Case Study

**Dic (17;20) (p11;q11) Preceded MLL Gene Amplification in a Patient with de novo Mixed-Lineage Leukemia**

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We report a case of acute mixed-lineage leukemia, as seen in a 65-year-old female with MLL gene amplification and biallelic loss of wild type p53 gene. The diagnosis was based on the findings that her bone marrow (BM) blasts expressed cytoplasmic CD3 (cyCD3), B-lineage antigens and myeloid antigens accompanied by clonal rearrangements of IgH gene. The BM blasts consisted of small-sized peroxidase-negative blasts (97%) and large-sized peroxidase-positive blasts (3%). The BM blasts showed a complex “karyotype,” including dic(17;20) (p11;q11), -5 and add (11q23). Add (11q23) abnormality was found in sideline karyotypes as well as the stemline abnormality of dic(17;20) (p11;q11). For the p53 gene, which is located at 17p13, fluorescence in situ hybridization analysis showed the loss of one of two p53 alleles. Furthermore, polymerase chain reaction-single-strand conformation polymorphism and following nucleotide sequencing showed that the p53 gene was mutated at codon 215, leading to an amino acid substitution from Ser to Arg. For the MLL gene, southern blot analysis showed that the MLL gene locus was amplified but not rearranged at its breakpoint cluster region, which is usually rearranged in balanced translocations with many partner genes. These findings suggest that MLL gene amplification may in this case be based on the genetic instability caused by the preceding biallelic loss of the wild type p53 gene.  

**Keywords:** MLL amplification, leukemia, fluorescence in situ hybridization, p53

**INTRODUCTION**

Acute leukemia of ambiguous lineage is defined as a new entity in the new WHO classification. This category of leukemia lacks the morphologic, cytochemical and immunophenotypic features sufficient to classify it as being of myeloid or lymphoid origin. This includes various types of leukemias that were formerly called such as acute undifferentiated leukemia, acute biphenotypic leukemia, mixed-lineage leukemia, or stem-cell leukemia, etc. Generally, the type of chromosomal aberration is closely related to the phenotype of leukemia. As for acute leukemia with ambiguous lineage, Ph1-abnormalities occupy a third of all cases, and cases with 11q23 abnormalities such as t(4;11) (q21;q23) are also frequently observed. On the other hand, acute leukemias with T/myeloid components or T/B components are infrequent and sometimes have complex karyotypes rather than Ph1 or 11q23 abnormalities.

Recently, a new clinicopathological entity in acute myeloblastic leukemia (AML)/myelodysplastic syndrome (MDS), 17p- syndrome, has been postulated. This new entity is characterized by the strong correlation between unbalanced translocations involving 17p deletion, less often monosomy 17 or i(17p), and typical dysgranulopoiesis combining pseudo-Pelger-Huët hypolobulation and small vacuoles in neutrophils, and p53 mutations. Notably, Soenen et al. reported that the deletion of one p53 allele, which is located at 17q13, was found in all cases with 17p- syndrome and point mutation of the non-deleted p53 allele in all but one. Thus, loss of germ line p53 might play an important role in 17p- syndrome.

Abnormalities in the MLL gene, located at 11q23, are among the most common recurring abnormalities in de novo and therapy-related hematologic disorders, including acute leukemias and MDS. Various types of MLL translocations generating chimeric proteins are closely associated with...
AML, acute lymphoblastic leukemia, or mixed lineage leukemia depending on the chimeric partners. Currently, three types of MLL gene aberrations other than reciprocal translocation are recognized in leukemia/MDS: partial tandem duplication (PTD) within MLL gene locus, internal deletion, and gene amplification. MLL gene amplification has recently been implicated as a potential mechanism of leukemia development. Moreover, it has been proposed that the precedent mutation of p53 due to previous exposure to alkylating agents in therapy-related myeloid malignancies may contribute to the genetic instability of the MLL gene, resulting in the amplification of this gene.

Here, we present a case of de novo mixed-lineage leukemia with p53 mutation and MLL amplification. This is the first case-report of mixed-lineage leukemia with biallelic p53 mutation and MLL amplification. This case provides new insights into the role of MLL amplification in leukemogenesis.

CASE REPORT

A 62-year-old woman was admitted to Kansai Medical University Hospital presenting pancytopenia (white blood cell count 1.0 × 10^9/μL, hemoglobin 6.6 g/dL, and platelet count 1.5 × 10^9/μL). A bone marrow (BM) aspirate showed 80% abnormal cells, 2% neutrophils, 16% lymphocytes and 2% erythroblasts. The abnormal cells in the BM consisted of 96% of small-sized blastic cells (less than three times the diameter of the erythrocytes) and 4% large-sized ones (more than three times the diameter of the erythrocytes). The small blasts were negative for myeloperoxidase (MPO) (< 0.1%), but the large blasts were MPO-positive (31%) (Fig. 1). Flow cytometric analysis revealed that the BM blasts were positive for cytoplasmic CD3 (cyCD3) and terminal deoxynucleotidyl transferase as well as CD13, CD19, CD22, CD24, but negative for surface CD3, CD4, CD20, CD33, CD34a/b, and CD56, as shown in Table 1. On gating, the large blasts were found to be also positive for cyMPO in addition to the antigens for which the small blasts were positive (data not shown). Southern blot analysis on BM blasts revealed that the immunoglobulin heavy chain (IgH) gene was monoclonally rearranged, whereas the T cell receptor (TCR) β chain gene was in germ-line configuration (Fig. 2). All these findings led to the diagnosis of mixed-lineage leukemia. The patient underwent induction chemotherapy with a combination of Adriamycin, predonizolone, cyclophosphamide, L-asparaginase, and vincristine. However, she failed to achieve complete remission, and became refractory to succeeding therapies such as high-dose cytarabine. Her condition progressively worsened and she died 7 months after the diagnosis.

Table 1. Cell-surface and cytoplasmic antigen expression on bone marrow blasts

<table>
<thead>
<tr>
<th>Cell-surface staining</th>
<th>Cytoplasmic staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 &lt; 1.0%</td>
<td>CD21 1.0%</td>
</tr>
<tr>
<td>CD2 &lt; 1.0%</td>
<td>CD22 95.0%</td>
</tr>
<tr>
<td>CD3 1.3%</td>
<td>CD23 1.8%</td>
</tr>
<tr>
<td>CD4 2.0%</td>
<td>CD24 35.3%</td>
</tr>
<tr>
<td>CD5 &lt; 1.0%</td>
<td>CD25 72.9 μ</td>
</tr>
<tr>
<td>CD7 &lt; 1.0%</td>
<td>CD30 1.5 μ</td>
</tr>
<tr>
<td>CD8 &lt; 1.0%</td>
<td>CD33 1.3 λ</td>
</tr>
<tr>
<td>CD10 &lt; 1.0%</td>
<td>CD34 99.0%</td>
</tr>
<tr>
<td>CD11a 93.7%</td>
<td>CD38 91.5%</td>
</tr>
<tr>
<td>CD11b 25.4%</td>
<td>CD41a 3.5%</td>
</tr>
<tr>
<td>CD11c 1.2%</td>
<td>CD41b &lt; 1.0%</td>
</tr>
<tr>
<td>CD13 94.0%</td>
<td>CD45 99.0%</td>
</tr>
<tr>
<td>CD14 2.4%</td>
<td>CD56 &lt; 1.0%</td>
</tr>
<tr>
<td>CD15 73.1%</td>
<td>CD71 74.8%</td>
</tr>
<tr>
<td>CD16a &lt; 1.0%</td>
<td>CD17 5.4%</td>
</tr>
<tr>
<td>CD19 99.0%</td>
<td>HLA-DR 99.0%</td>
</tr>
<tr>
<td>CD20 &lt; 1.0%</td>
<td></td>
</tr>
</tbody>
</table>

TdT, terminal deoxynucleotidyl transferase; MPO, myeloperoxidase

Fig. 1. Morphology of bone marrow (BM) blasts (~1000). The typical features on May-Giemsa (M-G) and peroxidase (POX) staining of bone marrow blasts are shown. Small blasts (~3 × erythrocyte in diameter) were negative for POX, whereas 31% of large blasts (~3 × erythrocyte in diameter) were positive for POX staining. (M-G & (POX), ~1,000.)
MATERIALS AND METHODS

Karyotyping and fluorescence in situ hybridization (FISH)

Cells from BM samples were processed for chromosome analysis by standard techniques, with 24- to 48-hr unstimulated cultures. Air-dried chromosome preparations on glass slides were G-banded. At least 20 metaphase cells were analyzed in each sample. Karyotypes were designated according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009).

FISH analysis was performed with D11Z1 (a marker probe for chromosome 11), CEP17 (a marker probe for chromosome 17), MLL, and p53 probes (Oncor, Gaithersberg, MD). All were hybridized and detected according to the manufacturers’ directions. These probes were biotin-labeled by nick translation (Biotin nick, GIBCO-BRL, Grand Island, NY). The hybridization solution for custom probes contained 0.2 μg labeled probe, 10 μg Cot-1 DNA (GIBCO-BRL), and 30 μg herring sperm DNA (GIBCO-BRL) in 15 μL of Hybrizol VII (Oncor) per slide. The probe cocktail was heat denatured at 70°C for 5 min and allowed to pre-anneal at 37°C for 2 hr. Chromosome preparations on slides were conditioned before hybridization by a 30-min 37°C bath in 2 × SSC, followed immediately by dehydration in 70%, 80%, and 95% EtOH (2 min each) at room temperature, and air-dried. The slides were then denatured in 70% formamide/2 × SSC at 70°C for 5 min, followed by serial dehydration at room temperature.

Hybridization was for 18 hr in a moist 37°C chamber. Slides were washed in 50% formamide/2 × SSC at 37°C for 30 min, followed by 2 × SSC at 37°C for 10 min. Slides were further washed three times at room temperature in a phosphate-buffered detergent before signal detection. Hybridized DNA was detected with Avidin-fluorescent isothiocyanate, followed by a single round of amplification according to the supplier’s instructions (Oncor).

FISH signals were captured by using a monochromatic CCD camera mounted on a Zeiss epifluorescence microscope with a LUDL filter wheel and a fixed, multi-bandpass beam splitter with the use of Macprobe software (PSI, Houston, TX).

Southern blot analysis

Southern blot analysis was performed according to the standard method. Briefly, 5 mg of DNA from the patient and normal control were digested separately or a combination of BamHI, HindIII, EcoRI, or EcoRV (Takara, Kyoto, Japan), electrophoresed through 0.8% agarose gel, and transferred to a nylon membrane. After hybridization with the labeled probe, the nylon membranes were washed at an appropriate stringency, and autoradiographed.

For TCRβ chain gene, the HindIII-EcoRI 3.5 Kb fragment of the TCRβ constant region was used as a Cβ1 probe. For IgH, the EcoRI-HindIII fragment of the IgH constant region was used as a JH probe. For testing the MLL gene, a 0.74 Kb BamHI fragment of the MLL gene was used as a MLL probe, which detects all rearrangements (including PTD) within the MLL breakpoint cluster region.8

Fig. 2. Southern blot analysis for IgH and TCRβ genes. A quantity (5 μg) of restriction enzyme digests of patient DNA was loaded in each lane. (2A) JH probe. lane 1; BamHI plus HindIII, lane 2; HindIII. Arrows indicate rearranged bands. (2B) Cβ1 probe. lane 1; BamHIl, lane 2; EcoRV, lane 3; HindIII.
Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and nucleotide sequencing

For detecting point mutations of the p53 gene, one hundred nanograms of genomic DNA (gDNA) was used to amplify the exons 5 to 8 of p53 gene. Primer sets for amplification of 4 exons of p53 were designed with fluorescence Cy-5 (Amersham Pharmacia Biotech, Little Chalfont, UK) at 5' site of primers according to benebank X54156. Table 2 shows the primer sequences used. PCR-SSCP analysis was performed according to Orita et al.13 with the Expand™ High Fidelity PCR System (Roche Molecular Biochemicals Diagnostic, Manheim, Germany). The PCR conditions of exons 5, 6 and 8 were at 94°C for 30 sec (denature), 60°C for 60 sec (annealing), and 72°C for 60 sec (extension) for 35 cycles, and those of exon 7 were at 94°C for 30 sec, 70°C for 60 sec, and 72°C for 60 sec for 35 cycles. The PCR products were diluted fifty-fold with 95% formamide and denatured at 80°C for 5 min followed by rapid cooling on ice. Denatured products were separated on 5% DNA sequencer ALF express (Amersham) and analyzed with Allele link software (Amersham).

For direct sequencing of the PCR product, an interim sequencing primer for exon 6 (Table 2) was set to detect point mutations within exon 6 of p53. The PCR product was re-amplified with the Expand™ High Fidelity PCR System. The PCR product was purified using the High Pure PCR product purification kit (Roche) and directly sequenced using the Thermosequencing kit (Amersham) with ALF express. The sequence was finally compared with the wild-type p53 gene.

RESULTS

Dic (17; 20) (p11; q11) and MLL amplification

Karyotype analysis of BM cells at the time of diagnosis (sample from June 27, 2002) demonstrated that 2 of 20 cells analysed had normal karyotypes while the remaining 18 cells had complex karyotypes with multiple structural and numerical abnormalities (Fig. 3). Four of the 18 cells showed the karyotype of 43, XX, -5, del(7) (q?), del(12) (p?), -16, dic(17; 20) (p11;q11) (Fig. 3A). The karyotype of the remaining 14 cells was 45, XX, -5, del(7) (q?), add(11) (q23), del(12) (p?), dic(17;20) (p11;q11), +mar (Fig. 3B). Thus, all the 18 cells with abnormal karyotypes had -5, del(7) (q?), del(12) (p?) and dic(17;20) (p11;q11) in common (Fig. 3A), suggesting that add(11) (q23) is a secondary chromosomal aberration. No double minute chromosomes, frequently accompanied by MLL gene amplification in AML/MDS cases,1,12 were detected in any of the 20 cells analyzed. Karyotype analysis on the refractory stage of disease (Dec 19, 2002) demonstrated

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Table 2. Primer sequences of p53 for polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and nucleotide sequencing

<table>
<thead>
<tr>
<th>Primers for PCR-SSCP</th>
<th>Exon 5</th>
<th>E5SF 5’-TTCCTCTTCTCTACAGTACTCC-3’</th>
<th>E5SR 5’-GCCCCAGCTGCTCACCATGCG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exon 6</td>
<td>E6SF 5’-CACTGATGCTCTTAGGTCTG-3’</td>
<td>E6SR 5’-AGTTGCAAAACCGAGACTCGAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Exon 7</td>
<td>E7SF 5’-CAAAAGCGACACTGGCTCATC-3’</td>
<td>E7SR 5’-TCAGCGGCAAGCAGAGGCTGG-3’</td>
</tr>
<tr>
<td></td>
<td>Exon 8</td>
<td>E8SF 5’-GCTATCTCTAGTATGTAGTAAAT-3’</td>
<td>E8SR 5’-GTCCTGCTCGCTTAACCTGC-3’</td>
</tr>
</tbody>
</table>

Primers for direct sequencing of exon 6

5’-GAGGGCCACTGACAACCACC-3’

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Fig. 3. Karyotype analysis (G-banding) of leukemic cells at the time of diagnosis (June 27, 2002). (3A) A stemline karyotype with no chromosome 11 abnormality is shown ; 43, XX, -5, del(7) (q?), del(12) (p?), -16, dic(17;20) (p11;q11). (3B) A sideline karyotype is shown ; 45, XX, -5, del(7) (q?), add(11) (q23), del(12) (p?), dic(17;20) (p11;q11), +mar.
the following karyotype: 48, XX, -5, del(7) (q?), add(11) (q23) × 2, del(12) (p?), dic(17;20) (p11;q11), +3mar, indicating further karyotypic evolution (data not shown).

Since the MLL gene is located on 11q23, we assumed that this gene might be involved in the 11q23 aberration observed in this case. FISH analysis demonstrated that some BM cells on the refractory stage of disease (Dec 19, 2002) had amplification of the MLL gene on chromosome 11 (Fig. 4A).

Southern blot analysis for the MLL gene on the refractory stage of disease revealed that this gene was of germ-line configuration (Fig. 4B). This result excluded the possibility of mutual translocations of the MLL gene with other partner genes. In addition, PTD of the MLL gene, which occasionally occurs in AML without cytogenetic aberrations at 11q23, is also unlikely to exist. Interestingly, the density of the MLL band was ten times that of the germ line band of normal control as detected by image analyzer (Fig. 4B), demonstrating the amplification of the MLL gene locus without gene rearrangement.

Loss of wild type p53 alleles by dic(17;20) (p11;q11) and single nucleotide mutation

To demonstrate the deletion of the p53 allele, which is located on 17p13, due to dic(17;20) (p11;q11), we performed FISH analysis using a probe specific for p53 on BM blasts. FISH analysis on BM blasts at the time of diagnosis (sample of June 27, 2002) showed that all of the cells analyzed lost one of two p53 alleles (Fig. 5). We further analyzed the status of another p53 allele using PCR-SSCP. PCR-SSCP analysis on BM blasts at the time of diagnosis (sample of June 27, 2002) for exons 5 through 8 of the p53 gene locus, which spans mutational hot spots in cancers, showed a shifted band on exon 6 (Fig. 6A). The direct sequencing of the PCR product for exon 6 revealed that AGT to AGG nucleotide conversion from Ser to Arg at least in some cells (Fig. 6B). On the other hand, the wild type codon 215 (AGT) was also detected, which may reflect the presence of cells with a normal karyotype. These results, combined with those from FISH analyses, demonstrated that both alleles of the wild type p53 gene were lost because of the deletion of one allele resulting from unbalanced translocation, dic(17;20) (p11; q11), and one point mutation at codon 215 at the time of initial presentation.

DISCUSSION

Deletions of 17p or 20q are well-recognized abnormalities in myeloid malignancies. Deletions of 17p are found in 4% of AML/MDS cases and are strongly associated with loss and mutation of the p53 gene, while deletions of 20q are found in 5% of myeloid disorders including AML/MDS and myeloproliferative disorders such as polycythemia vera. Recently, six cases of AML/MDS with a dicentric unbalanced translocation between chromosome 17 and 20, dic(17;20) (p11;q20), in which the segments distal to 17p11 and 20q12 are lost, have been reported. The cases are characterized by myelodysplastic features and poor prognosis. It is therefore postulated that dic(17;20) is a rare but recurrent abnormality in
myeloid malignancies. Interestingly, the deletion of the p53 gene on the dicentric chromosome with retention of the p53 on the normal copy of chromosome 17 has been demonstrated by FISH analysis in 4 of 4 AML cases with dic(17;20).16

Loss of 17p is frequently associated with complete or partial monosomy of chromosome 5 and a poor prognosis in AML/MDS.1-3 Moreover, a recurring abnormality of dic(5;17) (q11-13;p11) is reported in AML/MDS.17 In our case, hsr(11q) but not dmin was detected in the advanced stages of the disease, and the intra-chromosomal amplification of the MLL gene was confirmed by FISH analysis. The germ line configuration of the MLL gene in our case is in accordance with the reports that showed the duplicated or amplified MLL gene was in germ line configuration.24 A recent report on myeloid malignancies with 11q23 amplification showed that the MLL and DDX6 genes were identified as the most expressed genes among candidate oncogenes at 11q.25 The transcription levels for the MLL-regulated genes such as HoxA9 were also significantly enhanced. Furthermore, AML/MDS with 11q/MLL amplification shows a characteristic gene expression signature.26 Therefore, MLL is considered to be the main target gene for 11q23 amplification, and the gain in MLL function is suspiciously critical in leukemogenesis. Interestingly, MLL amplification has been reported almost exclusively in AML and MDS. This may imply that an abundance of the wild-type MLL protein may enhance the transcriptional activity of myeloid-specific genes in a hematopoietic precursor. Therefore, it is plausible that the myeloid phenotype on the large-sized blasts in our case are related to the MLL amplification, although we could not technically distinguish these blasts from the smaller and MPO-negative blasts based on cytogenetical analysis.

Taken together, we concluded that the mutated p53 alleles, one by deletion and the other by point mutation, may have induced genomic instability and caused MLL amplification. We believe that our case is very instructive in understanding the roles of p53 mutations and MLL amplification in hematologic malignancies.

A series of cytogenetical analyses of this case revealed that at the time of disease onset there was a stemline in which the 11q23 abnormality was absent but dic(17;20) (p11;q12) already existed. This suggests that the mutation of p53 caused by dic(17;20) (p11;q12) may precede MLL amplification manifested as 11q23 abnormality in our case, although the possibility of MLL amplification without karyotypic change can not be completely discounted. Therefore, we assume that the biallelic loss of p53 function was the earlier and crucial event, and that this event induced chromosomal instability that might trigger MLL amplification, although direct evidence is lacking.

The amplification of MLL is reported so far only in AML and MDS,9 while PTD of MLL is reported only in AML and T-acute lymphoblastic leukemia.22 Gene amplification of MLL is cytogenetically manifested as either a homogenously staining region (hsr) or a double minute chromosome (dmin) in AML/MDS.23 In our case, hsr(11q) but not dmin was detected in the advanced stages of the disease, and the intra-chromosomal amplification of the MLL gene was confirmed by FISH analysis. The germ line configuration of the MLL gene in our case is in accordance with the reports that showed the duplicated or amplified MLL gene was in germ line configuration.24 A recent report on myeloid malignancies with 11q23 amplification showed that the MLL and DDX6 genes were identified as the most expressed genes among candidate oncogenes at 11q.25 The transcription levels for the MLL-regulated genes such as HoxA9 were also significantly enhanced. Furthermore, AML/MDS with 11q/MLL amplification shows a characteristic gene expression signature.26 Therefore, MLL is considered to be the main target gene for 11q23 amplification, and the gain in MLL function is suspiciously critical in leukemogenesis. Interestingly, MLL amplification has been reported almost exclusively in AML and MDS. This may imply that an abundance of the wild-type MLL protein may enhance the transcriptional activity of myeloid-specific genes in a hematopoietic precursor. Therefore, it is plausible that the myeloid phenotype on the large-sized blasts in our case are related to the MLL amplification, although we could not technically distinguish these blasts from the smaller and MPO-negative blasts based on cytogenetical analysis.

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