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DDX43 promoter is frequently hypomethylated and may predict a favorable outcome in acute myeloid leukemia

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ABSTRACT

DEAD box polypeptide 43 (*DDX43*), a cancer/testis antigen (CTA), has been found to be overexpressed in various solid tumors and some hematologic malignancies. In the present work hypomethylation of the *DDX43* gene was detected in 15% (32/214) of primary acute myeloid leukemia (AML) using real-time quantitative methylation-specific PCR (RQ-MSP). The level of *DDX43* expression was correlated with *DDX43* hypomethylation ($R = 0.277$, $P = 0.014$). Moreover, bisulfite sequencing confirmed the significant correlation between the methylation density and the level of *DDX43* hypomethylation. Additionally, restoration of *DDX43* expression in the K562 cell line by 5-aza-2′-deoxycytidine treatment confirmed a direct contribution of methylation in regulating the *DDX43* gene. *DDX43* hypomethylation was observed more frequently in favorable group (21.4%) and intermediate group (15.8%) than in poor group (0%) ($P = 0.009$). AML patients with *DDX43* hypomethylation had a better overall survival (median not obtained) than those with *DDX43* methylation (median 8 months, 95% confidence interval 5.6–10.4 months) ($P = 0.014$). In summary, the *DDX43* gene is activated by promoter hypomethylation and *DDX43* hypomethylation may be a favorable prognostic factor in AML.

1. Introduction

Acute myeloid leukemia (AML) is a group of common disorders derived from haemopoietic progenitor cells, which lose the ability to differentiate normally and to respond to normal regulators of proliferation [1]. So far, it is well-known that genetic abnormalities play a pivotal role in the pathogenesis of AML [2,3]. Recently, it has been demonstrated that epigenetic aberrations, such as alterations in the DNA methylation, the histone modification patterns and miRNA expression are also involved in the development of leukemia [4–7]. Aberrant methylation of numerous genes, such as *p15*, *p73*, *HIC1*, *RARβ2*, *DAPK1*, *SOCS-1*, *E-cadherin*, *CTNNA1*, and *ER* have been identified in AML [8,9].

Cancer/testis antigens (CTAs) provide promising targets for cancer-specific immunotherapy due to their expression in a broad spectrum of cancers and limited expression in normal tissues such as testis and placenta [10]. A new member of the DEAD (Asp-Glu-Ala-Asp)-box family of helicases, *DDX43* (DEAD box polypeptide 43, also known as *HAGE*), was first identified together with sarcoma antigen (*SAGE*) as a tumor-specific CTA gene in a human sarcoma cell line [11]. So far, *DDX43* has been found with overexpression in various solid tumors such as salivary gland, colon, brain, lung and prostate cancers and hematologic malignancies (e.g., chronic myeloid leukemia and multiple myeloma) [10,12–16]. Recently, it was also shown that the abnormal hypomethylation of *DDX43* promoter was present in chronic myeloid leukemia (CML) and was associated with poor outcome [15]. However, the pattern of *DDX43* methylation has not been studied in AML. In this study, we aimed to analyze the methylation status of *DDX43* promoter and its clinical implications in primary AML patients.

2. 2 Materials and Methods

2.1. Patients and Samples

Two hundred and fourteen patients with primary AML presented at the Affiliated People's Hospital of Jiangsu University were selected for this investigation based on the availability of stored leukemic cells. Cytogenetic data were acquired from

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Table 1
The hypomethylation of *DDX43* gene promoter in patients with AML.

Patient's parameter	The status of *DDX43* methylation			
	Hypomethylated ($n=32$)	Methylated ($n=182$)	Total ($n=214$)	*P*-value
Age (years)[a]	48.5 (11–83)	47 (2–93)	47 (2–93)	0.870
Sex (male/female)	24/8	99/83	123/91	0.033
WBC ($\times 10^9$/l)[a]	17.2 (0.9–528.0)	14.1 (0.7–528.0)	14.1 (0.7–528.0)	0.996
Hemoglobin (g/l)[a]	78 (40–126)	71 (31–147)	74 (31–147)	0.233
Platelets ($\times 10^9$/l)[a]	35 (3–157)	38 (4–447)	38 (3–447)	0.909
FAB, no.				0.019
M1	3	23	26	
M2	6	80	86	
M3	10	21	31	
M4	7	32	39	
M5	4	20	24	
M6	2	6	8	
Risk grouping				0.009
Favorable	15	55	70	
Intermediate	16	85	101	
Poor	0	30	30	
Karyotyping				0.012
Normal	13	74	87	
t(8;21)	3	24	27	
inv(16)	0	1	1	
t (15;17)	10	18	28	
+8	0	7	7	
−5/5q-, −7/7q-	0	11	11	
t(11q23)	0	5	5	
t(9;22)	0	4	4	
Complex	0	9	9	
NPM1				1.000
Mutant	3	17	20	
Wild-type	29	162	191	
FLT3-ITD				1.000
Mutant	1	3	4	
Wild-type	31	176	207	
IDH1				1.000
Mutant	1	4	5	
Wild-type	31	175	206	
IDH2				0.698
Mutant	1	11	12	
Wild-type	31	168	199	
IDHI and IDH2				0.476
Mutant	1	15	16	
Wild-type	31	164	195	
DNMT3A				0.697
Mutant	1	12	13	
Wild-type	31	167	198	
DDX43 transcripts (%)[a]	977.96 (59.00–9843.00)	0.02 (0.00–128.00)	0.61 (0.00–9843.00)	<0.001

WBC, white blood cells; FAB, French–American–British classification; AML, acute myeloid leukaemia.
[a] Median (range).

201 patients. The diagnosis and classification of AML patients were made according to the revised French–American–British (FAB) classification and the 2008 World Health Organization proposal [17,18]. The risk grouping was taken according to the cytogenetical and molecular abnormalities [19]. Clinical and laboratory features of all patients were summarized in Table 1. After obtaining informed consent, the bone marrow (BM) aspirates from all patients were collected at the time of diagnosis. BM specimens obtained from 24 iron deficiency anemia (IDA) individuals were used as controls. Bone marrow mononuclear cells (BMNCs) were separated by density gradient centrifugation using Ficoll solution and washed twice with PBS.

The follow-up data were obtained for 124 cases. The median follow-up duration of the patients was 8 months (range, 1–73 months).

2.2. 5-aza-2′-Deoxycytidine Treatment

The leukemic cell line K562 was plated at a density of 1×10^6/ml and was cultured in 5 ml RPMI 1640 medium at 37 °C in a humidified atmosphere containing 5% CO_2. 5-aza-2′-deoxycytidine (DAC) (Sigma-Aldrich, Steinheim, USA) diluted in dimethyl sulfoxide (DMSO) was added in four flasks of K562 cells once a day at the same time at different final concentrations of 0.1 μM, 1 μM, 10 μM and 50 μM as experimental group. K562 cells only added with DMSO were set as the DAC controls, and the volume of DMSO was the same as that used in diluting DAC. K562 cells without treatment were also used as the control. All cells were cultured until harvested for extraction of RNA and DNA.

2.3. Real-Time Quantitative Methylation-Specific PCR

The basic methods were briefly described as previously reported [20]. Genomic DNA was obtained from BMNCs using DNA Purification Kit (Gentra, Minneapolis, MN, USA). 1 μg of genomic DNA was sodium bisulphite-modified as described in manufacturer instruction using the CpGenome™ DNA Modification kit (Chemicon, Ternecula, CA, USA). Modified DNA was used for real-time quantitative methylation-specific PCR (RQ-MSP) immediately or stored at −80 °C at once until analyzed.

RQ-MSP was performed using methylation-specific and unmethylation-specific primers and *ALU* repetitive sequence in a 7300 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Primer sequences for the methylated (M) RQ-MSP reaction were 5′-GGAGGAGTTTTTAAGGTTTTTACGT-3′ (forward) and 5′-GACAATTCC TCGTAACCAACG-3′ (reverse), and primer sequences for the unmethylated (U) RQ-MSP reaction were 5′-GGAGGAGTTTTTAAGGTTTTTATGT-3′ (forward) and 5′-ACAACAATTCCTCATAACCAACAA -3′ (reverse). *ALU* repetitive sequence was used as reference sequence. Each PCR reaction included 2 μl of modified DNA, $MgCl_2$ 2.0 mmol/l, 10 × PCR buffer, 1U Taq DNA polymerase (MBI Fermentas, Amherst, NY, USA), 200 nmol/l of each primer, 200 μmol/l dNTP, 20 × EvaGreen 1.2 μl, and 50 × ROX 0.5 μl. Both methylated and unmethylated PCR running protocols consisted of an initial denaturation step of 4 min at 94 °C, followed by a amplification program of 40 cycles at 94 °C for 30 s (denaturation), 61 °C for 30 s (anneaing), 72 °C for 30 s (extension), 82 °C for 30 s (data collection), and finally, a melting program of one cycle at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s and 60 °C for 15 s. Distilled water without DNA was used as negative control as well as recombined methylated and unmethylated *DDX43* plasmids were positive controls for each set of PCR. The