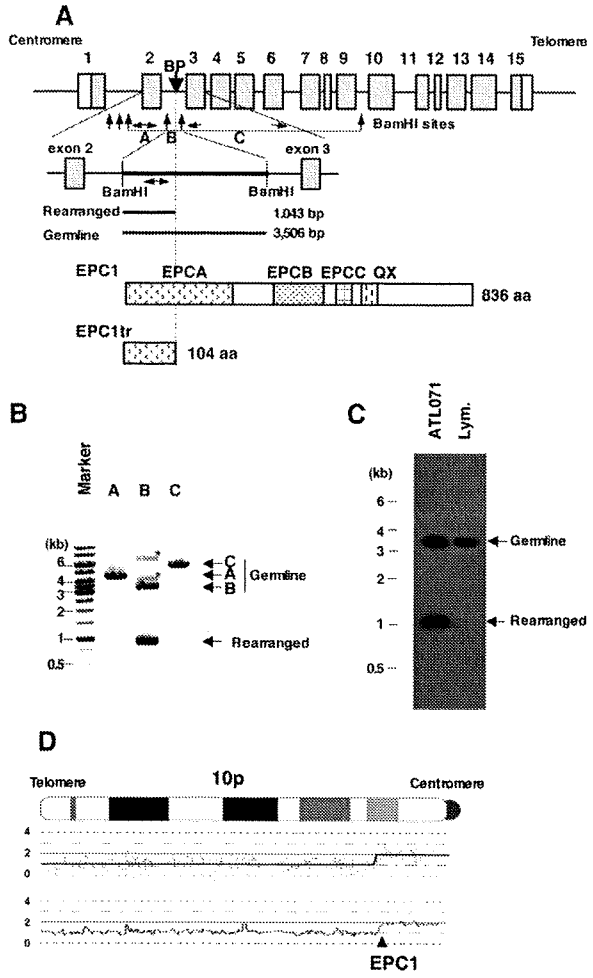


Figure 4. Identification of a chromosomal breakpoint with a genomic deletion in the *EPC1* locus of ATL071. (A) Schematic representation of the strategy for inverse PCR and genomic deletion of the *EPC1* locus. *EPC1* has 15 coding exons with a chromosomal breakpoint (BP) located in intron 2 (vertical arrow in the upper panel). Inverse vertical arrows represent *Bam*HI-restricted sites, and A to C fragments were used for inverse PCR. The second panel shows the precise mapping of fragment B. Horizontal arrows indicate the position of primers used for inverse PCR. A 3,506 bp germline fragment of B and a 1,043 bp rearranged fragment are indicated as narrow bars. The lower panel represents an entire EPC1 protein with 836 amino acid residues and a putative truncated EPC1 protein (EPC1tr), which is derived from coding exons 1 and 2 with 104 amino acid residues. (B) Detection of rearranged bands in *Bam*HI-digested fragment B in intron 2 of *EPC1* by inverse PCR. Major 3.5 kb and 0.9 kb bands were amplified by inverse PCR in fragment B, which corresponded to germline and rearranged fragments, respectively, whereas a single germline band was only amplified in fragment A or C regions. An asterisk denotes non-specific bands. (C) Southern blot analysis of the *Bam*HI-digested DNA from the patient (ATL071) and peripheral lymphocytes from healthy volunteers (Lym.) using a probe corresponding to 0.6 kb of the 5′ upstream sequence of fragment B. The 3.5 kb and 1 kb bands represent germline and rearranged fragments, respectively. (D) Array-CGH image of a DNA sample from ATL071. The DNA copy number indicated by digits was changed from 2 to 1 within the *EPC1* gene.

lymphocytes and ATL071, and an additional 1 kb band, possibly corresponding to the deleted *Bam*HI fragment of ATL071, also hybridized (Fig. 4C). This suggests that the chromosomal breakage was located within intron 2 and that the rest of the rearranged allele was deleted starting from the telomeric region of the breakpoint at 10p11.2 (Fig. 4D). Unfortunately, we could not obtain further genomic information beyond the chromosomal breakpoint in ATL071, since only a few micrograms of genomic DNA, and no mRNA or cell pellets, was available. Based on our results, we hypothesized that the putative truncated EPC1 protein containing a portion of the EPCA domains (104 amino acid residues) may be expressed in leukemia cells. We examined the function of the truncated EPC1 in the subsequent experiments (EPC1tr in Fig. 4A).

### Enhancement of Cell Growth by Expression of the *EPC1/ASXL2*-Fusion Gene or the Truncated *EPC1* Gene

To determine whether expression of the *EPC1/ASXL2* fusion gene or the truncated *EPC1* gene is pathogenically important in ATLL, *EPC1*, *ASXL2*, *EPC1/ASXL2a*, *EPC1/ASXL2b*, and the truncated *EPC1* gene (*EPC1tr*) were introduced into various leukemia cell lines (Fig. 5). Expression vectors containing FLAG-tagged cDNA for each variant were transiently transfected into the murine IL-2-dependent T-cell line CTLL2 using the Amaxa Nucleofector system, and cell proliferation of transfected cells was determined by cell counting with trypan blue staining under normal growth conditions. Expression of each cDNA in the transfected cells was confirmed by Western blot analysis using an anti-FLAG-tag antibody (Fig. 5A, right). EPC1- and ASXL2-transfected cells had the same growth rate as mock vector-transfected cells (Mock) and parental cells (Parental); however, EPC1/ASXL2a- and EPC1/ASXL2b-transfected cells grew significantly faster compared with the control cells (Fig. 5A, left). We next examined whether shRNA-mediated knock down of the *EPC1-ASXL2* fusion gene impacts growth of the SO4 cell line. We successfully knocked down the expression of *EPC1-ASXL2* by transient transfection of the EPC1-ASXL2 shRNA vector using Amaxa Nucleofector (Fig. 5B, right). The EPC1-ASXL2 shRNA-transfected cells exhibited a statistically significant decrease in the rate of cell growth compared to control parental cells or Luciferase shRNA-transfected cells (Fig. 5B, left). These results indicate that EPC1-ASXL2 expression confers a growth advantage to the SO4 cell line. Moreover, the growth rate of EPC1tr-transfected CTLL2 cells

was significantly faster than that of control cells, even although the growth rate of EPC1tr-transfected CTLL2 cells was slower than that of EPC1/ASXL2-transfected cells (Fig. 5C, left). As similar results were obtained by ectopic expression in HTLV-1-negative (Jurkat) and -positive (MT2) T-cell lines, overexpression of EPC1tr in T-leukemia cells appears to accelerate cell growth (Supporting Information Fig. 4). As CTLL2 is an IL-2-dependent cell line, we examined whether the expression of the fusion protein interferes with the IL-2 response. Cell survival assays showed that the growth rate of EPC1/ASXL2-transfected cells was not statistically different than that of control cells or EPC1-transfected cells at any concentration of IL-2 (Supporting Information Fig. 5).

As the PRC2 complex, which includes EPC1, is reported to possess both activating and repressive activities as a transcriptional regulator (Shimono et al., 2000; Tezel et al., 2002), transcriptional activities of the *EPC1/ASXL2* fusion gene or the truncated *EPC1* gene products were examined by reporter assays using GAL4-fusion proteins in the SO4 cell line (Fig. 5D). Expression constructs in which EPC1, ASXL2, EPC1/ASXL2a, EPC1/ASXL2b, or EPC1tr was fused with the GAL4-binding domain were cotransfected with a luciferase reporter vector containing GAL4-binding sites before the SV40 promoter (pG5proLuc; Nishikata et al., 2003). GAL4-fusion EPC1 and ASXL2 did not activate transcription of the luciferase gene; however, EPC1/ASXL2a and EPC1/ASXL2b enhanced expression of luciferase by around 10-fold, suggesting that the EPC1/ASXL2 fusion protein functions as a transcriptional enhancer. Interestingly, expression of the truncated EPC1 protein did not affect the transcription of the luciferase gene. Since ectopic expression of the truncated EPC1 protein enhanced cell growth, the truncated EPC1

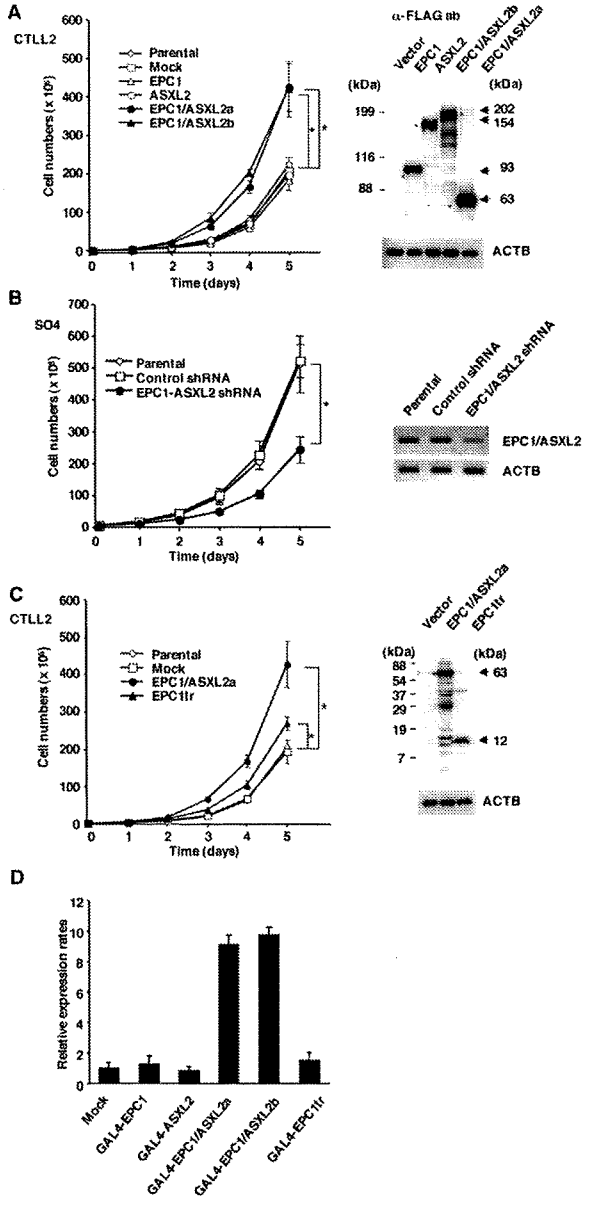


Figure 5. Effects on cell proliferation and/or transcriptional regulation caused by ectopic expression of the *EPC1/ASXL2* fusion gene or truncated *EPC1* in T-leukemia cell lines. (A) The left panel shows growth curves of CTLL2 cell lines transfected with each indicated plasmid (Mock, EPC1, ASXL2, EPC1/ASXLa, and EPC1/ASXL2b). Stars indicate statistically significant differences between EPC1/ASXL2a (or EPC1/ASXL2b)-transfected cells and control cells (Parental and Mock). A Student's *t* test ($P < 0.05$) was used for the statistical analysis. The right panel shows the protein expression of each transfected plasmid by Western blot analysis using anti-FLAG antibody. The expression level of β-actin (ACTB) is shown at the bottom as a control. (B) Left panels show a growth curve of the SO4 cell line transfected with either the control luciferase-specific shRNA vector or the EPC1/ASXL2-specific shRNA vector or untransfected parental cells. Stars indicate statistically significant differences between EPC1/ASXL2 shRNA-transfected cells and control cells (Parental and control shRNA-transfected cells). A Student's *t* test ($P < 0.05$) was used for the statistical analysis. Right panels show a semiquantitative RT-PCR analysis with mRNA isolated from SO4 cells untransfected (Parental) or transfected with either control luciferase or EPC1/ASXL2 shRNA vector. (C) Left panels show a growth curve of the CTLL2 cell line transfected with either mock, EPC1/ASXL2a or EPC1tr expression plasmids or untransfected parental cells. Stars indicate statistically significant differences between EPC1tr (or EPC1/ASXL2a)-transfected cells and control cells (Parental and Mock). A Student's *t* test ($P < 0.05$) was used for the statistical analysis. Right panels show protein expression of each transfected plasmid by Western blot analysis using the anti-FLAG antibody. (D) Transcriptional regulation by various types of GAL4-fusion *EPC1* and *ASXL2* genes. The luciferase reporter vector (pG5proLuc) containing GAL4-binding sites before an SV40 minimal promoter was transfected into the SO4 cell line with each GAL4-fusion expression plasmid (EPC1, ASXL2, EPC1/ASXL2a, EPC1/ASXL2b, EPC1tr). Data (mean ± SD) are from triplicate transfections expressed as relative luciferase activity normalized against cells transfected with pG5proLuc and GAL4-binding domain alone (Mock) whose value was taken as 1.

appears to have an unknown function unrelated to transcriptional enhancement in leukemia cells.

## DISCUSSION

In this article, the chromosomal breakpoints in an ATLL-derived cell line (SO4) and an acute-type ATLL patient were determined by genomic analysis to be located within the *EPC1* gene locus. In the SO4 cell line, *EPC1* was fused with the *ASXL2* gene at 2p23.3, leading to the generation of an *EPC1/ASXL2* fusion gene. In patient ATL071, a truncated *EPC1* gene product was generated by unbalanced translocation with a deletion of 10p. Although there is a discrepancy between the *EPC1/ASXL2* fusion gene and the *EPC1* truncated gene in their ability to regulate transcription, ectopic expression of these two genes enhanced cellular proliferation, suggesting that genetic alteration of the *EPC1* gene by chromosomal translocation has an important role in ATLL leukemogenesis.

Altered expression of polycomb group genes has been implicated in various hematological and epithelial cancers (Raaphorst, 2005). In endometrial stromal sarcoma, several chromosomal translocations involving gene fusions of the polycomb group (*JAZF1/SUZ12*, *JAZF1/PHF1*, and *EPC1/PHF1*) have been identified (Koontz et al., 2001; Micci et al., 2006). Among them, the *PHF1* gene, encoding a polycomb-like protein at 6p21, was found fused with the *EPC1* gene as the result of a three-way translocation t(6;10;10) (Micci et al., 2006). The breakpoint of the *EPC1/PHF1* fusion gene is located in intron 10 of *EPC1*, after the EPCC domain. The EPC1/PHF1 fusion has an open reading frame containing 581 amino acids from EPC1, six additional residues upstream of the initial EPC1 methionine, and the entire PHF1 sequence, consisting of 567 amino acids (Micci et al., 2006). PHF1 was previously shown to interact and modulate the activity of polycomb group protein EZH2, a histone methyltransferase whose activity is pivotal in gene repression (Sarma et al., 2008). The function of *EPC1/PHF1* in cancer progression is still unclear, but we speculate that the chimeric protein may modulate the associations of polycomb group complexes. Intriguingly, a region of 2p23.3 is known to be a recombination hot spot or fragile site associated with carcinogenesis (Mathew et al., 2000; Ramocki et al., 2003; Raaphorst, 2005). *ASXL2*, which is within this region, is a candidate gene for carcinogenesis and is reported to fuse to *MOZ* (monocytic leukemia zinc finger protein), a member of the MYST family of protein acetyltransferases (Hosoda et al., 2002 [UniProt, Entry: Q92794]). The ASXL2 protein also belongs to a polycomb gene family and contains conserved regions of unknown function found in the ASX family, known as ASXH, as well as a conserved COOH-terminal PHD domain (Fisher et al., 2006). Ectopic expression of the *EPC1/ASXL2* fusion gene in T-cell leukemia cells enhanced cell proliferation to a greater extent than did ectopic expression of the truncated *EPC1* gene, suggesting that the part of *ASXL2* in the *EPC1/ASXL2* fusion gene has an important role in cell proliferation. We have performed semiquantitative RT-PCR analysis on several cell cycle-related genes with mRNA isolated from CTLL2 cells transfected with an EPC1/ASXLa expression vector. We did not detect significant differences in expression of the cyclin B1, MYB, MYC, P15, or P21 genes between EPC1/ASXL2a-transfected cells and control cells (data not shown). Therefore, at present, it is not clear how the EPC1/ASXL2 fusion protein enhances cellular growth. EPC1 has four conserved domains termed EPcA, EPcB, EPcC, and a glutamine-rich (Qx) region (Shimono et al., 2000). When the EPcB domain or the COOH-terminal EPcC and Qx regions are targeted to a promoter by GAL4, they are able to repress transcription of reporter genes (Shimono et al., 2000; Tezel et al., 2002). In contrast, the EpcA region has been reported to contain transcription-activating ability, which is unique to EPC1 among polycomb group proteins (Shimono et al., 2000; Tezel et al., 2002). It is speculated that the truncated *EPC1* gene products detected in ATLL cells do not contain transcriptional repression activity. Therefore, these genetic alterations in the *EPC1* gene might be partially responsible for the leukemogenic process in ATLL.

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CASE REPORT

# Acute megakaryoblastic leukemia in a child with the *MLL-AF4* fusion gene

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**Abstract**

Mixed-lineage leukemia (*MLL*) rearrangements are commonly observed in childhood acute lymphoblastic and myeloid leukemia, as well as therapy-related leukemia. However, the occurrence of *MLL* rearrangements in acute megakaryoblastic leukemia (AMKL) is very rare. We report a pediatric case of AMKL with the *MLL-AF4* fusion transcript. *MLL-AF4* is derived from t(4;11)(q21:q23) and occurs exclusively in B-cell lineage leukemia. To our knowledge, *MLL-AF4* as well as t(4;11)(q21:q23) has not been reported in adult and childhood AMKL. Thus, our case provides new insight into the molecular mechanisms of *MLL-AF4*-associated leukemia.

**Key words** acute megakaryoblastic leukemia; 11q23 rearrangement; *MLL-AF4*

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Acute megakaryoblastic leukemia (AMKL) is a heterogenous subgroup of acute myeloid leukemia (AML) and recognized as AML M7 according to the French-American-British (FAB) cooperative group classification system (1). Previous studies on clinicopathological analyzes of AML suggest that AMKL is relatively rare, approximately 5–10% of all AML (2). Childhood AMKL is the most common form of Down syndrome-related leukemia, and its prognosis is excellent in this group of patients (2). AMKL without Down syndrome appears to be more heterogenous, and its prognostic factors have not been well defined (3). The t(1;22)(p13;q13) translocation forming the chimeric fusion transcript, *OTT-MAL*, is the most common chromosomal abnormality in infants with AMKL who do not have Down syndrome (4, 5). Infants with AMKL and this translocation usually have abdominal masses, myelofibrosis, and a relatively poor prognosis (6). However, other molecular genetic mechanisms in children with AMKL without Down syndrome are still elusive.

Chromosomal rearrangement of 11q23 involving the mixed-lineage leukemia (*MLL*) gene is commonly found in childhood lymphoid, myeloid, and *MLL*s (7). Recent cytogenetic and molecular studies have shown that *MLL* has more than 50 different partner genes, including *AF4* at 4q21, *AF9* at 9p21, *AF10* at 10p21, and *ENL/ELL* at 19p13 (7). The most frequent 11q23 abnormality in AML is t(9;11)(p22;q23); other common abnormalities include t(11;19)(q23;p13.3) and t(11;19)(q23;q13.1) (8). More than 15 additional aberrations on 11q23 with various partners, including t(6;11), t(10;11), and t(11;17), have been reported in AML (8, 9). These 11q23 aberrations mainly occur in AML M4 and M5 and are rare in AMKL (8, 10, 11).

Of the various partners, *AF4* at 4q21 is the most common partner for *MLL*, and the *MLL-AF4* fusion transcript has been detected almost exclusively in B-cell lineage leukemia (12). Involvement of the *MLL-AF4* fusion transcript in *de novo* myeloid-lineage leukemia is known to be very rare (3). We describe the first case

of a child with AMKL with the *MLL-AF4* fusion gene.

## Case report

A 3-year-old girl presented with fever and epistaxis and admitted to our hospital in January 2004. Physical examination showed anemia, hepatosplenomegaly, and petechiae on the trunk and extremities. Cervical, axillary, and inguinal lymph nodes were negligible. A peripheral blood cell count showed a white blood cell count of 6100/$\mu$L, with 34% mature granulocytes, 9% monocytes, 1% eosinophils, 38% lymphocytes, and 18% blasts. The hemoglobin concentration was 9.7 g/dL, and the platelet count was 14 000/$\mu$L. Bone marrow aspiration showed myeloid hyperplasia with 6% myeloid cells, 4.5% erythroid cells, 22% lymphoid cells, 0.5% monocyte cells, and 76% blasts (Fig. 1A). Blast cells were negative for peroxidase staining, and Auer rods were not found in the blasts. Approximately 40% of the blasts had cytoplasmic blebs (Fig. 1A). Immunophenotyping showed that blasts expressed the CD7, CD13, CD33, CD34, and CD41a antigens, and a diagnosis of AMKL (AML M7) was made. Chemotherapy following the Japanese Childhood AML Cooperative Study Group Protocol, AML99, for intermediate-risk AML (cytarabine, idarubicin, etoposide, and mitoxantrone) induced complete clinical and cytogenetic remission. Thereafter, the patient underwent six courses of intensification chemotherapy; however, hematological relapse occurred 2 months later. Although chemotherapy according to the AML99 protocol and the FLAG (fludarabin, cytarabine, and G-CSF) regimen was provided, the disease progressed, and the patient died 12 months after diagnosis. Informed consent for the genetic analyzes of leukemic cells from this patient was obtained from the parents.

## Cytogenetic studies

Chromosomal analyzes of leukemic blasts were performed at initial diagnosis, remission, and relapse using standard G-banding methods (13). Bone marrow blasts

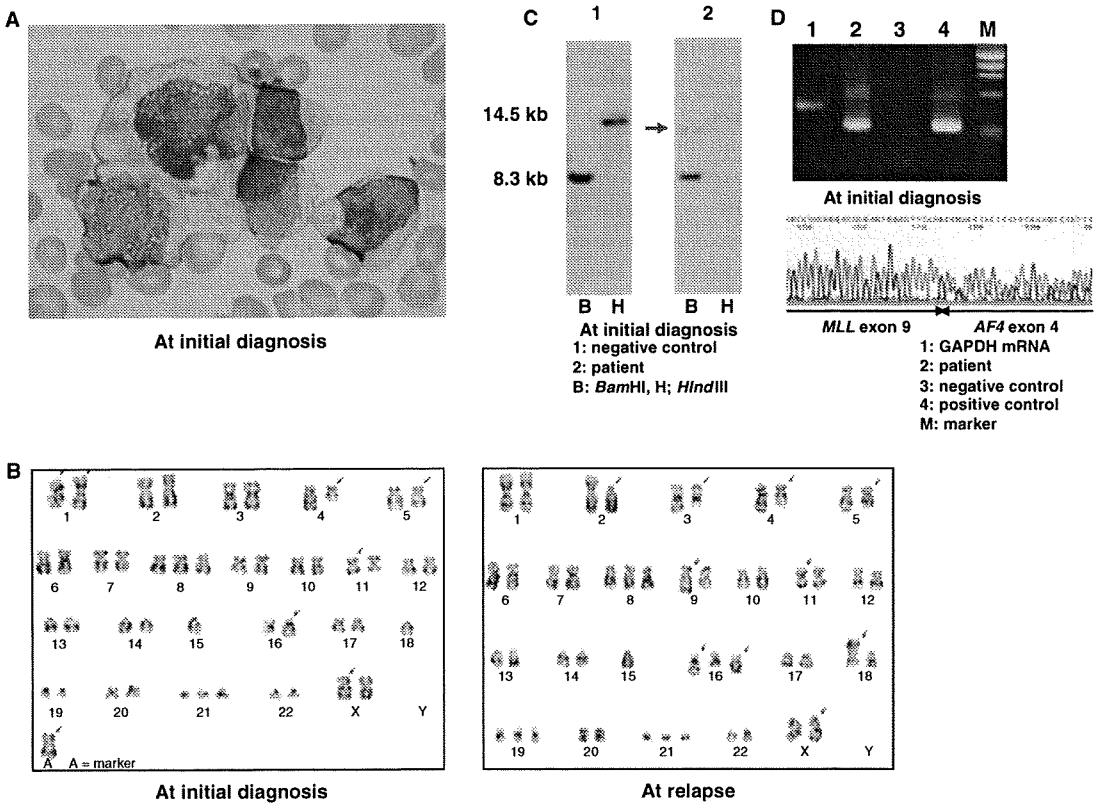


**Figure 1** Morphological and cytogenetic analyzes of leukemic cells from the patient. (A) May-Giemsa staining of bone marrow cells at initial diagnosis. The blasts had cytoplasmic blebs. (B) Karyotypic findings in the patient. Complex chromosomal aberrations, including add(X)(q26), add(4)(q21), del(5)(q23q32), +8, del(11)(q23), −15, add(16)(q22), −18, and +21, were detected in bone marrow aspirates at the time of initial diagnosis (left) and at relapse (right). (C) Southern blot analysis of the blasts at the time of initial diagnosis with an *MLL* cDNA probe, which showed the rearrangement of *MLL* with *Bam*HI digestion (arrowhead). (D) RT-PCR analysis of the *MLL-AF4* fusion transcript in the blast at the time of initial diagnosis. A direct sequencing of the clear band identified the fusion of exon 9 of the *MLL* and exon 4 of the *AF4*. A direct sequencing oh the faint band identified the fusion of exon 9 of the MLL and exon 4, which awas cosidered to be generated by alternative splicing.

at initial diagnosis and relapse showed complex chromosomal aberrations as follow; 47~48, X, add(X)(q26), add(1)(q32), add(1), add(4)(q21), del(5)(q23q32), +8, del(11)(q23), −15, add(16)(q22), −18, +19, +21, +mar, inc, (in eight cells of 20 investigated, at initial diagnosis) and 49~50, X, add(X)(q26), del(2)(q11), t(3;9)(q21;q34), add(4)(q21), del(5)(q23q32), +8, del(11)(q23), −15, add(16)(q22), t(1;16)(q25;q22), add(17)(p11), add(18)(p11), −18, +19, +21 (in all 20 cells investigated, at relapse) (Fig. 1B).

These complex chromosomal aberrations commonly including add(X)(q26), add(4)(q21), del(5)(q23q32), +8, −15, del(11)(q23), +18, and +21 in 24 of 40 cells investigated (Fig.1B). Although trisomy 21 was commonly observed in the blast cells, chromosomal analysis of the bone marrow aspiration at remission exhibited a normal karyotype, 46XX, which indicated that the patient did not have Down syndrome.

## Genetic analyzes

With an overall frequency of 15%, abnormalities of 11q23 are among the most frequent chromosomal aberrations in childhood AML, and recent molecular investigations of well-known 11q23 translocation have shown consistent involvement of the *MLL* gene (7). Therefore, because the cytogenetic studies conducted at the time of diagnosis and relapse in the bone marrow aspirate of this patient detected the common cytogenetic aberration del(11)(q23), we further investigated the status of the *MLL* gene in leukemic cells of this patient. Southern blot analysis using an *MLL* cDNA probe showed rearrangement of the *MLL* gene with *Bam*HI digestion (Fig. 1C). To identify the partner gene fused to *MLL*, we focused on the common chromosomal aberration add(4)(q21), because the *AF4* gene at 4q21 is one of the most common partner genes of *MLL* (7). RT-PCR analysis using the sense primer located in exon 8 of *MLL* and the antisense primer located in exon 6 of *AF4* showed the *MLL-AF4* transcript (Fig. 1D). The *OTT-MAL* fusion gene derived from t(1;22)(p13;q13) is predominantly detected in non-Down cases with AMKL; however, this fusion transcript was not detected in our case. In addition, although a mutational analysis of the *FLT3* and *GATA1* genes was performed as described previously (14), no mutations were detected in leukemic cells of these patients.

## Discussion

Chromosomal translocations involving the *MLL* gene at chromosome 11q23 are often associated with the phenotype for acute leukemia (7). For instance, *MLL* rearrangements are commonly found in infant acute lymphoblastic leukemia (ALL) and childhood *de novo* AML and in most patients with therapy-related leukemia (7). AML patients with *MLL* rearrangement tend to be young and often have hyperleukocytosis and myelomonocytic (FAB M4) or monoblastic (FAB M5) disease (3). However, the occurrence of the *MLL* rearrangement in AMKL (FAB M7) is very rare and limited to children (3, 8). To the best of our knowledge, only 16 cases of AMKL with *MLL* and/or 11q23 involvement have been reported (Table 1) (8, 9, 15). Thirteen of these 16 cases had *MLL* rearrangements, as detected by molecular studies; however, *MLL-AF4* fusion, t(4;11)(q21;q23) abnormalities, and abnormalities at 4q21 were not shown (8, 9, 15). The *MLL-AF4* fusion is the most commonly detected gene in infant ALL and appears to represent approximately 5% of ALL in older children and adults (16). Moreover, using conventional knockin or conditional invertor approaches, Mll-Af4 is capable of inducing B-cell lymphoma in mice models (17). However, our case suggests that *MLL-AF4* fusion is not exclusively associated with lymphoid malignancies.

Although rearrangements of 11q23 confer a poor prognosis in childhood ALL, the prognostic significance of 11q23 abnormalities in childhood AML is equivocal (7). Seven of 16 previously reported AMKL cases with 11q23 rearrangement and current case died, and one case relapsed 2 months after bone marrow transplantation (Table 1).

Previously it has been reported that a complex karyotype with multiple chromosomal abnormalities at diagnosis in AMKL would be a poor prognostic indicator (2). Interestingly, 12 of 16 previously reported AMKL cases with 11q23 aberrations and our case had variable complex karyotypes (Table 1). Thus, patients with AMKL with 11q23 aberrations tend to have a poor prognosis may be due to the complex karyotypes. Furthermore, of the 17 cases with AMKL with 11q23 aberrations (including our case), 15 cases were not associated with Down syndrome, and the status of the remaining two was unknown (8, 9, 15). These clinical and cytogenetic data suggest that childhood AMKL with 11q23 abnormalities might be a specific subtype of AMKL with a complex karyotype and poor prognosis and is not associated with Down syndrome. Allogenic bone marrow transplantation could be the best treatment for this group patients, but the too few number of case prevent to compare.

Although cytogenetic studies of leukemic cells of the present case showed common del(11)(q23) and add(4)(q21) abnormalities, the karyotypes shown in this case does not suggest it as a typical t(4;11) translocation. Because translocations of 11q23 are often not detected by standard G-banding methods, the frequency of *MLL* involvement in AMKL is still probably underestimated. Therefore, a combination of cytogenetic and molecular

**Table 1** Acute megakaryoblastic leukemia with 11q23 and/or the *MLL* rearrangement

| Case no. | Age (years) | Karyotype | *MLL* status | Outcome | Reference |
|---|---|---|---|---|---|
| 1 | 2 | 49,XX+6,t(10;11)(p13;q23),+21,+22 | nd | Dead | (16) |
| 2[1] | nd | 50,XX,+6,+8,t(9;11)(p21;q23),+20,+21 | nd | nd | (16) |
| 3 | nd | 46,XX,t(9;11)(p21;q23) | nd | Alive[2] | (16) |
| 4 | 12 | 51,XX,+6,+15,+17,+20,+21 | *MLL-AF10* | Dead | (16) |
| 5 | 5 | 52,XX,+X,+3,t(9;11)(p22;q23),+12,+15,+19,+21 | R | Alive[2] | (16) |
| 6 | 1 | 54,XX,del(3)(q13q21),+6,+7,+8,t(11;17)(q23;q23), +14,+19,+19,+21,+21 | R | Alive | (16) |
| 7 | 15 | 46,XX,t(9;11)(p22;q23) [6]/47,idem,+6[7]/92, idemx2[2]/94,idemx2,+6,+6[5] | R | nd | (16) |
| 8 | 1 | t(5;9;11)(q33;p22;q23) | R | Dead | (9) |
| 9 | 1.9 | 50,XX,+der(6)t(6;10;11)(q10;p10-12; q22-23), der(7)t(7;11)(p15;q23),der(10) ins(10;11)(p13;q22-23),der(11)t(7;11) ins(10;11),add(16)(q24),+19,+21,+22 | R | Dead | (9) |
| 10 | 2 | 51,X,t(X;11)(q22;q23),+6,+8,+19,+21,+21 | R | Dead | (9) |
| 11 | 0.7 | 48,Y,+X,t(X;19)(q26;q13),der(10)del(10)(p13)t (10;20;16)(q23;q11;p13),ins(15;5)(q11;q11q13), der(16)t(10;20;16),+19,der(20)t(10;20;16) | R | Dead | (9) |
| 12 | 4.9 | 46,XX,t(7;11)(q22;p15) | R | Dead | (9) |
| 13 | 15 | 92,XXXX,t(9;11)(p22;q23)[2]/94,XXXX, +6,+6,t(9;11)[5]/47,XX,+6,t(9;11)[7] | R | Alive[2] | (16) |
| 14 | 2 | 47,XY,der(4)(?::4p15 → 4qter),der(6) (6pter → 6q22::11q? → 11q?::4p15 → 4pter), der(10)(6qter → 6q26::15q21 → 15q24::11q2? → 11q23::10p12 → 10qter),del(11)(q14), der(15) (15pter → 15q21::6q22 → 6q26::10p12 → 10pl?::18q11.2 → 18qter),der(18) (18pter → 18q11.2::15q24 → 15qter),+21 | *MLL-AF10* | Relapse at 2 m from BMT | |
| 15 | nd | nd | *MLL-AF10* | Alive | (15) |
| 16 | nd | nd | *MLL-ENL* | Alive | (15) |
| 17 | 3.6 | 47~48,X,add(X)(q26),add(1)(q32),add(1),add(4)(q21), del(5)(q23q32),+8,del(11)(q23),−15,add(16)(q22), −18,+19, +21,+mar,inc, (at diagnosis) 49~50,X,add(X)(q26),del(2)(q11),t(3;9)(q21;q34), add(4)(q21),del(5)(q23q32),+8,del(11)(q23),−15, add(16)(q22),t(1;16)(q25;q22),add(17)(p11), add(18)(p11), −18,+19, +21 (at relapse) | *MLL-AF4* *MLL-AF4* | Dead | Present case |

R, rearranged; nd, not determined; m, months; BMT, bone marrow transplantation; AMKL, acute megakaryoblastic leukemia.
[1]Therapy-related AMKL.
[2]Short follow-up.

studies, such as Southern blot and RT-PCR analyzes, is better at identifying *MLL* involvement.

The *OTT-MAL* fusion transcript is closely associated with non-Down AMKL in infants, and the *GATA1* mutations are frequently detected in AMKL with Down syndrome (18). However, the *OTT-MAL* and *GATA1* mutations were not detected in our patient, which suggested that her disease was an independent subgroup of childhood AMKLs. Furthermore, *FLT3* is one of the most commonly mutated genes in AML, and AML patients with the *FLT3* mutation usually have a poor prognosis (19). In our patient, no *FLT3* mutation was detected, indicating that a pathway other than *FLT3* signaling would have existed in her aggressive disease. Because the prognostic factors for non-Down AML in children are still unclear, further data accumulation is necessary.

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CANCER GENETICS AND CYTOGENETICS

Short communication

# *NUP98–NSD3* fusion gene in radiation-associated myelodysplastic syndrome with t(8;11)(p11;p15) and expression pattern of NSD family genes

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**Abstract** Chromosomal 11p15 abnormality of therapy-related myelodysplastic syndrome (t-MDS)–acute myeloid leukemia (AML) is rare. *NUP98–NSD3* fusion transcripts have been detected previously in one patient with AML and one patient with t-MDS having t(8;11)(p11;p15). Here we present the case of a 60-year-old man with radiation-associated MDS (r-MDS) carrying chromosome abnormalities, including t(8;11)(p11;p15) and del(1)(p22p32). Fluorescence in situ hybridization analysis demonstrated that the *NUP98* gene at 11p15 was split by the translocation. Southern blot analysis of bone marrow cells showed both rearrangements of *NUP98* and *NSD3* genes. Reverse transcriptase–polymerase chain reaction (RT-PCR) followed by sequence analysis revealed the presence of both *NUP98–NSD3* and *NSD3–NUP98* fusion transcripts. Expression analysis by RT-PCR showed that *NSD3* as well as *NSD1* and *NSD2* was ubiquitously expressed in leukemic cell lines and Epstein–Barr virus transformed B lymphocyte cell lines derived from the normal adult lymphocytes examined. Two isoforms of *NSD3*, *NSD3S* and *NSD3L* (but not *NSD3L2*), were expressed in leukemic cell lines and were fused to *NUP98* in our patient, suggesting that qualitative change of these two isoforms of *NSD3* by fusion with *NUP98* might be related to leukemogenesis, although the function of each isoform of the *NSD3* gene remains unclear. 

## 1. Introduction

Myeloid malignancies with 11p15 translocations are likely to be related to the nucleoporin gene, *NUP98* [1]. These translocations produced fusion genes between *NUP98* and many different partner genes [1]. Four patients with t(8;11)(p11;p15) have been reported previously [2–5], and the four diagnosed with acute myeloid leukemia (AML) or therapy-related myelodysplastic syndrome (t-MDS). The *NUP98–NSD3* fusion gene was identified in only two of these four patients with t(8;11) [4,5].

Therapy-related myelodysplastic syndrome (t-MDS) is considered to be a heterogeneous disorder of pluripotent hematopoietic stem cells that have various findings of bone marrow (BM) failure, often evolve to AML, and have a poor prognosis [6,7]. Although the pathogenesis of t-MDS is unknown, many recurrent chromosomal abnormalities are involved in t-MDS [8,9]. Only 17 patients were identified with 11p15 chromosomal abnormality among 511 patients with t-MDS–AML [10]. In the survey of Japanese childhood t-MDS–AML, 5 of 81 children had 11p15 translocations involving *NUP98* rearrangements [11].

Here we describe the case of a 60-year-old patient with radiation-associated MDS (r-MDS) patient exhibiting translocation t(8;11) and a *NUP98–NSD3* fusion transcript. We also report the expression of NSD family genes *NSD1*, *NSD2*, and *NSD3* in several leukemia and normal Epstein-Barr virus transformed B lymphocyte (EBV-B) cell lines from healthy volunteers.

We note that in the international human gene nomenclature (http://www.genenames.org), *NSD1* is an approved gene symbol, but *NSD2* and *NSD3* are classified as aliases,

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for *WHSC1* and WHSC1L1, respectively. In the present report, however, for convenience of discussion we continue to use the *NSD* nomenclature for all three genes.

## 2. Case report

A 60-year-old man was admitted for assessment of anemia. He had been an atomic-bomb survivor in Nagasaki 44 years before. When he was 59 years old, he was diagnosed with sigmoid colon cancer and underwent operative resection. His father died of lung cancer. On examination, blood examination showed a white blood cell count of 6,250/μL with no leukemic blasts, a hemoglobin level of 11.1 g/dL, and a platelet count of 337,500/μL. The BM examination revealed a nuclear cell count of 127,500/μL with no leukemic blasts. He had megakaryocytes with multiseparated nuclei and mature neutrophils with pseudo-Pelger–Hüet anomaly. Conventional chromosomal analysis demonstrated 46,XY,t(8;11)(p11;p15),del(1)(p22p32) in all 20 BM cells examined. He was diagnosed with refractory anemia (RA), but was not treated; he developed AML, 1 year after the diagnosis of RA. Cytogenetic findings in the AML were the same as in the RA. He died of progressive disease 23 months after diagnosis of RA, despite low-dose cytarabine.

### 2.1. Fluorescence in situ hybridization analysis

The fluorescence in situ hybridization (FISH) analysis of the patient's leukemic cells using bacterial artificial chromosome (BAC) clone PK505 was performed as described previously [12]. We mapped this BAC clone to leukemic cells together with a whole-chromosome painting probe for chromosome 11 (WCP11) (Coatasome 11, digoxigenin-labeled; Oncor, Gaithersburg, MD).

### 2.2. Southern blot analysis

After obtaining informed consent from the patient, high molecular weight DNA was extracted from BM cells by proteinase K digestion and phenol–chloroform extraction [13]. Ten micrograms of DNA were digested with *Eco*RI and *Bgl*II restriction endonucleases, subjected to electrophoresis on 0.7% agarose gels, transferred to nylon membrane, and hybridized to cDNA probes$^{32}$ P-labeled by the random hexamer method [13]. The probes were an 837-bp *NUP98* cDNA fragment (nucleotide nt 1213 to 2049; GenBank accession no. U41815) and a 512-bp *NSD3* cDNA fragment (nt 929 to 1440; GenBank accession no. AJ295990).

### 2.3. Reverse transcriptase-polymerase chain reaction and nucleotide sequencing

*NUP98–NSD3* chimeric mRNA was detected by reverse transcriptase–polymerase chain reaction (RT-PCR) in essentially the same manner as described previously [14]. Total RNA was extracted from the leukemia cells of the patient using the guanidine thiocyanate–phenol–chloroform method [14]. Total RNA (4 μg) was reverse-transcribed to cDNA, using a cDNA synthesis kit (GE Healthcare Bio-Science, Piscataway, NJ) [14]. The PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan), using the reagents recommended by the manufacturer.

The primers used for detection of *NUP98–NSD3* fusion transcripts and the reciprocal fusion transcripts were NUP98–S10 (5′-TGGGACTCTTACTGGGCTT-3′) and NSD3-R4 (5′-CTCTCTGGCTGGTTGCTAAA-3′) for *NUP98–NSD3*, and NSD3-S1 (5′-CAAGATCTGAAGAGCG CAAG-3′) and NUP98–R13 (5′-TAGGGTCTGACATCG GATTC-3′) for *NSD3-NUP98*. The PCR amplification was performed with this mixture using a DNA thermal cycler (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 9 minutes, 40 cycles at 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by a final elongation at 72°C for 7 minutes.

For detection of *NUP98–NSD3L*, *NUP98–NSD3L2*, and *NUP98–NSD3S* fusions, nested RT-PCR was performed. The primers for first RT-PCR were NUP98–S10 and NSD3L-R (5′-ACCTGGGGTTGCAGATCTCT-3′), NUP983L2-R (5′-AATCTTCCACCTCTGGCAC-3′), NSD 3S-R (5′-ACGGAGCTGTCACTGAATCT-3′), respectively. The primers for second RT-PCR were NUP98–S11 (5′-CCTCTTGGTACAGGAGCCTT-3′) and NSD3-R4. The PCR conditions were as described above. The PCR products were subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and were sequenced by the fluorometric method using the Big Dye terminator cycle sequencing kit (Applied Biosystems).

### 2.4. Expression of three isoforms of the NSD3 gene and the NSD1 and NSD2 genes by RT-PCR in leukemic cell lines

To analyze the expression pattern of three isoforms of the *NSD3* gene (*NSD3L*, *NSD3L2*, and *NSD3S*) and the family genes *NSD1* and *NSD2* in leukemic cell lines, RT-PCR was performed. In all, 59 cell lines were examined, as follows [14]: 10 B-precursor ALL cell lines (NALM-6, NALM-24, NALM-26, UTP-2, THP-4, RS4;11, SCMC-L10, KOCL-33, KOCL-45, and KOCL-69), 9 B-ALL cell lines (BALM-1, BALM-13, BALM-14, BJAB, DAUDI, RAJI, RAMOS, BAL-KH, and NAMALVA), 9 T-ALL cell lines (RPMI-8402, MOLT-14, THP-6, PEER, H-SB2, HPB-ALL, L-SAK, L-SMY, and KCMC-T), 8 AML cell lines (YNH-1, ML-1, KASUMI-3, KG-1, inv-3, SN-1, NB4, and HEL), 6 acute monocytic leukemic cell lines (THP-1, IMS/M1, CTS, P31/FUJ, MOLM-13, and KOCL-48), 5 chronic myelogenous leukemia cell lines (MOLM-1, MOLM-7, TS9;22, SS9;22, and K-562), 2 acute

megakaryoblastic leukemia cell lines (CMS and CMY), and 10 EBV-B cell lines derived from normal adult peripheral lymphocytes. Five normal BM samples were also examined.

The RT-PCR mixtures and conditions were as previously described [13]. The primers used for RT-PCR were as follows: for *NSD3L* and *NSD3L2*, NSD3-2711F (5′-TCTGCCT GCTCTATGGAGAA-3′) (sense primer) and NSD3-3260R (5′-ACCTGGGGTTGCAGATCTCT-3′) (antisense primer); for *NSD3S*, NSD3-1779F (5′-GCCTGGATTTGCAGAAGT GT-3′) (sense primer) and NSD3-2220R (5′-ACGGAGCTGT CACTGAATCT-3′) (antisense primer), for *NSD1*, NSD1-4941F (5′-AACCTGTCATGCCGCTAATCC-3′) (sense primer) and NSD1-5495R (5′-ATCTTATCCTTGCTGCTCACG-3′) (antisense primer); and for *NSD2*, NSD2-2811F (5′-TCAAACC-CAAGGCCGTCAAA-3′) (sense primer) and NSD2-3365R (5′-GACTCTTCCGATCCCTCTGA-3′) (antisense primer).

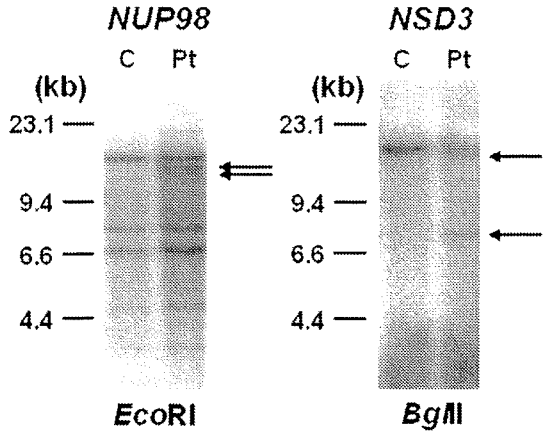


Fig. 2. Southern blotting of the *NUP98* gene with *Eco*RI and the *NSD3* gene with *Bgl*II restriction endonuclease. Arrows indicate rearranged bands. Pt, patient; C, control.

## 3. Results

Chromosomal abnormalities of the patient's leukemic cells revealed the karyotype as 46,XY,t(8;11)(p11;p15),-del(1)(p22p32), suggesting that the *NUP98* gene located in 11p15 was rearranged. A FISH analysis using the probe containing *NUP98* detected the split signals on both der(11)t(8;11)(p11;p15) and der(8)t(8;11)(p11;p15), in addition to normal chromosome 11 (Fig. 1). To date, the *NSD3* gene on chromosome 8p11 has been reported as a fusion partner gene of *NUP98* in the t(8;11)(p11;p15) anomaly [4].

Southern blot analysis of DNA from leukemic cells of the patient using the *NUP98* probe and the *NSD3* probe showed rearranged bands (Fig. 2). We performed RT-PCR for *NUP98–NSD3* chimeric mRNA and obtained one RT-PCR product of 247 bp. Sequence analysis of the PCR product showed an in-frame fusion transcript of exon 11 of *NUP98* to exon 4 of *NSD3*. Two reciprocal *NSD3–NUP98* transcripts were also detected. Sequence analysis of these PCR products showed that one product was an in-frame fusion transcript of exon 3 of *NSD3* to exon 12 of *NUP98*; the other was an in-frame fusion transcript of exon 3 of *NSD3* to exon 13 of *NUP98*. We also examined which of the *NSD3* isoforms (*NSD3L*, *NSD3S*, and *NSD3L2*) were fused to the *NUP98* gene. We identified two types of chimeric transcripts, *NUP98–NSD3L* and *NUP98–NSD3S*, but not *NUP98–NSD3L2*.

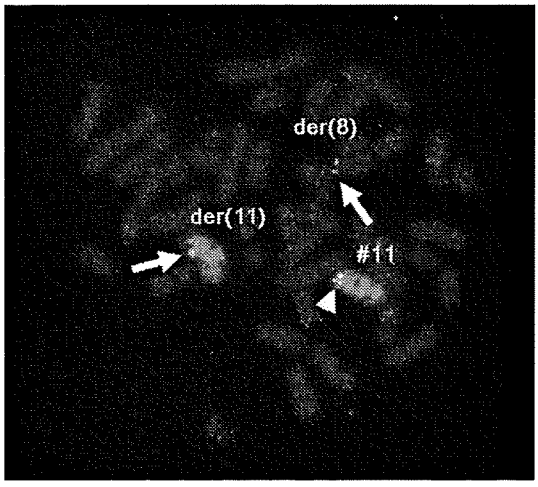


Fig. 1. FISH analysis of *NUP98* rearrangement in a leukemic metaphase. Split signals (arrows) of bacterial artificial chromosome clone PK505 containing *NUP98* were observed on the boundary between painted and unpainted regions of der(11)t(8;11) and der(8)t(8;11). An intact PK505 signal was observed on the normal chromosome 11 (arrowhead).

We next examined the *NSD3* gene and the family gene expression by RT-PCR analysis in 49 leukemic cell lines and 10 EBV-B cell lines (Fig. 3). There are three isoforms of the *NSD3* gene: *NSD3L* (full length), *NSD3L2* (lacking exon 14), and *NSD3S* (from exon 1 to exon 9a, which is completely different from exon 9 of *NSD3L*). There are also two *NSD* family genes (*NSD1* and *NSD2*) in addition to *NSD3*. *NSD1* is located on chromosome region 5q35 and *NSD2* is located on 4p16.3. Two of the three *NSD3* isoforms (i.e., except for *NSD3L2*) were expressed in all leukemic cell lines, the EBV-B cell line, and normal BM cells. The *NSD3L2* isoform was not expressed in any samples examined. *NSD1* and *NSD2* genes were expressed in all samples examined.

## 4. Discussion

*NUP98–NSD3* fusion transcripts have been detected only in a patient with AML and a patient with t-MDS having t(8;11)(p11;p15) [4,5]. All patients reported were diagnosed with adult-onset myeloid malignancies, and had a poor prognosis [4,5]. The present patient died of disease progression. As fusion genes between *NUP98* and isoforms of *NSD* in hematological malignancies, there are two other fusion transcripts; one is the *NUP98–NSD1*