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. [*J Clin Exp Hematopathol* 50(1) : 51-58, 2010]

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.¹ 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.^{2,3} 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.² 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.⁴⁻⁷ Various types of *MLL* translocations generating chimeric proteins are closely associated with

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AML, acute lymphoblastic leukemia, or mixed lineage leukemia depending on the chimeric partners.⁸ 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.⁹⁻¹¹ 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.¹²

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 $1.0 \times 10^9/\mu\text{L}$, hemoglobin 6.6 g/dL, and platelet count $1.5 \times 10^9/\mu\text{L}$). 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, CD41a/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

Cell-surface staining		Cytoplasmic staining	
CD1	< 1.0%	CD21	1.0%
CD2	< 1.0%	CD22	95.0%
CD3	1.3%	CD23	1.8%
CD4	2.0%	CD24	35.3%
CD5	< 1.0%	CD25	72.9
CD7	< 1.0%	CD30	1.5
CD8	< 1.0%	CD33	1.3
CD10	< 1.0%	CD34	99.0%
CD11a	93.7%	CD38	91.5%
CD11b	25.4%	CD41a	3.5%
CD11c	1.2%	CD41b	< 1.0%
CD13	94.0%	CD45	99.0%
CD14	2.4%	CD56	< 1.0%
CD15	73.1%	CD71	74.8%
CD16a	< 1.0%	CD117	5.4%
CD19	99.0%	HLA-DR	99.0%
CD20	< 1.0%		

TdT, terminal deoxynucleotidyl transferase; MPO, myeloperoxidase

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

Fig. 2. Southern blot analysis for *IgH* and *TCRβ* genes. A quantity (5 μ g) 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) C β 1 probe. lane 1 ; BamHI, lane 2 ; EcoRV, lane 3 ; HindIII.

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, Gaithersburg, MD). All were hybridized and detected according to the manufacturers' directions. These probes were biotin-labeled by nick translation (Biotinick, GIBCO-BRL, Grand Island, NY). The hybridization solution for custom probes contained 0.2 μ g labeled probe, 10 μ g Cot-1 DNA (GIBCO-BRL), and 30 μ g herring sperm DNA (GIBCO-BRL) in 15 μ L of Hybridol 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 $\times$ 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 $\times$ 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 $\times$ SSC at 37°C for 30 min, followed by 2 $\times$ 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 *Bam*HI, *Hind*III, *Eco*RI, or *Eco*RV (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 *Hind*III-*Eco*RI 3.5 Kb fragment of the *TCRβ* constant region was used as a *Cβ*1 probe. For *IgH*, the *Eco*RI -*Hind*III fragment of the *IgH* constant region was used as a *JH* probe. For testing the *MLL* gene, a 0.74 Kb *Bam*HI fragment of the *MLL* gene was used as a *MLL* probe, which detects all rearrangements (including PTD) within the *MLL* breakpoint cluster region.⁸

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, Mannheim, 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

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

Primers for PCR-SSCP		
Exon 5	E5SF	5'-TTCCTCTTCTACAGTACTCC-3'
	E5SR	5'-GCCCCAGCTGCTCACCATCGC-3'
Exon 6	E6SF	5'-CACTGATTGCTCTTAGGTCTG-3'
	E6SR	5'-AGTTGCAAACAGACCTCAGG-3'
Exon 7	E7SF	5'-CCAAGGCGCACTGGCCTCATC-3'
	E7SR	5'-TCAGCGCAAGCAGAGGCTGG-3'
Exon 8	E8SF	5'-CCTATCCTGAGTAGTGGAAT-3'
	E8SR	5'-GTCCTGCTTGCTTACCTCGCT-3'
Primers for direct sequencing of exon 6		
5'-GAGGGCCACTGACAACCACCC-3'		

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

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) $\times$ 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.¹¹ 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 substitution occurred at codon 215, resulting in amino acid 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,^{1,2} while deletions of 20q are found in 5% of myeloid disorders including AML/MDS and myeloproliferative disorders such as polycythemia vera.¹⁴ Recently, six cases of AML/MDS with a dicentric unbalanced translocation between chromosome 17 and 20, dic(17;20) (p11;q12), in which the segments distal to 17p11 and 20q12 are lost, have been reported.^{15,16} These cases are characterized by myelodysplastic features and poor prognosis. It is therefore postulated that dic(17;20) is a rare but recurrent abnormality in

Fig. 4. Fluorescence *in situ* hybridization and southern blot analyses demonstrate multiple copies of *MLL*. (**4A**) Metaphase cells were double-stained with *MLL* and *D11Z1* probe, a marker probe for chromosome 11. Red signal (arrow) indicates *MLL*, and blue signal (arrowhead) indicates chromosome 11. Marked amplification of *MLL* gene at chromosome 11 is noted. (**4B**) Southern blot analysis for *MLL* gene. *MLL* probe was hybridized with *Bam*HI digest. *MLL* gene was of germ line configuration. Thick band for *MLL* gene segment shows the amplification of the *MLL* gene.

Fig. 5. Fluorescence *in situ* hybridization analysis demonstrates loss of a *p53* locus. Metaphase cells were double-stained for *p53* and *CEP17* probe, a marker probe for chromosome 17. Red signal indicates *p53*, and blue signal indicates chromosome 17. One of the two *p53* signals is lost.

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 cases.¹⁷ Therefore, a possible cooperation of *p53* gene located at 17p13 and a putative tumor suppressor gene at 5q13 has been proposed.^{17,18} On the other hand, several cases of 17p- syndrome resulting from dic(17;20) in AML/MDS cases have also been reported.^{15,16} This unique karyotypic combination of 17p- and monosomy 5 is detected at the initial presentation in our case. Since the reported cases carrying 17p- or dic(17;20) have been confined to AML/MDS cases, our case is the first, to the best of our knowledge, of *de novo* mixed-lineage leukemia with dic(17;20). Although clinical and phenotypic features may not be linked to 17p- syndrome in our case, the genetic features such as del(17p), del(5) and *p53* mutation of

both alleles are quite similar to the genetic characteristics of 17p- syndrome.

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 AML¹⁹⁻²¹ and internal deletion of *MLL* at exon 8 is reported only in T-acute lymphoblastic leukemia.²² Gene amplification of *MLL* is cytogenetically manifested as either a homogeneously 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 supposedly 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.

Fig. 6. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and direct sequencing of *MLL* gene. (**6A**) PCR-SSCP analysis for exon 5 through 8 of *p53* gene demonstrated a shifted peak in exon 6 (red arrow). (**6B**) Direct sequencing of exon 6 showed the single nucleotide mutation at codon 215 (red arrow, AGT to AGG), resulting in amino acid conversion from Ser to Arg.

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