Homology modeling and molecular dynamics studies of Wilms' tumor gene 1 frameshift mutations in exon 7

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Abstract. As a transcription factor, the Wilms' tumor 1 (WT1) gene plays an important role in leukemogenesis. The impact of WT1 gene mutations has been reported in acute myeloid leukemia (AML). However, the number of available studies on the spatial configuration changes following WT1 mutation is limited. In this study, we sequenced the mutation in exon 7 of the WT1 gene in 60 children with newly diagnosed AML and the spatial configuration of WT1 with frameshift mutations in exon 7 was evaluated using the software for homology modeling and optimization of molecular dynamics. Three cases with frameshift mutations in exon 7 were identified (3/60; mutation rate, 5%). One case had a mutation that had been previously described, whereas the remaining two mutations were first described in our study. Of the three cases, one case presented with antecedent myelodysplastic syndrome (MDS) and the remaining two cases exhibited primary resistance to induction chemotherapy. The spatial configuration analysis demonstrated that the three mutations affected the spatial structure of exon 7 and even affected exon 8 compared to its wild-type. This study demonstrated that the frameshift mutation in exon 7 of the WT1 gene is a poor prognostic factor for children with AML, partly through the spatial configuration changes following frameshift mutations of WT1, which highlights the structure-based function analysis and may facilitate the elucidation of the pathogenesis underlying WT1 gene mutations.

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Introduction

The Wilms' tumor 1 (WT1) gene has been extensively used in monitoring minimal residual disease (MRD) in acute myeloid leukemia (AML) as a panleukemic marker, due to its overexpression (1-3), particularly in cases lacking known fusion genes (4). The WT1 gene, which is considered to be an oncogene, is mainly involved in promoting stem cell proliferation and hampering cell differentiation in AML (5,6). However, the number of studies investigating WT1 mutations has been on the increase, although their prognostic significance in predicting the outcome in AML has been controversial (7-11). WT1 mutations are encountered in ~10% of AML patients and the resulting defect affects the interaction of WT1 with other transcription factors, which may contribute to leukemogenesis and resistance to chemotherapy (7). The hotspots of WT1 mutations are mainly located in exon 7 (85.41-87.06%) (9,10), with frameshift mutations leading to the formation of a premature stop codon and a truncated protein lacking the C-terminal zinc fingers. Mutations in exon 9 (7.06-8.33%) (9,10) are rare and predominantly of the missense type, which interrupt DNA binding capacity by affecting the amino acid residues directly involved in DNA binding or essential to the structure of the zinc finger motif (12).

In this study, we screened the rate of WT1 gene mutations in exon 7 and analyzed their effect on pediatric AML. Furthermore, we used the software [ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html)] for homology modeling and optimization of molecular dynamics to evaluate the spatial configuration of WT1 with frameshift mutations in exon 7.

Homology modeling, also referred to as comparative or knowledge-based modeling, develops a three-dimensional model from a protein sequence based on the structures of homologous proteins. Evolutionarily related proteins have similar sequences and naturally occurring homologous proteins have similar structures. It has been demonstrated that the three-dimensional protein structure is evolutionarily more conserved than would

Table I. Clinical and genetic characteristics of the 60 children with newly diagnostic acute myeloid leukemia.

FAB subtype	Cases	Gender (% female)	Median age (years)	Median WBC (x10^9/l)	Karyotype (% normal)
M1	2	100	4	19.9	50
M2	12	56	6	8.7	33
M3	21	53	8	11.7	10
M4	13	22	8.5	31.2	25
M5	12	55	11	38.6	38

FAB, French-American-British classification; WBC, white blood cell.

be expected due to sequence conservation (13). By utilizing this method, we demonstrated that WT1 frameshift mutations in exon 7 affected the spatial configuration of WT1.

Materials and methods

Patients and samples. Cryopreserved bone marrow samples collected at diagnosis from 60 patients with newly diagnosed AML were provided by the Children's Hospital of Soochow University. The diagnosis and classification of AML were based on morphological, cytogenetic and immunophenotypic criteria according to the WHO classification. Children with AML were treated according to the protocol for Chinese AML children, as determined by the Subspecialty Group of Hematology, Society of Pediatrics, Chinese Medical Association (14). More detailed information is provided in Table I.

The study was approved by the ethics committee of the hospital and informed consent was provided by the parents or the legal guardians of the patients. The procedures were approved by the hospital's Institutional Review Board.

Polymerase chain reaction (PCR). For mutation analysis of the exon 7 of the WT1 gene, PCR amplification was performed with the use of specific primers (15): 7F: 5'-CTCCAG TGCTCACTCTCCCTC-3'; 7R: 5'-CCTTAGCAGTGTGAG AGCCTG-3'.

The following PCR conditions were used: 2 min at 50˚C, 10 min at 95˚C, 35 cycles for 10 sec at 95˚C and 30 sec at 60˚C, with a final extension step for 30 sec at 72˚C. The wild-type amplicon comprised 309 base pairs. The purified PCR products were directly sequenced from the two strands using the described primers and analyzed on the Applied Biosystems 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis. The sequence data were analyzed using Chromas software version 2.31. According to the structure of the Wilms' tumor suppressor protein zinc finger domain bound to DNA (16), homology modeling and optimization of molecular dynamics was performed with the ExPASy Translate Tool to investigate the spatial configuration of the WT1 gene with frameshift mutations in exon 7.

Results

Study population. A total of 60 newly diagnosed AML samples collected from The Children's Hospital of Soochow University, were screened for WT1 gene mutations. The patient characteristics are provided in Table I.

Mutation analysis of the WT1 exon 7. We analyzed the samples for mutations in the exon 7 of the WT1 gene. Of the 60 cases of patients with AML, only three cases harboured a frameshift mutation, which accounts for 5%. The three cases are described in detail below.

Case 1 had antecedent myelodysplastic syndrome (MDS). Case 1, a 3-year-old female was diagnosed as AML-M1 with a white blood cell (WBC) count of 19.9x10^9/l and an abnormal karyotype 46,XX,t(2;11)(q31;p15),del(12)(p12) (15). The patient had a history of MDS for 6 months prior to the diagnosis of AML-M1. Complete remission (CR) was achieved after the first induction therapy. The patient has been on consolidation therapy since CR. The c.[1319delG] was detected, which caused a frameshift mutation compared to the wild-type sequence (Table II). Alanine (Ala) coded by GCC was replaced by proline (Pro) coded by CCC, since 1319 G was deleted and the 1320 C behind G moved forward to form a new codon with 1317 C and 1318 C. Accordingly, all the amino acids behind Ala were changed due to the recombined codons. This mutation was previously reported by Gaidzik *et al* (15), although not in association with patient characteristics (16). The spatial configuration alterations due to the mutation compared to the wild-type may be visualized based on homology modeling and optimization of molecular dynamics (Fig. 1A and B).

Cases 2 and 3 exhibited primary resistance to chemotherapy. Case 2, a 2-year-old male, was diagnosed as AML-M3 with a WBC count of 53.3x10^9/l and a normal karyotype (46,XY). PML-RARα was negative. CR was achieved after 4 cycles of chemotherapy and the patient relapsed 6 months later. The patient had a mutation in c.[1342delA; 1349-1350insA; 1353delG; 1355-1356insG; 1392delG; 1400-1401insT; 1405delT];c.[1415-1416insC];c.[1431delG];c.[1433-1434insA] (Table II), which had not been reported before.

Case 3, a 6-year-old female, was diagnosed as AML-M2a with a WBC count of 43.6x10^9/l and a complex karyotype 46,XX,t(8;12;21)/45,idm,-X (1,8). No positive fusion genes were detected. The ratio of blast cells in the bone marrow was 22% following administration of daunorubicin, cytarabine and etoposide as induction therapy for 7 days, and even increased to 57% after a prolonged 3 days of cytarabine. The patient had a mutation in c.[1345delT;1349-1350insA; 1353delG;

Table II. Sequence of exon 7 of the WT1 gene (wild-type and cases with frameshift mutations).

Exon 7	Sequence
Wild-type	1295-GAT GTG CGA CGT GTG CCT GGA GTA GCC CCG ACT CTT GTA CGG TCG GCA TCT GAG ACC AGT GAG AAA CGC CCC TTC ATG TGT GCT TAC CCA GGC TGC AAT AAG AGA TAT TTT AAG CTG TCC CAC TTA CAG ATG CAC AGC AGG AAG CAC ACT G-1445
Case 1	1295-GAT GTG CGG CGT GTG CCT GGA GTA **(del G)**CC CCG ACT CTT GTA CGG TCG GCA TCT GAG ACC AGT GAG AAA CGC CCC TTC ATG TGT GCT TAC CCA GGC TGC AAT AAG AGA TAT TTT AAG CTG TCC CAC TTA CAG ATG CAC AGC AGG AAG CAC ACT G-1445
Case 2	1295-GAT GTG CGA CGT GTG CCT GGA GTA GCC CCG ACT CTT GTA CGG TCG GC**(del A)** TCT GAG A**(ins A)**CC A **(del G)**T G**(ins G)**AG AAA CGC CCC TTC ATG TGT GCT TAC CCA GGC T**(del G)**C AAT AAG AGA T**(ins T)**AT TT**(del T)** AAG CTG T**(ins C)** CC CAC TTA CAG AT**(del G)** CA **(ins A)**C AGC AGG AAG CAC ACT G -1445
Case 3	1295-GAT GTG CGG CGT GTG CCT GGA GTA GCC CCG ACT CTT GTA CGG CCG GCT TC**(del T)** GAG A**(ins A)**CC A**(del G)**T G**(ins G)**AG AAA CGC CCC TTC ATG TGT GCT TAC CCA GGC TGC A**(del A)**T AAG AGA TAT TTT AAG CTG TCC CAC TTA CAG ATG CAC AGC AGG AAG CAC ACT G-1445

del, deletion; ins, insertion; WT1, Wilms' tumor 1 gene. Bold lettering highlights the position and type of mutation.

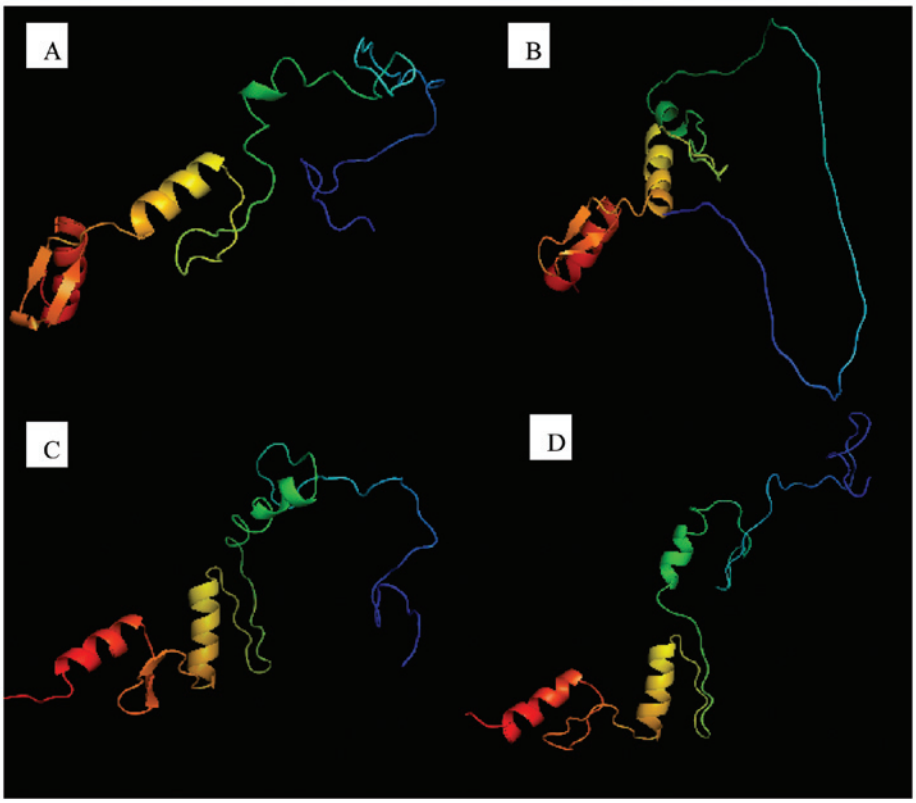

Figure 1. Spatial configuration of exon 7 demonstrated by homology modeling and optimization of molecular dynamics. (A) Wild-type; (B) case 1; (C) case 2; (D) case 3. Blue color, N-terminal coding regulatory regions of other transcription factors; green color, exon 7; orange color, exon 8; red color, exon 9. Compared to the wild-type, the three cases exhibit distinct alterations in their spatial configuration.

1355-1356insG; 1392delA], which was first reported by our group (Table II).

The mutations in these two cases affected the spatial configuration of exon 7 (Fig. 1C and D), although to a lesser extent compared to case 1 (Fig. 1B).

Discussion

The WT1 gene is located on 11p13, encoding a protein of 429 amino acids (17). The protein is constituted of the C-terminal of the DNA-binding region and the N-terminal

Table III. Mutation panel of AML patients.

ID	Gender	Age	FAB	NPM1	FLT3[a]	C-kit	DNMT3A 882AA	WT1E7	WT1E9	CEBPA
1	F	12	M3v	P	N	N	N	N	N	N
2	M	8	**M2**	N	N	N	N	N	N	P
3	M	1	M4Eo	N	N	N	N	N	N	N
4	F	3	M4	N	**P**	N	N	N	N	N
5	F	6	M2-relapse	N	N	N	N	N	N	N
6	F	9	AML	N	**P**	N	N	N	N	N
7	M	11	M3	N	N	N	N	N	N	N
8	F	1	M4Eo	N	N	P	N	N	N	N
9	M	13	M2a	N	**P**	N	N	N	N	N
10	F	3	M5b	N	N	N	N	N	N	N
11	F	3	M5	N	N	N	N	N	N	N
12	F	2	M5	N	N	N	N	N	N	N
13	M	8	M4	N	**P**	N	N	N	N	N
14	M	9	M2	N	**N**	N	N	N	N	P
15	F	11	M5	N	P	N	N	N	N	N
16	F	8	M2	N	N	P	N	N	N	N

[a]Either FLT3-ITD or FLT3-TKD. AML, acute myeloid leukemia; F, female; M, male; FAB, French-American-British classification; NPM1, nucleophosmin; DNMT3A 882AA, DNA (Cytosine-5-)-methyltransferase 3α at amino acid postion R882; WT1, Wilms' tumor 1 gene; E7, exon 7; E9, exon 9; CEBPA, CCAT/enhancer binding protein-α double mutation; N, negative; P, positive. Bold lettering highlights mutation.

of the transcriptional regulatory region. A previous study by King-Underwood *et al* (1) was the first to report WT1 gene mutations in AML and their potential effect on AML. Since then, WT1 mutations have been sporadically reported until recently, when the results on WT1 mutations from cohort studies (8-11,15) have begun to attract attention again. However, the effect of WT1 gene mutations on the prognosis of AML varies widely among different groups, even leading to opposite conclusions (8-11). This inconsistency may be attributed to differences in the populations investigated, such as age (children vs. adults), karyotype (normal vs. abnormal), combination with other mutations such as *FLT3-ITD*, or different ethnicities (17). The mutation rate in exon 7 in our study was 5% (3/60), which was lower compared to that reported by previous studies on pediatric AML (9,12). This may be due to our limited patient sample or due to the fact that M3 was included in this cohort. Our data demonstrated that the three cases with frameshift mutations had a short term survival which indicated that frameshift mutations may be related to their initial characterics (antecedent MDS or resistance to chemotherapy). Hollink *et al* (10) reported similar results, according to which WT1 gene mutations conferred an independent poor prognostic significance. By contrast, Ho *et al* (9) expanded the population to 842 patients and observed that WT1 mutations alone are of no independent prognostic significance in predicting the outcome in pediatric AML. From the data of that study it was indicated that the *FLT3-ITD* status affected the evaluation of WT1 mutations. We investigated *FLT3-ITD*, *FLT3-TKD*, *NPM1*, *CEBPA*, *C-kit*, *DNMT3A 882AA* and *WT1* exon 7 and 9 mutation in 16 cases of AML and did not observe WT1 mutation overlapping with any other mutations (Table III).

Gene expression regulated by transcriptional factors is one of the important regulatory mechanisms in the proliferation and differentiation of hematopoietic cells (18). The WT1 gene is a transcription factor which is crucial in the early differentiation of hematopoietic cells. WT1 may inhibit blood-related gene transcription, such as (Bcl-2, c-Myc and CSF-1) and is closely associated with hematological disorders (19,20). Stoll *et al* (16) demonstrated that exon 7 (zinc finger 1) played an important role in enhancing the WT1 binding activity with its target DNA in a non-specific manner. Zinc finger structure is a supersecondary structure that is able to regulate transcription by specifically combining with nucleic acid binding sites or by forming connections between the zinc finger proteins. In our study, three cases with WT1 frameshift mutations in exon 7 were demonstrated to exhibit spatial configuration alterations, which may disturb the interaction with other transcription factors, conferring transformation of MDS into AML or leukemia cell resistance to chemotherapy. Further investigations of the effect of WT1 exon 7 mutations on the mechanism of AML are required, with the use of bioinformatics technology.

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