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平成２０年度学位申請論文

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ABSTRACT

P53 **mutation has been reported in various solid tumors, acute leukemia and myelodysplastic syndrome (MDS). However, the diagnostic significance of p53 in MDS remains to be determined. We examined *p53* mutation and immunostaining of the same patients, since there have been few reports of simultaneous analysis of these markers. Seven *p53* mutations were observed among 37 MDS and 11 cases of overt leukemia transformed from MDS (MDS-OL). Mutated *p53* mainly observed in high-risk MDS showed more intense p53 staining than those of MDS cases with wild-type *p53* overexpression. Aplastic anemia (AA) showed no p53 staining. The percentage of p53 staining of MDS (71%) was higher than that of mutated *p53* cases (11%) but did not reach 100% of MDS cases studied. Therefore, we tried to differentiate MDS, especially RA and AA using a combination of p53 immunostaining, HbF immunostaining and chromosome abnormality, because Hemoglobin F (HbF) of erythroblasts was reportedly observed in MDS refractory anemia (RA) but not in AA. Most MDS/MDS-OL (47/48) showed at least one positive marker, whereas 11 AA cases proved totally negative except for two positive HbF cases. Our results suggest that the combination of these three markers is useful to discriminate MDS from AA.**

Key words: aplastic anemia, differential diagnosis, HbF, immunostaining, karyotype analysis, myelodysplastic syndrome, p53 mutation study, p53 protein

INTRODUCSION

Myelodysplastic syndrome (MDS) is a heterogeneous disease characterized by bi- or pancytopenia. MDS-associated chromosomal abnormalities such as monosomy 7, 5q-, and trisomy 8 have been reported, suggesting the involvement of these abnormalities in its pathogenesis.[1] Compared to *de novo* leukemia whose pathogenesis is adequately explained by chromosomal translocation, the molecular events involved in the pathogenesis of MDS are far from being completely elucidated. This must involve an important issue, i.e., disease progression as certain percentage of patients deteriorate to overt leukemia. Such progression may constitute a transition among the subtypes classified by the percentage of blast cells, which is regarded as one of many steps toward acute leukemia.[2]

Though pancytopenia is also a feature of aplastic anemia (AA), a substantial decline in the hematopoietic stem cell population, which is not the case with MDS, is considered fundamental to the pathogenesis of AA. The effectiveness of immunosuppressive therapy for AA patients suggests that most, if not all, AA cases are caused by immunological disorder.[3] Therefore, the treatment strategy is quite different between MDS refractory anemia (RA) and AA, although recent reports have suggested the limited effectiveness of immunosuppressive therapy for some RA patients.[4]

Because of the paucity of reliable RA markers, the differential diagnosis between RA and atypical AA is sometimes very difficult. Morphological diagnosis is one of the best ways,[5] but the quantitative evaluation of morphological dysplasia is not always easy. Recent progress in the microarray analysis of a purified blast population,[6,7] whole genome painting, [8] or array-based comparative genomic hybridization[9] has made it possible to detect a unique message expression pattern and chromosomal aneuploidy as well as defects in small genomic regions. Analysis using these methods might uncover a reliable marker for differential diagnosis between RA and AA. These analyses, however, require special equipment and involve costly measurements. Furthermore, the significance of MDS-specific gene detected by microarray analysis such as delta-like protein (Dlk)[6] remains to be determined. Therefore, these latest technologies cannot be immediately introduced into the laboratory routine. More cost-effective and practical methods are required for the differential diagnosis of MDS RA and AA.

In the clinical practice of MDS diagnosis, simple laboratory examination is desirable. One of the candidates is *p53*, a well-known tumor suppressor gene whose expression and activation were enhanced by extracellular stresses. p53 regulates cell cycles through p21 protein and activates a process of either DNA repair or apoptosis depending on the severity and the kind of the stresses imposed on the cells.[10] Furthermore, mutation and/or loss of *p53*

gene are the single most common genetic events observed in human cancer as well as in hematological malignancies,[11,12] and these genetic alterations are known to eliminate the p53 function itself. According to a previous report, there is a large variety of p53 expressions in MDS bone marrow.[13] Of particular interest in the report is the presence of a discrepancy between the mutation and protein expression of the *p53* gene.[13,14] This raises the question of whether such a discrepancy is a common feature of MDS. Only limited information, however, is available regarding this issue.

The immunostaining of p53 has several advantages in clinical practice than mutation survey of *p53* such as WAVE method following direct sequence because of its easy applicability. However, it is known that the simple immunostaining of p53 is not informative in a substantial number of tumors. P53 positive staining means either mutated p53 or increased normal p53 protein as the stress response. To clarify the difference between *p53* mutation and the percentage of p53 stained positive cells, we tested both *p53* mutation and p53 protein expression in the same clot samples of MDS.

Our current data suggested that p53 immunostaining was not sufficient to completely discriminate among low-risk MDS, especially RA from AA. We previously reported that HbF-positive erythroblasts are increased in the bone marrow of MDS patients compared to those of AA patients.[15,16] A typical karyotype abnormality involving either chromosome 5, 7 or 8 will definitely confirm that the patient is suffering from MDS. It should be noted that chromosome abnormality is included as the index of an IPSS system for MDS.[17] We can get these data as the patient characteristics. Therefore, we explored HbF expression and karyotypes in search of markers used to distinguish between RA and AA, since the point of interest in this study is whether the status of p53 or HbF expression provides any useful information in the diagnosis of MDS for patients with the normal karyotype. Although we had only three hypoplastic RA cases in the present study, these cases are presented and discussed in terms of three indexes we proposed.

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MATERIALS AND METHODS

Clinical samples

Bone marrow clot sections (from the bone marrow case files of the Department of Pathology, Nagoya University Hospital, May 2001~May 2005) were selected from 37 patients with MDS (19 RA, 1 RARS, 13 RAEB, 3RAEB-T, 1 CMML), 11 with overt leukemia transformed from MDS (MDS-OL) according to the FAB classification, and 11 with AA. Most of the marrow clots were taken from patients in the Department of Hematology and Oncology, Nagoya University School of Medicine, and used after obtaining informed consent. Samples were obtained at their initial diagnosis or from patients without prior therapy. Each clot was subjected to both immunohistochemistry and DNA analyses.

Immunohistochemistry

Serial sections of paraffin-embedded bone marrow clot were immunohistochemically stained for HbF and p53 using the streptavidin-biotin complex method. We used an anti-HbF antibody that we recently developed[15] and a mouse monoclonal anti-p53 antibody (NCL-p53 DO7; Novacastra, Newcastle upon Tyne, UK) that recognizes both wild and mutant type p53. Antibodies against mdm2 (SMP14) and p21WAF1/Cip1 were purchased from Dakocytomation (Carpinteria, CA, USA). For the detection of HbF (+) erythroblast, sections were treated with 0.1% trypsin in 0.05M Tris buffer (pH 7.0) at 37^{o}C for 30 min. To examine the p53 expression, sections were microwaved in citrate buffer, pH 7.0, for 15 min for antigen retrieval. Known positive controls (29-week gestated fetal liver for HbF, and a colorectal carcinoma for p53) as well as negative controls (sections in which the primary antibody was replaced by non-immune mouse or rabbit serum) were also stained. We determined that p53 was positively expressed when more than 5% of cells were positively stained. The staining of the p53 signals was divided into two categories: weak, light brown nuclear staining and strong, dark brown staining as shown in Figure 2. Positive expressions of mdm2 and p21 were determined if positive cells were present in the section observed. To test HbF expression, 1000 marrow cells were examined, and the presence of at least one HbF-expressing erythroblast prompted the conclusion that the bone marrow was HbF-positive.

Detection of p53 mutations

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks of bone marrow using DEXPAT (Takara Bio Inc., Kyoto, Japan). *p53* gene (from exon 5 to exon 8) was amplified with PCR. The sense and antisense primers for each exon were as follows:

p53 exon 5: sense 5'-TTCCTCTTCCTACAGTACTC-3'

antisense 5’-GCAACCAGCCCTGTCGTCTC-3’

exon 6: sense 5’-ACCATGAGCGCTGCTCAGAT-3’

antisense 5’-AGTTGCAAACCAGACGTTCGA-3’

exon 7: sense 5’-GTGTTGTCTCCTAGGTTCGC-3’

antisense 5’-CAAGTGGCTCCTGACCTAGGTTCGC-3’

exon 8: sense 5’-CCTATCCTGAGTAGTGGTAA-3’

antisense 5’-TGAATCTGAGGCATAACTGC-3’

Subsequently, each PCR product was subjected to WAVE denaturing high-performance liquid chromatography analysis (Transgenomic Inc., Omaha, NE, USA) for screening of mutations. This method is based on ion-pair, reverse-phase high-performance liquid chromatography and temperature-modulated hetero-duplex analysis. Sequence variations create mismatch hetero-duplexes during reannealing of PCR products containing wild-type and mutant sequences. The difference in melting temperature between hetero-duplexes and homo-duplexes allowed for their separation by ion-pair, reverse-phase high-performance liquid chromatography and the identification of the presence of mutations. PCR products with mutant sequences were detected with the protocol specified by the manufacturer. First, 5 μl of PCR product was tested under non-denaturing conditions to determine the DNA concentrations. The PCR sample was then mixed with an equal amount of PCR-amplified control DNA without a mutation to assure detection of a mutation upon the loss of heterozygosity (LOH). The mixture was denatured at 95℃ for 4 min and gradually cooled down to 25^{o}C for 45 min. The denatured and re-annealed sample was run on the WAVE instrument. The melting temperatures were 63^{o}C for p53 exons 5 and 8, and 62^{o}C for exons 6 and 7. The sensitivity of WAVE analysis was evaluated using DNA samples with a known *p53* mutation that were sequentially diluted by DNA with the wild type *p53* samples. Results of the WAVE analysis were confirmed by direct sequencing. Detection of a heterogeneous pattern by direct sequencing could have been the results of either heterozygosity in the leukemic cells or the presence of nonleukemic cells in the specimen.

Statistical analysis

Statistical analysis was performed with Microsoft Excel and attached software.

RESULTS

Mutation study and immunohistochemical analysis of p53 gene in MDS and MDS-OL

The characteristics (sex, age, FAB classification, bone marrow cellularity, *p53* mutation, p53 immunostaining (IHC), HbF staining, karyotype) of patients enrolled in this study were summarized in Table 1. DNA samples extracted from their bone marrow clot section were analyzed for *p53* mutation using the WAVE system. Hetero-duplex formation was evident in samples with mutated *p53* samples, which was not the case in those with wild type *p53* (precise data not shown). We detected 7 *p53* mutations (3: MDS-OL; 4: MDS) out of 48 analyzed. Mutations were present predominantly in exon 5, with the percentage of *p53* mutation being 14% in our MDS/MDS-OL cohort (Table 1). Three out of 4 MDS patients with *p53* mutation had been diagnosed as RAEB, while 5 out of the 6 patients carrying the mutated *p53* also have chromosomal abnormality. It should be noted that no *p53* mutation was detected in patients with AA (Table 1).

Using the same clot section, p53 protein expression was tested by immunostaining. The sensitivity of the p53 staining had been confirmed using a control cell line either with or without *p53* mutation. Representative results obtained from patients' clot sections showing p53-negative, -weak positive, and -strong positive are illustrated in Figure 2A. p53 positive cells were mononuclear cells with nucleolus, fine chromatin structure and scanty cytoplasm, suggesting them as the myeloblast like cells (data not shown). Based on our cut-off criteria, 34 out of 48 MDS/MDS-OL cases (71%) showed p53 positive staining, while we failed to detect p53 expression in any of the 11 AA patients (Table 1). As shown in Figure 1A, the frequency of p53 positive cells seemed to be correlated to the percentage of blast cells. Mutated *p53* was apparently related to the strong p53 signal (1B and data not shown). Of particular interest is the fact that p53 was overexpressed in the bone marrow of 27 MDS/MDS-OL patients in the absence of gene mutation, suggesting that overexpressed wild type p53 was involved with positive staining. The reason for the negative p53 staining in AA is not clear at present, however, it might suggest the possibility that stress response observed in some MDS cases was not present in our AA cases whose disease severity was mostly of the moderate grade.

Based on these results, the involvement of wild type p53 overexpression in the apoptotic process (ineffective hematopoiesis in MDS) might be considered. Our rationale was that the expression level of normal p53 protein is regulated by its partner protein, mdm2,[14] and that the expression of p21 is up-regulated by activated wild type p53 in cells under stress. To explore the mechanisms underlying p53 overexpression, we carried out mdm2 and p21 immunostaining. Unexpectedly, mdm2 was mostly expressed in MDS-OL (7/11 cases),

whereas positive mdm2 staining was rare in cases of both high-risk MDS (RAEB and RAEB-T; 1/16) and low-risk MDS (RA and RARS: 1/20) cases. No relationship was observed between *p53* mutation and mdm2 expression (data not shown). We failed to detect p21 in any of the samples including clot sections of normal bone marrow, although p21 was well stained in normal buccal mucosa (data not shown).

Differential diagnosis between MDS and AA

Cost effectiveness and easy applicability of the examination method are points of importance in the practice of laboratory medicine. The data shown above suggested that no single examination including p53 immunostaining was sufficient for a full differential diagnosis between whole MDS and AA patients. We next tried to analyze whether the combination of some markers is advantageous for differential diagnosis. As the second marker, we adopted HbF staining, which was based on our previous observation.[16] An example of positive HbF staining was shown in Fig. 2. HbF-positive erythroblasts were observed in 35 out of 47 MDS patients (74%, data not available in one case, Table 1), a percentage similar to that of our previous observation[11] which was obtained from the analysis of a different group of patients. Two out of 11 AA cases were shown to be HbF-positive (18%). We previously reported that positive HbF staining in hypoplastic RA denotes ineffective erythropoiesis.[18] However, HbF staining in high-risk MDS was also observed in the present investigation.

Results of karyotype analysis are illustrated in Table 1. The percentage of abnormality in MDS was 43% in total, with CMML and MDS-OL showing a higher percentage of abnormality, while there was no significant difference in chromosomal abnormality among RA and RARS (35%), RAEB (33%) and RAEB-T (33%). The percentage of chromosome abnormality with poor prognosis as suggested in IPSS (more than three anomalies and/or any anomalies of chromosome 7) was 2/19 in RA, 4/16 of RAEB/RAEB-t, and 5/12 in OL. AA patients showed no chromosomal abnormality whatsoever.

Results of the combination of these three markers (chromosomal abnormality, HbF staining and p53 staining) are illustrated in Figure 3. Using this combination, only one out of 48 MDS /MDS-OL patients were negative for all three markers. We then focused on the comparison between MDS RA and AA, since discrimination of these two is the most important and difficult task. All MDS-RA patients showed at least one of these three markers. However, nine out of 11 AA patients showed no positive marker at all. Two AA patients showed positive HbF. No p53 staining was observed in the AA group. The difference between MDS/MDS-OL and AA or between MDS RA and AA were both highly statistically significant ($p<0.0001$).

Among 19 RA patients, three showed hypo-cellular marrow with bone marrow cellularity of 30% or less. The p53 staining of hypoplastic RA case 8 and of a representative AA bone

marrow smear was shown in Figure 2. Case 1 showed 10% of p53 positive staining and subsequent bone marrow examination (one month later) revealed der (1:9), +16. Case 8 was transferred to another hospital after the initial survey, and we could not obtain further information. However, his bone marrow smear showed dysplasic findings in both erythroid and megakaryocyte lineages. Case 15 was trisomy 8 and HbF positive staining.

DISCUSSION

P53 is a potent tumor suppressor gene, and its genetic mutation is considered to be a major oncogenic step in many human malignancies. Based on this, the mutation of *p53* gene has been investigated in MDS bone marrow by a number of hematological researchers. Its frequency, however, is not consistent among the reports.[1,13] In this study, we reported that *p53* mutation was observed in 14% of all MDS/MDS-OL patients. Similar results can be found in previous reports.[11,13] Of note is that 5 out of 7 mutations were detected in patients with high risk MDS or MDS-OL that carry chromosomal abnormality (Table 1), suggesting that p53 mutation is involved in the disease progression of MDS rather than being the initial event in the pathogenesis of MDS. A similar speculation regarding the role of p53 in MDS has been mentioned by Elghetany.[19]

Previous immunohistochemical analysis of bone marrow specimens revealed that p53 protein was expressed in 14 to 20% of MDS patients.[20] However, the selection of anti-p53 antibody reportedly affects the positive percentage of p53 immmunostaining.[21] Using the same anti-p53 antibody that we used, Elghetany[19] reported that 57% of hypo RA and 79% of hyper RA were stained p53 positive, while no p53 was detected in AA. Our data confirmed his results.[19] In our previous preliminary report focusing on hypo MDS RA, p53 expression of hypo RA was 75%, while AA and hematological normal bone marrow showed 6% and 0%, respectively.[16] In the present study, two out of 3 hypo MDS RA cases showed positive p53 staining, whereas AA cases did not show p53 positive.

There were a few reports analyzing the *p53* mutation and p53 immunostaining of MDS samples simultaneously.[20] In agreement with their data, we observed that about 80% of p53 over-expressions occurred in the absence of mutation (27/34 patients). The immunohistochemical signals of p53 were generally strong in patients with a mutated *p53* (Figure 1B). This in turn suggests the possibility that one can predict the presence of mutated *p53* by the strength of p53 staining. Strong signals, however, were also detected in a small number of patients without *p53* mutation (Figure 1B).

To explore the mechanisms underlying p53 overexpression in the absence of mutation, we tested the expression of mdm2 immunohistochemically, since the half-life of wild type p53 protein is controlled by mdm2. Considering this regulatory mechanism, the best scenario would have been that reduced mdm2 allowed the accumulation of wild type p53 in bone marrow. In our preliminary study (data not shown), we observed that mdm2 was aberrantly expressed in some MDS-OL but not in normal control bone marrow as well as MDS, which may suggest that aberrant mdm2 expression occurred in the late phase of leukemogenesis. Therefore, it is less likely that decreased mdm2 expression is the cause of normal p53

overrexpression in MDS, although it may not be ruled out. Another possibility is that some stress induced p53 in MDS but not in AA bone marrow. However, further analysis is necessary to elucidate our observed phenomenon.

For the differential diagnosis between MDS and AA, especially MDS-RA and AA, we propose a combination of three markers, the immmunostaining of p53 and HbF, and the presence of chromosomal abnormalities. Serum HbF is increased and is of prognostic significance of MDS.[22] In HPLC analysis, Kudo et al. [23] reported that the HbF/total Hb ratio is highest among various hematological diseases. With the same anti-HbF antibody that was also used in the present study, Choi et al. [15,16] showed that HbF-positive erythroblasts are increased in the bone marrow of MDS patients compared to those of AA patients and that these increases might be correlated with the increase of apoptosis in MDS. The percentage of positive HbF cases in that paper was reported to be around 80% of MDS based on the same criteria that we used in this experiments. In the current study, the percentage was 71% in total.

In a recent paper analyzing a large number of MDS cases[24], chromosome abnormality was observed in 52% of patients. In our cases, 36% of MDS and 60% of MDS-OL patients showed chromosome abnormality. Figure 3 clearly showed that three markers, i.e., immunostained p53 and HbF, and karyotype, are informative for a differential diagnosis between MDS and AA, and especially between MDS-RA and AA. These examinations can be performed in a routine clinical setting requiring no special and expensive equipment.

Hypoplastic MDS were reported to consist of 15% of all adult MDS[25] and be less anemic, more neutropenic and thrombocytopenic. However, these unique characteristics are not of help to distinguish between each hypoplastic MDS and AA upon their presentation. Vardiman reviewed the difficulty in differential diagnosis between AA and hypoplastic MDS.[26] He described the multi-parameter flow cytometry and the percentage of Ki-67 positive cells as well as HbF cells[16] as methods for further examination. In the current study, all our cases possessed at least one positive finding in three examinations, whereas 2 out of 11 cases of AA only showed HbF-positive. We only experienced three cases of hypoplastic MDS. Two out of three cases showed two positive markers and another case showed p53 positive staining and definitive morphological dyplasia, which is excluded from our present combination of differential markers. Although further analysis using a much larger patient sample is needed, our current results suggest the clinical usefulness of the combination of these three markers in the diagnosis of hypoplastic MDS as an easily applicable clinical laboratory examination.

Taken together, our present data clearly show that immunostaining for p53 and HbF as well as karyotype analysis are useful for the differential diagnosis between MDS-RA and AA.

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Table 1. Characterization of MDS and MDS-OL patients enrolled in this study

UPN	Sex	Age	FAB	BM cellularity	p53 mutation	p53 IHC	HbF IHC	Karyotype
1	F	30	RA	I	−	+	−	Normal
2	M	48	RA	II	−	2+	+	Int
3	F	27	RA	II	+	+	+	Good
4	M	65	RA	II	−	+	+	Normal
5	M	72	RA	III	−	+	+	Normal
6	M	39	RA	III	−	2+	+	Int
7	M	55	RA	II	−	+	+	Poor
8	M	35	RA	I	−	+	−	Normal
9	F	53	RA	III	−	−	+	Normal
10	M	63	RA	II	−	−	+	Normal
11	F	74	RA	III	−	−	+	Normal
12	F	69	RA	II	−	2+	+	Poor
13	F	53	RA	II	−	−	−	Int
14	F	63	RA	II	−	+	+	Normal
15	M	74	RA	I	−	−	+	Int
16	F	74	RA	III	−	−	+	Normal
17	M	59	RA	II	−	−	+	Normal
18	F	75	RA	II	−	−	+	Normal
19	F	57	RA	III	−	−	+	Normal
20	M	43	RARS	III	−	+	−	Normal
21	M	57	RAEB	III	−	2+	+	Poor
22	M	70	RAEB	II	−	+	+	ND
23	M	60	RAEB	III	−	2+	+	Poor
24	F	57	RAEB	III	−	+	−	Normal
25	F	71	RAEB	III	−	+	ND	Normal
26	F	46	RAEB	III	−	2+	+	Normal
27	M	75	RAEB	II	−	+	−	Normal
28	F	67	RAEB	III	−	+	+	Normal
29	F	55	RAEB	III	+	+	−	Int
30	M	64	RAEB	III	−	−	+	Normal
31	F	57	RAEB	III	−	−	+	Normal
32	M	84	RAEB	II	+	+	−	Normal
33	M	74	RAEB	II	+	+	+	Poor
34	M	42	RAEB-T	III	−	+	+	Normal
35	M	54	RAEB-T	III	−	+	+	Normal
36	M	53	RAEB-T	III	−	2+	+	Poor
37	M	67	CMML	III	−	2+	+	Int
38	M	69	OL	III	+	2+	+	Poor
39	M	61	OL	III	−	2+	−	Poor
40	F	79	OL	II	−	−	+	Poor
41	M	61	OL	III	+	2+	+	Poor
42	M	56	OL	III	−	2+	+	Good
43	M	55	OL	III	−	+	+	Normal
44	M	58	OL	III	−	+	+	Normal
45	M	59	OL	III	−	+	+	Normal
46	M	53	OL	III	−	−	−	Normal
47	F	81	OL	II	+	2+	−	dry tap
48	F	56	OL	III	−	−	−	Poor

Abbreviations: IHC, immunohistochemistry; RA, refractory anemia; RARS, refractory anemia with ringed sideroblast; RAEB, RA with excessive blast; RAEB-t, RAEB in transformation; CMML, chronic myelomonocytic leukemia; OL, overt leukemia transformed from MDS; AA, aplastic anemia

Grading of BM cellularity was as follows. I: < 30%, II: 30~60%, III: >60%

Good, Intermediate and Poor karyotypes were according to IPSS classification.

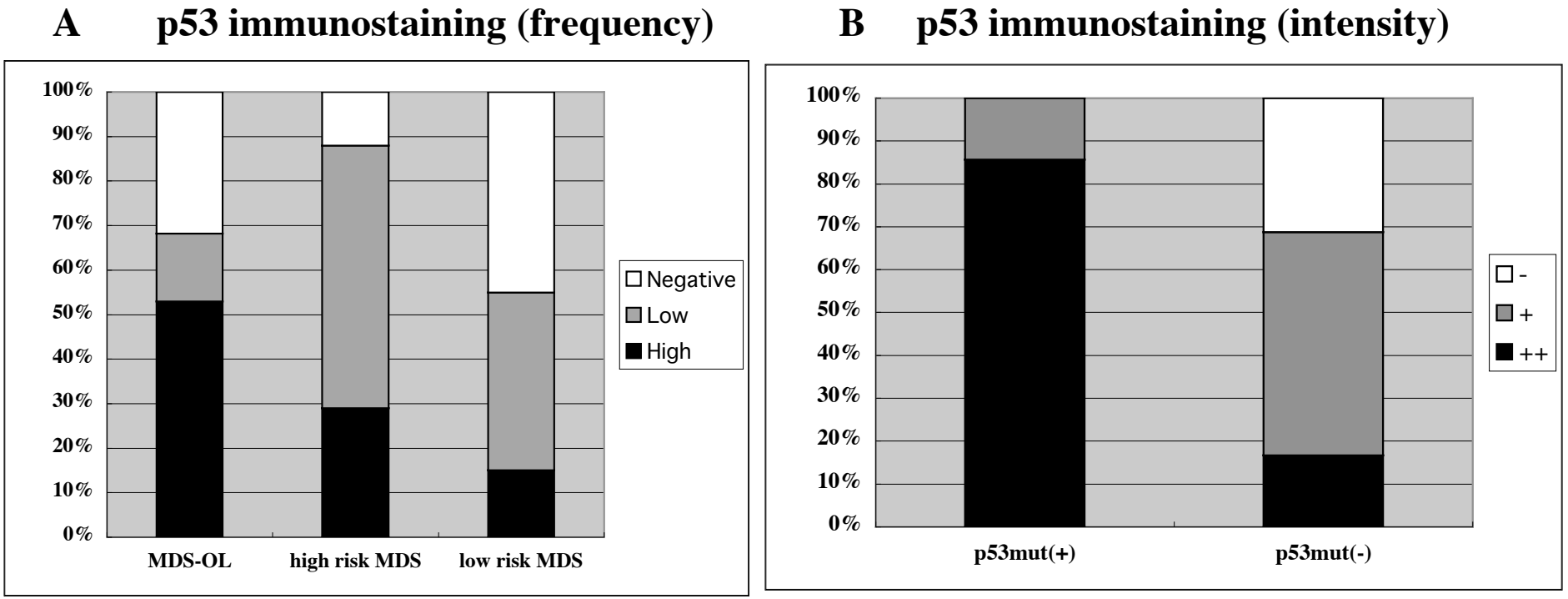
Figure legends

Figure 1 (A) Relationship between frequency of p53 positive cells and MDS stage. Relationships among disease status, MDS-OL, high-risk MDS (RAEB and RAEB-T), low-risk MDS (RA and RARS) and frequency of p53 positive cells are shown. High (solid column) denotes more than 20% of p53 positive cells. Low (grey column) denotes p53 positive cells are more than 5% and less than 20%. Open column shows negative staining. (B) Distribution of intensity of p53 staining among cases with/without *p53* mutation. Weak (+) and strong (++) p53 staining was determined according to criteria described in the Materials and Methods.

Figure 2 Immunohistochemical staining of p53 and HbF. (A) Representative cases of negative, weak and strong positive p53 immunostainings were shown. (B) Comparison of p53 staining observed in hypoplastic RA case 8 and a representative AA case. (C) HbF; Typical positive and negative HbF staining patterns are shown. Black bar denotes 10 μM.

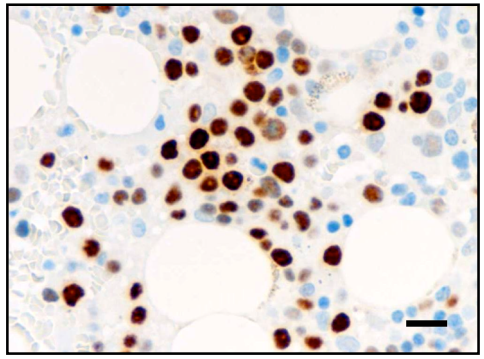
Figure 3 Classification of patients according to markers, i.e., positive p53 staining, positive HbF staining and chromosomal abnormalities. Patient groups in this classification were: Total MDS/MDS-overt leukemia (MDS-OL) (48 cases): MDS-RA/PARS (20 cases): and AA (11 cases). Number of cases is indicated in appropriate area. Each circle encloses the positive number of cases.

Figure 1

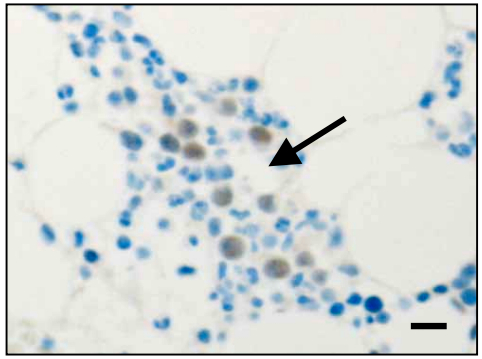

A p53 immunostaining (frequency)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
MDS-OL
high risk MDS
low risk MDS
Negative
Low
High
B p53 immunostaining (intensity)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
p53mut(+)
p53mut(-)
-
+
++

A. P53

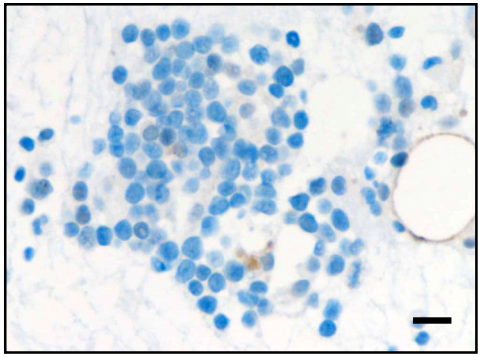
strong positive Case 47



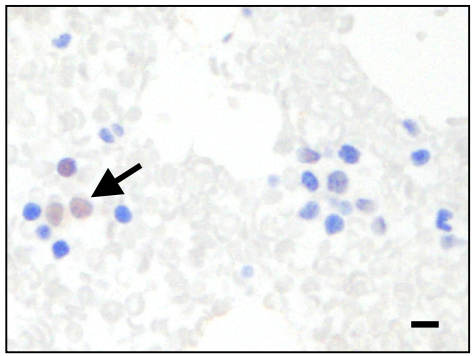
weak positive Case 22



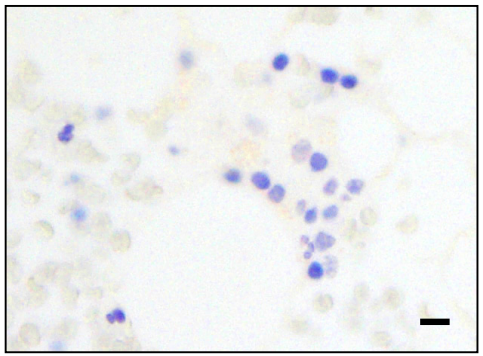
negative Case 30



Hypo MDS Case 8

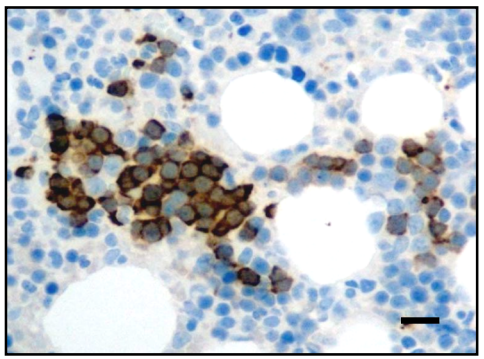


AA



B. HbF

positive Case 26



negative Case 39

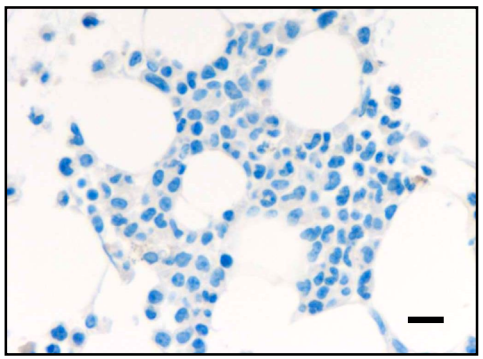
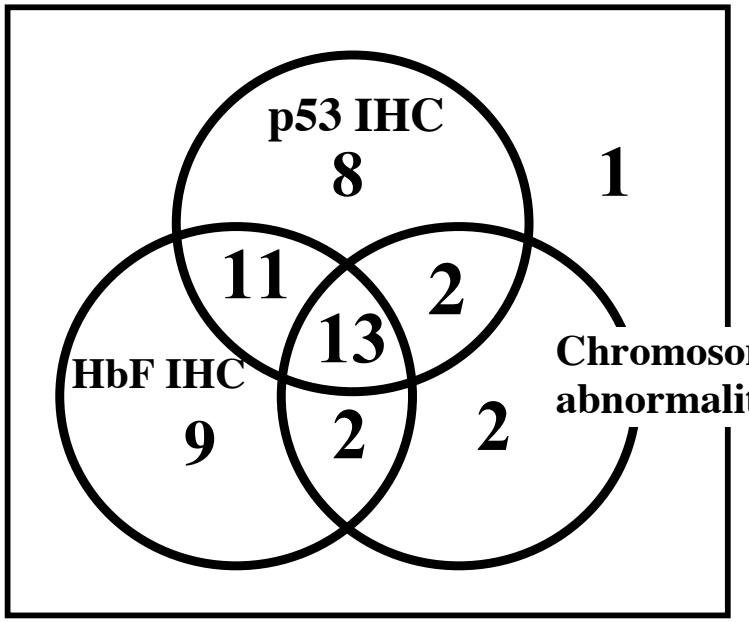
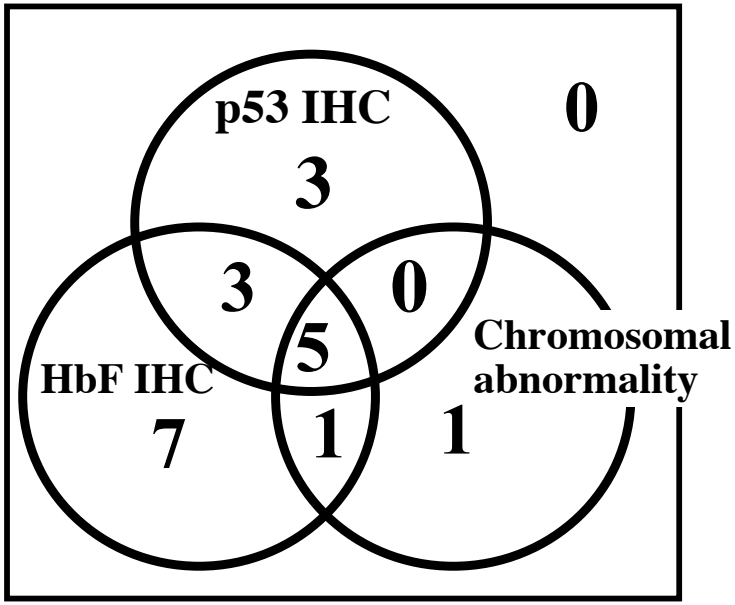


Figure 3



MDS/MDS-OL (48 cases)

MDS RA/RARS (20 cases)



AA (11 cases)