Original Article

Epigenetic Silencing of Eyes Absent 4 Gene by Acute Myeloid Leukemia 1-Eight-twenty-one Oncoprotein Contributes to Leukemogenesis in t(8;21) Acute Myeloid Leukemia

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Abstract

Background: The acute myeloid leukemia 1 (AML1)-eight-twenty-one (ETO) fusion protein generated by the t(8;21)(q22;q22) translocation is considered to display a crucial role in leukemogenesis in AML. By focusing on the anti-leukemia effects of eyes absent 4 (*EYA4*) gene on AML cells, we investigated the biologic and molecular mechanism associated with AML1-ETO expressed in t(8;21) AML.

Methods: Qualitative polymerase chain reaction (PCR), quantitative reverse transcription PCR (RT-PCR), and Western blotting analysis were used to observe the mRNA and protein expression levels of EYA4 in cell lines. Different plasmids (including mutant plasmids) of dual luciferase reporter vector were built to study the binding status of AML1-ETO to the promoter region of *EYA4*. Chromatin immunoprecipitation assay was used to study the epigenetic silencing mechanism of *EYA4*. Bisulfite sequencing was applied to detect the methylation status in *EYA4* promoter region. The influence of *EYA4* gene in the cell proliferation, apoptosis, and cell clone-forming ability was detected by the technique of Cell Counting Kit-8, flow cytometry, and clonogenic assay.

Results: *EYA4* gene was hypermethylated in AML1-ETO$^+$ patients and its expression was down-regulated by 6-fold in Kasumi-1 and SKNO-1 cells, compared to HL-60 and SKNO-1-siA/E cells, respectively. We demonstrated that AML1-ETO triggered the epigenetic silencing of *EYA4* gene by binding at AML1-binding sites and recruiting histone deacetylase 1 and DNA methyltransferases. Enhanced EYA4 expression levels inhibited cellular proliferation and suppressed cell colony formation in AML1-ETO$^+$ cell lines. We also found EYA4 transfection increased apoptosis of Kasumi-1 and SKNO-1 cells by 1.6-fold and 1.4-fold compared to negative control, respectively.

Conclusions: Our study identified *EYA4* gene as targets for AML1-ETO and indicated it as a novel tumor suppressor gene. In addition, we provided evidence that *EYA4* gene might be a novel therapeutic target and a potential candidate for treating AML1-ETO$^+$ t (8;21) AML.

Key words: Acute Myeloid Gene 1-Eight-twenty-one; Acute Myeloid Leukemia; Epigenetics; Eyes Absent 4

Introduction

The t(8;21)(q22;q22) is the second most common chromosomal translocation of acute myeloid leukemia (AML), leading to the fusion between the *AML1* (also known as *RUNX1*) gene and eight-twenty-one (*ETO*, also known as *RUNX1T1*) gene, and produces the chimeric gene *AML1-ETO*.[1] It accounts for 10–15% cases of discernible translocations in AML and induces the M2 leukemia according to French-American-British (FAB) classification, and is associated with a relatively favorable prognosis.[2,3] *AML1* gene is located on the 21st chromosome and serves as a DNA-binding transcription factor while *ETO* gene is located on the 8th chromosome and serves as a corepressor molecular. The formation of the chimeric gene *AML1-ETO* encodes the AML1-ETO fused protein, a transcription factor.[4] Studies have shown that 40% of M2 AML cases according to FAB classification are associated with the translocation t(8;21)

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that leads to the *AML1-ETO* fusion gene.[1] Many studies also documented the multifunction of AML1-ETO fusion protein including differentiation inhibition, subsequent apoptosis, and signals activation for cell proliferation.[5,6] However, the *AML1-ETO* fusion gene alone is not sufficient for inducing leukemia and there probably involves other additional genetic abnormalities.

Eyes absent 4 (*EYA4*) gene, a member of the *EYA* gene family (*EYA1–4*), is a transcriptional activator.[7] The *EYA* gene family operates in a network of transcriptional regulators which are required for the formation of many organs and tissues. The *EYA* gene family encodes EYA family proteins, which contain the EYA domain (ED). The ED is a large, highly conserved C-terminal domain of EYA family proteins, containing 271 amino acids [Figure 1a].[7,8] The ED contains the phosphatase catalytic domain and plays an important role in the interaction with other proteins, including sine oculis homeobox homolog (SIX) proteins.[9-11] EYA has been extensively characterized as a transcriptional coactivator, which operates in association with Sine oculis (SO/SIX) proteins. Several studies have confirmed that *EYA* genes express in certain regions of the embryo to produce the visual system, and EYA proteins play an essential role in the vertebrate eye.[12] It is validated that the mutations of *EYA* genes developed no eyes and were responsible for progressive postlingual hearing loss at the deafness.[7,13,14] Previous studies showed that the EYA4 protein acted through its protein phosphatase activity and mutations in *EYA4* gene were associated with progressive hearing loss.[13,15]

However, the function of *EYA4* gene in hematological malignancies has not yet been determined and the epigenetic mechanisms in the leukemogenesis of *AML1-ETO* prompted us to investigate the possible role of *EYA4* in AML carrying this chimeric protein. In this study, we provided evidence that *AML1-ETO* triggers the epigenetic silencing of *EYA4* gene, contributing to leukemogenesis in t(8;21) AML. Our findings also identified *EYA4* gene as a novel potential therapeutic target of *AML1-ETO*$^+$ t(8;21) AML.

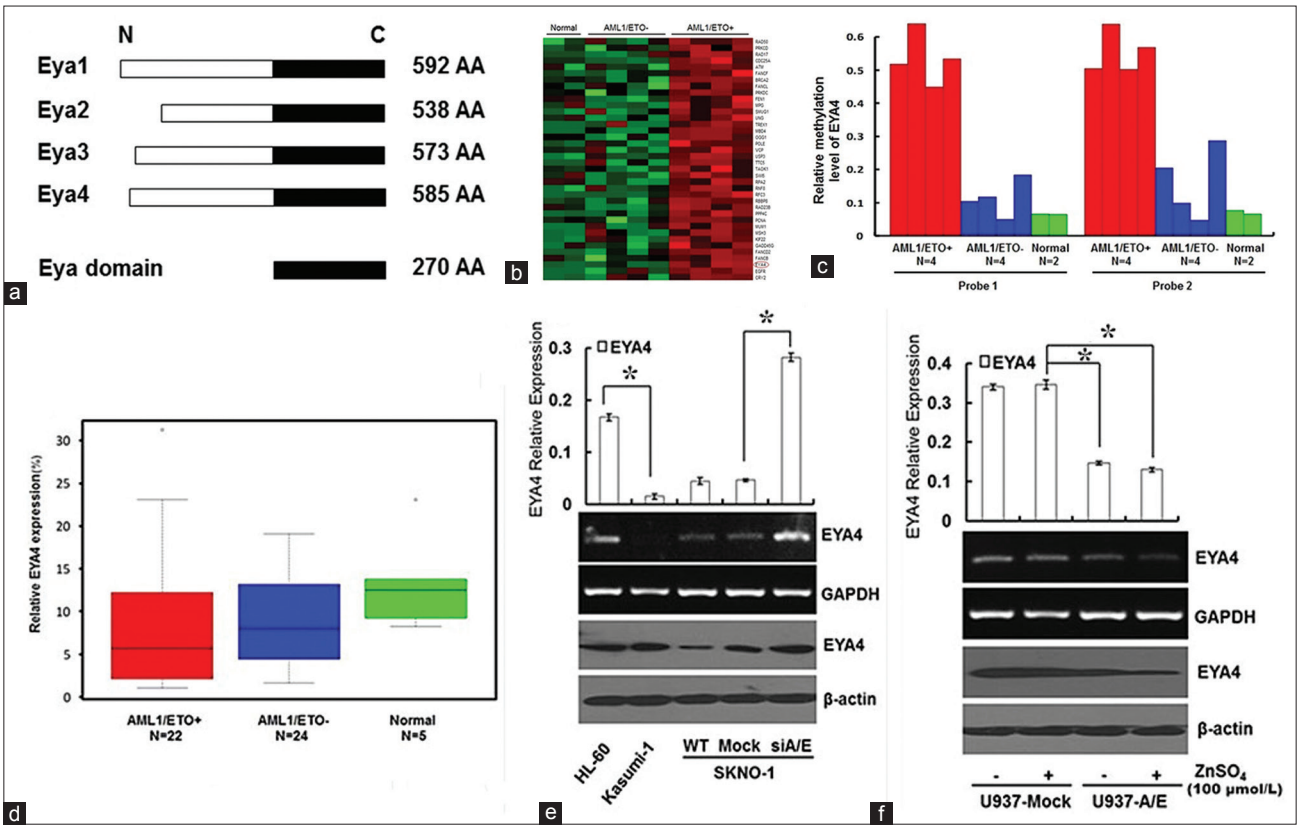

Figure 1: EYA4 levels in leukemia and cell lines. (a) The structure of EYA family proteins and EYA domain. The methylation status of 38 genes in mononucleated cells isolated from four AML1/ETO$^+$ patients, four AML1/ETO$^-$ patients and two healthy donors using 450 K Infinium Methylation BeadChIP of Illumina (b; EYA4 is showed in a red circle) and the relative methylation level of *EYA4* in these samples (c). (d) Relative qRT-PCR quantification of *EYA4* expression level in mononucleated cells isolated from 22 AML1/ETO$^+$ patients, 24 AML1/ETO$^-$ patients and five healthy donors. AML1/ETO$^+$ cases had lower *EYA4* levels. (e) Top and middle panels: Relative quantification of *EYA4* levels in HL-60, Kasumi-1, SKNO-1 (wild-type, mock and siA/E) cells. The results represent mean of three independent evaluations ± standard deviation (*$P < 0.05$). Bottom panels: Immunoblot analysis for EYA4 and AML1/ETO with an antibody against EYA4. β-actin was used as a protein loading control. (f) Top and middle panels: Relative quantification of *EYA4* levels in U937 (mock and A/E-HA) cells. The results represent mean of three independent evaluations ± standard deviation (*$P < 0.05$). Bottom panels: Immunoblot analysis for EYA4 and AML1/ETO with an antibody against EYA4. β-actin was used as a protein loading control. The expression of AML1/ETO in U937 cells was increased by Zn^{2+} treatment (100 μmol/L for 16 h). EYA: Eyes absent; AML: Acute myeloid leukemia; ETO: Eight-twenty-one; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; ChIP: Chromatin immunoprecipitation.

Methods

Clinical samples

This study obtained approval from the Human Subject Ethics Committee in Chinese PLA General Hospital and was carried out in accordance with principles of *Declaration of Helsinki*. Leukemia blasts were obtained from the bone marrow (BM) of patients with leukemia, who were diagnosed as AML according to FAB classification.[16] Normal mononuclear cells were isolated from the BM of consenting healthy donors. BM samples of the AML1-ETO positive and negative patients were collected from 1990 to 2008 in Chinese PLA General Hospital.

Cell lines and cell cultures

AML1-ETO$^-$ HL-60, SKNO-1-siA/E-RNA and U937, AML1-ETO-inducible human myeloid leukemia U937-A/E-HA, AML1-ETO$^+$ Kasumi-1 and SKNO-1 cell lines were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA, SH40007-13) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT, USA, SV30087.02), 50 μg/ml streptomycin (Solarbio, Beijing, China, P1400-100) and 50 IU penicillin (Solarbio, Beijing, China, P1400-100). To silence *AML1-ETO* in SKNO-1 cells, we used pRRLcPPT.hPGK, a lentiviral vector which encoded the previously mentioned siAGF1 oligonucleotides, to infect SKNO-1 cells. The siAGF1 oligonucleotides (sense, 5'-CCUCGAAAUCGUACUGAGAAG-3'; antisense, 5'-UCUCAGUACGAUUUCGAGGUU-3') were against the AML1-ETO mRNA fusion site.[17-20] U937-A/E-HA clone was obtained by electroporating an HA-tagged *AML1-ETO* cDNA subclone into a vector, which contains the ZN^{2+}-inducible mouse MT-1 promoter, and then into U937 wild-type (WT) cells.[19] Human embryonic kidney (HEK) 293T cells were maintained in DMEM medium (Hyclone, Logan, UT, USA, SH30022.018) supplemented with 10% FCS, 50 μg/ml streptomycin, and 50 U penicillin.

RNA extraction and analysis

Total RNA was extracted from BM cells and cDNA was reversely transcribed from total RNA using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA, 15596-018). cDNA was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using SYBR Green (TaKaRa, Japan, DRR041A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control in the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Western blotting analysis

Total protein was extracted from cell lines using radioimmunoprecipitation assay buffer (Sigma-Aldrich, St Louis, MO, USA, R0278). Protein expression was analyzed by Western blot using anti-EYA4 (Abcam, Cambridge, USA, ab47990) on total cell lysates (50 μg). The anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, USA, sc-47778) was used as the basis of detection to normalize the amount of samples analyzed. Immunoreactivity was then determined by the ECL method (Amersham Biosciences, Piscataway, USA, RPN2232).

Transactivation assays

DNA fragments were amplified by PCR from human genomic DNA. Primer sequences are shown in Supplementary Table 1. All fragments were then inserted in the pGL3-LUC reporter vector (Promega, Madison, WI, USA, E1751). The mutant was generated by the overlap-extension PCR method. HEK293T cells were plated in 24-well plates (each well consists of 2×10^5 cells) and were transiently cotransfected by the SuperFect reagent (QIAGEN, Valencia, CA, USA, 301305) with 10, 50, or 100 ng of the pcDNA3.0 vectors with or without the *AML1-ETO* cDNAs and 400 ng of the LUC reporter constructs as described above. A cotransfected pRL-TK renilla luciferase reporter vector (Promega, Madison, WI, USA, VQP0126) was simultaneously used as an internal control. All cells were harvested 48 h after the transfection and then assayed by the dual luciferase assay (Promega, Madison, WI, USA, E1910) according to the instructions of the manufacturer.

Chromatin immunoprecipitation assay

Formaldehyde (1% final concentration) was added into cells (2×10^6 cells). Cells were then incubated for 10 min at 37°C to crosslink proteins to DNA. After sonication, 5 μg of antibodies recognizing the following AML1 (Santa Cruz Biotechnology, Santa Cruz, USA, sc-28679), ETO (Santa Cruz Biotechnology, Santa Cruz, USA, sc-9737), histone deacetylase 1 (HDAC1) (Abcam, Cambridge, USA, ab7028), DNA methyltransferase 1 (DNMT1) (Abcam, Cambridge, USA, ab13537), DNMT3a (Abcam, Cambridge, USA, ab13888) and DNMT3b (Abcam, Cambridge, USA, ab13604) were immunoprecipitated with the chromatin overnight. Chromatin immunoprecipitation (ChIP) was performed on the naked and sonicated DNA extracted from SKNO-1, SKNO-1-siA/E, U937, and U937-A/E cell lines and then assayed with the EZ-ChIP kit (Millipore, Billerica, MA, USA, 17-371) according to the instructions of the manufacturer. Genomic *EYA4* upstream regions, which were close to the putative AML1-binding site, were amplified. Primers sequences are shown in Supplementary Table 1. GAPDH was used as a control for nonspecific precipitated sequences.

Bisulfite modification and genomic sequencing

The methylation status of CpG dinucleotides in regions from nt −707 to −356 relative to the *EYA4* gene were analyzed. The bisulfite sequencing assay was then performed on the bisulfite-treated genomic DNA (1 μg) from indicated cell lines. The fragments of interest were amplified after the bisulfite conversion performed with the use of EpiTech bisulfite kit (QIAGEN, Valencia, CA, USA, 59104). Primer sequences are shown in Supplementary Table 1. PCR products were then gel purified and cloned into pGEM-T vector systems (Promega, Madison, WI, USA, A1360). The individual bacterial colonies were performed for PCR with vector-specific primers. The products were then sequenced to analyze DNA methylation.

Small interfering RNA targeting

si-EYA4 (Santa Cruz Biotechnology, Santa Cruz, USA, sc-41952) cell transfection was performed with the use of HiPerFect Transfection Reagent (QIAGEN, Valencia,

CA, USA, 301705) according to the instructions of the manufacturer.

Cell proliferation, apoptosis and colony forming unit analysis

Posttransfection (5×10^3) cells were plated in 96-well plates. Cell proliferation was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan, CK04) according to the instructions of the manufacturer. For cell apoptosis assay, the cells were stained with propidium iodide and annexin V (Biolegend, San Diego, CA, USA, 640906), and then analyzed by FACSCalibur flow cytometer machine (Becton Dickinson, Franklin Lakes, NJ, USA). Colony forming unit assays were performed with the use of the methylcellulose H4230 culture system (StemCell Technologies, Vancouver, Canada, 4230) according to the instructions of the manufacturer. After incubation at 37°C and 5% CO_2 in a humidified atmosphere for 10 days, clusters showing morphologic hematopoietic characteristics (more than 50 cells) were counted as colonies.

Bioinformatics and statistical analysis

All AML cases were found based on the Gene Expression Omnibus databases (www.ncbi.nih.gov/geo, GSE6891), including their clinical, molecular, and cytogenetic information. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze all the data. All data were showed as mean ± standard deviation (SD), and were from at least three separate experiments. Student's *t*-test was used to compare the variables between two groups. The value of $P < 0.05$ was considered statistically significant.

Results

Eyes absent 4 expression was down-regulated in acute myeloid leukemia 1-eight-twenty-one positive acute myeloid leukemia cell lines

To determine the epigenetic regulation role of DNA methylation in t(8;21) AML, we collected ten BM samples from eight patients with t(8;21) AML-M2 subtype (4 AML1-ETO$^+$, 4 AML1-ETO$^-$) and two healthy donors. Using 450 K Infinium Methylation BeadChIP of Illumina, we found that *EYA4* gene showed higher methylation status in AML1-ETO$^+$ patients than those in AML1-ETO$^-$ patients and healthy donors [Figure 1b]. To confirm this observation, the relative methylation levels of *EYA4* gene were quantified from these samples using two different *EYA4* probes. We found out that the methylation levels of *EYA4* gene were significantly higher in AML1-ETO$^+$ patients [Figure 1c]. In analogy to this, we studied the expression of *EYA4* gene in 51 BM samples from 46 patients with t(8;21) AML-M2 subtype (22 AML1-ETO$^+$, 24 AML1-ETO$^-$) and five healthy donors. The result showed that *EYA4* gene was expressed at higher levels in healthy donors than that in patients with t(8;21) AML-M2 subtype [Figure 1d].

To verify the putative role of AML1-ETO in the regulation of *EYA4* expression, AML1-ETO was knocked-out in the SKNO-1 cells (SKNO-1-siA/E) with the use of a lentiviral vector. It was calculated that the expression of endogenous levels of *EYA4* gene increased by 6-fold in SKNO-1 siA/E cells compared with those in SKNO-1 and mock cells. Similar results were observed in HL-60 cells compared with Kasumi-1 cells [Figure 1e]. In U937 cells ectopically expressing an hemagglutinin (HA)-tagged AML1-ETO (U937-A/E-HA) in the zinc-inducible manner,[17,19] *EYA4* measurable levels were reduced up to 37% relative to U937 mock cells [Figure 1f]. It was demonstrated that decitabine (DAC), the most common demethylating drug, could significantly upregulate the expression of *EYA4* gene [Supplementary Figure 1a]. These results suggested that a negative functionally association existed between *EYA4* gene and AML1-ETO levels.

Acute myeloid leukemia 1-eight-twenty-one protein localized at an acute myeloid leukemia 1-biding site and triggered epigenetic silencing of eyes absent 4 genomic region

A bioinformatics search (http://www.cbrc.jp/research/db/TFSEARCH.html) of the 5'-end of the predicted "core promoter" sequence showed the presence of one putative AML1-binding sites surrounded by the CpG islands on the *EYA4* upstream region (nt −1000 to +1 relative to *EYA4*) [Figure 2a]. We built luciferase reporter constructs containing WT (EYA4-P1 and EYA4-P2) and mutated (EYA4-M) sequences of the *EYA4* regulatory region [Figure 2a, bottom panel], and then co-transfected them with increasing amounts of empty or AML1-ETO-containing vectors into 293T cells. We then observed that the expression of AML1-ETO could cause a dose-dependent decrease in the luciferase reporter activity of the EYA4-P1, but not in its mutant EYA4-M and EYA4-P2 that lack any functional AML1 binding site [Figure 2b]. These results suggested that the proximal AML1-binding site on the *EYA4* putative regulatory region contributed to the AML1-ETO-dependent silencing of *EYA4*.

A ChIP assay was performed in SKNO-1, SKNO1-siA/E, U937, and U937-A/E using specific antibodies against AML1, ETO, HDAC1, DNMT1, DNMT3a and DNMT3b. The results showed the presence of HDAC1, DNMT1, DNMT3a, and DNMT3b at the *EYA4* chromatin regulatory regions surrounding the AML1-binding site [Figure 2c]. In this region, the frequency of the methylated CpG dinucleotides which encompassed the endogenous *EYA4* gene sequences was demonstrated using genomic bisulfite sequencing in the following samples: normal BM (0.04%), 2 AML1-ETO$^+$ M2 patients (74.30% and 79.30%, respectively) and 2 AML1-ETO$^-$ M2 patients (8.60% and 7.10%, respectively). These results indicated a higher frequency of the methylated CpG dinucleotides encompassing the endogenous *EYA4* gene sequences was observed in AML1-ETO$^+$ blasts compared to AML/ETO$^-$ blasts and normal BM blasts [Figure 2d]. Therefore, the chromatin remodeling complex which was aberrantly formed by AML1-ETO, and the hypermethylation of the CpG islands which were presented in the AML1 binding sites on the *EYA4* regulatory region appeared to be the key regulatory mechanisms for the transcriptional silencing of *EYA4*.

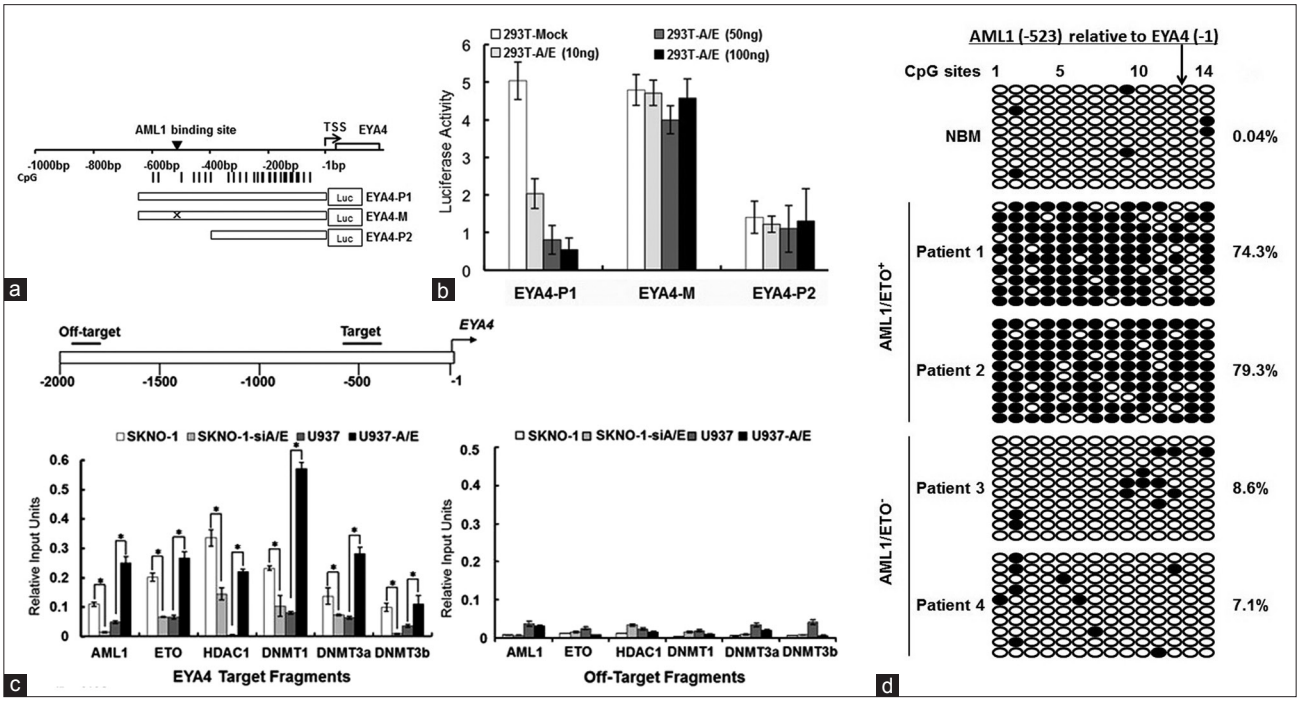

Figure 2: AML1/ETO/HDACs/DNMTs complex acting on the AML1 DNA-binding site presents on the upstream sequence of *EYA4* gene and alters its epigenetic status. (a) Schematic diagrams of the AML1-binding site and the CpG islands along the *EYA4* gene. The numbers are the nucleotides relative to *EYA4* (−1). The vertical arrow indicates the AML1 DNA-binding site. The vertical lines indicate CpG dinucleotides, and the horizontal bar below the CpG sites shows the region analyzed by the bisulfite sequencing. (b) Human 293T cells were cotransfected transiently for 48 h with luciferase reporter containing the wild-type sequence of the *EYA4* regulatory regions or its counterpart mutants, and with increasing amounts (10, 50 and 100 ng) of pcDNA3.0 with or without AML1/ETO cDNA. (c) Chromatin was immunoprecipitated by the use of the indicated antibodies. The horizontal lines indicate the location of the primers that are used in the ChIP assay. To evaluate the specificity of protein binding, qRT-PCR was performed using "Target" primers designed for the amplification of DNA sequences surrounding the proximal AML1-binding site, and "Off-target" primers designed for the amplification of a distal region on *EYA4* gene without containing the predicted AML1 binding site. Input shows the amplification from the sonicated chromatin. The amplification of glyceraldehyde-3-phosphate dehydrogenase was used as a control for nonspecific precipitated sequences. $*P < 0.05$. (d) Genomic bisulfite sequencing was performed to find out the methylation status of the DNA sequences surrounding the AML1-binding site (−523 bp) in *EYA4* from the indicated leukemic blasts. Each row of circles represents the sequence of a single clone. Black and empty circles represent methylated and unmethylated CpG dinucleotides, respectively. For each sample, the specific percentages of global methylation level of these regions on *EYA4* gene are indicated. AML: Acute myeloid leukemia; ETO: Eight-twenty-one; HDAC: Histone deacetylase; DNMT: DNA methyltransferases; EYA: Eyes absent; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; ChIP: Chromatin immunoprecipitation.

Eyes absent 4 inhibited cell proliferation, induced apoptosis and suppressed colony formation in acute myeloid leukemia 1-eight-twenty-one positive cell lines

To further confirm the biological effects of *EYA4* gene in AML, we assessed whether overexpressing *EYA4* would perturb the growth curve of AML cell lines. Kasumi-1 cells were transfected with pcDNA3.0-EYA4 and pcDNA3.0, while HL-60 cells were transfected with synthetic small interfering RNA (siRNA) against *EYA4* (EYA4-siRNA) and luciferase. The cell proliferation was then measured by CCK-8 assay. It was shown that restoring *EYA4* expression in Kasumi-1 inhibited the cell proliferation while the silencing of *EYA4* in HL-60 promoted it with respect to the negative control, respectively [Figure 3a and 3b]. Cell apoptosis was measured by annexin V assay. *EYA4* transfection increased apoptosis of Kasumi-1 and SKNO-1 cells at 48 h by 1.6-fold and 1.4-fold compared to negative control, respectively, whereas the silencing of *EYA4* decreased apoptosis of HL-60 and SKNO-1-siA/E cells at 48 h by 0.6-fold and 0.5-fold compared with negative control, respectively [Figure 3c and 3d]. In analogy to this, the apoptosis of Kasumi-1 cell and SKNO-1 cell was highly increased after treated with DAC. However, after treated with DAC with knocking down of *EYA4* by EYA4-siRNA, the result of apoptosis remained approximately the same as the negative control [Supplementary Figure 1b and 1c]. The number and size of colonies formed were markedly reduced in Kasumi-1 cells transfected with pcDNA3.0-EYA4, while a significant increase in colony formation was observed in HL-60 cells transfected with EYA4-siRNA [Figure 3e and 3f]. Taken together, these results suggested that *EYA4* gene inhibited cell proliferation, induced apoptosis and suppressed colony formation in AML1-ETO$^+$ cell lines.

Discussion

In the current study, we explored the functional role of *EYA4* gene in t(8;21) AML. In general, patients with t(8;21) AML represent a favorable risk group, for its

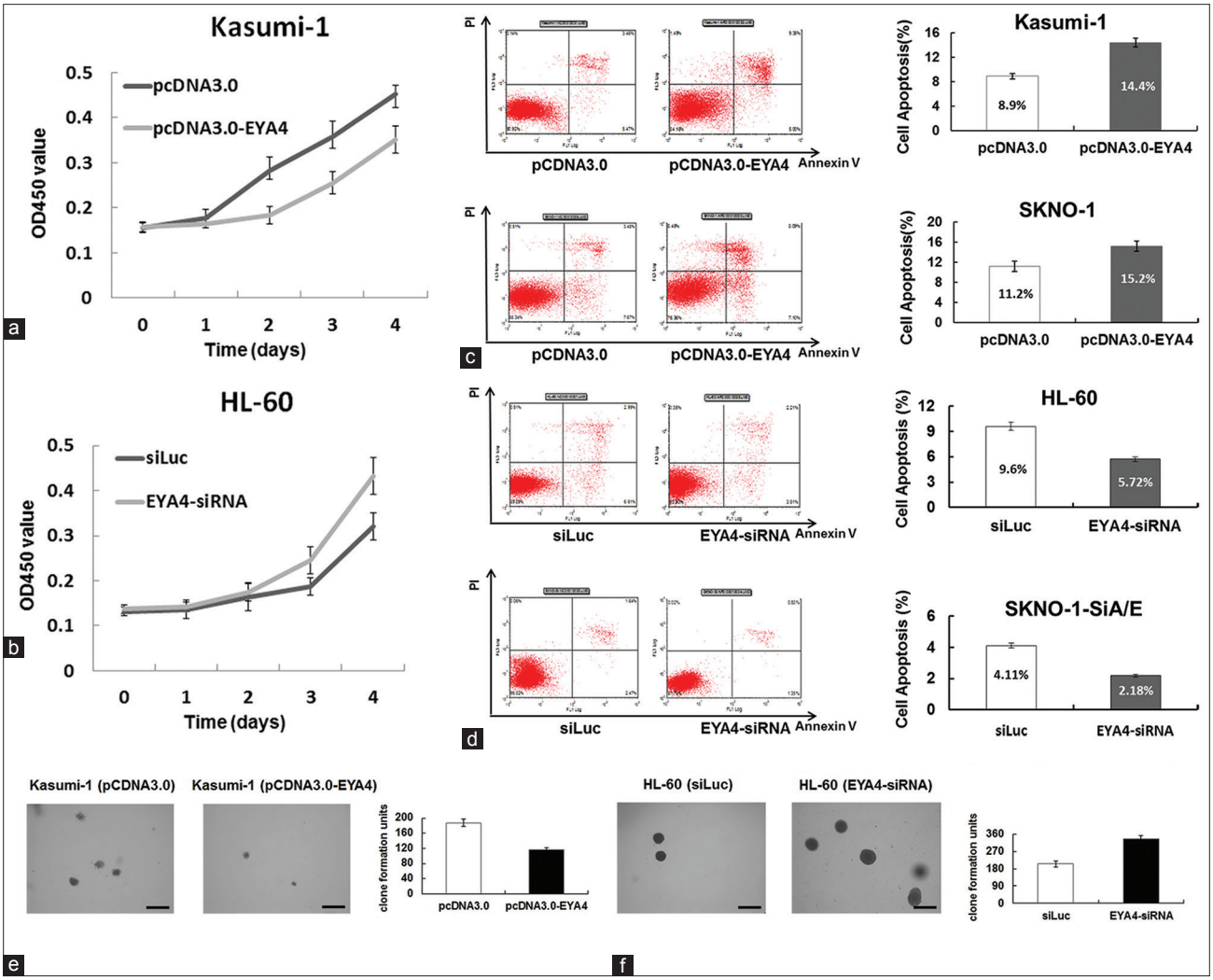

Figure 3: EYA4 inhibits cell proliferation, induces apoptosis and suppresses colony formation in AML1/ETO$^+$ cell lines. (a) Growth curve of Kasumi-1 cells transfected with pcDNA3.0-EYA4 and pcDNA3.0. The number of viable cells was assessed by the CCK-8 assay. (b) Growth curve of HL-60 cells transfected with EYA4-siRNA and siLuc. The number of viable cells was assessed by the CCK-8 assay. (c) Flow cytometry analysis of apoptosis in Kasumi-1 and SKNO-1 cells at 48 h after transfected with 1 μg of pcDNA3.0 and pcDNA-EYA4. (d) Flow cytometry analysis of apoptosis in HL-60 and SKNO-1-siA/E cells at 48 h after transfected with 750 μg of EYA4-siRNA and siLuc. (e) Colony formation assay of Kasumi-1 cells at 48 h after transfected with 1 μg of pcDNA3.0 and pcDNA-EYA4 (scale bar = 1 mm). (f) Colony formation assay of HL-60 and SKNO-1-siA/E cells at 48 h after transfected with 750 μg of EYA4-siRNA and siLuc (scale bar = 1 mm). AML: Acute myeloid leukemia; ETO: Eight-twenty-one; EYA: Eyes absent; CCK-8: Cell Counting Kit-8; siLuc: Small interfering luciferase; siRNA: Small interfering RNA.

excellent responsiveness to the induction chemotherapy and the high complete remission rate.[21-23] Although the overall disease-free survival rate is around 60% in t(8;21) AML, 30–40% of patients relapse after the standard intensive chemotherapy and half of them become treatment resistant.[24-28] As t(8;21) AML is a heterogeneous disease with a poor survival rate in a subgroup of patients, approaches to find novel epigenetic targets are needed.

A previous study has shown that DNA methylation profiling identified *EYA4* gene functioned as a prognostic molecular marker in hepatocellular carcinoma (HCC). The aberrant hypermethylation and subsequent down-regulation of *EYA4* gene might promote tumor progression in HCC.[29] More recently, *EYA4* gene was shown to act as a new tumor suppressor gene in colorectal cancer. EYA4 transfection could lead to the inhibition of cell proliferation both in colony assays and xenograft studies in colorectal cancer. Furthermore, EYA4 was shown to associate with the Wnt and MAPK signaling pathways.[30] *EYA4* gene was also found to be frequently hypermethylated and down-regulated in esophageal and colon cancers.[31-33] Methylated EYA4 was enriched for functions such as cell death/apoptosis and reduced EYA4 expression was consistently and significantly associated with poor survival in lung cancer.[33,34] In hematopoietic malignancy, *EYA4* gene was previously highlighted as potential markers for clinical outcome in two subtypes of acute lymphoblastic leukemia.[35]

In this study, we reported that EYA4 expression was dramatically and specifically down-regulated in AML1-ETO$^+$ AML cell lines. Notably, we demonstrated that the heterochromatic silent state of *EYA4* gene contributed to t(8;21) AML leukemogenesis. Furthermore, AML1-ETO

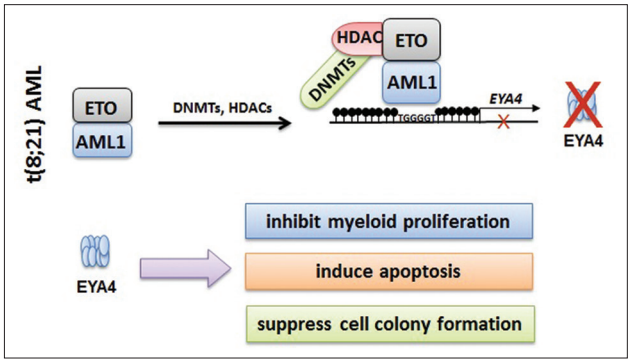

Figure 4: Schematic model for epigenetic silencing of EYA4 by AML1/ETO and its role in t(8;21) AML. AML: Acute myeloid leukemia; ETO: Eight-twenty-one; EYA: Eyes absent; DNMTs: DNA methyltransferases; HDAC: Histone deacetylase.

targeted *EYA4* through the interaction with the AML1 binding sites at the *EYA4* upstream regions where they recruited *DNMTs* and *HDACs*. The AML1-ETO-associated complex reset the *EYA4* genes through changes in DNA methylation to a repressed ground state, contributing to the cell apoptosis and proliferation block [Figure 4].

In conclusion, our results provided new insights into the role of *EYA4* gene in AML leukemogenesis. Our study firstly demonstrated that *EYA4* gene was one of the tumor suppressor genes in t(8;21) AML and played a crucial role in inhibiting cell proliferation, inducing apoptosis and suppressing cell colony formation. We showed that EYA4 was negatively regulated by oncoprotein AML1-ETO at the mRNA level, suggesting that the down-regulation of EYA4 by AML1-ETO might contribute to accelerate the leukemogenesis of AML. On these observations, we speculat that dysregulation of EYA4 plays an important role in the development of t(8;21) AML and the strategies to regulate EYA4 expression would be a valuable adjunctive therapy for t(8;21) AML.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

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Conflicts of interest

There are no conflicts of interest.

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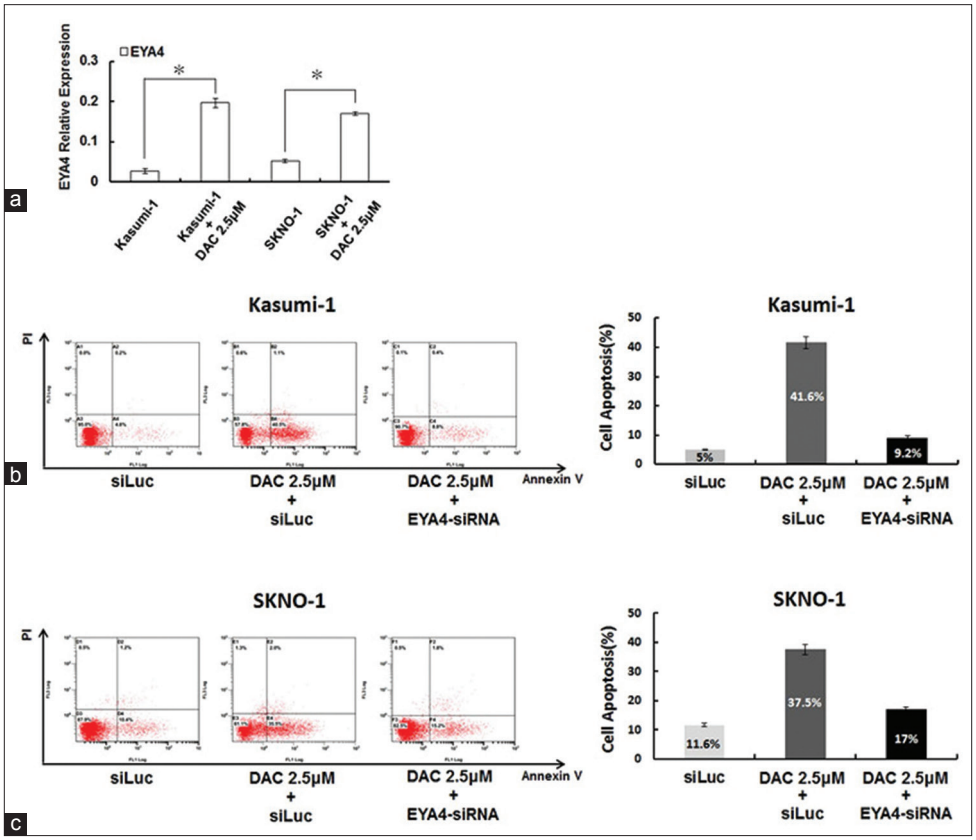

Supplementary Figure 1: The effects of demethylating drugs for treatment of AML1-ETO$^+$ leukemia cells. (a) Relative quantification of *EYA4* levels in Kasumi-1 and SKNO-1 cells with/without 2.5 μmol/L demethylating drug decitabine, respectively. The results represented mean of three independent evaluations ± standard deviation (*$P < 0.05$). (b) Flow cytometry analysis of apoptosis in Kasumi-1 cells at 48 h after treatment of transfection with 750 μg of siLuc, treatment of 2.5 μmol/L decitabine and transfection with 750 μg of siLuc, and treatment of 2.5 μmol/L decitabine and transfection with 750 μg of EYA4-siRNA. The analysis of apoptosis in Kasumi-1 cells treated with 2.5 μmol/L decitabine at 48 h after transfected with 750 μg of EYA4-siRNA and siLuc. (c) Flow cytometry analysis of apoptosis in SKNO-1 cells at 48 h after transfected with 750 μg of siLuc, and the analysis of apoptosis in SKNO-1 cells treated with 2.5 μmol/L decitabine at 48 h after transfected with 750 μg of EYA4-siRNA and siLuc. EYA4: Eyes absent 4; DAC: Decitabine; AML-ETO: Acute myeloid leukemia 1-eight-twenty-one; siLuc: Small interfering luciferase; siRNA: Small interfering RNA.

Supplementary Table 1: Sequences of primers used in this study

Names	Sequence from 5' to 3'	Product size (nt)
RT-PCR and qRT-PCR		
RT-EYA4		
Sense	TCGTTGTGTTTGCATGGTTT	190
Antisense	CCTGACTCCAGGATCCACAT	
GAPDH		
Sense	TTGATTTTGGAGGGATCTCG	238
Antisense	GAGTCAACGGATTTGGTCGT	
EYA4 transactivation assays		
EYA4-P1		
Sense	CGGGGTACCTTCGCAGCACAGCCTATCCCCAGA	759
EYA4-P2		
Sense	CGGGGTACCACGGAGATTACGGCGGCGCCACC	365
EYA4-M		
Sense	TCTCCTCCCTTCGCGAAAGTGGAAA	608
Antisense for EYA4-P1, P2, M	CCCAAGCTTACCCCGGCTTTTCCCGCAGCTCT	
ChIP assay		
Unrelated-EYA4-ChIP		
Sense	CCAGAATGTGCTCTCAACCA	172
Antisense	CAGTCGTTGCTGCTCTCATC	
Related-EYA4-ChIP		
Sense	ACGGATGCCTATACCTGCAC	217
Antisense	CTTACCCCAAGGGAGGAGAC	
GAPDH		
Sense	GAGTCAACGGATTTGGTCGT	238
Antisense	TTGATTTTGGAGGGATCTCG	
Bisulfite modification and genomic sequencing		
EYA4-BSP		
Sense	GTAGTATTGGAAGGGGTTTAGG	337
Antisense	ACTACAACCTCCAAACTAAA	
Full-length EYA4 cDNA for transfection		
EYA4		
Sense	CGGGGTACCATGGAAGACTCCCAGGATTTAAATGAACAATC	1761
Antisense	CCGCTCGAGTTACAAATACTCTAATTCCAGTGCTTGGTGGA	

RT-PCR: Reverse transcription polymerase chain reaction; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; EYA4: Eyes absent 4; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ChIP: Chromatin immunoprecipitation.