DNMT3A mutations in Chinese childhood acute myeloid leukemia

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Abstract

Background: DNA methyltransferase 3A (DNMT3A) mutations have been found in approximately 20% of adult acute myeloid leukemia (AML) patients and in 0% to 1.4% of children with AML, and the hotspots of mutations are mainly located in the catalytic methyltransferase domain, hereinto, mutation R882 accounts for 60%. Although the negative effect of DNMT3AR882 on treatment outcome is well known, the prognostic significance of other DNMT3A mutations in AML is still unclear. Here, we tried to determine the incidence and prognostic significance of DNMT3A mutations in a large cohort in Chinese childhood AML.

Methods: We detected the mutations in DNMT3A exon 23 by polymerase chain reaction and direct sequencing in 342 children with AML (0–16 years old) from January 2005 to June 2013, treated on BCH-2003 AML protocol. The correlation of DNMT3A mutations with clinical characteristics, fusion genes, other molecular anomalies (FLT3 internal tandem duplication [FLT3-ITD], Nucleophosmin 1, C-KIT (KIT proto-oncogene receptor tyrosine kinase), and Wilms tumor 1 mutations), and treatment outcome were analyzed.

Results: DNMT3A mutations were detected in 4 out of 342 (1.2%) patients. Two patients were PML-RARA positive and 1 patient was FLT3-ITD positive. The mutations in coding sequences included S892S, V912A, R885G, and Q886R. Furthermore, there was 1 intronic mutation (c.2739+55A>C) found in 1 patient. No association of DNMT3A mutations with common clinical features was found. Two patients with DNMT3A mutations died of relapse or complications during treatment. One patient gave up treatment due to remission induction failure in day 33. Only 1 patient achieved continuous complete remission.

Conclusions: DNMT3A mutations were rare in Chinese children with AML including PML-RARA positive APL. The mutation positions were different from the hotspots reported in adult AML. DNMT3A mutations may have adverse impact on prognosis of children with AML.

Abbreviations: AML = adult acute myeloid leukaemia, CCR = continuous complete remission, CR = complete remission, DNMT3A = DNA methyltransferase 3A, EFS = event-free survival, FLT3-ITD = FLT3 internal tandem duplication, M1 = acute myeloblastic leukemia without maturation, M2 = acute myeloblastic leukemia with granulocytic maturation, M3 = acute promyelocytic leukemia, M4 = acute myelomonocytic leukemia, M4EO = acute myelomonocytic leukemia together with bone marrow eosinophilia, M5 = acute monoblastic leukemia, M6 = acute erythroid leukemia, M7 = acute megakaryoblastic leukemia, MPAL = mixed phenotype acute leukemia.

Keywords: childhood acute myeloid leukemia, DNA methyltransferase 3A mutations, FLT3 internal tandem duplication, prognosis

1. Introduction

Acute myeloid leukemia (AML) accounts for 20% of childhood leukemias. Although the 5-year event-free survival (EFS) for children with newly diagnosed AML approaches 50% to 60%, relapsed or refractory AML remains the most challenging problems in treatment of this disease.[1–4] Leukemia is a heterogeneous disease characterized by specific genetic alterations and related multiple epigenetic changes.[5] DNA methyltransferase 3A (DNMT3A) mutations have been found in approximately 20% to 25% of adult AML patients and only 0% to 1.4% of childhood AML.[1–20] The hotspots of mutations are mainly located in the catalytic methyltransferase domain, hereinto, mutation R882 accounts for 60%, which conferred significant adverse prognostic impact.[13–19,21] However, the prognostic significance of other DNMT3A mutations in AML is to be determined. Here, we reported the incidence and prognostic significance of DNMT3A mutations in a large cohort of Chinese children with AML.

2. Materials and methods

2.1. Patients

A total of 342 children with AML, including 3 cases with acute myeloblastic leukemia, without maturation (M1), 142 cases with acute myeloblastic leukemia with granulocytic maturation (M2),
76 cases with acute promyelocytic leukemia (M3), 43 cases with acute myelomonocytic leukemia (M4) or together with bone marrow eosinophilia (M4EO), 33 cases with acute monoblastic leukemia (M5), 11 cases with acute erythroid leukemia (M6), 29 cases with acute megakaryoblastic leukemia (M7), and 5 cases with mixed phenotype acute leukemia (M0/AML), were enrolled in this study. The patients were diagnosed as AML from January 2005 to June 2013 at Beijing Children’s Hospital. There were 208 boys and 134 girls, aged from 1 to 16 years with a median of 7 years. One hundred and ninety-seven patients were found positive for different types of fusion genes including AML1-ETO, PML-RARA, and CBFβ-MYH11, MLL rearrangements, DEK-CAN and TLS-ERG fusion. The patients were treated in accordance with the AML BCH-2003 Protocol. Informed consents were obtained from all the children’s parents or legal guardians.

2.2. Nucleic acid extraction

Ficoll 400 (MD Pacific Technology Co., Ltd., Tianjin, China) was used to isolate bone marrow mononucleated cells. The cells were stored at −70°C until use. We used the DNA Extraction Kit (Ugene Co., Ltd., Anhui, China) to extract genomic DNA. Trizol Reagent (Invitrogen, Carlsbad, CA, Promega, Madison, WI) was used to extract total RNA. Then the RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen; Promega).

2.3. Detection of DNMT3A mutations

In this study, the mutations in exon 23 as well as adjacent intronic regions were focused, because most of DNMT3A mutations were concentrated in exon 23. The polymerase chain reaction (PCR) mixture was 50 μL, containing 100 ng of genomic DNA, 5 μL of 10 times buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 pmol of upstream and downstream primers, and 1 to 2 U of Taq DNA polymerase (Promega, USA). The primers’ sequences could be found in Table 1. The cycling condition was as follows: 5 minutes at 95°C for predenaturation, 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, and 10 minutes at 72°C for final extension. The PCR products were sent to Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. and directly sequenced using AB PRISM 3730 Automated Sequencer. Four PCR products with unsatisfactory sequencing results were subcloned into pEASY™-T5 Zero cloning vector. The recombinant plasmids were transformed into Trans 5α chemically competent cell (Transgene, Beijing, China). Five to 10 clones for each product were selected for plasmid sequencing after incubation at 37°C for 15 hours.

2.4. Analysis and interpretation of DNMT3A mutations

Mutation Surveyor software (SoftGenetics, PA) was used to analyze the sequencing results for existence of mutations. Variant effect predictor (Ensembl, Sanger Institute, Cambridge, United Kingdom) online tool and mutation taster online tool were used to analyze the possible effect on protein function for every mutation of DNMT3A. The National Center for Biotechnology Information record NM_175629 was used as the reference sequence of DNMT3A.

2.5. Detection of the other genetic aberrations and mutations

Twenty-nine types of fusion genes resulting from chromosome translocations were detected in cDNA samples as described previously.[22] The other common genetic mutations in AML were also detected in genomic DNA samples including Nucleophosmin 1 (NPM1), C-KIT, Wilms tumor 1 (WT1) mutation, and FLT3 internal tandem duplication (FLT3-ITD), as described elsewhere.[2–4,24–28] The primers sequences were presented in Table 1.

2.6. Statistical analysis

The difference in clinical characteristics between the 2 groups of patients with or without DNMT3A mutations was tested using Fisher exact test. SPSS 16.0 software (SPSS Inc., Chicago, IL) package was used to statistically analyze all of the data. P < .05 was appointed statistically significant.

3. Results

3.1. DNMT3A mutations in childhood AML

In total, 4 out of 342 (1.2%) patients with newly diagnosed AML were identified with DNMT3A mutations by direct sequencing and clone sequencing for exon 23. PML-RARA fusion gene was detected in 2 patients’ leukemic blasts. No other fusion gene was found in the other 2 patients. Three missense mutations and one synonymous mutation in exon 23 including V912A, R885G, Q886R, and S892S were detected in 4 patients respectively (Fig. 1 and Table 2). In patient 3, we also detected one mutation in intrinsic regions: c.2739+55A>G, as described elsewhere.[27,32] The other 4 were all novel variants (Table 3).

However, the most common mutation R882S was not found in our series of cases. Of note, the DNMT3A mutations detected in diagnostic samples did not exist in corresponding complete remission (CR) samples. These findings indicated that these mutations were leukemia specific and were not germline.

Table 1

| Primers for mutation detection in childhood AML. |
|-----------------------------------------------|
| Forward primer (5'–3') | Reverse primer (5'–3') | Product size (bp) | Sequencing primer (5'–3') |
|------------------------|------------------------|------------------|--------------------------|
| DNMT3A (exon23)        | TGCCTGGTGGTGTTAGACG     | TTACCTCCTGTGTTGTT | 380                      | F                       |
| FLT3-ITD (exon14-15)   | TATTTGAGTTAGAAAGCCACG   | CTCTCCTTCTTGTTTTC | 329                      | R                       |
| NPM1 (exon12)          | TATGGTGGTTGTTAGACGAGA   | CAGAATCTTTTGAATCTTCAC | 560                    | R                       |
| WT1 (exon7)            | AGGCGCTCGTGTTAGACG      | GTGCGTGAACACCTGTTC | 225                      | R                       |
| C-KIT (exon8)          | TAGGCGCGGCTGGTTAGACG    | ATCTTTTCACTCAGCTTTC | 204                      | R                       |
| (exon17)               | CAGAAGCTGTTAGAGCGCACAGA | CAGAAGCTGTTAGAGCGCACAGA | 630                    | F                       |
|                        | GGAGACCATATATATAGGATCT   | GGAGACCATATATATAGGATCT | 666                    | F                       |

F = forward primer, FLT3-ITD = FLT3 internal tandem duplication, NPM1 = nucleophosmin 1, R = reverse primer, WT1 = wilms tumor 1.
In order to assess the possible effect of the 5 mutations on protein structure and function, we analyzed the mutations with variant effect predictor and mutation taster. The 3 missense mutations, V912A, R885G, and Q886R, changed amino acid sequence as well as splice site, with moderate impacts. In addition, the latter 2 might affect protein features. Although S892S and c.2739+55A>C made no effect on amino acid sequence, these 2 mutations changed splice site. Interestingly, the former might also affect protein features according to analysis result of mutation taster (Table 3).

3.2. Correlation of DNMT3A mutations with clinical characteristics

We firstly analyzed the association of DNMT3A mutations with other 4 gene (NPM1, C-KIT, WT1, and FLT3-ITD) mutations respectively, but found no significant correlation with them (Fisher exact test, \( P > .05 \), Table 4). Furthermore, no correlation was found between the common clinical features including age, sex, diagnostic white blood cell count, leukemia subtype, karyotype, fusion genes, and DNMT3A mutations (Table 4).

3.3. Clinical outcome of patients’ with DNMT3A mutations

DNMT3A mutations were detected in 4 out of 342 (1.2%) patients. Of those with DNMT3A mutated, patient 1 (man, 6 years old, FAB M4) harboring a S892S mutation was diagnosed as leukemic infiltration of the gastrointestinal tract, and gave up treatment after 38 days due to remission induction failure at day 33. Patient 2 (woman, 12 years old, APL) harboring a V912A mutation and PML-RARA fusion gene achieved continuous complete remission for 60 months. Patient 3 (man, 1 year old, FAB M5) harboring a R885G mutation and 1 intronic mutations (c.2739+55A>C) was diagnosed as AML with testicular and CNS involvement. After 10 months, the patient underwent hematopoietic stem cell transplant but relapsed again in testicle due to leukemic infiltration of the gastrointestinal tract in the early phase of treatment, we were not sure about the association of S892S with prognosis. V912A mutation was a fairly subtle amino acid change and did not affect the protein features according to mutation taster tool (University of Berlin, Germany). However, Pt.1 with DNMT3A[S892S] gave up treatment due to leukemic infiltration of the gastrointestinal tract, and died of retinoic acid syndrome 22 days later (Table 2).

4. Discussion and conclusions

In former reports, DNMT3A mutation has been found in approximately 20% of adult AML patients and in 0% to 1.4% childhood AML. The hotspots of mutations are mainly located in the catalytic methyltransferase domain, hereinto, R882 mutation accounts for 60% in adults and 13% in children, 19]. Thus, in the present study, we focused on the mutations in exon 23 which encoded the catalytic methyltransferase domain of DNMT3A. We found that DNMT3A mutations can be found in 1.2% of Chinese children with AML. This incidence was in accordance with the previous results. However, the mutation positions were different from those reported in adult AML (Fig. 1 and Table 2): of the 5 mutations (S892S, V912A, R885G, Q886R, and c.2739+55A>C), only the synonymous mutation, S892S, has been reported in ExAC (28/121344, 0.02307% in Latino), the other 4 mutations were all reported for the first time to our knowledge (Table 3). These results indicated the distinctive features of DNMT3A mutations in childhood AML.

S892S mutation changed the splice site and might affect the protein features according to mutation taster tool. Neither was the mutation position almost never found in APL. However, Pt.1 with DNMT3A[S892S] gave up treatment due to leukemic infiltration of the gastrointestinal tract, and died of retinoic acid syndrome 22 days later (Table 2).

There have been few reports of DNMT3A mutations in patients with PML-RARA. A study has figured out that APL patients were generally excluded from many studies because of existence of the unique fusion gene PML-RARA and the favorable prognosis in contrast to other subtypes. Moreover, DNMT3A gene mutation was almost never found in APL.

In order to assess the possible effect of the 5 mutations on protein structure and function, we analyzed the mutations with variant effect predictor and mutation taster. The 3 missense mutations, V912A, R885G, and Q886R, changed amino acid sequence as well as splice site, with moderate impacts. In addition, the latter 2 might affect protein features. Although S892S and c.2739+55A>C made no effect on amino acid sequence, these 2 mutations changed splice site. Interestingly, the former might also affect protein features according to analysis result of mutation taster (Table 3).

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### Table 2

Clinicobiological characteristics and treatment outcome of childhood AML with DNMT3A mutations.

| Case | Gender | Age (y) | WBC (×10⁹/L) | Hemoglobin (g/L) | Platelet (×10⁹/L) | Karyotype | Fusion gene | Mutation of DNMT3A | Diagnosis | FLT3-ITD | NPM1 | C-KIT | WT1 | Outcome |
|------|--------|---------|--------------|-----------------|-----------------|------------|------------|-------------------|-----------|----------|-------|--------|------|---------|---------|
| 1    | M      | 6       | 1            | 81              | 52              | 46XY (2)   | Neg        | c.2676A>G/p.S892S | AML-M4    | Neg      | Neg   | Neg    | Neg  | Leukemic infiltration of the gastrointestinal tract, gave up after 38 days |
| 2    | F      | 12      | 5.8          | 97              | 17              | 46XX (5)   | PML-RARA (S form) | c.2735T>C/p.V912A | AML-M3    | Neg      | Neg   | Neg    | Neg  | CCR for 60 months |
| 3    | M      | 1       | 7.7          | 112             | 34              | 46XY       | Neg        | c.2653A>G/p.R885G c.2739+55A>C | AML-M5    | Neg      | Neg   | Neg    | Neg  | Relapsed in testicle 39 days after HSCT and died 8 months later |
| 4    | F      | 2       | 169          | 102             | 18              | 46XX(1)/46,XX,t(15;17)(q22;q21)(1) | PML-RARA (S form) | c.2657A>G/p.Q886R | AML-M3    | Pos      | Neg   | Neg    | Neg  | Dead of retinoic acid syndrome 22 days later |

CR = continuous complete remission; FLT3-ITD = FLT3 internal tandem duplication; HSCT = hematopoietic stem cell transplant; NPM1 = nucleophosmin 1; WBC = white blood cells.

*According to AML BCH-2003 protocol; M3: acute promyelocytic leukemia; M4: acute myelomonocytic leukemia; M5: acute monoblastic leukemia.

### Table 3

The interpretation of mutations of DNMT3A.

| Case | Mutations of DNMT3A | Variant effect predictor | Consequence | Impact | Known variant | Mutation taster | Disease causing |
|------|---------------------|-------------------------|-------------|--------|---------------|-----------------|----------------|
| 1    | c.2676A>G/p.S892S   | synonymous_variant      | Low         |        | EvAC:23/12134 = 0.0012307 in Latino population | Splice site changes protein features (might be) affected |
| 2    | c.2735T>C/p.V912A   | missense_variant        | Moderate    |        | Variant was neither found in EvAC nor 1000G | Amino acid sequence changed |
| 3    | c.2653A>G/p.R885G   | missense_variant        | Moderate    |        | Variant was neither found in EvAC nor 1000G | Amino acid sequence changed |
| 3    | c.2653A>G/p.R885G   | 3_prime_UTR_variant    | Modifier    |        | Variant was neither found in EvAC nor 1000G | Amino acid sequence changed |
| 4    | c.2657A>G/p.Q886R   | missense_variant        | Moderate    |        | Variant was neither found in EvAC nor 1000G | Amino acid sequence changed |

DNMT3A = DNA methyltransferase 3A.
However, in our study, DNMT3A mutations were found in 2 patients (Pt.2 and Pt.4) with APL and PML-RARA fusion gene, indicating the requirement of detection of DNMT3A mutations in childhood APL. Now, more and more studies have shown that DNMT3A is a haploinsufficient tumor suppressor gene in myeloid leukemias, when cooperating mutations are present.\(^{30-32}\) In the absence of high-risk cytogenetics, DNMT3A mutation status has an adverse impact on outcome in the presence of FLT3 and/or NPM1 mutations.\(^{\text{[13]}}\) The R882H mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers.\(^{\text{[13]}}\) DNMT3A loss drives enhancer hypomethylation in FLT3-ITD-associated leukemias.\(^{\text{[14]}}\) Coexistence of DNMT3A R882 mutation and FLT3-ITD was an extremely poor prognostic factor in patients with normal-karyotype AML after allogeneic hematopoietic cell transplantation.\(^{\text{[15]}}\) Notably in our study, the poor treatment outcome of Pt.4 in this study was consistent with the above results. Although both of Pt.2 and Pt.4 have PML-RARA fusion gene and mutated DNMT3A, the outcome of these 2 patients was quite different. Pt.4 (PML-RARA, DNMT3A\textsuperscript{R886R}, and FLT3-ITD positive) died at early phase of treatment, whereas Pt.2 (PML-RARA and DNMT3A\textsuperscript{R882}) had been in CCR for 60 months, suggesting that FLT3-ITD acts as a key role to accelerate the progress in Pt.4 with APL and DNMT3A\textsuperscript{R886R}. DNMT3A mutations alone may not induce AML, only acts as an initial lesion and requires an additional genetic event to increase susceptibility to leukemic development.

The clinical significance of adult AML with DNMT3A mutations seems to be age dependent.\(^{\text{[10,21]}}\) DNMT3A-R882 mutation are associated with adverse prognosis in older patients (≥60), and non-R882-DNMT3A mutations are associated with adverse prognosis in younger patients (18 < and <60). This finding may also be available and suitable in childhood AML. In this study, the median age of the 4 patients with non-R882 DNMT3A mutations was 7 years old. In the 3 patients younger than 7, 2 patients (Pt.3 and Pt.4) died of relapse or complications, the other patient gave up treatment because of remission induction failure in day 33. In contrast, the only 1 patient older than 7 (Pt. 2) were in CCR up to 60 months. The mechanisms behind this phenomenon were worth investigating.

### Table 4

| Characteristic | Number (%) | Positive | Negative | P |
|----------------|------------|----------|----------|---|
| Age, years median | 7 | 206 | 206 | .763 |
| Gender | | | | .646 |
| Male | 206 (60.8) | 2 | 206 | |
| Female | 134 (39.2) | 2 | 132 | |
| WBC (<10\(^{9}\)L\(^{-1}\)) | | | | 1 |
| <50 | 284 | 3 | 281 | |
| ≥50 | 58 | 1 | 57 | |
| FAB-subtype | | | | .338 |
| M1 | 3 (0.9) | 3 | | |
| M2 | 142 (41.5) | 142 | | |
| M3 | 76 (22.2) | 2 | 74 | |
| M4 or M4Eo | 43 (12.6) | 1 | 42 | |
| M5 | 33 (9.6) | 1 | 32 | |
| M6 | 11 (3.2) | 11 | | |
| M7 | 29 (8.5) | 29 | | |
| MPAL | 5 (1.5) | 5 | | |
| Fusion gene | | | | .775 |
| Negative | 145 (42.4) | 145 | | |
| AML1-ETO | 72 (21.1) | 72 | | |
| PML-RARA | 72 (21.1) | 2 | 70 | |
| CBX4-MYH11 | 13 (3.8) | 13 | | |
| MLL-AF9 | 12 (3.5) | 12 | | |
| MLL-AF10 | 8 (2.3) | 8 | | |
| DEX-CAN | 5 (1.5) | 5 | | |
| cdimML | 4 (1.2) | 4 | | |
| MLL-AF9 | 3 (0.9) | 3 | | |
| TLS-ERG | 3 (0.9) | 3 | | |
| MLL-AF6 | 2 (0.6) | 2 | | |
| NPM-AUK | 1 (0.3) | 1 | | |
| BCR-ABL | 1 (0.3) | 1 | | |
| MLL-AF1 | 1 (0.3) | 1 | | |
| FLT3-ITD | 39 (11.4) | 38 | | .385 |
| NPM1 mutation | 13 (3.8) | 13 | 1 | |
| C-KIT mutation | 35 (10.2) | 35 | 1 | |
| WT1 mutation | 15 (4.4) | 15 | 1 | |

FAB = French-American-British, FLT3-ITD = FLT3 internal tandem duplication, M1 = acute myeloblastic leukemia without maturation, M2 = acute myeloblastic leukemia with granulocytic maturation, M3 = acute promyelocytic leukemia, M4 = acute myelomonocytic leukemia, M4Eo = acute myelomonocytic leukemia together with bone marrow eosinophilia, M5 = acute monocytic leukemia, M6 = acute erythroid leukemia, M7 = acute megakaryoblastic leukemia, MPAL = mixed phenotype acute leukaemia, NPM1 = nucleophosmin 1, WBC = white blood cells, WT1 = Wilms tumor 1.
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