Int J Hematol (2012) 96:229–233
DOI 10.1007/s12185-012-1104-z

ORIGINAL ARTICLE

Screening for mutation R882 in the *DNMT3A* gene in Chinese patients with hematological disease

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Received: 21 March 2012 / Revised: 25 April 2012 / Accepted: 11 May 2012 / Published online: 29 June 2012
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Abstract The DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*) gene is actively involved in epigenetic regulation. Mutations in this gene affect disease progression and response to therapy, particularly in hematological disease. Recent studies have demonstrated that *DNMT3A* gene mutations affecting codon R882 are actively involved in acute myelogenous leukemia (AML), and cause abnormal alteration of DNA methylation and poor survival. In this study, we screened DNMT3A mutations in a total of 389 Chinese patients with hematological disease by sequencing the coding region of exon 23 covering residue R882. Three heterozygous mutations (p.R882S, p.R882C and p.R882H) were identified in three of 61 AML patients, whereas none of patients with other hematological disorders harbored any mutation. Our results support the notion that DNMT3A R882 was a frequent mutation in AML patients but rare in other types of hematological disorder.

Keywords *DNMT3A* gene · AML · Heterozygous mutation · Hematological disease · Chinese

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Introduction

Pathological mechanism of hematological disease is subjected to aberrant cascade of signaling pathway activation and gene expression [1]. Abnormal alteration of epigenetic regulation, as implemented by these epigenetic genes, such as *DNMT* (DNA methyltransferases), *MBD* (methyl-CpG-binding domain proteins) and *HAT* (histone acetyltransferases) [2], have been showed to affect the development and progress of these diseases [3, 4].

The DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*) gene encodes a DNA methyltransferase that is said to function in de novo methylation and affects the expression of many genes and genome stability [5]. DNMT3A protein can directly methylate targeted DNA fragment (e.g. promoter region) and leads to subsequent alteration of gene expression [6, 7]. This protein has been reported to be associated with colorectal cancer cell proliferation and serves as a switch in doxorubicin-induced colorectal cancer cell senescence and apoptosis [8, 9]. Genetic variants in either the promoter region or coding region of the *DNMT3A* gene have been shown to be associated with genetic susceptibility of gastric cancer [7] and acute myeloid leukemia (AML) [6, 10]. In particular, the residue 882 (R882) in DNMT3A protein is the most frequently mutated site in patients with AML [6, 10]. This mutation is located in a catalytic domain which binds to the targeted DNA fragment. Hitherto, four different mutations

(p.R882H, p.R882C, p.R882S, and p.R882P) at codon 882 have been identified in patients with different AML subtypes [6, 10, 11]. These R882 mutants greatly reduce DNA methylation activity of DNMT3A and are associated with poorer survival [6, 12].

In this study, we screened DNMT3A R882 mutation in a cohort of 389 Han Chinese patients with different types of hematological disease from Kunming, with an intention to uncover the frequency of R882 mutation in these diseases.

Materials and methods

Patients and genomic DNA isolation

Patient specimens (bone marrow or whole blood) from 389 Han Chinese patients at diagnosis of hematological disease were collected from the First Affiliated Hospital of Kunming Medical College. One AML patient (2011-KM06) with R882 mutation was sampled twice during his first and second visits to our clinical center. Part of these patients had been analyzed for *IDH* gene mutations and *CASP8* gene variants in our recent studies [13, 14]. The detailed information of these patients was shown in Table 1. We did not have a preset rational for selecting patients in this study, and the analyzed patients represented random patients who were diagnosed and/or received treatment at our clinical center during January 2008 to December 2011 and agreed to participate in this study. The study conformed to the tenets of the Declaration of Helsinki and informed consent was obtained from each donor prior to the study. The institutional review board of the Kunming Institute of Zoology approved this study.

Genomic DNA from bone marrow or whole blood was extracted using standard phenol–chloroform method or the AXYGEN DNA extraction kit (AxyPrep Blood Genomic DNA Miniprep Kit, USA) according to the manufacturer's protocol.

PCR amplification and sequencing

A fragment of 845 bp covering the entire coding region of exon 23 of the *DNMT3A* gene was amplified using primer pair hDNMT3Af (5′-AGGAGTTGGTGGGTGTGAGT-3′)/hDNMT3Ar (5′-TGCTCCTATCTGATCAGGCT-3′) that was newly designed in this study. PCR reactions were performed in a volume of 50 μL containing 30 ng genomic DNA, 400 μM dNTP, 1× *LA Taq*™ PCR Buffer, 2.5 U of TaKaRa *LA Taq*™ and 0.2 μM of each forward and reverse primer. The following conditions were used for PCR amplification: a denaturation cycle at 94 °C for 3 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 50 s, then followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 50 s, and ended with a final extension at 72 °C for 7 min. PCR products were purified using Genomic DNA Purification Kits (Tiangen, China) and were sequenced using

Table 1 DNMT3A R882 mutations in Han Chinese patients with different types of hematological disease from Kunming, Yunnan

Disease	No.	DNMT3A R882 mutation				
		p.R882S	p.R882C	p.R882H	p.R882P	Wild type
Neoplastic samples						
NHL	130	0	0	0	0	130
AML	61	1	1	1	0	58
MM	40	0	0	0	0	40
CLL	2	0	0	0	0	2
CML	44	0	0	0	0	44
ALL	34	0	0	0	0	34
MDS	20	0	0	0	0	20
Non-neoplastic samples						
Anemia	28	0	0	0	0	28
Thrombocytopenia	25	0	0	0	0	25
Pancytopenia	5	0	0	0	0	5

We collected all patients who were diagnosed and/or received treatment at our clinical center and agreed to participate in this study during January 2008 to December 2011, with no other bias for sampling various types of disorders of myeloid and lymphatic origin. The exact disease of those patients who were grouped as non-neuplastic was unclear and we presented the disorder as anemia, thrombocytopenia, or pancytopenia. Note that such a classification may not make much sense since different disorders can underlie these conditions

NHL non-Hodgkin's lymphoma, *AML* acute myelogenous leukemia, *MM* multiple myeloma, *CLL* chronic lymphocytic leukemia, *CML* chronic myelogenous leukemia, *ALL* acute lymphoblastic leukemia, *MDS* myeloproliferative disorder

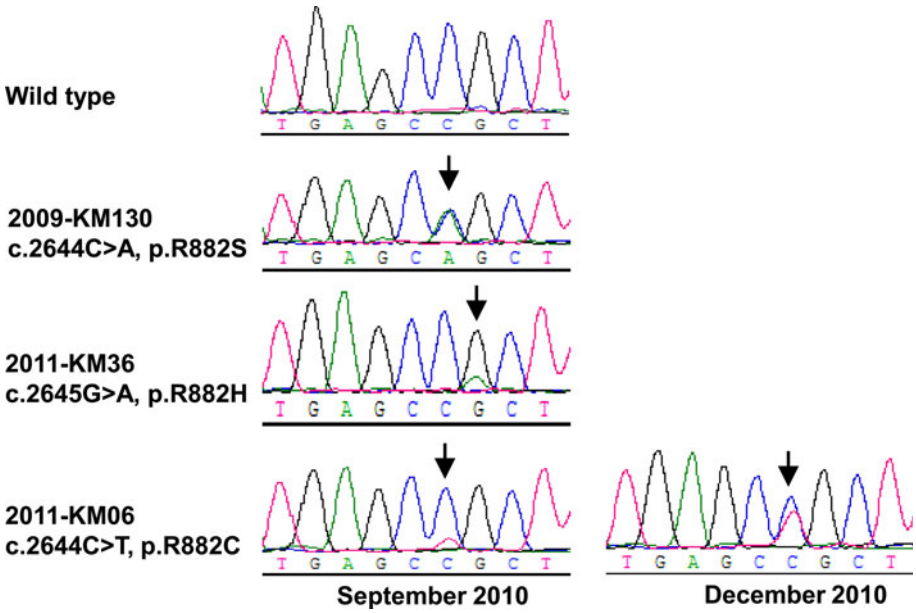

Fig. 1 Sequencing results showing AML patients with DNMT3A R882 heterozygous mutations. Genomic DNA isolated from bone marrow or whole blood was used for mutation screening. One patient, 2011-KM06 was sampled twice in September and December in 2010

the PCR primers and the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) on an ABI Prism 3730 DNA sequencer (Applied Biosystems). The identified mutation was further confirmed by sequencing with an inner primer 5′-CTCTTCTGGGTGCTGATACT-3′.

Evolutionary conservation analysis

Evolutionary conservation analysis was performed by aligning amino acid sequences of the DNMT3A protein of eight vertebrate species, including *Homo sapiens* (*DNMT3A*, GenBank accession No. NM_022552.3), *Pan troglodytes* (*DNMT3A*, XM_001148246.1), *Canis familiaris* (*DNMT3A*, XM_540110.2), *Bos taurus* (*DNMT3A*, XM_867643.3), *Mus musculus* (*Dnmt3a*, NM_007872.4), *Rattus norvegicus* (*Dnmt3a*, NM_001003958.1), *Gallus gallus* (*DNMT3A*, NM_001024832.1), and *Danio rerio* (*Dnmt6*, NM_001018140.1) from GenBank.

Results and discussion

In this study, DNMT3A R882 mutations were examined in a cohort of Han Chinese patients with various types of hematological disease of myeloid and lymphatic origin (Table 1) by sequencing the entire coding region of exon 23 of the *DNMT3A* gene. We identified three heterozygous mutations (p.R882S, p.R882H and p.R882C) in three AML patients (Fig. 1; Table 2), albeit the level of mutation load varied in different patients. The overall frequency of DNMT3A R882 mutation in our AML patients (3/61) was lower than those of previous studies (37/281, Ley et al. [10]; 12/182, Lin et al. [11]; 33/355, Yan et al. [6]). There was no mutation in patients with Non-Hodgkin's lymphoma (NHL), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) or other types of hematological disorders, albeit the sample size for each disease may be too small. We did not find any of those reported genetic variants that were listed in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), such as rs141606269 (c.2601A > C, p.V867 V), rs181757577 (c.2688A > G, p.P896P), rs61758432 (c.2697C > T, p.R899R) and rs149095705 (c.2711C > T, p.P904L) in our sequenced fragments. Evolutionary conservation analysis showed that DNMT3A codon R882 is highly conserved in different vertebrate species (Fig. 2).

By searching DNMT3A R882 mutation data in recent reports [6, 10] and the "Wellcome Trust Sanger Institute Cancer Genome Project" (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=DNMT3A&start=1&end=913&coords=AA:AA), we found that DNMT3A p.R882S mutation has been observed in AML M2 and M5 patients. In particular, DNMT3A R882 mutation was found in patients with MDS and secondary AML [15]. Among the four DNMT3A p.R882 mutations, p.R882H occurred at all stages of AML from M0 to M5, followed by p.R882S mutation, which was found in patients with AML M2 [6, 10], AML M4 (this study), and AML M5 [6, 10], whereas the other mutations were mainly identified in patient with AML at the late stage. Recent functional assay showed that methylation activity of the DNMT3A in the presence of R882 mutation was lower than the wild type, and different R882 mutations had different levels of

Table 2 Clinical data of three Han Chinese patients with DNMT3A R882 mutations

Patient	Sex/age	Diagnosis	WBC ($\times 10^9$/L)	Hemoglobin (g/L)	Platelet ($\times 10^9$/L)	DNMT3A mutation	Current status
2009-KM130	M/60	AML-M4	111.37	102	44	p.R882S	Received treatment of hydroxycarbamide tablets (2 g/day *po* d1–d5) after initial diagnosis and died 2 months later.
2011-KM06	M/48	AML-M5	187.9	84	108	p.R882C	Received treatment of hydroxycarbamide tablets (2 g/day *po* d1–d2, 3 g/day *po* d3–d9) in December, 2010 and died 1 week later.
2011-KM36	M/40	AML-M4	3.2	108	275	p.R882H	Received treatment of cytosine arabinoside (200 mg/day *iv* d1–d7) and mitoxantrone (10 mg/day *iv* d1–d3) after initial diagnosis and still alive after survived for 10 months.

Subtypes (M4 and M5) of acute myeloid leukemia (AML) were defined according to the French-American-British (FAB) classification

WBC white blood cell count

Fig. 2 Evolutionary analysis of DNMT3A codon R882 in different vertebrate species. Sequences for vertebrate species were retrieved from GenBank

reduced methylation activity [6]. Whether the occurrence of different R882 mutations in AML had different biological significance awaits further study.

One intriguing question is whether the DNMT3A R882 mutations evolved during the progress of the disease. We were able to collect samples from donor "2011-KM06" at two different time points. Upon initial diagnosis in September 2010, this patient had a relatively low level of p.R882C mutation load. He declined to receive chemotherapy but was forced to visit our clinic in December 2010 because of deteriorated health condition. His blood harbored a much higher p.R882C mutation load at this time. He received treatment of hydroxycarbamide tablets (2 g/day *po* d1–d2, 3 g/day *po* d3–d9) and died around 1 week later (Fig. 1). The different mutation load was most likely caused by the discrepancy of DNMT3A R882 mutation frequency among different types of blood cells and different percentages of leukemia blasts. Indeed, recent reports have showed that the mutation load of DNMT3A R882 varied in different peripheral blood cells (monocyte, B-lymphocyte and T-lymphocyte). Meanwhile, DNMT3A R882 mutation might be acquired in bone marrow after birth [16, 17].

The current study had several limitations. First, the sample size for patients with certain disease was small and the exact disease for those patients grouped as non-neuplastic was unclear. Second, we did not screen the entire *DNMT3A* gene for mutation. There is a possibility that other mutation in this gene may be equally pathogenic, or abnormal expression of *DNMT3A* gene can cause hematological disorders. Nonetheless, the current study may provide a profile for the occurrence of R882 mutations in Chinese patients with hematological disorders. Further study with large sample size and incorporation of detailed

clinical and experimental data will be essential to uncover the role of *DNMT3A* gene in the development of AML.

Acknowledgments We thank patients for participating in this study. This study was supported by the National Natural Science Foundation of China (30925021 and 81060046), Yunnan Province (2009CI119 and 2010CD161), and the Chinese Academy of Sciences.

Conflict of interest The authors declare no conflicts of interest.

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