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Original Article

Down-regulation of TET2 in CD3$^+$ and CD34$^+$ cells of myelodysplastic syndromes and enhances CD34$^+$ cells proliferation

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Abstract: Aims and background: To investigate the expressions of TET2 mRNA in bone marrow CD3$^+$ and CD34$^+$ cells of the patients with myelodysplastic syndromes (MDS) and to study the effect of silencing TET2 by small interfering RNA (siRNA) on the biological characteristics of CD34$^+$ cells. Methods: CD3$^+$ and CD34$^+$ cells were sorted by magnetic activated cell-sorting system from bone marrow of MDS patients and controls. The mRNA expressions of TET2 in bone marrow CD3$^+$ and CD34$^+$ cells of 28 MDS patients and 20 controls were detected by qPCR. The silencing effect of RNA interference (RNAi) on TET2 expression in CD34$^+$ bone marrow cells of normal control was identified by qPCR and Western blot analysis. The cell cycle kinetics and cell apoptosis were then detected by flow cytometry. Results: The expression of TET2 mRNA in CD3$^+$ and CD34$^+$ cells was down-regulated in MDS compared with that in controls [(0.16±0.11) vs. (1.05±0.32) (P<0.001); (0.58±0.26) vs. (1.25±0.94) (P<0.005)]. The siRNA targeting TET2 suppressed the expression of TET2 in normal CD34$^+$ cells. Meanwhile, the proliferation activity was significantly enhanced [G0/G1: (87.82±8.25)% vs. (92.65±7.06)% and (93.60±5.54)%, P<0.05; S: (11.50±8.31)% vs. (6.92±7.04)% and (5.95±5.53)%, P<0.05]and the apoptosis rate was declined [(21.28±9.73)% vs. (26.17±9.88)% and (26.20±9.78)%] in the cells which transfected with TET2 siRNA as compared to those in the cells transfected with scrambled siRNA and control cells. Conclusions: The TET2 expression of in CD3$^+$ and CD34$^+$ cells of MDS patients was decreased. Suppression of TET2 expression renders the CD34$^+$ cells harboring more aggressive phenotype. This preliminary finding suggests that CD34$^+$ cells lowering expression of TET2 may play an oncogenic role on myeloid tumor and CD3$^+$ T cells of MDS patients may be derived from the malignant clone.

Keywords: Myelodysplastic syndromes, CD3$^+$ T cells, CD34$^+$ cells, TET2, siRNA, Biological effect

Introduction

Myelodysplastic syndromes (MDS) is a group of clonal malignant hematopoietic disorders, characterized by ineffective hematopoiesis and frequent progression to acute myeloid leukemia (AML). Recent studies suggest that MDS is a stem-cell disorder in which aberration within a hematopoietic stem cell (HSC) gives rise to the entire disease just as in AML [1, 2] and that TET2 was the most frequently mutating gene in MDS known so far. The highest expression of TET2 mRNA was in myeloid cells CD33$^+$ or monocytes CD14$^+$, and also immature CD34$^+$ cells of healthy persons [3, 4].

Based on this evidence, the present study aimed to examine the hypothesis that TET2 performed important roles in the tumorigenesis of CD34$^+$ cells. This hypothesis represents a novel perspective on CD34$^+$ cell differentiation induced by TET2 knockdown. This study has the potential not only to elucidate the role of TET2 in the regulation of CD34$^+$ cell cycle and apoptosis, but also to provide mechanistic insights into the progression of CD34$^+$ cell malignancy.

Materials and methods

Patients

A total of 28 untreated patients (18 males, 10 females), who had been newly diagnosed with MDS according to WHO classification [5] were enrolled in the present study. The median age was 59 years (range, 29-77 years). According to the WHO criteria, patients were classified as follows: refractory anemia (RA; including RA with

Table 1. Characteristics of MDS patients

Case	Age/sex	Diagnosis	Blast smear, %	Cytogenetics	IPSS
1	60/M	RA	0.5	Int	0.5
2	49/M	RA	1	Good	0
3	52/F	RARS	1.5	Good	0
4	29/M	RARS	0.5	Poor	1
5	39/F	RCMD	3.5	Good	0.5
6	77/F	RCMD	1.5	Good	0.5
7	57/M	RCMD	4.5	Poor	1.5
8	63/M	RCMD	4.5	Poor	1.5
9	46/F	RCMD	2	Good	0
10	59/M	RCMD	2	Int	1
11	47/M	RCMD	2.5	Poor	1.5
12	61/F	RCMD	4.5	Int	1
13	75/F	RCMD	3	Poor	1.5
14	53/M	RCMD	2	Good	0.5
15	51/M	RAEB-I	8	Good	1
16	55/M	RAEB-I	5	Int	1.5
17	49/F	RAEB-I	9.5	Poor	2
18	67/M	RAEB-II	14	Poor	3
19	70/F	RAEB-II	10.5	Poor	3
20	68/M	RAEB-II	16	Good	2
21	38/M	RAEB-II	10	Poor	3
22	44/F	RAEB-II	10	Int	2
23	56/M	RAEB-II	12.5	Int	2.5
24	50/M	RAEB-II	13	Good	2
25	64/M	RAEB-II	10	Int	2
26	66/F	RAEB-II	12	Good	2
27	58/M	RAEB-II	17	Good	2
28	49/M	RAEB-II	13	Poor	3

ring sideroblasts, RARS; n=4), refractory cytopaenia with multilineage dysplasia (RCMD; n=10), refractory anemia with excess blasts 1 (RAEB-1; n=3) and refractory anemia with excess blasts 2 (RAEB-2; n=11). Patients' features are listed in supplementary material **Table 1**. Written informed consent was obtained from each patient prior to entering the trial. The study complied with the acceptable international standards outlined in the Declaration of Helsinki, and was approved by the Institutional Ethics Committee of Tianjin Medical University (Tianjin, China).

There were 36 healthy volunteers with a median age of 35 years (range 19 to 48, supplementary material **Table 2**). All the healthy volunteers were provided with written informed consent before entering the study.

Magnetic sorting CD3+ and CD34+ cells

CD34+ cells from MDS bone marrow were obtained from the mononuclear cell fraction (Ficoll density separation) followed by immunomagnetic bead selection with monoclonal murine antihuman CD3 and CD34 antibodies using the Auto MACs automated separation system (Miltenyi Biotec, Monchengladbach, Germany). Yield and purity of the positively selected CD3+ and CD34+ cells were evaluated by flow cytometry (FACS Calibur) (Bio-rad, Hercules, CA, USA).

Cell culture and transfection

CD34+ cells were cultured in X-VIVO 10 medium supplemented with 10% fetal bovine serum. All cultures were maintained at 37°C in a moist atmosphere containing 5% CO2. The cells were plated in 6-well plates at 2×10^5 cells/well. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacture instructions. The siRNA sequence targeting TET2 (siRNA-TET2) was 5'-CUG CUU CUG UUC UCA AUA ATT dTdT-3', 5'-UUA UUG AGA ACA GAA GCA GTT dTdT -3'. The siRNA sequence for scrambled siRNA (siRNA-scr) was 5'-UUG AAG UUA UGU AUC CUC CUU-3', 5'-CUG AAG CUG CUG GGA GUA AUU-3'. Blank transfection served as the control.

Real-time quantitative PCR assay

Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA, USA) 48 hours after transfection. Total RNA (1 μg) was processed directly to cDNA by reverse transcription with AMV reverse transcriptase (Takara, Osaka, Japan) according to manufacture protocols. The TET2 was amplified under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. β-actin served as a reference. Oligonucleotide sequences for TET2: forward 5'-GCC AAG TCG TTA TTT GAC CA-3', reverse 5'-CTG AAG AAG TTG TTT GCT GCT CTA-3'; for β-actin: forward 5'-CAC GAT GGA GGG GCC GGA CTC ATC-3', reverse 5'-TAA AGA CCT CTA TGC CAA CAC AGT-3'. The expression ratio was calculated as 2n, where n is the CT value difference for each patient normalized by the average CT differ-

Table 2. Characteristics of normal controls

Case	Age/sex	Granulocyte ($\times10^9$/L)	Hemoglobin (g/L)	Platelet ($\times10^9$/L)	Abnormal chromosome
1	38/F	7.65	127	160	Absence
2	36/M	9.46	160	195	Absence
3	28/F	6.98	132	263	Absence
4	24/M	4.85	148	214	Absence
5	39/F	6.45	134	136	Absence
6	35/F	6.56	126	252	Absence
7	43/M	9.12	158	186	Absence
8	27/M	6.98	147	265	Absence
9	28/F	4.56	143	186	Absence
10	42/F	6.27	136	210	Absence
11	39/M	8.36	139	286	Absence
12	29/F	7.53	140	184	Absence
13	41/M	5.66	160	245	Absence
14	37/F	8.12	141	216	Absence
15	24/F	9.03	136	145	Absence
16	19/M	5.89	140	208	Absence
17	26/F	9.45	129	210	Absence
18	39/M	8.26	151	169	Absence
19	25/M	4.76	149	178	Absence
20	29/M	4.78	138	259	Absence
21	21/F	6.91	127	240	Absence
22	45/F	7.10	119	296	Absence
23	38/M	7.38	157	290	Absence
24	40/M	5.98	148	243	Absence
25	19/F	9.68	130	150	Absence
26	47/M	8.21	146	186	Absence
27	22/M	5.70	132	249	Absence
28	37/F	4.95	125	209	Absence
29	42/F	6.17	120	258	Absence
30	33/M	9.01	134	195	Absence
31	46/M	7.33	153	249	Absence
32	27/F	6.59	143	167	Absence
33	36/M	8.92	154	245	Absence
34	48/M	6.77	155	135	Absence
35	42/F	8.14	139	243	Absence
36	34/F	7.80	125	140	Absence

ence of the samples from control subjects (ΔΔCT method) [6].

Western blotting

Cell lysates were prepared on ice in RIPA lysis buffer containing 50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1% Nonidetp-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1-2 mM PMSF. 100 μg of whole cell lysate proteins were used for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF membrane. After blocking by incubation with 5% BSA in Tris-buffered saline for 1 hr at room temperature, the membrane was incubated using polyclonal antibody against TET2 (Santa Cluz, USA) and anti-GAPDH antibody (Xiangtian, China).

Flow cytometry

At 48 hours after transfection, cells were collected and prepared in a suspended solution. The cells were fixed with 250 μl solution A at room temperature for 10 minutes. The cells were incubated with 200 μl solution B at room temperature for 10 minutes, followed by solution C for 10 minutes at 4°C in the dark. Cell cycles were analyzed using FACS Calibur flow cytometry.

When 48 hours passed after transfection, cells were collected and washed with cold PBS. The cells were incubated with 5 ul Annexin V and 5 μl PI at room temperature for 15 minutes in the dark. Cell apoptosis were analyzed using FACSCalibur flow cytometry.

Statistical analysis

SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were expressed as Mean ± SD. Paired T test analysis of variance was used to analyze significance between groups. Least significant difference method of multiple comparisons with parental and control groups was used when the probability for analysis of variance was statistically significant. A level of $P<0.05$ was determined to be significant.

Results

Expression of TET2 mRNA in the CD3+ and CD34+ cells of patients with MDS and normal control

The expressions of TET2 mRNA of the CD3+ and CD34+ cells of twenty-eight MDS patients and twenty normal controls were measured. TET2 mRNA relative expression was found to be lower expressed in the CD3+ and CD34+ cells of

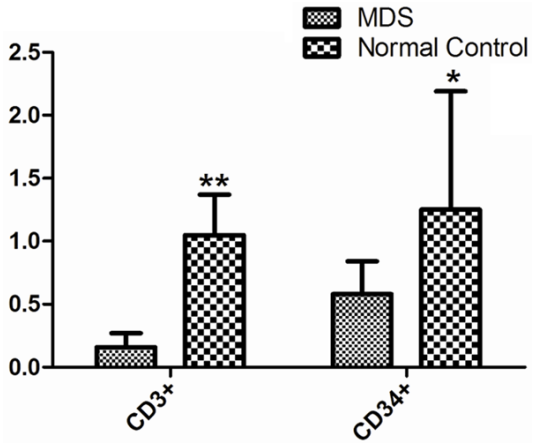

Figure 1. TET2 expression on CD3+ and CD34+ cells in MDS and healthy controls. CD3+ and CD34+ expression of TET2 in MDS and healthy controls. (Data was analyzed by t-test. *group compared with normal controls, P<0.005; **group compared with normal controls, P<0.001).

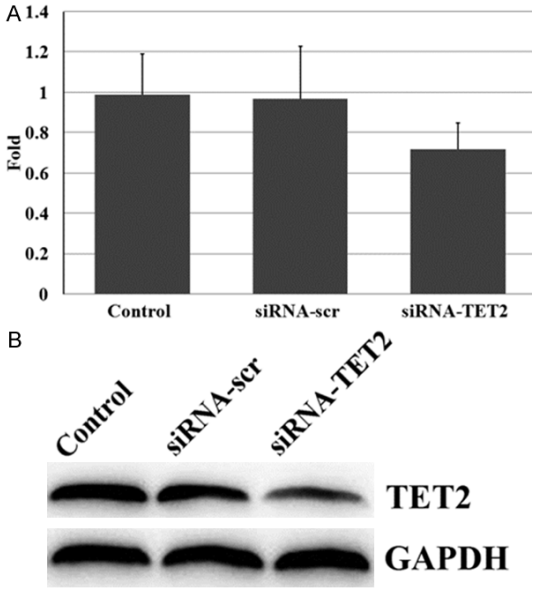

Figure 2. A. TET2 mRNA expression in CD34+ cells, as determined by real-time quantitative polymerase chain reaction. Relative expressions of the groups are 0.99 in control, 0.97 in siRNA-scr, and 0.70 in siRNA-TET2. The mean of the ratio is shown. B. TET2 Protein expression in CD34+ cells, as determined by Western Blot. Results were compared with GAPDH. Relative expressions of the groups are 1.07 in control group, 0.99 in siRNA-scr group, and 0.59 in siRNA-TET2 group. siRNA-scr, small interfering RNA sequence for scrambled siRNA; siRNA-TET2, siRNA sequence targeting TET2.

MDS patients than that in normal controls [(0.16±0.11) vs. (1.05±0.32) (*P*<0.001); (0.58±0.26) vs. (1.25±0.94) (*P*<0.005)] (**Figure 1**).

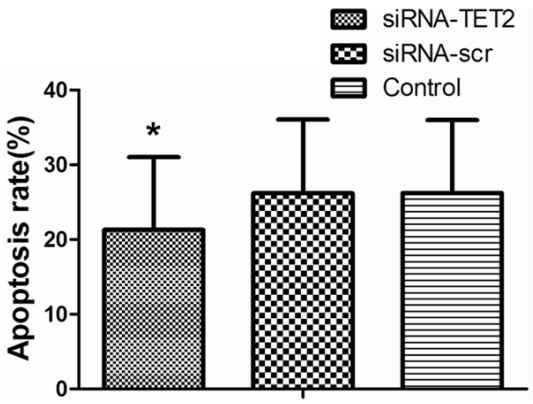

Figure 3. Apoptosis rate was detected by flow cytometry. Cells from the siRNA-TET2 group were lower apoptosis than the control group. Significant differences between groups are shown as *P<0.05. siRNA-scr, small interfering RNA sequence for scrambled siRNA; siRNA-TET2, siRNA sequence targeting TET2.

Inhibition of TET2 expression with siRNA

Following the transfection of the siRNA-TET2, the mRNA and protein expression levels of TET2 were inhibited. Real-time polymerase chain reaction (RT-PCR) amplification revealed significantly decreased TET2 mRNA expression due to the siRNA targeting TET2 (**Figure 2A**). Western-Blot analysis revealed significantly decreased TET2 protein expression (**Figure 2B**). Therefore, TET2 expression was effectively inhibited by RNA interference.

Apoptosis in TET2 knockdown cells

The rate of apoptosis was measured by flow cytometry. The apoptosis rate was decreased when TET2 was knocked down compared with siRNA-scr and control groups [(21.28±9.73)% vs. (26.17±9.88)% and (26.20±9.78)%, *P*<0.05, **Figure 3**].

Cell cycle in TET2 knockdown cells

Flow cytometry assay revealed promotion of cell cycle to S phase and demotion of cell cycle to G0/G1 phase when TET2 was knocked down compared with siRNA-scr and control groups [G0/G1: (87.82±8.25)% vs. (92.65±7.06)% and (93.60±5.54)%, *P*<0.05; S: (11.50±8.31)% vs. (6.92±7.04)% and (5.95±5.53)%, *P*<0.05, **Figure 4**].

Discussion

Recent studies showed that TET2 was the most frequently mutating gene in MDS known so far

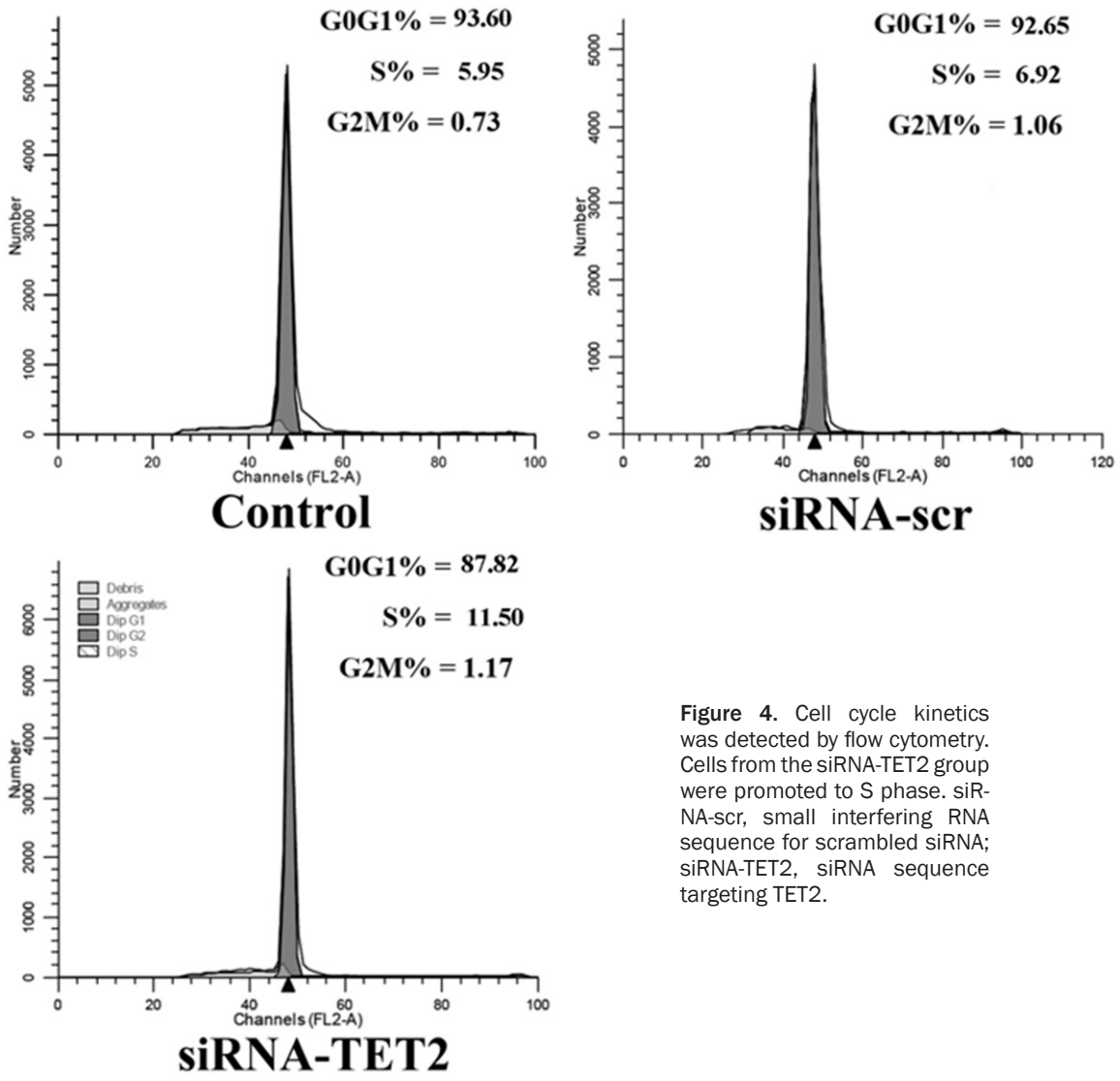

Figure 4. Cell cycle kinetics was detected by flow cytometry. Cells from the siRNA-TET2 group were promoted to S phase. siRNA-scr, small interfering RNA sequence for scrambled siRNA; siRNA-TET2, siRNA sequence targeting TET2.

[3, 4]. Molecular and cytogenetic approaches can identify the TET2 gene in a common 500-kb minimal deleted region. The TET2 gene contains 11 exons spreading over 150 kb [3]. The 2002 amino acids of TET2 protein exhibits 2 evolutionary conserved regions that is related to the hydroxylase family and depends on iron and 2 oxoglutarate [7, 8]. TET2 mutations were also reported in other myeloid malignancies [3, 9, 10]. Decreased TET2 expression was reported in patients with hematologic malignancy [11].

Cell cycle analysis revealed that TET2 inhibiting $CD34^+$ cells exhibited more progress through G0/G1 phase into S phase than controls. In addition, $CD34^+$ cells' apoptosis rate was reduced when TET2 expression was inhibited. These findings reflected the importance of TET2 gene in inducing cell differentiation and apoptosis.

It was recently identified the TET proteins TET1, TET2 and TET3 as a new family of enzymes that alter the methylation status of DNA [12, 13]. TET proteins are 2-oxoglutarate (2OG)- and Fe(II)-dependent dioxygenases that catalyze the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine (5 hmC) in DNA. A highly homologous catalytic domain shared among the three proteins catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine in TET1 [13], which could epigenetically regulate gene expression by altering methylation-driven gene silencing. Given its homology to TET1, TET2 is hypothesized to act as a tumor suppressor gene (TSG) by similarly regulating DNA methylation and epigenetic control of gene

expression at critical loci important for myelopoeisis and leukemogenesis [14].

TET1 and TET2 are both implicated in cancer: TET1 is an MLL partner in rare cases of acute myeloid (AML) and lymphoid (ALL) leukemias, and loss-of-function of TET2 is strongly associated with AML as well as a variety of myelodysplastic syndromes and myeloproliferative disorders [15]. Together, these data suggest that dysregulation of DNA methylation via TET proteins and hmC may have a role in ESC pluripotency, oncogenic transformation (especially of hematopoietic stem cells toward the myeloid lineage), and neuronal function.

In conclusion, our studies indicate that suppression of TET2 expression renders the CD34+ cells harboring more aggressive phenotype. Impaired differentiation and reduced hematopoietic cell production are important features of MDS hematopoiesis. Our results support further investigation of the role of TET2 in abnormal hematopoiesis in MDS.

Acknowledgements

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Disclosure of conflict of interest

None.

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