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Mutation Analysis of the *WT1* Gene in Myelodysplastic Syndromes

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The *WT1* tumor suppressor gene was examined for mutations in a panel of 44 patients with myelodysplastic syndromes (MDS) including acute myelogenous leukemias (AML) secondary to MDS, using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and sequencing analysis. A *WT1* mutation was detected in one out of 17 cases of AML secondary to MDS. This mutation exists upstream of the zinc finger region and is predicted to produce a truncated WT1 protein lacking the zinc finger region. No mutations were detected in 27 MDS patients who had not progressed to AML. This is the first report of analysis for *WT1* mutations in a large number of MDS patients, suggesting that *WT1* mutations are uncommon in MDS. Abnormalities in this gene may, however, contribute to a small proportion of cases showing progression from MDS into AML.

Key words: Tumor suppressor gene — *WT1* — Mutation — Hematological disease — Myelodysplastic syndrome

The tumor suppressor gene *WT1* on chromosome 11p13 was first identified by cytogenetic deletion analysis of patients with Wilms' tumors.[1, 2] It encodes a zinc-finger transcription factor that either activates or represses transcription of many target genes, and has the potential to control cell growth and differentiation.[3–5] *WT1* is expressed in tissues of mesodermal origin, including the kidney, gonads, mesothelial lining of the gut, heart, lung, spleen and immature cells in bone marrow. *WT1* mutations have been reported in Wilms' tumors, non-asbestos-related mesothelioma, a juvenile granulosa cell tumor of the ovary, desmoplastic small round cell tumor, and a secondary leukemia in a WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) patient.[6, 7] Most recently, *WT1* mutations were found in acute leukemias *de novo*.[8, 9]

WT1 appears to be active in immature hematopoietic cells that are less differentiated, with high rates of cell proliferation. *WT1* is expressed in normal $CD34^+$ bone marrow stem cells, but is hardly expressed in normal mature blood cells.[10] It is expressed in the acute phase of leukemias and in hematopoietic cell lines,[11–14] and is down-regulated during differentiation.[15, 16] A recent study has suggested that WT1 may play a role in apoptosis.[17] Although the exact role of WT1 in hematopoiesis and leukemogenesis is still unclear, these lines of evidence suggest that WT1 may play a role in hematopoiesis and leukemogenesis.

Myelodysplastic syndromes (MDS) comprise a stem cell disorder characterized by tri-lineage dysplasia of peripheral blood and bone marrow cells and ineffective hematopoiesis. There is a tendency for the disease to develop into acute leukemias by accumulation of heterogenous mutations of oncogenes or tumor suppressor genes. The role of WT1 in MDS is unknown. To examine whether *WT1* mutations could also be identified in MDS, we have screened 44 cases of MDS including acute myelogenous leukemias (AML) secondary to MDS for *WT1* mutations. We detected a *WT1* mutation in only one case of AML secondary to MDS, and no mutation in any case of MDS that had not progressed to AML.

MATERIALS AND METHODS

Patients After informed consent had been obtained from patients, mononuclear cells were isolated from bone marrow samples from 44 patients with various types of MDS. They were 12 cases of refractory anemia (RA), 9 of refractory anemia with excess of blasts (RAEB), 2 of refractory anemia with excess of blasts in transformation (RAEB in T), 4 of chronic myelomonocytic leukemia (CMMoL), and 17 of AML secondary to MDS. The diagnosis of MDS was made according to the French-American-British (FAB) classification. Samples were taken at the time of diagnosis. Genomic DNAs of these cells were extracted as previously described.[18]

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis Point mutations were sought in 10 exons of the *WT1* gene using PCR-SSCP analysis as previously described.[7] PCR amplifications were performed with 50 ng of DNA samples and 10 pmol of primer pairs, incorporating [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK). The primer pairs for each exon of the *WT1* gene and conditions for PCRs were

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taken from the literature.[7] Only the 3′ end of exon 1 was amplified, because the exon is extremely GC-rich.[3, 8] The SSCP gels were run at 40 W for 3 to 4 h at 15°C. Autoradiography was done by the standard method. Where abnormally migrating bands were detected, the PCR-SSCP procedure was repeated to confirm any shifts.

Sequencing analysis Mutations in the PCR products that showed abnormally migrating bands in SSCP analyses were confirmed by sequencing analyses. The PCR products were subcloned into the pCR 2.1 Vector (Invitrogen, San Diego, CA) and at least four independent clones were sequenced using Sequenase Version 2.0, 7-deaza-dGTP Kit (Amersham, Buckinghamshire, UK). Direct sequencing of PCR products was also carried out using a Sequenase PCR Product Sequencing Kit (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions.

RESULTS

All 10 exons of *WT1* were analyzed by PCR-SSCP in a total of 44 samples of MDS or AML secondary to MDS. One sample of AML secondary to MDS had a *WT1* mutation (Table I).

SSCP analysis of exon 7 showed a complex band pattern because of the existence of a polymorphism in this exon (Fig. 1). The SSCP patterns in patient 85 and patient 373 represent the wild-type. Those in patients 79, 84, 87, 340, 458, 354 represent a previously described silent polymorphism (Arg 301, A→G) (data not shown).[19] Patient 83 was heterozygous for the polymorphism. Although the polymorphism (Arg 301, A→G) appears to be present in the majority of the patients shown in Fig. 1, the frequency of the polymorphism was 42.0% in our study. On the other hand, one sample (patient 86, a case of AML secondary to MDS) showed a different pattern of abnormal mobility in SSCP compared with the samples carrying the polymorphism (Fig. 1). Direct sequencing analysis of this PCR product revealed an insertion of TCGG after the first base of codon Ala 314 (Fig. 2). This causes a frameshift, and three novel amino acids are followed by a stop codon. The same mutation has been described in one case of AML *de novo*.[8] No wild-type *WT1* sequences were obtained in patient 86, either in direct sequencing analysis or in sequencing analyses of 10 independent clones after subcloning (data not shown).

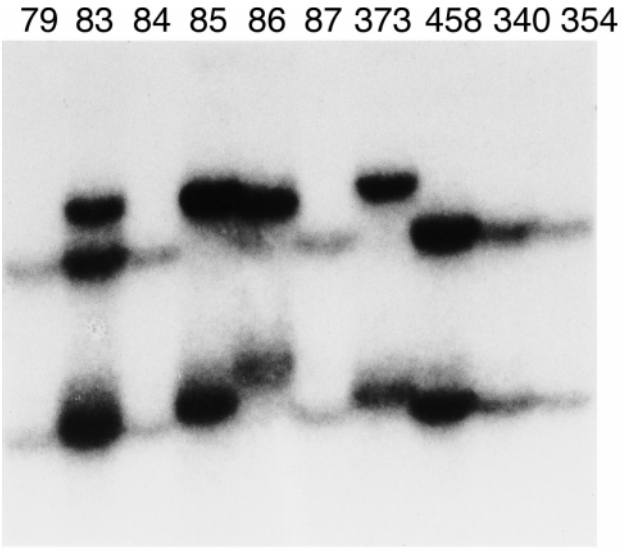

Fig. 1. SSCP analysis of *WT1* exon 7. Numbers of the lanes indicate the patient number. The SSCP patterns in patients 85 and 373 represent the wild-type. The abnormally migrating bands of patients 79, 83, 84, 87, 458, 340 and 354 show a consistent pattern of mobility. All of them were later shown to represent a polymorphism of Arg 301 (A→G) by sequencing analyses. On the other hand, patient 86 shows a different pattern of abnormal mobility compared with other patients. No normal bands were detected in patient 86. Patient 86 was later shown to carry a homozygous 4-base insertion by sequencing analysis.

Table I. Frequency of *WT1* Mutations in MDS

RA	0/12
RAEB	0/9
RAEB-T	0/2
CMMoL	0/4
MDS-AML	1/17
Total	1/44

In the other nine exons, no mutations were detected in any of the 44 samples.

DISCUSSION

This is the first study, to our knowledge, in which a large number of patients with MDS including AML secondary to MDS has been examined for *WT1* mutations. As shown in Table I, none of the 27 cases with RA, RAEB, RAEB in T, or CMMoL, which have a better prognosis than AML secondary to MDS, had *WT1* mutations. Although we can not rule out the possibility that some mutations may have been missed because of the limitations of the SSCP technique, with which only 90% of all mutations may be detected, our study suggests that *WT1* mutations occur infrequently in MDS.

On the other hand, one out of 17 cases of AML secondary to MDS, which has a poor prognosis, carried a *WT1* mutation. This is the first report of a *WT1* mutation in a patient with AML secondary to MDS. The *WT1* mutation observed in patient 86 is an insertion of 4 bp after the first

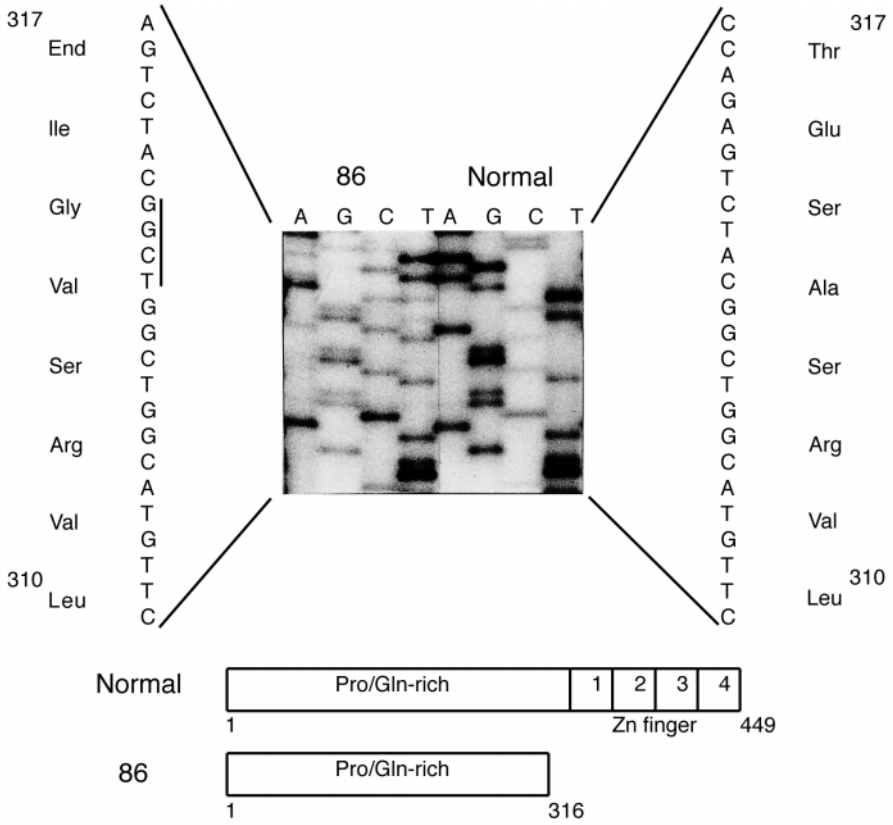

Fig. 2. Direct sequencing of PCR products from patient 86 and a normal individual, showing part of *WT1* exon 7. The inserted four nucleotides in patient 86 are lined. A schematic diagram of the resulting truncated WT1 protein in patient 86 is included.

base of codon 314, causing a frameshift termination at codon 317, resulting in loss of the entire zinc finger region. This mutation has been previously reported as a heterozygous mutation in a case of AML (M1) *de novo*.[8] In our study, more than 95% of the cells processed for DNA isolation from patient 86 were leukemia cells. No wild-type sequences were obtained either in direct sequencing analysis or in sequencing analyses of 10 independent clones, suggesting loss of heterozygosity in the leukemia cells of patient 86.

The majority of the previously reported *WT1* mutations in patients with Wilms' tumors, Denys-Drash syndromes (DDS), and AML *de novo* cause disruption of the zinc fingers, which abolishes the DNA binding capacity of the transcriptional regulator WT1. The zinc finger region contains 92% of *WT1* mutations in DDS, 72% of the *WT1* mutations in Wilms' tumors, and 80% of the mutations in AML.[6] The majority of other mutations upstream of the zinc fingers also result in disruption of the zinc fingers. The mutation found in our case of AML secondary to MDS is predicted to inactivate WT1, suggesting that abnormalities in the *WT1* gene may contribute to a small proportion of cases showing progression from MDS into AML.

However, it should be noted that the frequency of *WT1* mutations in our samples from AML secondary to MDS patients (5.9%) is low. Although the previous findings in acute leukemias *de novo* showed that about 15% of adult AML cases carried inactivating mutations of *WT1*,[8, 9] the role of WT1 in leukemogenesis is still undefined. MDS is likely to be a heterogenous disease, as regards the molecular mechanisms of development and leukemic transformation. Many other genes, including N-*ras*, *p53*, c-*fms*, *NF1*, *Rb*, *p16*, *EVI-1* and *IRF-1*, have also been examined to see whether they are involved in the development of MDS and in the progression to AML. However, the full spectrum of mutational events that occur during the progression of MDS has not been elucidated. To clarify the roles of *WT1* and other genes in the development and the progression of MDS, further examination of mutations of the *WT1* and other genes in a larger MDS panel would be required.

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