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Research paper

The synergy of Vitamin C with decitabine activates TET2 in leukemic cells and significantly improves overall survival in elderly patients with acute myeloid leukemia

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ABSTRACT

Background: Decitabine is widely used in the treatment of acute myeloid leukemia (AML) in elderly patients. Low-dose Vitamin C has also been indicated to induce DNA demethylation at the cellular level. However, little is known whether low-dose Vitamin C has a synergistic effect with decitabine in clinic.

Methods: The effect of combined low-dose Vitamin C and decitabine on cell proliferation, the cell cycle, apoptosis and the expression level and activity of TET2 was investigated in HL60 and NB4 human leukemic cells. Additionally, we analyzed the clinical outcomes of 73 elderly AML patients who received A-DCAG (intravenous Vitamin C [IVC] plus DCAG [n = 39]) or DCAG (n = 34) treatment.

Results: We found that low-dose Vitamin C and decitabine has a synergistic efficacy on proliferation, apoptosis, TET2 expression and activity, compared to drug–alone treatment in HL60 and NB4 cell lines in *vitro*. In clinic, feasibility and safety evaluations revealed that patients who received A-DCAG regimen have a higher complete remission (CR) rate than those who received the DCAG regimen (79.92% *vs.* 44.11%; P = 0.004) after one cycle of chemotherapy. The median overall survival (OS) was better in the A-DCAG group compared with the DCAG group (15.3 months *vs.* 9.3 months, P = 0.039). Patients with adverse cytogenetics did benefit from CR. There was no clinically significant additional toxicity observed with the addition of IVC.

Conclusion: On the basis of these results, the addition of IVC at low doses to DCAG appeared to improve CR and prolong OS, compared with DCAG, in elderly patients with AML.

1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder of hematopoiesis characterized by dysregulated gene expression and abnormal patterns of DNA methylation. Aberrant DNA methylation that silences the expression of tumor suppressor genes (TSGs) occurs frequently in patients with AML, and can be used to predict the outcome of therapy [1]. Decitabine has recently become the mainstay of different types of AML and myelodysplastic syndrome (MDS) due to its ability to induce objective remissions and prolong overall survival (OS) compared with low-dose cytarabine, even in patients with adverse cytogenetics [2]. TSGs that are epigenetically silenced in AML cells were induced synergistically by the combination epigenetic therapy, providing a rationale for clinical trials using a combination of epigenetic or cytotoxic therapies [3]. Our previous study demonstrated its combined effect and safety with low-dose decitabine prior to aclarubicin and cytarabine (DCAG) in elderly patients with AML, in which it appeared to achieve a complete remission (CR) rate of 76.3% and a median overall survival of 10 months [4].

Potentially, one mechanism for the success of treatment with hypomethylating agents is the upregulation of ten-eleven-translocation2 (TET2) proteins themselves [5]. Indeed, the associations between TET2 and clinical outcome have been newly investigated [6,7]. TET2, a member of the TET family genes that modify DNA by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), is located at chromosome 4q24 and is frequently mutated in myeloid

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malignancies [8]. Loss-of-TET2 protein function occurs frequently in AML and MDS [8,9]; which inhibits the TET-mediated conversion of 5mC to 5hmC, a known intermediate in DNA demethylation pathways [6]. Interestingly, decitabine resulted in the paradoxical increase in 5hmC levels, while it reduced the global levels of 5mC *in vitro* [10]. Recent studies have demonstrated that ascorbic acid (Vitamin C), as a direct regulator of TET activity, may enhance 5hmC generation, most likely by acting as a co-factor for TET methylcytosine dioxygenase to hydroxylate 5mC [11,12]. Notably, the global increase in 5hmC was rapidly lost after three days of Vitamin C withdrawal, whereas promoter 5mC gradually increased after Vitamin C removal *in vitro* [11]. Increasing evidences indicate that high concentrations (0.5–5 mM) of intravenous Vitamin C (IVC) *in vivo* appears to have therapeutic potential in cancer patients, with an outstanding safety profile and a spectrum of clinical benefits [13–15]. Importantly, pharmacological concentrations of Vitamin C (10–1000 μM) induced a time- and dose-dependent increase in 5-hmC [12]. Furthermore, Vitamin C does not inhibit but rather enhances the activity of chemotherapeutic drugs [16]. Recently, Cimmino et al. demonstrated Vitamin C also drives DNA hypomethylation and expression of a TET2-dependent gene signature in human leukemia cell lines [17]. Nevertheless, little is known whether low-dose IVC has a synergetic effect with decitabine in clinic. Welch et al. reported that low-dose Vitamin C increases the apoptotic effect of arsenic trioxide on AML cells *in vitro*, and can be safely combined with decitabine and arsenic trioxide *in vivo* [18]. It has been considered that patients with leukemia, especially elderly AML patients, may have a higher risk of IVC-associated prothrombotic risks [19]. In this study, Vitamin C with low-concentrations of 0.3 mM *in vitro* and low-doses of IVC (50–80 mg/kg/day) *in vivo* were used. These were within the pharmacological concentration window that may be achieved after the intravenous administration in AML patients. In this study, we investigated the combinatorial effect of low-dose Vitamin C with decitabine in AML cells, and compared the outcomes of elderly AML patients treated with low-dose IVC in combination with decitabine-based chemotherapy (A-DCAG) or DCAG regimen.

2. Materials and methods

2.1. Cell culture and chemicals

Human AML cell line with TET-wild-type (WT), NB4 and HL60 were obtained from the Cell Bank (Chinese Academy of Medical Sciences, Shanghai, China). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. Decitabine and Vitamin C were obtained from Sigma-Aldrich. Decitabine was added from 0.2 M of stock solution with dimethylsulfoxide (DMSO) to a final concentration of 2.5 μM at different time points, while Vitamin C was added from 0.2 M of stock solution with phosphate buffered saline (PBS) to a final concentration of 0.3 mM. Target or effector cells were incubated at different time points with 2.5 μM of decitabine alone or combined with 0.3 mM of Vitamin C before measurement, with untreated cells as controls in all experiments.

2.2. Viability test

NB4 and HL60 cells (2.0×10^4/well) were plated and treated in 96-well plates (three wells per group). Cells were treated with 2.5 μM of decitabine, 0.3 mM of Vitamin C and its combinations for 24, 48, or 72 h. Then, 10 μl of cell counting kit-8 (CCK8; Dojindo, Kumamoto, Japan) was added to the cells before the test. Absorbance was measured at 490 nm in a spectrophotometer (BioTek, Winooski, VT, USA), according to manufacturer's instructions. Cell viability was calculated and the dose-response curves were measured to evaluate the cytotoxic effect.

2.3. Apoptosis

NB4 and HL60 cells were treated with specified concentrations of 2.5 μM of decitabine, 0.3 mM of Vitamin C and its combinations for 48 h. Cells were washed in PBS and resuspended in 100 μl of binding buffer containing 5 μl of Annexin V and 5 μl of propidium iodide (PI) (BD Pharmingen, SanDiego, CA, USA). Cells were analyzed by flow cytometry (Becton Dickinson, CA, USA) after the addition of Annexin V, which bonded to cells that expressed phosphatidylserine on the outer layer of the cell membrane; while PI stained the cellular DNA of these cells with a compromised cell membrane. Cells were considered to be apoptotic if they were either Annexin V+/PI− (early apoptotic) or annexin V+/PI+ (late apoptotic).

2.4. Cell cycle

NB4 and HL60 cells were treated with 2.5 μM decitabine, 0.3 mM Vitamin C and their combinations for 48 h. 1×10^6 cells were suspended in 100 μl ice-cold PBS and fixed in 1 ml 75% ethanol at −20 °C. For the analysis, the suspension was washed twice with PBS, re-suspended in PI-staining solution (0.1% [v/v] Triton X-100, 100 μg/mL of RNaseA, 50 μg/mL of PI), and kept for 30 min at room temperature in the dark. The treated cells were subjected to BD flow cytometry (Becton Dickinson, CA, USA) for cell-cycle distribution analysis.

2.5. RNA isolation and qRT-PCR

After cells were treated with compounds for 48 h, total RNA was extracted from 5×10^6 cells using Trizol reagent (Invitrogen, Shanghai, China), according to manufacturer's instructions. Complementary DNA (cDNA) was generated from 0.5 μg of total RNA using a Reverse Transcription System Kit (Takara, Dalian, China), and the cDNA was amplified by qPCR with the FastStart Universal SYBR Green Master (ROX) (Takara, Dalian, China). The primers were: TET2: 5′-ACG CTT GGA AGC AGG AGA T -3′ (forward); 5′- CAC AAG GCT GCC CTC TAG TT −3′ (reverse); GAPDH: 5′-TGT TGC CAT CAA TGA CCC CTT-3′ (forward); 5′- CTC CAC GAC GTA CTC AGC G-3′ (reverse). Reaction mixtures were prepared using 2 μl of cDNA, 10 μl of SYBR Premix Ex Taq, 0.4 μl of ROX Reference Dye, 0.8 μl of TET2 primers (10 μM) or 0.8 μl of GAPDH primers (10 μM), and deionized water to a total volume of 20 μl. Cycle conditions were one cycle for 5 min at 95 °C, 40 cycles for 5 s at 95 °C and 30 s at 60 °C, 15 s at 72 °C, and one cycle for 10 min at 72 °C. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold, and each sample was normalized according to its endogenous GAPDH RNA content. The relative expression of TET2 mRNA was $2^{-\Delta\Delta CT}$.

2.6. Western blotting

Total proteins were extracted from 5×10^6 cells treated with drugs for 48 h. Cells were washed with ice-cold PBS and resuspended in 100 μl of lysis buffer for 30 min at 4 °C, and centrifuged at 12,000*g* for 30 min at 4 °C. After protein concentration assay (Bradford) supernatants were mixed with 4 × laemmli sample buffer and boiled for 5 min, 20 mg protein were subjected to SDS-PAGE and transferred into nitrocellulose membranes (Hybond PVDF, Amersham). After blocking with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween 20, PVDF membranes were incubated with antibodies against human TET2 (Abcam, Cambridge, UK) at 1:1000 dilutions and GAPDH (Abcam, Cambridge, UK) at 1:10,000 dilutions overnight at 4 °C, followed by incubation with horseradish peroxidase-linked secondary antibody (CST; 1:5000 dilutions) for 1 h at room temperature. Blots were visualized using an enhanced chemiluminescence reagent (Pierce, Rockford, IL) before exposure to X-ray film. Relative protein expression was evaluated by GeneTools Software and statistical analysis was performed in GraphPad software.

2.7. Enzyme-linked immunosorbent assay (ELISA) for measuring the activity of total 5mC hydroxylase TET2 enzyme

The epigenase 5mC hydroxylase TET2 activity assay kit (Epigentek, USA) is suitable for measuring the activity of total 5mC hydroxylase TET2 enzymes using nuclear extracts. The *EpiQuik*™ nuclear extraction kit (Epigentek, USA) was chosen to prepare the nuclear extracts from mammalian cells. Briefly, TET2 assay standard (20 μg/mL) and 80 μl of binding solution were added into a 96-well plate and incubated at 37 °C for 90 min. Then, 1–4 μl of nuclear extracts (5 μg) was added at 37 °C for 90 min, followed by 50 μl of capture antibody at room temperature for 60 min. Absorbance was read at 450 nm. TET2 activity was calculated based on the standard curve generated using kit controls. We calculated the TET2 activity using the equation: TET2 activity (RFU/min/mg) = 1000 × (sample RFU − blank RFU)/(Protein Amount (μg)* × min**). * nuclear extracts added into the reaction; ** incubation time after nuclear extracts added.

2.8. Patient clinical features

Patients who were ≥60 years old and were newly diagnosed *de novo* or secondary AML according to the International Working Group (IWG) criteria [20] were analyzed. From November 2010 to April 2016, a total of 73 newly diagnosed elderly patients with AML who were deemed unfit for or refused intensive chemotherapy were included into the study. All the patients were randomly divided into two treatment arms (A-DCAG, 39; DCAG, 34). The median age of these patients was 68.2 years old (range: 60–87 years old) with a male-to-female ratio of 1.2:1. Baseline characteristics are shown in Table 1. All patients had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2. The study procedures and informed consent forms were approved by Institutional Review Board. This study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent.

2.9. Treatment regimens

Patients received one of the two induction courses: DCAG or A-DCAG. DCAG consisted of 15 mg/m^2 of decitabine (intravenously, days 1–5) and 300 μg/day of granulocyte colony-stimulating factor (G-CSF, days 0–9) for priming its combination with 10 mg/m^2 of cytarabine (*q12h*, days 3–9), 8 mg/m^2 of aclarubicin (days 3–6, DCAG), or IVC (50–80 mg/kg/day, days 0–9; A-DCAG). The G-CSF priming was discontinued when white blood count (WBC) was ≥20 × 10^9/L. Treatment responses were defined according to the modified 2003 IWG criteria [20]. Up to two cycles of induction therapy were allowed when a response was not achieved. Patients with progressive disease after two cycles were excluded from the study. Patients with refractory diseases were offered alternative re-induction regimens. Patients who achieved CR or PR were treated the same regime as post-remission therapy for 4–6 cycles. The next cycle of treatment was given until the recovery of hematopoiesis and resolution of all toxicities. Treatment was continued until relapse or progressive disease, death, or unacceptable toxicity occurred. All patients received antimicrobials, supportive care and transfusions of blood products according to institutional guidelines. Blood routine was monitored every 1–2 days during and after the chemotherapy. Red cell and platelet concentrations were given if hemoglobin was under 70 g/L or platelet under 20 × 10^9/L, respectively. Bone marrow aspiration was taken at the time of recovery of peripheral hemogram, or three weeks later after chemotherapy.

2.10. Statistical analysis

All statistical analyses were performed using SPSS software 22.0. Data from the *in vitro* study were reported as mean ± standard deviation, and the statistical significance of the difference among groups was determined by two-sided Student's *t*-test or one-way ANOVA. CR rate was analyzed using Chi-square test. Kaplan-Meier product-limit estimator was used to describe OS, and death was a competing risk factor. OS was defined as from the date of diagnosed to date of death, irrespective of cause. A *P*-value ≤0.05 was considered statistically significant, when compared between groups.

Table 1
Characteristics of elderly patients with AML.

Characteristics	DCAG + VC (n = 39)	DCAG (n = 34)	P
Age years			< 0.001
median	69	65	
range	60–87	60–77	
Sex			0.919
Male	21	19	
Female	18	15	
ECOG			0.161
0–2	32	32	
≥3	7	2	
Dignosis (WHO)			0.613
M0	1	2	
M1	7	5	
M2	17	16	
M4	2	4	
M5	8	5	
M6	4	2	
AML type			0.956
AML, de novo	24	28	
AML, Secondary	15	6	
Primary karyotype			0.842
Good	0	2	
Intermediate	31	27	
Poor	8	3	
Unavailable	0	2	
NPM1 mutation	5	4	0.891
CEBPA mutation	5	5	0.815
FLT3 mutation	3	3	0.861
myeloid sarcoma	0	0	1.000
WBC (×10^9/L)			0.251
median	5.1	10.8	
range	0.41–240	0.5–186.25	
percent blasts in the bone marrow (%)			0.401
median	52.4	62.8	
range	22–92.8	20–95.2	
LDH (U/L)			0.080
median	473	258	
range	199–8246	153–2635	

3. Results

3.1. Vitamin C improves decitabine-induced cytotoxicity in NB4 and HL60 cells

The combinatorial effect of decitabine and Vitamin C on cell viability was investigated by CCK-8 assay. AML cell lines NB4 and HL60 were treated with different concentrations of decitabine and Vitamin C for 24, 48, or 72 h. NB4 cells were more potently inhibited, suggesting that the anti-proliferative effect of these drugs could be cell-type dependent. Based on the growth inhibition assay, 2.5 μM of decitabine and 0.3 mM of Vitamin C were used in the subsequent experiments. Moreover, both cells revealed a significant decrease in cell viability after 48 h and 72 h of treatment ($P = 0.002$ for NB4, $P = 0.019$ for HL60 after 48 h, and $P < 0.001$ for every after 72 h), which was shown in Fig. 1. These data demonstrated that the cytotoxicity of decitabine was enhanced by Vitamin C in AML cells.

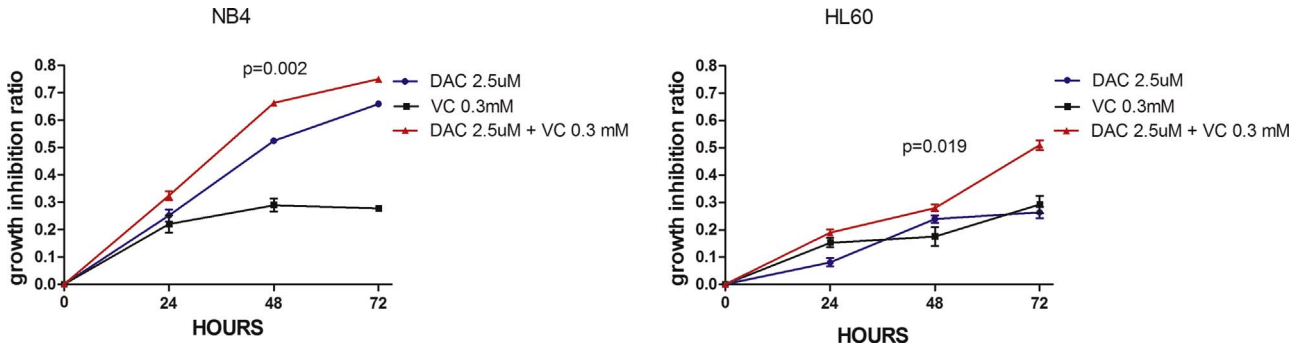

Fig. 1. The increasing growth inhibition after Decitabine(DAC) and Vitamin C(VC) treatment. The growth inhibition results of NB4 and HL60 after 2.5 μM DAC and 0.3 mM VC for 24–48–72 h was shown in Fig. 1. A significant inhibition could be observed after incubation for 48 and 72 h.

3.2. Vitamin C increases decitabine-induced apoptosis in NB4 and HL60 cells

In order to investigate whether cytotoxicity was related to apoptosis, NB4 and HL60 cells were treated with 2.5 μM of decitabine and 0.3 mM of Vitamin C for 48 h, with untreated cells as controls. Apoptotic cells double labeled with Annexin V/PI were analyzed by flow cytometry. Annexin V positive cells increased after treatment with 2.5 μM of decitabine ($P < 0.001$, each). However, this tendency was not observed in 0.3 mM of Vitamin C ($P = 0.118$ for NB4, $P = 0.250$ for HL60), compared with untreated cells. Moreover, Annexin V positive cells increased due to the co-treatment with decitabine and Vitamin C in both cells ($P < 0.001$, each). All above was shown in Fig. 2A and B. These data indicates that Vitamin C increased decitabine-induced apoptosis in AML cells.

3.3. Vitamin C increases decitabine-induced G2-M phase cell cycle arrest in NB4 and HL60 cells

The distribution of cell cycle phases was monitored by the PI staining of ethanol-fixed cells after 2.5 μM of decitabine alone or combined with 0.3 mM of Vitamin C for 48 h. There was no obvious change in cell cycle distribution after treatment with Vitamin C alone. However, decitabine arrested a progressively greater number of cells in

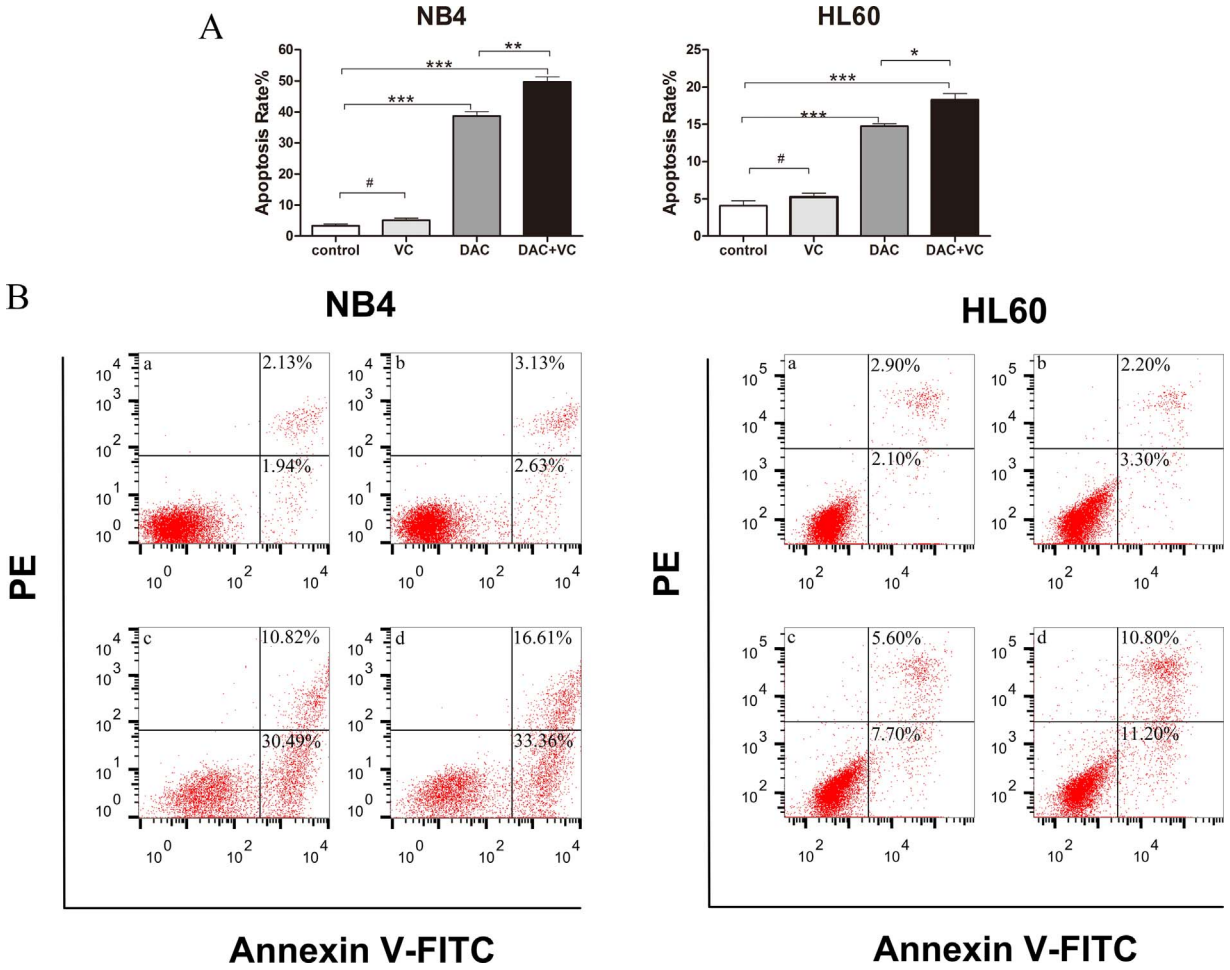

Fig. 2. Decitabine(DAC) and Vitamin C(VC) induced apoptosis after 48 h treatment. Annexin V positive cells were detected by flow cytometry. (a: control; b: 0.3 mM VC; c: 2.5 μM DAC; d: 2.5 μM DAC + 0.3 mM VC). Results represent the mean ± S.D. for three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

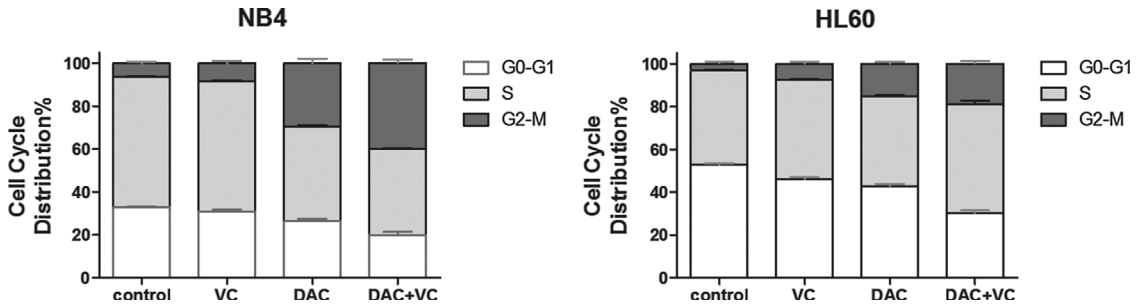

Fig. 3. The cell cycle distribution after Decitabine(DAC) and Vitamin C(VC) for 48 h. 0.3 mM VC caused no obvious change of cell cycle distribution; while 2.5 μM DAC alone or combined with 0.3 mM VC could increase G2-M phase cell fraction after 48 h treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the G2-M phase in both cell lines ($P < 0.001$, each) and this phenomenon became more significant after the addition of Vitamin C ($P < 0.05$, each; Fig. 3).

3.4. TET2 expression and TET2 enzyme activity changed after decitabine and Vitamin C treatment

After treatment with 2.5 μM of decitabine and 0.3 mM of Vitamin C for 48 h, the mRNA and protein expression were measured by qRT-PCR and western blotting. The mRNA and protein expression of TET2 remained unchanged after Vitamin C treatment ($P = 0.494$, 0.538 for NB4 and HL60 mRNA, Fig. 4A; $P = 0.333$, 0.402 for NB4 and HL60 protein, Fig. 4B and C). However, decitabine treatment resulted in the upregulation of the mRNA and protein expression of TET2 in both NB4 and HL60 cells ($P = 0.007$, 0.013 for NB4 and HL60 mRNA, Fig. 4A; $P < 0.001$, 0.027 for NB4 and HL60 protein, Fig. 4B and C), and this was not enhanced by the addition of Vitamin C to decitabine ($P = 0.780$, 0.653 for NB4 and HL60 mRNA, Fig. 4A; $P = 0.258$, 0.183 for NB4 and HL60 protein, Fig. 4B and C; compared to decitabine treatment). Furthermore, TET2 enzyme activity was also detected by ELISA, which was shown in Fig. 4D. Both decitabine ($P < 0.001$, each) and Vitamin C alone ($P = 0.003$, < 0.001 for NB4 and HL60) can enhance TET2 enzyme activity in NB4 and HL60 cells after 48 h. The combined administration of decitabine and Vitamin C resulted in the most significant activity of TET2 enzymes ($P < 0.001$ for each, compared to controls; $P = 0.003$, 0.002 for NB4 and HL60, compared to decitabine treatment). Overall, TET2 mRNA and protein expression and TET2 enzyme activity can be increased after the simultaneous treatment of Vitamin C and decitabine in NB4 and HL60 cells (Fig. 4).

3.5. Efficacy of A-DCAG versus DCAG in elderly AML patients

The CR rate after one and two induction cycles was higher in the A-DCAG arm compared with the DCAG arm (79.9% *vs.* 44.1% [$P = 0.004$] and 84.6% *vs.* 70.6% [$P = 0.148$], respectively). There was a good tendency in the overall CR ratio after adding IVC to DCAG, although there was no significance between these two arms (Table 2). In patients with adverse cytogenetics, the CR rate was comparable between the A-DCAG group ($n = 8$, 75%) and DCAG group ($n = 3$, 66.7%).

With a median follow-up of 13.8 months (range: 1.5-66.7 months), A-DCAG produced a better median OS (15.3 months *vs.* 9.3 months, HR: 0.47, 95% CI: 0.263–0.838, $P = 0.039$; Fig. 5); which was accounted for the achievement of CR after one cycle (Table 2). Because of small sample size of good or poor genetic prognosis, the median OS between two arms was not analyzed in details for these patients. The estimated 3-year survival rate was 28.6% in the A-DCAG group and 12.5% in DCAG group ($P < 0.001$). The median OS was 18 months in A-DCAG and 15 months in DCAG for patients who achieved CR/PR after one or two cycles of induction therapy. In this study, the higher rate of early CR and prolonged OS in the A-DCAG arm support the anti-leukemic synergy effect of IVC and DCAG in elderly AML patients.

3.6. Treatment-related toxicity

Both regimens of DCAG and A-DCAG were well tolerated. In patients with CR, no significant differences between the two arms could be demonstrated with respect to the median time for platelet recovery ($\geq 20 \times 10^9$/L) and granulocyte recovery ($\geq 0.5 \times 10^9$/L). The most common grade 3 or 4 toxicities included neutropenia, thrombocytopenia, constipation, diarrhea, hemorrhage and thrombosis. The incidence of non-hematological toxicities was low. Infection was the most frequent grade 3 or 4 non-hematologic adverse events, with no significant differences observed for the A-DCAG arms compared with the DCAG arm (Table 2). During convalescence, thrombotic complications were observed in only one patient receiving A-DCAG regimen, who had deep venous thrombosis; and platelet count was 37×10^9/L.

4. Discussion

At present, decitabine has been widely used for AML patients who are not suitable for conventional intensive chemotherapy, although the precise molecular mechanism remains not fully understood. Furthermore, only a fraction of patients with hematopoietic malignancies such as MDS or AML respond to treatment with hypomethylating agents [5,21]. Decitabine treatment can cause global hypomethylation in primary AML cells. However, the patterns of methylation and gene expression were primarily determined by the intrinsic properties of primary cells, rather than the decitabine treatment itself [22]. It has been well documented that decitabine, in combination with cytotoxic agents, can achieve better outcomes with good toleration in older or relapsed patients with AML [4,18,23,24]. Our previous study also suggested that the DCAG regimen appears to be safe and effective for elderly AML patients [4]. Additionally, the combination of low-dose Vitamin C and decitabine revealed an additive or synergistic effect on cell apoptosis in HL-60 and NB4 cells. Our understanding has developed overtime; that is, Vitamin C works as an anticancer agent. However, not all studies that combined Vitamin C with chemotherapy have shown improved outcomes. Conversely, Vitamin C supplementation may detrimentally affect therapeutic response by protecting mitochondria from cytotoxic agents [14,15]. Based on emerging data, IVC could be a potential anticancer agent when reaching pharmacological concentrations [13]. Based on the present understanding, Vitamin C can upregulate TET activity [12,25], which in turn increases 5hmC levels, and potentially sensitizes patients to decitabine. To date, no prospective trials that compared Vitamin C combined with decitabine alone or decitabine-based treatment has been reported in AML. In our study, A-DCAG was an encouraging clinical and biologic activity in elderly patients with AML. With A-DCAG treatment, the CR rate after one or two cycles of therapy was 79.9% and 84.62%, respectively; while CR rate was 75% for patients with adverse karyotype. The median OS was 15.3 months, with an estimated 3-year survival rate of 28.6%. Compared with DCAG treatment, the CR rate after one or two cycles of therapy was only 44.1% and 70.6%,

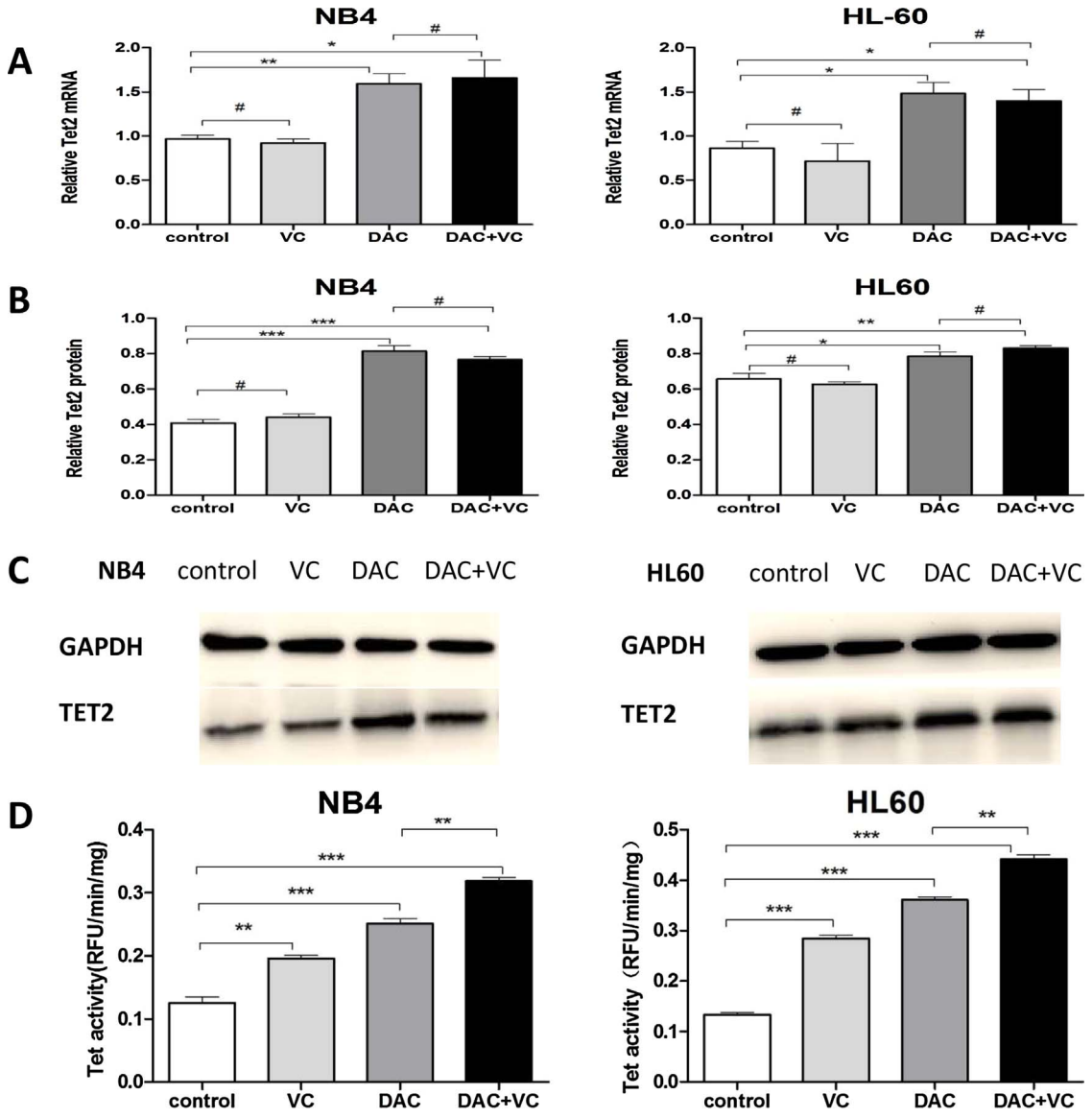

Fig. 4. Decitabine(DAC) and Vitamin C(VC) increased TET2 expression and enzyme activity. 0.3 mM VC could not change TET2 expression, but 2.5 μM DAC alone or combined with 0.3 mM VC increased TET2 expression after 48 h treatment. qRT-PCR and Western Blotting were used to determine TET2 relative level using GAPDH as an internal control, which was shown in A (qRT-PCR), in B and C (Western Blotting). DAC or VC Monotherapy and the combination increased the TET2 enzyme activity after 48 h by ELISA, as shown in D. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2
Efficacy and safety of A-DCAG and DCAG treatment.

Groups	A-DCAG n (range or%)	DCAG n (range or%)	p-value
CR after first induction	30(76.9)	15(44.1)	0.004
Overall CR after 2 cycle	33(84.6)	24(70.6)	0.148
Three-year OS probability	28.6	12.5	< 0.001
Induction therapy			
Neutropenia (Grade III/IV)	29(74.4)	20(58.8)	0.159
Thrombocytopenia (Grade III/IV)	36(92.3)	29(85.3)	0.339
Neutropenia recovery (d)	21(15–42)	22(17–40)	0.642
Thrombocytopenia recovery (d)	17(7–45)	17(8–47)	0.743
Infection accident (n)	34(87.2)	31(91.2)	0.586

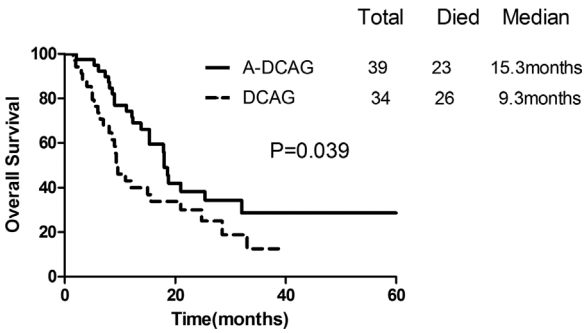

Fig. 5. A-DCAG prolonged OS of elderly AML patients with newly diagnosed.

respectively. The median OS was 9.3 months, with an estimated 3-year survival rate of 12.5%.

Most notably, for responders, the median OS was 18 months for A-DCAG and 15 months for DCAG. We also found that CR, especially in achieving CR after one cycle of therapy, might be required for prolonged survival no matter what kind of regimen the patients have received; which was consistent with previous studies [2,4].

Due to the absence of important side effects in high-dose IVC as a

sole therapy [13], an interesting perspective exists in its combination with decitabine and other cytotoxic agents. Furthermore, particular attention has been given to the risk of thrombosis in patients with leukemia [19]. Decitabine revealed its pro-maturation effect on mega karyocytes and promoted platelet release [26]. Jung et al. reported that 16% of patients with AML and MDS had increased platelet count [27], and we previously found that platelets peaked above 500×10^9/L during convalescence in some responders [4]. In this study, we observed that only one patient (2.6%), who received the A-DCAG regimen, had deep venous thrombosis; while platelet count was 37×10^9/L. The data in this present study indicates that the additional treatment of low-dose IVC to DCAG could increase efficacy without increasing toxicities in almost all categories of toxicity chemotherapy-associated side effects.

Based on recent findings, 5hmC, the downstream derivatives of 5mC, or the upregulation of the TET proteins themselves, has the possibility to serve as a biomarker for monitoring the clinical outcome of patients who received decitabine [5,10]. Importantly, decitabine, TET and Vitamin C epigenetic processes have been dynamically linked [12]. In this study, we investigated whether the cytotoxic effect of low-dose Vitamin C was enhanced when combined with DCAG treatment. We found that the concurrent administration of decitabine and low-dose Vitamin C exhibited a combination effect in TET WT cell lines HL60 and NB4, which lead to a mild inhibition on the rate of cell proliferation, a significant change in cell cycle and apoptotic profile (Figs. 2–4), and an increase in the expression level and activity of TET2 (Fig. 5), compared to decitabine treatment alone.

Biochemical and *in vitro* experiments clearly indicate that Vitamin C treatment caused a global increase in 5hmc levels associated with the demethylation of promoters and increased germline gene expression [5,11]. Interestingly, the level of DNA methylation treated with Vitamin C was similar to the combination treatment with Vitamin C and decitabine [28]. Furthermore, the rapid effect of Vitamin C on 5hmC was specific and likely mediated by the activation of the enzymatic activity of TET proteins, and not TET protein synthesis; suggesting that the intracellular accumulation of Vitamin C is sufficient to enhance the activity of available TET [12,25]. Hence, it is worthy to investigate whether Vitamin C has a therapeutic effect on leukemic cells by enhancing TET activity. The results in this study were clearly consistent with these studies. Although Vitamin C treatment did not change the amount of TET2 proteins and the activity of TET2 significantly increased, this is obviously the synergic effect of Vitamin C and decitabine.

5. Conclusion

The data presented in this study indicate that decitabine, in combination with low-dose Vitamin C, shows a synergistic anti-neoplastic action against AML cells through the modulation of TET2 expression and activity. A-DCAG was found to be likely effective and safe in treating elderly AML patients. CR after one cycle of therapy might be an important index to predict the survival probability of elderly AML patients. Given the advantage of the low toxicity of IVC, larger clinical trials need to be performed to definitively adjust the dosage of IVC and examine the benefits of the co-treatment with IVC to decitabine-based chemotherapy.

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References

[1] M.E. Figueroa, S. Lugthart, Y. Li, C. Erpelinck-Verschueren, X. Deng, P.J. Christos, et al., DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia, Cancer Cell 17 (2010) 13–27.
[2] H.M. Kantarjian, X.G. Thomas, A. Dmoszynska, A. Wierzbowska, G. Mazur, J. Mayer, et al., Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia, J. Clin. Oncol. 30 (2012) 2670–2677.
[3] R.L. Momparler, S. Côté, L.F. Momparler, Y. Idaghdour, Epigenetic therapy of acute myeloid leukemia using 5-aza-2'-deoxycytidine (decitabine) in combination with inhibitors of histone methylation and deacetylation, Clin. Epigenet. 6 (2014) 19.
[4] J. Li, Y. Chen, Y. Zhu, J. Zhou, Y. Xu, Y. Li, et al., Efficacy and safety of decitabine in combination with G-CSF, low-dose cytarabine and aclarubicin in newly diagnosed elderly patients with acute myeloid leukemia, Oncotarget 6 (2015) 6448–6458.
[5] Y. Huang, A. Rao, Connections between TET proteins and aberrant DNA modification in cancer, Trends Genet. 30 (2014) 464–474.
[6] R. Scopim-Ribeiro, J.A. Machado-Neto, P. de Melo Campos, F.S. Niemann, I. Lorand-Metze, F.F. Costa, et al., Low Ten-eleven-translocation 2 (TET2) transcript level is independent of TET2 mutation in patients with myeloid neoplasms, Diagn. Pathol. 11 (2016) 28.
[7] R. Bejar, A. Lord, K. Stevenson, M. Bar-Natan, A. Pérez-Ladaga, J. Zaneveld, et al., TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients, Blood 124 (2014) 2705–2712.
[8] R. Scopim-Ribeiro, J.A. Machado-Neto, M. Campos Pde, C.A. Silva, P. Favaro, I. Lorand-Metze, et al., Ten-Eleven-Translocation 2 (TET2) is downregulated in myelodysplastic syndromes, Eur. J. Haematol. 94 (2015) 413–418.
[9] M. Ko, Y. Huang, A.M. Jankowska, U.J. Pape, M. Tahiliani, H.S. Bandukwala, et al., Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2, Nature 468 (2010) 839–843.
[10] B. Chowdhury, A. McGovern, Y. Cui, S.R. Choudhury, I.H. Cho, B. Cooper, et al., The hypomethylating agent Decitabine causes a paradoxical increase in 5-hydroxymethylcytosine in human leukemia cells, Sci. Rep. 5 (2015) 9281.
[11] K. Blaschke, K.T. Ebata, M.M. Karimi, J.A. Zepeda-Martı'nez, P. Goyal, S. Mahapatra, et al., VitaminC induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells, Natrue 500 (2013) 222–226.
[12] E.A. Minor, B.L. Court, J.I. Young, G. Wang, Ascorbate induces ten-eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine, J. Biol. Chem. 288 (2013) 13669–13674.
[13] Y. Ma, J. Chapman, M. Levine, K. Polireddy, J. Drisko, Q. Chen, High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy, Sci. Transl. Med. 6 (2014) 222ra18.
[14] G. Perrone, T. Hideshima, H. Ikeda, Y. Okawa, E. Calabrese, G. Gorgun, et al., Ascorbic acid inhibits antitumor activity of bortezomib in vivo, Leukemia 23 (2009) 1679–1686.
[15] M.L. Heaney, J.R. Gardner, N. Karasavvas, D.W. Golde, D.A. Scheinberg, E.A. Smith, et al., Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs, Cancer Res. 68 (2008) 8031–8038.
[16] J. Verrax, P.B. Calderon, Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects, Free Radic. Biol. Med. 47 (2009) 32–40.
[17] L. Cimmino, I. Dolgalev, Y. Wang, A. Yoshimi, G.H. Martin, J. Wang, et al., Restoration of TET2 function blocks aberrant self-renewal and leukemia progression, Cell 170 (2017) 1079–1095.
[18] J.S. Welch, J.M. Klco, F. Gao, E. Procknow, G.L. Uy, K.E. Stockerl-Goldstein, et al., Combination decitabine, arsenic trioxide, and ascorbic acid for the treatment of myelodysplastic syndrome and acute myeloid leukemia: a phase I study, Am. J. Hematol. 86 (2011) 796–800.
[19] K. Kim, O.N. Bae, S.H. Koh, S. Kang, K.M. Lim, J.Y. Noh, et al., High-dose vitamin C injection to cancer patients may promote thrombosis through procoagulant activation of erythrocytes, Toxicol. Sci. 147 (2015) 350–359.
[20] B.D. Cheson, J.M. Bennett, K.J. Kopecky, T. Büchner, C.L. Willman, E.H. Estey, et al., Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia, J. Clin. Oncol. 21 (2003) 4642–4649.
[21] P. Wongtrakoongate, Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries, World J. Stem Cells 7 (2015) 137–148.
[22] J.M. Klco, D.H. Spencer, T.L. Lamprecht, S.M. Sarkaria, T. Wylie, V. Magrini, et al., Genomic impact of transient low-dose decitabine treatment on primary AML cells, Blood 121 (2013) 1633–1643.
[23] X. Jiang, Z. Wang, B. Ding, C. Yin, Q. Zhong, B.Z. Carter, et al., The hypomethylating agent decitabine prior to chemotherapy improves the therapy efficacy in refractory/relapsed acute myeloid leukemia patients, Oncotarget 6 (2015) 33612–33622.
[24] Y. Zhu, H. Zhao, X. Zhang, Y. Wu, Y. Xie, Y. Li, et al., Decitabine before low-dose cytarabine-based chemotherapy combined with human leukocyte antigen-mismatched stem cell microtransplantation improved outcomes in elderly patients with newly diagnosed acute myeloid leukemia, Biol. Blood Marrow Transplant. 23 (2017) 830–835.
[25] K.M. Dickson, C.B. Gustafson, J.I. Young, S. Züchner, G. Wang, Ascorbate-induced generation of 5-hydroxymethylcytosine is unaffected by varying levels of iron and 2-oxoglutarate, Biochem. Biophys. Res. Commun. 439 (2013) 522–527.
[26] J. Wang, Z. Yi, S. Wang, Z. Li, The effect of decitabine on megakaryocyte maturation and platelet release, Thromb. Haemost. 106 (2011) 337–343.
[27] H.A. Jung, C.H. Maeng, M. Kim, S. Kim, C.W. Jung, J.H. Jang, Platelet response during the second cycle of decitabine treatment predicts response and survival for myelodysplastic syndrome patients, Oncotarget 6 (2015) 16653–16662.
[28] M.A. Ruiz, A. Rivers, V. Ibanez, K. Vaitkus, N. Mahmud, J. DeSimone, et al., Hydroxymethylcytosine and demethylation of the γ-globin gene promoter during erythroid differentiation, Epigenetics 10 (2015) 397–407.