

Methylation status of the promoter region of the human frizzled 9 gene in acute myeloid leukemia

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Abstract. The FZD9 gene is located at chromosome 7q11.23, and has been indicated to be a tumor suppressor gene. The present study examined the involvement of FZD9 promoter methylation in the downregulation of FZD9 expression in leukemia cells. The expression of the FZD9 gene was absent in various leukemic cell lines, while it was restored following treatment with DNA demethylating agent 5-aza-2'-deoxycytidine. Bisulfite sequencing analysis of the FZD9 promoter region showed that it was partially methylated in cell lines in which FZD9 gene was not expressed. Thus, DNA methylation in the promoter region may lead to inactivation of the FZD9 gene, which may represent an aberration associated with leukemia, since DNA was not methylated in normal peripheral blood mononuclear cells. Methylation-specific polymerase chain reaction analysis revealed that the promoter region of the FZD9 gene was frequently methylated in primary or relapse acute myeloid leukemia (52.9%; excluding acute promyelocytic leukemia); however, methylation was infrequent in B-cell acute lymphocytic leukemia (5.6%). In conclusion, the present study indicated that the methylation profile of the FZD9 gene corresponded to that of a candidate tumor-suppressor gene in acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is a clonal disorder arising from uncontrolled proliferation of hematopoietic progenitor or stem cells (1). Genetic and epigenetic aberrations, including promoter hypermethylation and covalent histone modification, have been implicated in the pathogenesis of leukemia (2),

leading to enhanced proliferation and self-renewal, evasion of apoptosis and differentiation arrest of leukemia stem cells.

Frizzled 9 is a protein encoded by the FZD9 gene in humans. Members of the 'frizzled' gene family encode seven transmembrane domain proteins that are receptors for Wnt signaling proteins. Wnt proteins secrete glycoprotein to regulate early B-cell growth and survival (3). The Wnt/FZD signaling pathway has important roles in development (4), tumorigenesis (5) and lymphoid maturation (6,7). The FZD9 gene is located within a region of chromosome 7, which is commonly deleted in Williams Beuren syndrome (8-10). FZD9 is expressed predominantly in the muscle, kidney, bones, eyes, testis and brain, and has an important role in the maintenance of stem cell populations in the blood, skin and gut (11-14). FZD9-deficient mice show a reduction in the number of pro/pre-B cells at developmental stages (15). In addition, aberrant DNA methylation has been identified to occur during the transformation of myelodysplastic syndrome (MDS) to AML (16). In addition, non-small cell lung cancer cell lines showed a decrease of Wnt7a expression (17,18), while restoration of Wnt7a and FZD9 expression inhibited cell proliferation and anchorage-independent growth, thus promoting cellular differentiation and reversing the transformed phenotype.

Aberrant Wnt signaling has been studied in a number of leukemia types, including lymphoid and myeloid lineages, as this pathway has an important role in the renewal of normal hematopoietic stem cells and its dysregulation may lead to leukemia (19). Transcriptional repression by DNA promoter hypermethylation has been shown to affect Wnt antagonists in several human malignancies, including leukemia. The loss of function of Wnt antagonists due to promoter hypermethylation contributes to the activation of the Wnt pathway in AML and may be involved in its pathogenesis, in addition to representing a possible prognostic factor (20,21).

DNA methylation in the promoter region leads to epigenetic gene inactivation by transcriptional silencing (22-24). Aberrant DNA methylation of candidate tumor suppressor genes (TSGs) has been found in various tumor types and may represent an alternative pathway of TSG inactivation (25-27). The present study assessed aberrant DNA methylation of the promoter region of the FZD9 gene in hematological malignancies and examined the association between DNA methylation of the FZD9 gene and its gene expression.

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Materials and methods

Samples and DNA/RNA preparation. Total DNA was isolated from bone marrow mononuclear cells (BMMCs) (28) of 78 patients, including 51 patients with AML (excluding acute promyelocytic leukemia), 18 patients with B-cell acute lymphocytic leukemia (B-ALL), 9 patients with chronic myeloid leukemia (CML), at the time of initial diagnosis or relapse stage, who were seen at The First Affiliated Hospital of Harbin Medical University, (Nangang, China) from 2011 to 2014, and grouped according to criteria of the French-American-British classification (29). DNA was also isolated from peripheral blood mononuclear cells (PBMCs) (30) of six healthy volunteers. The patients and volunteers provided informed consent to participate in this study. In addition, 10 hematological cell lines were examined. U937, JURKAT, K562, SUP-1, THP-1, MOLT4, HL60 and NB4 cells were provided by the Blood and Lymphatic Tumor Research Center of Nagoya University (Nagoya, Japan). NALM6 and RPMI8226 were provided by the Internal Hematology Laboratory of China Medical University (Shenyang, China). Cells were maintained in RPMI 1640 culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin, and were incubated in a 5% CO₂-humidified incubator at 37°C. Total RNA was extracted using the Aqua-SPIN RNA Isolation Mini kit (Watson, Shanghai, China) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized from 1 µg total RNA using random hexamers as primers and Moloney murine leukemia virus-H-reverse transcriptase (Gibco; Thermo Fisher Scientific, Inc.). PCR was then performed using first-strand cDNA as a template. The PCR thermocycling program was as follows: Initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C and extension for 30 sec at 72°C, followed by a final extension for 10 min at 72°C. The primers FZD9 forward (5'-TCAAGGTCAGGCAAGTGAGCA-3') and FZD9 reverse (5'-AGCTTCAGAGGAACGCAACA-3') were used to generate 249-bp products. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining (Shanghai Tuo Yang Biotechnology Co., Ltd., Shanghai, China). As the control, GAPDH cDNA was amplified by RT-PCR (25 cycles) in separate tubes by using the primers GAPDH1 (5'-CCATGGAGAAGGCTGGGG-3') and GAPDH2 (5'-CAAAGTTGTCATGGATGACC-3') to generate 225-bp products.

Culture with 5-aza-2-deoxycytidine. On day two after seeding, U937 and K562 cells (2x10⁵/ml) were incubated with 5-aza-2'-deoxycytidine (0, 5 or 10 µM; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The cells were then washed with phosphate-buffered saline and incubated for another 72 h. Finally, the cells were harvested on day five to assess mRNA expression and DNA methylation of the FZD9 gene.

Bisulfite sequencing. A total of 200 ng genomic DNA extract from PBMCs of healthy volunteers, cell lines and clinical samples were modified by sodium bisulfite using a

DNA modification kit (MethylampTM DNA Modification kit; Epigentek, New York, NY, USA). Modified DNA (25 ng) was amplified by PCR using the primers FZD9 forward (5'-GTT TTTTTTTAGAGTAAAATGAGG-3') and FZD9 reverse (5'-ACCRAAACTACCAACCCC-3') to obtain the promoter sequence of the FZD9 gene. The methylation status of the FZD9 gene was assessed at 501-246 bp upstream of the transcription initiation site. PCR products were separated on 1.5% low-melting agarose gels, excised and then digested with β-agarase (New England Biolabs, Beverly, MA, USA). The digestion products were sub-cloned into the pMD18-T vector as previously described (31), and a minimum of 8 clones from each product were subjected to cycle sequencing (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and analyzed using the ABI 310 sequencer (Applied Biosystems).

Methylation-specific PCR (MSP). The modified DNA was selectively amplified by PCR with the primers FZD9 forward and FZD9 reverse under the conditions described above, followed by MSP analysis with the use of primer sets specific for unmethylated (U) DNA (MSP-U forward, 5'-GATTTAGTTTGAGAGTGTGGGTATG-3' and reverse, 5'-CAAACTCCAAAAAACACA-3') and methylated (M) DNA (MSP-M forward, 5'-ATTTAGTTTGAGAGTGTGGGTACG-3' and reverse, 5'-GAAACTCCGAAAAACACGC-3'). The thermocycling conditions were as follows: Initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 58°C/62°C and extension for 40 sec at 72°C, followed by a final extension for 10 min at 72°C. Each PCR was 'hot-started' at 95°C, and the products were separated on 2% agarose gels and then visualized by ethidium bromide staining as described above.

Statistics and analysis. Each experiment was repeated 3 times. X-test, one-way analysis of variance and logistic analysis were conducted using the SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). P=0.05 was set as the test standard and P<0.05 was considered to indicate a statistically significant difference.

Results

FZD9 expression is lost in a proportion of leukemia cell lines. The expression of the FZD9 gene in 10 leukemic cell lines and 6 normal PBMC samples was evaluated by RT-PCR. The results showed that the FZD9 gene was not expressed in six of the cell lines (U937, K562, SUP-1, THP-1, NB4 and RPMI8226) (Fig. 1). Although these cell lines were derived from diverse hematological malignancies, the loss of FZD9 gene expression tended to be more frequent in myeloid leukemia cell lines. However, the FZD9 gene was expressed in all normal PBMC samples.

FZD9 gene expression in leukemia cells is restored by 5-aza-2'-deoxycytidine. The mechanism of the observed downregulation of the expression of the FZD9 gene was examined. The U937 and K562 cell lines, which did not express FZD9, were exposed to 5-aza-2'-deoxycytidine (0, 5 or 10 µM). The results showed that the expression of the FZD9

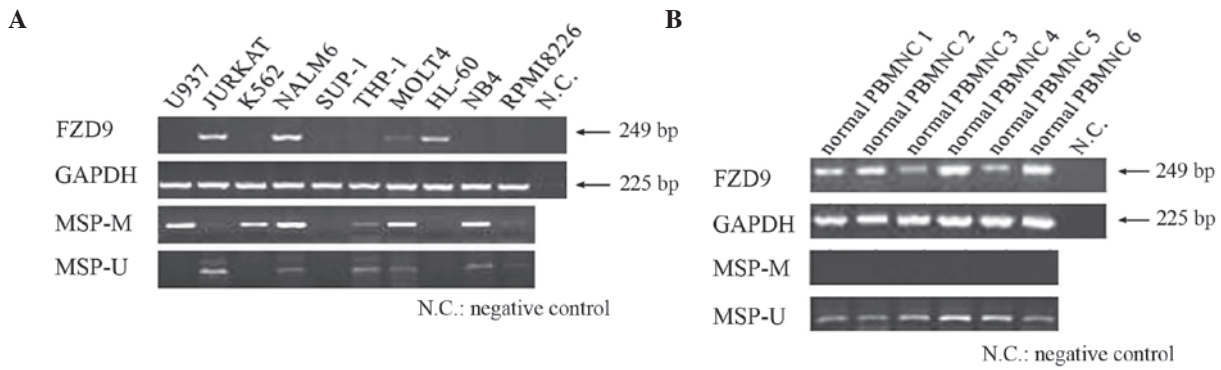


Figure 1. Reverse-transcription PCR analysis of FZD9 gene expression and MSP analysis of FZD9 gene promoter region in (A) various cell lines and (B) normal PBMNC. PCR, polymerase chain reaction; MSP, methylation-specific PCR; MSP-M/U, MSP using primer specific for methylated/unmethylated DNA; N.C., negative control; PBMNC, peripheral blood mononuclear cells.

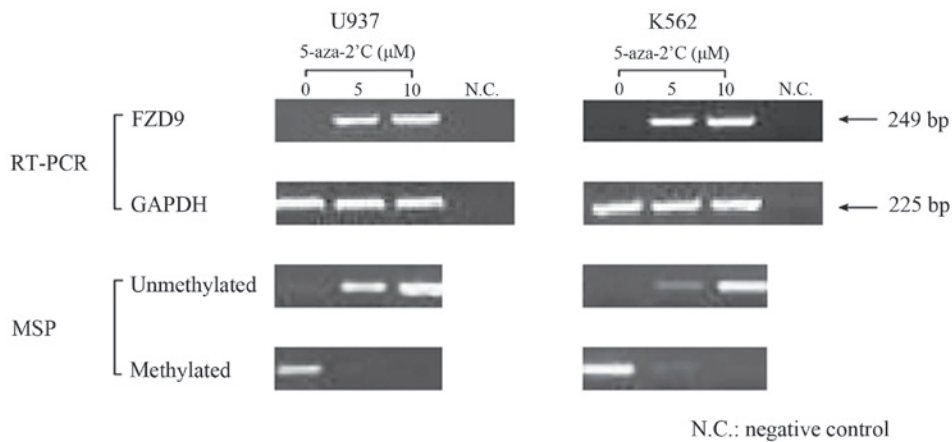


Figure 2. Restoration of FZD9 gene expression and demethylation by 5-aza-2'-deoxycytidine in U937 and K562 cell lines as indicated by RT-PCR and MSP. RT-PCR, reverse-transcription polymerase chain reaction; MSP, methylation-specific PCR; 5-aza-2'C, 5-aza-2'-deoxycytidine; N.C., negative control.

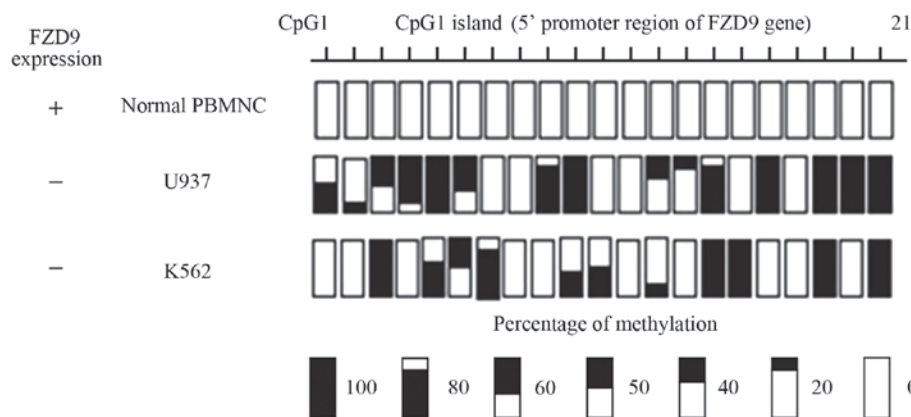


Figure 3. Methylation of the promoter region of the FZD9 gene in CpG islands and in one normal PBMNC sample, as well as in two cell lines determined by sequencing after bisulfite modification of genomic DNA. CpG 1 to 21 correspond to what is depicted in Fig. 1. Expression of the FZD9 gene was detected by reverse-transcription polymerase chain reaction, and the percentage of methylation was determined from ratios of methylated cytosine in 8 to 10 sequenced clones. White represents the amount of unmethylated and black represents methylated regions. PBMNC, peripheral blood mononuclear cells.

gene was restored in these cells following treatment with 5-aza-2'-deoxycytidine (Fig. 2).

The FZD9 gene promoter region is partially methylated in leukemia cell lines with loss of FZD9 expression. The association between DNA methylation and FZD9 gene expression was

further examined by bisulfite genomic sequencing of 21 CpG sites at the promoter region of the FZD9 gene. PBMNCs from a healthy volunteer and the U937 and K562 cell lines, which did not express the FZD9 gene, were analyzed (Fig. 1). The normal PBMNCs expressed the FZD9 gene and the 21 CpG sites of the promoter region were completely unmethylated. By contrast,

Table I. Frequency of FZD9 gene methylation in various hematological malignancies.

Malignancy	Methylation rate of FZD9 gene	Frequency (%)
AML (except M3)	27/51	52.9
AML-M1	2/6	33.3
AML-M2	13/22	59.0
AML-M2 (ETO positive)	5/6	83.3
AML-M4	2/5	40.0
AML-M5	5/10	50.0
AML-M6	0/2	0.0
CML	3/9	33.3
B-ALL	1/18	5.6

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; B-ALL, B-cell acute lymphocytic leukemia.

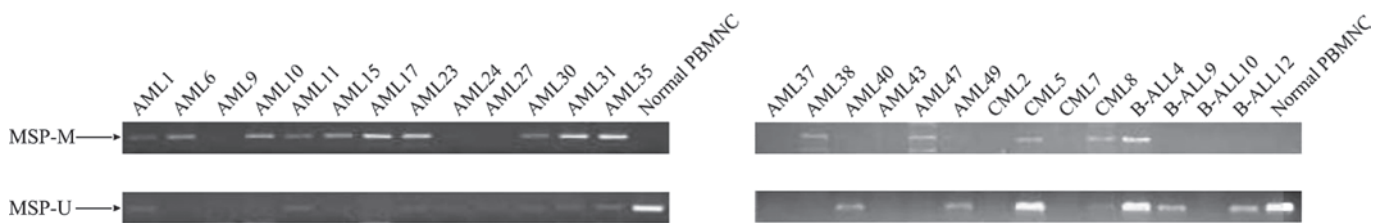


Figure 4. Analysis of FZD9 gene methylation in AML and other hematological malignancies using MSP. MSP, methylation-specific polymerase chain reaction; MSP-M/U, MSP using primer specific for methylated/unmethylated DNA; PBMNC, peripheral blood mononuclear cells.

the U937 and K562 cell lines did not express the FZD9 gene and their promoter regions were partially methylated (Fig. 3).

Methylation of FZD9 gene promoter region detected by MSP. The DNA methylation in the promoter region of the FZD9 gene was further screened using the MSP method. FZD9 gene expression in 10 leukemic cell lines was examined using MSP analysis. In analogy with the results of the bisulfite sequencing analysis, MSP analysis revealed that the DNA in the promoter regions of the FZD9 gene was methylated in the U937 and K562 cell lines. Furthermore, the NALM6, MOLT4, THP-1, NB4 and RPMI8226 cell lines showed partial methylation in their FZD9 promoter regions. However, the FZD9 promoter region of Jurkat cells was not methylated, while neither methylation nor unmethylation was detected in SUP-1 and HL-60 cell lines. In addition, analysis of the normal PBMNCs by MSP did not show any methylated bands (Fig. 1B). Analysis of patient samples showed that the methylation rate of the FZD9 gene promoter was 52.9, 33.3 and 5.6% in AML samples, CML samples and ALL samples, respectively (Fig. 4). The frequencies of FZD9 gene methylation in various hematological malignancies are shown in Table I. These data suggested that aberrant DNA methylation of the FZD9 gene was present in certain types hematological malignancies, and that the frequency of this methylation was higher in AML compared with that in CML or B-ALL.

Discussion

In addition to point mutation or gene deletion, transcriptional repression by the hypermethylation of promoter sequences

may represent an alternative mechanism of TSG inactivation in cancers (32). The FZD9 gene has been indicated to be a TSG, which has, however, remained to be directly evidenced. FZD9 expression has been shown to be decreased in chronic lymphocytic leukemia (21), myelodysplastic syndromes and non-small cell lung cancer (17,18), indicating its TSG role in hematogenic malignancies.

The results of the present study suggested that the methylation status of the FZD9 gene in leukemia cell lines was reversed after treatment with the demethylating agent 5-aza-2'-deoxycytidine. While methylated bands in the K562 cell line remained after exposure to 5 μ M 5-aza-2'-deoxycytidine, they disappeared in U937 cells, along with a restoration of FZD9 expression. These results suggested that DNA methylation of the FZD9 gene or genes that transcriptionally regulate FZD9 may represent a mechanism of the inactivation of the FZD9 gene in leukemia.

However, it is likely that silencing of FZD9 is not exclusively mediated via DNA methylation in the promoter region. Jiang *et al* (16) assessed the contribution of aberrant DNA methylation to TSG silencing during disease progression by DNA methylation microarray and high-density single-nucleotide polymorphism array karyotyping methods, revealing that FZD9 gene methylation was common in early MDS, and that the methylation ratio increased with the progression of the disease. Thus, despite the absence of genetic mutations, FZD9 is likely to be a TSG, which is silenced during the genesis and progression of cancers by epigenetic modifications.

MSP is a simple and sensitive method to detect DNA methylation and requires only small specimens containing limited amounts of DNA (33). In the present study, analysis of the SUP-1 and HL-60 cell lines showed that neither methylated

nor unmethylated FDZ9 was present, indicating that FZD9 expression may be deactivated by mechanisms other than DNA methylation of its gene promoter region, such as deregulation of transcription upstream of the FZD9 gene, histone deacetylation or genetic mutations. Analysis of clinical specimens revealed that the promoter region of FZD9 was 52.9% methylated in BMMCs from patients with AML, 33.3% methylated in BMMCs from patients with CML, but only 5.6% methylated in BMMCs from patients with B-ALL, suggesting that aberrant FZD9 gene methylation occurred more frequently in primary or relapse AML compared with other types of leukemia. In patients with B-ALL, FZD9 expression may be silenced through other mechanisms, such as gene mutations. FZD9 gene methylation was frequent in patients with AML, which was AML1/ETO fusion gene positive. Whether the methylation status of the FZD9 promoter in AML with translocation (8,21) is indicative of chromosomal translocation, patient prognosis or drug resistance requires further study. An understanding of the association between methylation of the FZD9 gene promoter and the clinical outcome of primary or relapse AML may aid in the elucidation of the clinical significance of such epigenetic alterations. As only limited clinical data of the subjects were available, these associations could not be examined in the present study.

According to a previous study, FZD9 knockout mice showed no obvious features of Williams Beuren syndrome, but revealed that the FZD9 gene had an important role in lymphoid development and maturation (15). FZD9 knockout mice showed thymic atrophy, pronounced splenomegaly and lymphadenopathy with accumulation of plasma cells in lymph nodes. These results showed that the FZD9 gene was associated with abnormal B-cell development. However, the underlying mechanisms of neoplastic transformations in humans may differ from those in mice.

In conclusion, the present study indicated that the methylation profile of the FZD9 gene corresponded to that of a candidate tumor-suppressor gene in AML, and that demethylation of FZD9 may represent a novel therapeutic strategy.

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