Genetic analysis of *TP53* in childhood myelodysplastic syndrome and juvenile myelomonocytic leukemia

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Abstract

Among 9 children with myelodysplastic syndrome (MDS) and 18 children with juvenile myelomonocytic leukemia, one MDS patient with der(5;17)(p10;q10) exhibited deletion of the *TP53* gene in one allele and mutation (410 T>A) in the other allele in myeloid and erythroid cells. Since the mutation was not detected in peripheral blood leukocytes 9 months before the diagnosis, biallelic somatic inactivation of the *TP53* gene might play an important role in the occurrence of MDS. His poor outcome might be associated with resistance to chemotherapy/radiation of a minor clone with both *TP53* gene alteration and *MLL* duplication that already existed at onset.

Keywords; MDS, JMML, *TP53*, childhood, clone, biallelic inactivation, *MLL* duplication

Introduction

The tumor suppressor gene, *TP53*, located on the short arm of chromosome 17 (17p13.1 band), encodes a 53-kDa nuclear phosphoprotein that functions as a negative regulator of cell proliferation. Several types of DNA damage such as those due to certain anticancer drugs and gamma radiation activate the p53 protein, resulting in p53-dependent cell cycle arrest at the G1 and G2 cell cycle checkpoints, allowing time for DNA repair. If the DNA is not repaired, the p53-dependent apoptotic pathway is activated.

Inactivation of p53 has been reported in hematologic malignancies in association with progression of disease. In adult patients with de novo myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML), somatically acquired mutations of *TP53* are observed in less than 10% of cases, and are often associated with loss of the short arm of chromosome 17, a complex karyotype, resistance to chemotherapy, and a short survival[1-5].

There has been controversy regarding the frequency of pediatric hematologic malignancies with the *TP53* abnormalities. Felix et al.[6] demonstrated that only one patient showed an inherited 2-base-pair deletion in exon 6 of the *TP53* gene among 19 children with therapy-related leukemia or MDS. On the other hand, Silveira et al.[7] reported that, in a total of 19 pediatric MDS patients including 6 patients who possessed deletion involving 17p13.1, 18 children (94.7%) had deletion of the *TP53* gene according to FISH analysis.

It has been demonstrated that patients with juvenile myelomonocytic leukemia (JMML) have mutually exclusive abnormalities in the GM-CSF signaling pathway (inactivation of

the *NF1* tumor suppressor gene or aberrations in *NRAS*, *KRAS*, *PTPN11*, or *CBL* genes) [8,9]. Recently, Sugimoto et al.[10] sequenced exon 12 of ASLX1 in 49 JMML patients, and found 2 novel heterozygous mutations, one occurring as a sole lesion, the other was in conjunction with a PTPN11 mutation. It remains unclear whether the *TP53* abnormalities are observed in JMML patients with genetic abnormalities in the GM-CSF signaling pathway.

In the present study, we examined the presence of *TP53* mutations and deletions in 9 children with MDS or AML with myelodysplasia-related changes and 18 children with JMML by means of DNA sequence, FISH, and immunocytochemical analyses.

Materials and Methods

The study was approved by the Institutional Review Board of Shinshu University School of Medicine. Informed consent was obtained from parents and patients over 12 years of age.

Isolation of bone marrow (BM) and peripheral blood (PB) mononuclear cells

A total of 9 MDS patients and 18 JMML patients, whose clinico-histopathologic features met the World Health Organization diagnostic criteria[11], were enrolled in this study. BM or PB mononuclear cells were separated and frozen with liquid nitrogen until the experiment.

Detection of *TP53* mutations

Exons 5-9 of *TP53* were amplified by polymerase chain reaction (PCR) using the following primers: exons 5-6: sense, 5’- GTTTCTTTGCTGCCGTCTTC-3’; antisense, 5’-

CTTAACCCCTCCTCCCAGAG -3'; exon 7: sense,

5'-CTTGCCACAGGTCTCCCCAA-3'; antisense,

5'-AGGGGTCAGAGGCAAGCAGA-3'; exons 7-8: sense,

5'-CTTGGGCCTGTGTTATCTCC-3'; antisense, 5'- TGCTAGGAAAGAGGCAAGGA -3; exons 8-9: sense, 5'-TTGGGAGTAGATGGAGCCT-3'; antisense,

5'-AGTGTTAGACTGGAAACTTT-5'. The PCR products were subjected to direct sequencing in both directions on an automatic DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA) according to the procedure described previously[5]. Mutational analyses of *NRAS, KRAS, PTPN11, CBL, FMS-like tyrosine kinase-3 receptor, c-kit, CSF1R, BRAF, IDH1, and IDH2* genes were performed using primers described previously[9,12-20].

Immunohistochemical analysis

Reactions with mouse monoclonal anti-p53 antibody (NCL-p53 DO-7; Novocastra, Newcastle upon Tyne, UK) were detected using the Ventana BenchMark LT (Ventana Medical System Inc., AZ) according to the manufacturer's recommendations. The cut-off value was defined as the mean + 2 S.D. obtained from PB cells of normal controls.

Fluorescence in situ hybridization (FISH) analysis

FISH analysis was performed according to a procedure described previously[21]. We obtained probes specific for the *TP53* (LSI p53 Spectrum Orange Probe), *MLL* (LSI MLL Dual Color, Break Apart Rearrangement Probe), *PML*, and *RARα* genes (PML/RARα Dual Color, Dual Fusion Translocation Probe) from Vysis (Abbott/Vysis, Downers Grove, IL). At least 100 nuclei were scored for the number of hybridization signals of all the genes

except for the *MLL* gene in GM or erythroid colony-constituent cells. The cut-off value of 1 signal for the *TP53* gene was defined as the mean + 2 S.D. obtained from normal controls.

Spectral kayotyping

Spectral karyotyping (SKY) analysis was performed according to the instructions provided with the SKY probe kit (Applied Spectral Imaging, Carlsbad, CA), as reported previously[22]. An SD200 Spectracube (Applied Spectral Imaging) was used for image acquisition.

Clonal cell culture

Clonal cell cultures were carried out in a dish containing methylcellulose medium supplemented with granulocyte-macrophage (GM) colony-stimulating factor, stem cell factor, interleukin-3, and erythropoietin (Methocult GF H4434, Stemcell Technologies Inc., Vancouver, Canada). Dishes were incubated at 37℃ in a humidified atmosphere with 5% CO_2. On day 14, GM colonies and erythroid colonies were individually lifted and prepared as single-cell suspensions, as described previously[12]. Then, sequence and FISH analyses were performed on GM or erythroid colony-constituent cells.

Results

***TP53* gene analysis and immunohistochemical analysis of p53 in patients with MDS**

We performed *TP53* gene analysis and immunohistochemical analysis of p53 in 6 patients with *de novo* MDS (cases no. 1, 2 and 4-7), one patient with therapy-related MDS (case no. 3), and 2 patients with AML with myelodysplasia-related changes (cases no. 8, 9).

As presented in Table 1, all of them had chromosomal abnormalities such as monosomy 7 and trisomy 8. Among 9 patients, one patient (case no. 6) exhibited deletion and mutation of the *TP53* gene. According to immunostaining, approximately 20% of his BM cells on paraffin-embedded sections (Figure 1A) or half of his BM mononuclear cells spread on glass slides showed profound overexpression of p53.

Clinical and genetic characteristics of case no. 6 with deletion and mutation of *TP53* gene

Case no. 6 was an 18-month-old boy who was admitted to our hospital in June 2007 because of a leukocyte count of 5.87 x 10^9/l with 1% blasts, a hemoglobin level of 10.1 g/dl, and a platelet count of 33 x 10^9/l. Coupled with the findings that 5.2% of BM nucleated cells were blasts, he was diagnosed with MDS (refractory anemia with excess of blasts-1). He had a markedly short stature and mental retardation, but no first-degree relatives with malignancies (including breast carcinoma and leukemia) at an early age. According to G-banding analysis, 17 of 20 metaphase marrow cells revealed der(5;17)(p10;q10), and the remaining cells did normal karyotype. Spectral karyotyping (SKY) analysis on GM colony- and erythroid colony-constituent cells grown with hematopoietic growth factors confirmed the same chromosomal abnormality (Figure 2A). On the other hand, PB mononuclear cells stimulated with phytohemagglutinin were of normal karyotype. Deletion of the *TP53* gene was found in half of BM cells by means of FISH analysis with a probe for the *TP53* locus (Figure 3). As presented in Figure 1B, direct sequencing revealed the presence of heterozygous *TP53* mutation (410 T>A) in BM and PB cells, but not in nail. The *TP53* mutation was not detected in PB leukocytes 9 and

13 months before the diagnosis of MDS. PB cells of his father and mother showed wild-type *TP53* gene. The patient possessed the wild-type *NRAS, KRAS, PTPN11, CBL, FMS-like tyrosine kinase-3 receptor, c-kit, CSF1R, BRAF, IDH1, and IDH2* genes. He did not have the clinical features of neurofibromatosis type I. Additionally, the chromosomal breakage test using diepoxybutane and mitomycin C on PB lymphocytes was negative. Serum trypsinogen and lipase levels were normal, and computed tomography scan showed no pancreatic lipomatosis. After a preparative regimen of total body irradiation, fludarabine, and cyclophosphamide, BM transplantation (BMT) from HLA-matched unrelated donor was performed in December 2008. Since grade Ⅲ acute GVHD occurred on day 67, he was treated with tacrolimus and methylprednisolone. Since short tandem repeat-PCR detected reappearance of a recipient-specific allelic signal on day 197, the first course of donor lymphocyte infusion was administered. However, on day 252, his BM cells were found to contain 24% blasts positive for CD13, CD33, CD34, CD41, and CD71. Three more courses of donor lymphocyte infusion combined with chemotherapy resulted in only temporary stabilization of the disease. A second BMT from his HLA-haploidentical mother was performed 441 days after 1st BMT, but he died on 134 days after 2nd BMT because of disease progression.

To elucidate whether or not the *TP53* gene abnormalities were restricted to the myeloid lineage, we performed cytogenetic analysis of circulating myeloid, B, and T cells obtained at diagnosis. CD14^{+}/CD15^{+} PB cells possessed homozygous *TP53* mutation according to direct sequencing (Figure 1C). FISH analysis revealed loss of one *TP53* allele in 90% of the CD14^{+}/CD15^{+} cells. On the other hand, CD19^{+} and CD3^{+} PB cells possessed the

wild-type of *TP53* gene. In addition, a large proportion of both lymphocytes had 2 signals for the gene. To examine whether the *TP53* abnormalities occurred at the progenitor level, we picked up GM colonies and erythroid colonies grown from $CD34^+$ BM cells at onset under stimulation with a combination of hematopoietic growth factors. The *TP53* homozygous mutation was detected in all of 9 GM colonies and in all of 8 erythroid colonies. Moreover, 99 of 100 pooled GM colony-constituent cells and 98 of 100 pooled erythroid colony-constituent cells had 1 signal for *TP53*. Mutated *TP53* gene and a loss of *TP53* gene remained stable in BM cells after the first and second transplantations.

Since Anderson et al.[23] reported that 7 of 8 therapy-related MDS or AML patients with mutations of *TP53* had duplication or amplification of the *MLL* gene, we examined the *MLL* gene copy number before and after BMT. FISH analysis revealed that only 1% of pre-transplant PB cells had 3 signals of the *MLL* gene in patient no. 6. The frequency of cells with extra copies of the *MLL* gene was 0.8%, and that of cells with 2 copies was 98% in 5 normal control PB cells. Interestingly, the der(7)t(7;11) as well as der(5;17) was found in 2 of three GM colony-constituent cells and in 4 of five erythroid colony-constituent cells grown from $CD34^+$ BM cells at onset under stimulation with hematopoietic growth factors according to SKY analysis (Figure 2B). FISH analysis revealed 3 signals for *MLL* in 4 of five GM colony-constituent cells and in 65 of 100 erythroid colony-constituent cells (Figure 2C). These results suggested the existence of the extra *MLL* gene on der(7)t(7;11). Eight months after the 1st BMT, FISH analysis revealed that 8% of BM cells had 3 signals for *MLL*. The frequency of PB cells with 3

MLL signals increased to 88% three months after the 2nd BMT.

***TP53* gene analysis and immunohistochemical analysis of p53 in patients with JMML**

We then performed *TP53* gene analysis and immunohistochemical analysis of p53 in a total of 18 children with JMML (7 *PTPN11* mutations; 4, *KRAS* mutations; 5, *NRAS* mutations; 2, NF1 individuals). All JMML patients examined had neither *TP53* gene aberration nor p53 overexpression (Table 1).

Discussion

There have been a few reports regarding pediatric patients with hematologic malignancies who possessed *TP53* abnormalities. Felix et al.[6] demonstrated that only one patient showed a 2-base-pair deletion in the *TP53* gene among 19 children with therapy-related leukemia or MDS. The other investigators also described a lack or very small numbers of *TP53* aberrations in more than 30 pediatric MDS patients[18, 24]. On the other hand, in a recent study of 19 pediatric MDS patients, 18 children (94.7%) had deletion of the *TP53* gene according to FISH analysis[7]. Our results supported the low frequency of the *TP53* abnormalities in childhood MDS. One possible explanation for the major differences in the frequency of TP53 abnormalities among the reports is the different frequency of MDS patients with deletion involving 17p13.1. In JMML, the *TP53* aberrations do not appear to be related to leukemogenesis.

On the basis of markedly short stature and mental retardation in patient no. 6, we evaluated the possibility that hematological abnormalities are a manifestation of germline *TP53* mutation. DNA obtained from his nails and lymphocytes showed the wild type of

the *TP53* gene, while DNA from myeloid cells revealed a point mutation. Thus, the patient appeared to have a somatic mutation, but not a germline mutation, in the *TP53* gene. Coupled with no history of malignancy in first-degree relatives, the patient did not fulfill the Chompret criteria for Li-Fraumeni syndrome. De Filippi et al.[25] reported a JMML patient with a constitutional mutation of *NRAS* gene who had short stature and some dysmorphic features. Niemeyer et al.[26] demonstrated a dominant developmental disorder resulting from germline missense *CBL* mutations, which is characterized by impaired growth, developmental delay, cryptorchidism and a predisposition to JMML. Nevertheless, these genes of patient no. 6 were of the wild-type. Additionally, he might not have had Schwachman-Diamond syndrome because of normal pancreatic function. However, we cannot exclude a possibility of germline mutation of genes other than those described above.

In patient no. 6, $CD14^+/CD15^+$ PB cells possessed homozygous *TP53* mutation, and 90% of them revealed loss of one *TP53* allele. All of 9 GM progenitors and all of 8 erythroid progenitors generated progenies with the *TP53* homozygous mutation. Additionally, a great majority of pooled GM or erythroid colony-constituent cells had 1 signal for p53. On the other hand, $CD19^+$ and $CD3^+$ PB cells had neither mutation nor deletion of the gene. These results imply that an abnormal clone with deletion of the *TP53* gene in one allele and mutation in the other allele originated from hematopoietic stem cells with the potential to differentiate into myeloid and erythroid lineages, but not the lymphoid lineage. The loss of 17p is often accompanied by a *TP53* mutation in AML and MDS[1,2]. It has been demonstrated that *TP53* mutations with or without a loss of

one TP53 allele are strongly associated with a complex aberrant karyotype in AML and MDS, when compared with the values of the patients without a complex aberrant karyotype[3,27]. Since AML with markedly abnormal karyotypes may be genetically unstable, and contain a high frequency of point mutations in some genes, it is generally held that inactivation of *TP*53 is mainly correlated with an advanced clinical stage[5]. In the contrast, in patient no. 6, der(5;17)(p10;q10) was recognized as the sole chromosomal abnormality at onset. Additionally, the *TP53* mutation was not detected in PB leukocytes at least 9 months before the diagnosis of MDS. Therefore, biallelic inactivation of the *TP53* gene might have played an important role in the occurrence of MDS in this case. Nevertheless, cooperation between loss of *p53* function and loss of a putative tumor suppressor gene at 5q cannot be excluded[28].

Serial FISH analyses showed that the abnormal clone with 3 copies of the *MLL* gene was at a negligible level before 1st BMT, but the clone became a major population of leukemic cells after the 2nd HSCT. Interestingly, according to the SKY and FISH analyses of cultured cells grown from CD34^{+} BM cells under stimulation with hematopoietic growth factors, the patient had two clones at diagnosis: one clone with der(5;17), and the other clone with both der(5;17) and der(7)t(7;11), which appeared to contain the extra copy of the *MLL* gene. Taken together with our previous report on JMML patients who had 6-mercaptopurine-refractory clone[11], it is suggested that the minor clone with both *TP53* gene alteration and *MLL* duplication already existed at onset, and that poor outcome was associated with resistance of this clone to chemotherapy and radiation.

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Authors' Contributions

K.K. and S.S. designed and performed research, analyzed data, and wrote the paper; K.M. designed and performed research, collected samples, analyzed data; C.T. and K.S.

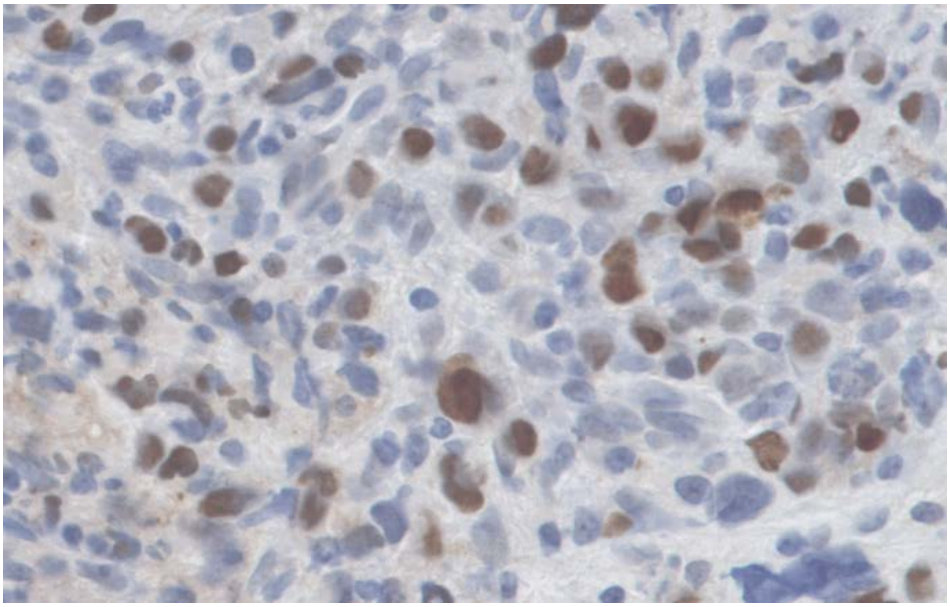
performed research and analyzed data; M.T-Y., R.Y., Y.N., K.S., and M.S. collected samples and analyzed data.

Conflict of Interst

The authors disclose no actual or potential conflict of interest.

Figure 1

A



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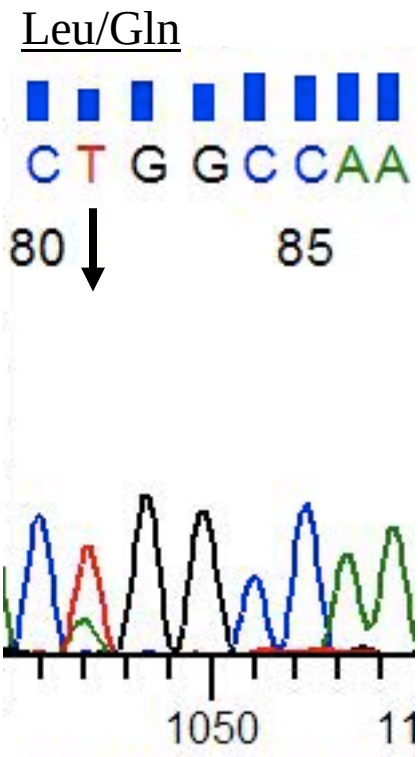
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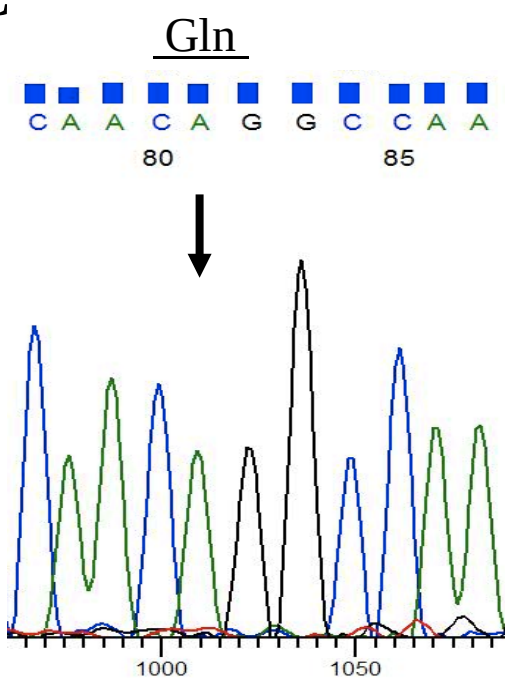
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Figure 2

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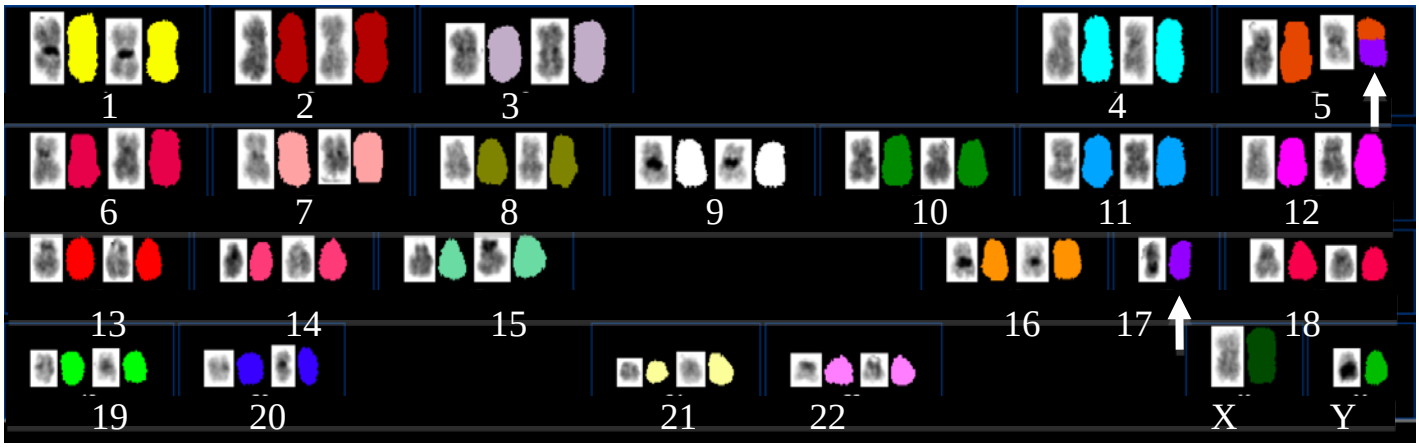
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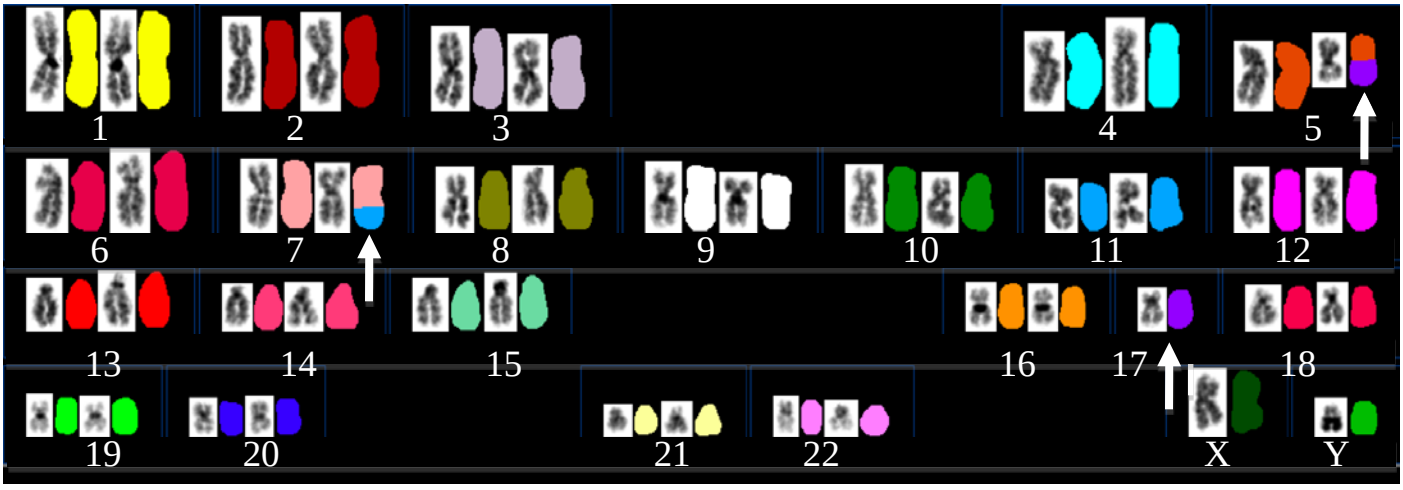
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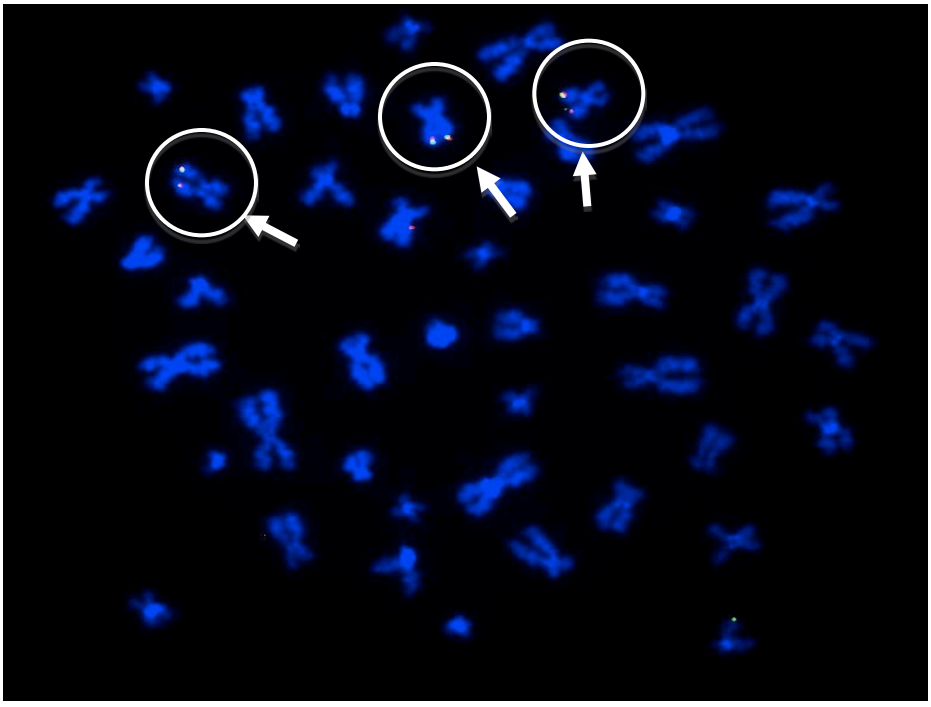

Figure 3

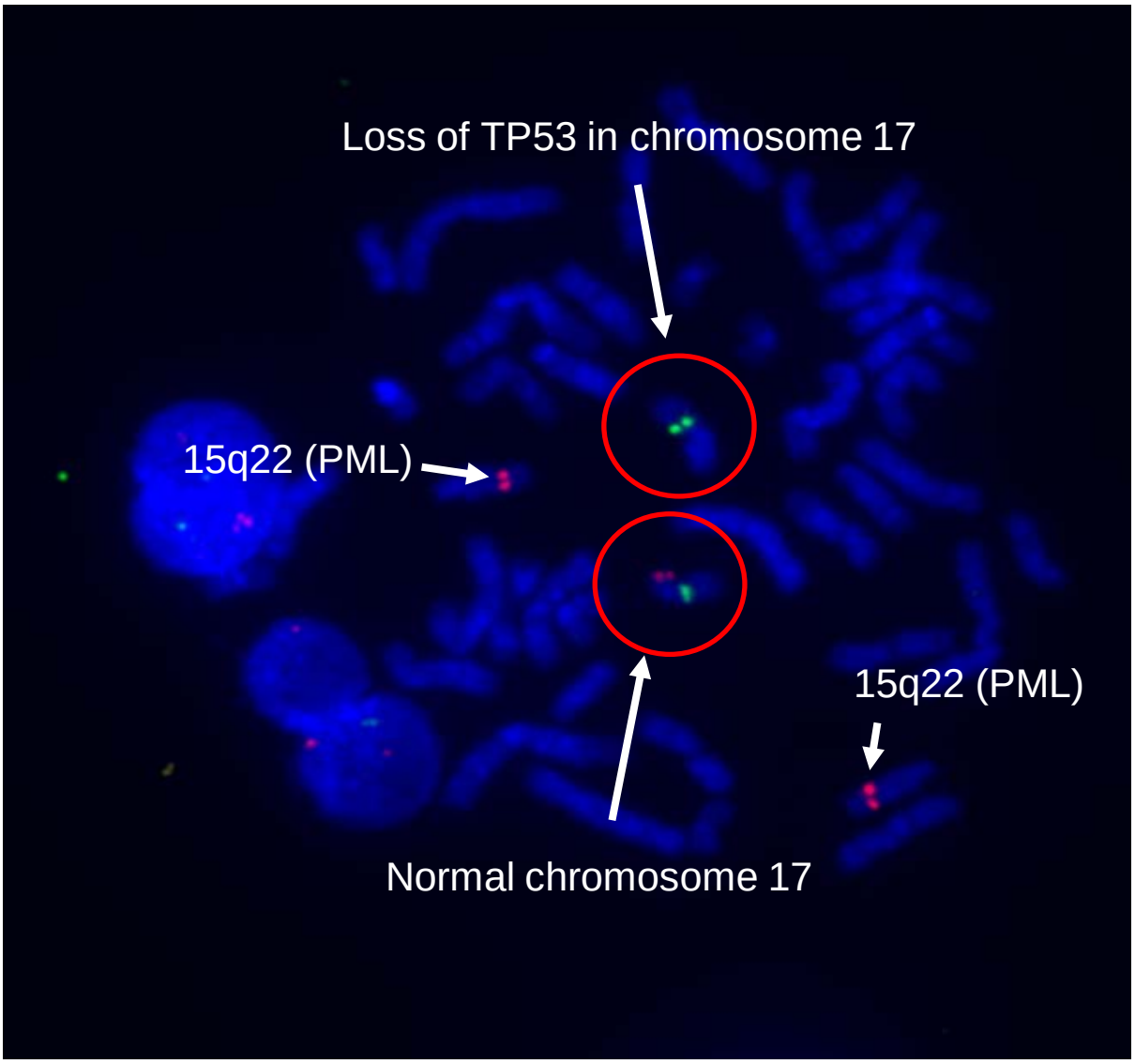
Loss of TP53 in chromosome 17
15q22 (PML)
15q22 (PML)
Normal chromosome 17

Figure legends

Figure 1. Immunocytochemical staining for p53 and direct sequencing analysis of TP53 gene at diagnosis in patient no. 6.

(A) Paraffin-embedded BM section. Approximately 20% of BM cells were positive for p53. (B, C) The direct sequencing traces showing exon 5 of TP53 gene in circulating mononuclear and $CD14^+/CD15^+$ cells. The heterozygous missense mutation was observed in PB mononuclear cells, whereas the homozygous mutation was observed in $CD14^+/CD15^+$ PB cells.

Figure 2. der(7)t(7;11) and *MLL* gene duplication in GM colony-constituent cells grown from $CD34^+$ BM cells at onset under stimulation with a combination of hematopoietic growth factors in patient no. 6.

(A, B) Sky analysis revealed that one of three GM colony-constituent cells possessed der(5;17) and the remaining 2 cells had both der(5;17) and der(7)t(7;11). (C) FISH analysis revealed 3 signals for *MLL* in 4 of five GM colony-constituent cells.

Figure 3. FISH analysis of BM cells using *TP53*, *RARα*, and *PML* probes in patient no. 6.

Half of BM cells had 1 signal for *TP53*. *TP53* on 17p13.1, red; *RARα* on 17q21, green; *PML* on 15q22, red.

Table 1 Karyotypic and genetic abnormalities in bone marrow or peripheral blood mononuclear cells of 27 patients with MDS or JMN

Case (No.)	Age(y)/ Sex	WHO type	Karyotype (G-banding)	TP53 gene Deletion	TP53 gene Mutation	p53 overexpression (cytospin)	Mutations PTPN11	Mutations KRAS	Mutations NRAS	Mutations Others
1	10/M	RCC	47,XY,+8 [6], 46,XY[13]	-	-	1.0%	wt	wt	wt	-
2	1/F	RCC	47,XX,+6,add(11)(q13)[4], 46,XX[17]	-	-	ND	wt	wt	wt	-
3	14/M	t-MDS	46,XY,der(7)t(1;7)(q12;q11)[6] 46,X,del(Y)(q11.2), der(21)t (1;21)(q25;q22) [5], 46,XY[8]	-	-	1.0%	wt	wt	wt	-
4	17/M	RCC→RAEB-2	45,XY,-7[19]	-	-	3.0%	wt	wt	wt	-
5	13/M	RAEB-2	47,XY,+X[18]	-	-	0.5%	1508G>T	wt	wt	-
6	1/M	RAEB-1→AML-	45,XY,der(5;17)(p10;q10)[17],	51.6%	410T>A	51.5%	wt	wt	wt	-
7	3/F	RAEB-2	46~48,XX,-7,+8,add(9)(p24), add(18)(q21),+19,+r[cp21]	-	-	ND	wt	wt	wt	-
8	4/F	AML-MRC	47,XX,+8[19]	-	-	0.5%	227A>C	wt	wt	-
9	5/M	AML-MRC	45,XY,-7[18]	-	-	0.5%	1508G>A	wt	wt	FLT3-ITD
10	2/F	JMML	46, XX	-	-	0.5%	182A>T	wt	wt	-
11	0/M	JMML	ND	-	-	0.0%	214G>A	wt	wt	-
12	4/M	JMML	46,XY	-	-	2.5%	226G>A	wt	wt	-
13	0/M	JMML	46,XY	-	-	1.5%	226G>A	wt	wt	-
14	2/F	JMML	46,XX	-	-	3.5%	226G>A	wt	wt	-
15	1/M	JMML	46,XY	-	-	1.5%	227A>G	wt	wt	-
16	5/M	JMML	46,XY	-	-	3.5%	227A>G	wt	wt	-
17	1/F	JMML	46,XX	ND	-	1.0%	wt	35G>A	wt	-
18	0/F	JMML	46,XX	-	-	0.0%	wt	35G>A	wt	-
19	0/F	JMML	46,XX	-	-	2.0%	wt	34G>A	wt	-
20	1/F	JMML	46,XX	ND	-	1.5%	wt	38G>A	wt	-
21	0/M	JMML	46,XY	-	-	1.0%	wt	wt	34G>A	-
22	0/M	JMML	46,XY	-	-	1.5%	wt	wt	34G>A	-
23	1/F	JMML	46,XX	-	-	1.0%	wt	wt	35G>A	-
24	2/M	JMML	46,XY	-	-	1.0%	wt	wt	35G>A	-
25	0/F	JMML	46,XX	-	-	1.0%	wt	wt	38G>A	-
26	0/M	JMML(NF1)	47,XY, +8[2], 46,XY[18]	-	-	1.5%	wt	wt	wt	-
27	3/M	JMML(NF1)	46,XY	-	-	1.0%	wt	wt	wt	-

Since 0.94 ± 0.43% (0% to 1.81%) of bone marrow or peripheral blood mononuclear cells had 1 signal for TP53 gene in 17 normal controls, the cut-off value of TP 1.8%. Since 1.25±1.18% (0-3.6%) of PB mononuclear cells spread on glass slides were stained with anti-p53 monoclonal antibody in 6 normal controls, the cut-off was defined as 3.61%.

AML-MRC, AML with myelodysplasia-related changes; F, female; FISH, fluorescence in situ hybridization; FLT3-ITD, FMS-like tyrosine kinase 3 internal tanden myelomonocytic leukemia; M, male; ND, not detected; NF1, neurofibromatosis type 1; RAEB, refractory anemia with excess blasts; RCC, refractory cytopenia of cl

Table legend

Table 1. Karyotypic and genetic abnormalities in bone marrow or peripheral blood mononuclear cells of 27 patients with MDS or JMML.

Since 0.94±0.43% (0% to 1.81%) of bone marrow or peripheral blood mononuclear cells had 1 signal for TP53 gene in 17 normal controls, the cut-off value of TP53 deletion was defined as 1.8%. Since 1.25±1.18% (0-3.6%) of PB mononuclear cells spread on glass slides were stained with anti-p53 monoclonal antibody in 6 normal controls, the cut-off value of p53 overexpression was defined as 3.61%.

AML-MRC, AML with myelodysplasia-related changes; F, female; FISH, fluorescence in situ hybridization; FLT3-ITD, FMS-like tyrosine kinase 3 internal tandem dupulication; JMML, juvenile myelomonocytic leukemia; M, male; ND, not detected; NF1, neurofibromatosis type 1; RAEB, refractory anemia with excess blasts; RCC, refractory cytopenia of childhood; t-MDS, therapy-related myelodysplastic syndrome; wt, wild type;