Short Communication

Study of *p53* in Elderly Patients with Myelodysplastic Syndromes by Immunohistochemistry and DNA Analysis

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We analyzed the tumor suppressor gene product, *p53*, in elderly patients with myelodysplastic syndromes (MDS) and in overt leukemia patients after transformation from MDS using immunohistochemical techniques. We examined 52 MDS patients (mean age 79 years, range 68 to 96) from the time of initial diagnosis to death or development of overt leukemia. *p53* protein was detected by immunohistochemistry (IHC) in 8/52 patients (15%) at initial diagnosis: 1/26 with refractory anemia (RA), 0/4 with RA with ringed sideroblasts, 3/11 with RA with an excess of blasts (RAEB), 3/8 with RAEB in transformation, and 1/3 with chronic myelomonocytic leukemia. We also analyzed gene mutations in patients with positive IHC. *p53* mutations were detected in 3/8 (38%) patients. IHC-positive patients had a significantly higher incidence of leukemic transformation and the presence of a complex karyotype with monosomy 17. IHC-positive cells included blasts as well as mature myeloid cells, erythroblasts, and megakaryocytes. Scrutiny of our data in combination with previous data revealed that patients with positive IHC in multilineage cells were older than those in whom positivity was noted mostly in myeloblasts. This suggests that *p53* IHC positivity with a multilineage pattern may be a characteristic of MDS in older patients. (*Am J Pathol* 1999, 155:717-721)

The myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic disorders predominantly affecting the elderly and often progressing to overt leukemia. Once designated as preleukemia, MDS have been studied as a model for multistep carcinogenesis. Although recent research has revealed oncogene muta-

tions, such as *RAS* and *FMS*, in MDS,^{1,2} the roles of tumor suppressor genes have been largely unclear.

p53 is a tumor suppressor gene located on the short arm of chromosome 17,³ and *p53* mutations have been found among many kinds of malignancies.⁴ Normal *p53* protein is a multifunctional protein that participates in cell cycle regulation, apoptosis, cell immortality, and cancer cell response to chemotherapeutic agents.⁵⁻⁷ Mutations of *p53* have been found in MDS patients, but not as frequently as in other malignancies, and it has been speculated that they play important roles in the progression of the disease, development of overt leukemia, and therapeutic responsiveness.⁸⁻²¹

In the present study, we examined *p53* in elderly patients with MDS and leukemia arising from MDS in order to clarify its clinical significance and the molecular mechanism of the evolution of this disease. We analyzed *p53* protein by immunohistochemistry (IHC) and *p53* gene mutations using single-strand conformation polymorphism of polymerase chain reaction (SSCP-PCR) products. The wild-type *p53* protein has a short half-life and usually cannot be detected by IHC. However, mutated *p53* typically has a prolonged half-life and is detectable in tissue sections of bone marrow or in peripheral blood.^{21,22} IHC of blood or bone marrow slides is a sensitive method for early detection of *p53* mutations, especially in heterogeneous cell populations, such as in MDS.²³ We report the characteristics of *p53* IHC and the clinical significance of *p53* expression in elderly MDS patients.

Materials and Methods

We analyzed 72 samples obtained from 52 patients diagnosed with MDS from 1986 to 1995, including 26 patients with refractory anemia (RA), 4 with RA with ringed sideroblasts (RARS), 11 with RA with excess of blasts

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Table 1. Characteristics of Patients with Positive Immunohistochemistry

Case	Age/Sex	FAB	Staining pattern	%p53-positive cells	Survival (days)	Chemotherapy response	Gene mutation	Karyotype
1	71/F	RA→M2	A	4 (40)*	2613 [†]	NR	exon 7: codon 249 (AGG to ATG) (Arg to Met)	45XX,+3,-5,+6q-,-7,-7 [‡] -11,-17,+mar1,+mar2 and variable abnormalities
2	69/M	RAEB-t→M2	A	18 (37)*	257 [†]	NR	exon 7: codon 237 (ATA to ATG) (Ile to Met)	42-44XY,-7,-12,-15,-17+mar1,+mar2 and variable abnormalities
3	80/M	RAEB	B	8	1227	CR	ND	46XY
4	76/F	RAEB-t	B	8	374	PR	ND	—
5	73/M	RAEB-t→M2	C	26 (28)*	320 [†]	NR	splice donor site (GT to GC)	44-46XY,add(2)(q?),-5,-7-17,-18,+ring,+mar1,+mar2,+mar3 and variable abnormalities
6	95/F	CMML→M4	C	34 (93)*	1346 [†]	—	ND	46XX
7	84/M	RAEB	C	32	46	—	ND	46XY
8	79/F	RAEB→M2	C	55 (71)*	367 [†]	PR	ND	47XX,+8

ND, not detected; CR, complete remission; PR, partial remission; NR, no remission; FAB, French-American-British classification.

*Numbers in parentheses indicate percentage of p53-positive cells in the leukemic phase.

[†]Days until death.

[‡]Karyotype analysis was done at the RAEB-t phase and not at diagnosis.

(RAEB), 8 with RAEB in transformation (RAEB-t), and 3 with chronic myelomonocytic leukemia (CMML). Average age was 79 ± 7 (mean $\pm$ SE) years (range, 68–96). Fourteen of the 52 patients transformed to overt leukemia within 28 to 2543 days (530 ± 186 days).

This study used bone marrow obtained by aspiration at the time of initial diagnosis. Bone marrow tissues were fixed in 10% neutral formalin, embedded in paraffin, and sectioned at a thickness of 4 μ m. Sections were immunostained as previously reported²⁵ with microwave oven pretreatment using the peroxidase LSAB kit (DAKO, Glostrup, Denmark) and visualized with diaminobenzidine. The primary antibody was anti-human p53 mouse monoclonal antibody (clone DO-7, DAKO) which recognizes both the wild-type and mutated p53 protein. Endogenous peroxidase was quenched using hydrogen peroxide. Counterstaining was performed using hematoxylin. Cells with nuclei stained brown were judged to be positive. The percentage of positive cells was determined among all nucleated cells. Negative controls were immunostained with normal mouse IgG antibodies.

In patients with positive IHC, p53 gene mutations were examined using an SSCP-PCR method and direct DNA sequencing. DNA was extracted from bone marrow samples at the initial diagnosis. DNA extraction and SSCP-PCR were performed as previously described.²⁵ Exons 5, 6, 7, and 8 of the p53 gene were screened for point mutations. PCR products with abnormal SSCP-PCR profiles were subjected to direct DNA sequencing.

Survival duration and other statistical examinations were calculated according to the Kaplan-Meier method, log-rank test, χ^2 test, Fisher's exact method, and the Mann-Whitney U test. Values of $P < 0.05$ were considered significant.

Results

Positive nuclear staining for human p53 was detected in 8/52 (15%) patients: RA 1/26 (4%), RARS 0/4 (0%), RAEB

3/11 (27%), RAEB-t 3/8 (38%), and CMML 1/3 (33%) at initial diagnosis (Table 1). One of 30 patients with early stage MDS (RA and RARS) and 6 of 19 patients with late stage MDS (RAEB and RAEB-t) were positive for a significantly higher incidence in the late stage ($P = 0.01$). The percentage of p53-positive cells was 4 to 55%. Positively stained cells included blasts with a high nuclear/cytoplasmic ratio, mature myeloid cells (including myeloid cells with pseudo-Pelger anomaly), erythroblasts, and megakaryocytes. We found three patterns of positive IHC. Positive blasts were observed among other, mostly negative cells, and comprised pattern A. Positive staining in mature myeloid cells and erythroblasts, including myeloblasts, comprised pattern B. The addition of positive megakaryocytes to pattern B constituted pattern C. Cases 1 and 2 were classified as pattern A, cases 3 and 4 as pattern B, and cases 5 to 8 as pattern C. Although the patients with pattern B or C (73 to 95 years, 81 ± 8 years, $n = 6$) were rather older than those with pattern A (69 and 71 years, $n = 2$), the difference was not statistically significant, with a marginal P value ($P = 0.0528$). The immunostaining results of cases 1 (pattern A) and 6 (pattern C) are shown, respectively, in Figure 1, A and C. Myeloid cells with pseudo-Pelger anomaly, erythroblasts, and megakaryocytes were positive in case 6 (Figure 1, C and E). Case 1 transformed to leukemia after 7 years and case 6 transformed to leukemia after 4 years. In case 1, positive blasts increased with progression to leukemia (4% at the RA phase, 25% at RAEB-t, and 40% at leukemia) (Figure 1B). In case 6, positive cells increased from 34% to 93% with progression to leukemia (Figure 1E). Monocytic blasts also were stained in case 6 (Figure 1E).

Of note among the staining patterns is that the two pattern A patients had a complex karyotype, including monosomy 17, gene mutations, and resistance to chemotherapy, that was observed in only one out of six patients with patterns B and C.

As for IHC positivity in patients with leukemic transformation, 5 of 14 (36%) patients who transformed to leuke-

Figure 1. p53 protein (brown) stained using peroxidase method. **A:** Case 1 (RA); **arrowheads** indicate positive myeloblasts. **B:** Case 1 (leukemic phase); positive myeloblasts increased. **C:** Case 6 (CMML); **arrow** indicates positive megakaryocyte. **Arrowheads** indicate positive myeloid cells with pseudo-Pelger anomaly. **D:** Case 6 (negative control). **E:** Case 6 (leukemic phase); positive blasts markedly increased. **Arrow** indicates positive erythroblasts. **Arrowheads** indicate positive myeloblast and monocytic blast. Original magnification, $\times 400$.

mia were judged positive at initial diagnosis. Five of 8 (63%) patients with positive IHC transformed to leukemia, compared with only 9 of 44 (20%) with negative stain, which represented a significantly higher incidence in the positive group ($P = 0.0254$). The presence of a complex karyotype with monosomy 17 was recognized in 3 of 7 (43%) karyotyped IHC-positive patients (one IHC-positive patient, case 4, was not karyotyped) compared with none of 21 patients with negative IHC, a rate significantly higher in the positive group ($P = 0.0107$). Among the 6 IHC-positive patients who received chemotherapy, 3 (50%) achieved complete or partial remission, and among the 9 negative patients who received chemother-

apy, 4 (44%) achieved complete or partial remission. No significant difference in chemotherapy response was observed. There was no significant difference in survival duration (positive patients, $n = 8$, 819 ± 306 days versus negative patients, $n = 44$, 795 ± 104 days, $P = 0.7894$).

As for DNA analysis, p53 gene mutations were detected in 3/8 patients (38%) with positive IHC. All p53 gene mutations were point mutations. Two were missense mutations at codons 249 (AGG to ATG) and 237 (ATA to ATG), and one was a rare mutation in a splice donor site at the 5' end of the fifth intron. We had previously reported two of these mutations.^{26,27} All patients with a mutation had the presence of a complex karyotype

with monosomy 17, occurrence of leukemic transformation, and chemotherapy resistance.

Discussion

We examined the tumor suppressor gene, *p53*, in elderly patients with MDS using both IHC and SSCP methods. We found positive IHC in 8 of 52 (15%) patients and *p53* gene mutations in 3 of the 8 (38%) patients with positive IHC.

Previous studies revealed that the incidence of mutations in MDS was 0–25%. Sixty-five mutations (8%) were found among 776 patients previously reported and almost all were point mutations.^{8–20} Although we applied SSCP only for those IHC-positive patients, the estimated minimum incidence of mutations in our elderly patients was comparable to that in previous studies.^{8–20} All detected mutations were point mutations. Two were missense mutations (in codon 249²⁶ and 237) and one was a mutation in a splice donor site.²⁷ The mutation in codon 249 occurred in the early phase. This mutation may have occurred in the presence of the wild-type allele and might have contributed to the onset of the second event as discussed previously.²⁶ The loss of *p53* function through these mutations may contribute to the evolution of MDS.^{5,6,28,29}

The IHC *p53*-positive rate in previously reported studies ranged from 14 to 20%.^{23,30,31} Twenty patients (18%) were found to be positive among 113 previously reported patients. The positive rate by IHC is slightly higher than that by SSCP, especially in the early stage. Discordant results between these two methods have been reported^{23,32,33} and may derive from the differences between these methods.^{4,13,21,32–34} For example, Lepelley et al reported one IHC-positive MDS patient whose SSCP was initially negative but turned positive in the leukemic phase, when the population of the mutated clone reached the level of detectability by SSCP.²³ Although the concordance between a *p53* gene mutation and the accumulation of *p53* protein cannot be perfect, IHC positivity in some patients may be a harbinger of *p53* with altered function.

Although most of the patients with a chain-terminating mutation have been reported to be IHC-negative,^{21,23} an accumulation of *p53* protein was detected in our patient with an intronic mutation. Such accumulations have been reported in ovarian cancer³⁵ and in a cell line.³⁶

In the present study, we observed positive staining not only in myeloblasts but also in mature myeloid cells, erythroblasts, and megakaryocytes. Most of the previous studies, which were comprised of patients rather younger than ours, reported that positively stained cells were almost always limited to myeloblasts or myeloid cells, although uneven *p53* expression has been noted among myeloblasts and among the myeloid lineage cells at different maturation stages.^{23,30,31} As we noticed that IHC-positive patients with pattern B or C (range 73–95, 81 ± 8 years) were older than the two pattern A patients (69 and 71 years), we scrutinized the expression patterns in the previously reported IHC-positive patients. We found one therapy-related 80-year-old MDS patient with pattern

C in addition to pattern A patients (range 34–75, 58 ± 14 years, $n = 8$) in the report by Orazi et al.³⁰ All the patients reported by Kitagawa et al were pattern A,³¹ with a mean age of 64 ± 19 years (range 21–78, $n = 7$) (Kitagawa M, personal communication). Combining these data, we found that the patients with pattern B or C, ie, multilineage pattern (MLP) (range 73–95, $n = 7$), were significantly older than the patients with pattern A (range 21–78, $n = 17$, $P = 0.0005$). Furthermore, MLP was more frequently found in patients older than 75 years than in those 75 years of age or younger (6/7 vs. 1/17, $P = 0.0003$). These data suggest that MLP of *p53* IHC may be a characteristic of older MDS patients. These findings also suggest that response to or effects of accumulated *p53* proteins seem to differ according to cell type and stage of maturation.^{31,37} However, the biological and clinical significance of MLP in older MDS patients has yet to be clarified.

As to the relationship between IHC and clinical data, the incidence of leukemic transformation was significantly higher in patients with positive IHC than those with negative IHC, in accordance with the previous reports.³¹ Although the *p53* mutation itself was associated with a high incidence of leukemic transformation,^{15,17} our results indicate that the IHC-positive population, including mutation-positive cases, is at risk for a high incidence of leukemic transformation. Therefore, it might be worthy of consideration to take *p53* IHC results into account, especially in the early stage, for estimations of the likelihood of leukemic transformation.

All of our IHC-positive patients with mutations and monosomy 17 transformed to leukemia and were resistant to chemotherapy, which is consistent with previously reported patients.^{26,27} Survival of patients with a *p53* mutation after the detection of monosomy 17 was significantly shorter than in patients without such a mutation ($n = 3$, 255 ± 38 days, versus $n = 49$, 782 ± 97 days, $P = 0.0088$), which is in accordance with previous reports.^{26,38}

As to the relationship between karyotype abnormalities and IHC, the occurrence of monosomy 17 and a complex karyotype were higher in patients with positive IHC, which is consistent with previously reported patients.^{26,27}

Our data suggest that *p53* analysis is useful for predicting the occurrence of leukemic transformation in MDS. Although IHC analysis alone is useful for predicting occurrence of leukemic transformation, analysis of *p53* by IHC in combination with gene analysis may provide instructive information on leukemic transformation, chemotherapy resistance, and clinical outcome.

Accumulated *p53* protein with mutations may be detectable by IHC in early stages when the population of mutated clones is still too small to be detected by SSCP. IHC analysis of *p53* may provide information on the status of *p53* function different from gene sequences, such as conformational changes without mutations, differences among cell lineages, and differences among differentiation stages. We observed positive staining not only in myeloblasts but also in mature myeloid cells, erythroblasts, and megakaryocytes in some patients. Scrutiny of our data combined with previous data revealed that IHC-

positive patients with MLP (pattern B or C) were significantly older than those with pattern A and that MLP occurred more frequently in patients who were older than 75 years than in those who were younger. These data suggest that p53 IHC positivity with MLP may be a characteristic of older MDS patients.

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