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Original Article

Regulation of expression of MLAA-34 gene through transcriptional factors E2F1 and MZF-1

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Abstract: Approximately 20% of adult patients with acute myeloid leukemia fail to achieve remission with initial induction chemotherapy, and around half ultimately experience relapse after achieving complete remission. Relapse continues to be a major hurdle in achieving cure after obtaining remission with induction chemotherapy in patients with acute myeloid leukemia. In last two decades, the immunogenic vaccine, involving peptide, protein, or DNA, has brought new perspectives for tumor immunotherapy. MLAA-34 is a newly identified monocytic leukemia-associated antigen. Downregulation of MLAA-34 expression significantly suppressed the proliferation of U937 cells in vitro and increased the spontaneous apoptosis of leukemia. However, the regulatory mechanisms of MLAA-34 gene are still unknown at present. Analysis of the promoter region of the MLAA-34 gene and reporter gene assays revealed that 600 bp core region was responsible for its regulation. In addition, our study indicated that E2F1 acts as a transcription repressor and MZF-1 acts as a transcription activator of the MLAA-34 gene.

Keywords: Monocytic leukemia-associated antigens, MLAA-34, leukemia, E2F1, MZF-1

Introduction

Leukemia, is the second most common blood cancer worldwide after lymphoma (NCI, 2014). In 2012, leukemia have developed in 352,000 people globally and caused 265,000 deaths [21]. Treatment of acute myeloid leukemia (AML) in younger patients (usually less than 60 years) consists of cytotoxic "chemotherapy" and might cure 20-75%, depending primarily on leukemia-cell cytogenetics [7]. However, treatment for elderly patients (variously defined as 55 to 60 years or older) remains very unsatisfactory [1]. Remission rates are lower than in younger patients because of both higher treatment-related toxicity and higher rates of resistant disease. For those patients achieving remission, the ideal post-remission therapy is still unclear. Generally speaking, elderly patients generally do not tolerate intensive consolidation chemotherapy with high-dose cytarabine, and do not seem to benefit from this kind of treatment [10, 17]; (Weick et al., 1994). Acute monocytic leukemia (M5), a subtype of AML, is largely incurable with high relapse rates, infiltration and a median remission duration of approximately six months [12]. Moreover, M5 has been reported to have a worse prognosis than any other subtypes of AML [19]. These situations above clearly require a novel and more effective therapy. Thus, understanding the specific mechanisms and signal pathway involved in leukemia tumor cell death may allow the identification of novel drug targets and the further development of more specific agents that are designed to specifically induce the apoptotic machinery of tumor cells, may intended particularly for older patients or M5. However, none of above are currently available for practical use.

One of the mechanisms through which tumor cells are believed to acquire resistance to apoptosis is by overexpression of inhibitors of apoptosis proteins (Meinhardt, 2000); [15]. In last two decades, the immunogenic vaccine, involving DNA, protein, or peptide has brought new perspectives for tumor immunotherapy [5, 11,

Table 1. Sequence of the primers for generating the reporter plasmids containing various portions of 2069 bp upstream of the MLAA-34 gene

Name of the promoter-reporter plasmid	Length(bp)	Forward Sequence (5'-3')
pGL3-Basic/LUC/stap2	1500	GGGGTACC TGGTGTTAATATTTCAGAGAGT
pGL3-Basic/LUC/stap3	1000	GGGGTACC ATATCTTTCCTTACTTAATTAC
pGL3-Basic/LUC/stap4	800	GGGGTACC GCCGCCCCTTTGACAACCTCAG
pGL3-Basic/LUC/stap5	600	GGGGTACC GCTCCGCGGCGTTTTCAGCTCC
pGL3-Basic/LUC/stap6	402	GGGGTACC AAACCTCCCGAGCGCAGGTGCC
pGL3-Basic/LUC/stap7	200	GGGGTACC GGAGCCCATTGATTGGTCGCACT

14, 16]; (Ophir et al., 2015). One of monocytic leukemia-associated antigens called MLAA-34, which exclusively reacts with sera from allogeneic leukemia patients but not with normal donor sera, had been identified by our lab using the method called serologic analysis of recombinant cDNA expression library on acute monocytic leukemia (Zhang et al., 2009). Also, our previous studies showed that the down-expression of MLAA-34 significantly suppressed the proliferation of U937 cells in vitro, and increased the spontaneous apoptosis of these leukemia cells (Zhao et al., 2011). However, the mechanisms of MLAA-34 gene expression are not completely elucidated at present.

Our present study was conducted to elucidate the regulatory mechanisms of MLAA-34 gene expression. Analysis of the MLAA-34 promoter region revealed that the 600 bp core region up start codon was responsible for gene regulation and identified both E2F1 and MZF-1 as the transcription regulator of MLAA-34 gene. In addition, we suggested that E2F1 acts as a transcription repressor to down-regulate and MZF-1 acts as a transcription activator to up-regulate the MLAA-34 gene when they are present. These two transcriptional factors are closely associated with MLAA-34 during apoptosis process in the U937 cell model.

Methods and materials

Cell culture

U937 and HEK293 cell lines were maintained in our laboratory and cultured in RPMI-1640 or DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin solution (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid construction

According to GenBank AY288977.20, the 2069 bp MLAA-34 upstream promoter region was amplified by polymerase chain reaction (PCR) from U937 cells genomic DNA using the forward primer 5'-GGGGTACCTTAGACATGAGGTCTATGTTGC-3' and reverse primer 5'-CCAAGCTTAACAGCTGCGCCACCACTCCAGT-3'. The PCR product was isolated by agarose gel electrophoresis and digested with *Kpn* I and *Hind* III (restriction site underlined in the primers) to generate the sticky ends. The final digested PCR product was subcloned into an upstream portion of the luciferase reporter gene in the pGL3-basic vector (Promega, Madison, WI, USA). Promoter region sequence of generated vectors was confirmed by restriction digestion and Sanger sequencing. The new constructed plasmid was named pGL3-Basic/LUC/stap. A series of reporter gene plasmids containing various portions of the 2069 bp MLAA-34 promoter region were generated using pGL3-Basic/LUC/stap as a template. The forward PCR primer sequences used for each promoter reporter construct and name are listed in **Table 1**. The reverse primer was the same with full length promoter region. The length shown in the row of each plasmid in **Table 1** represents the approximate distance from the translation start site in the MLAA-34 gene. All constructs were also verified by restriction enzyme digestion and Sanger sequencing.

Luciferase reporter assay

Luciferase transcription was quantitated by transient transfection of various pGL3 constructs containing the respective DNA sequences corresponding to the promoter regions of MLAA-34 gene as well as the firefly luciferase reporter gene. U937 and HEK293 cells were seeded in a 24-well plate at 5×10^4 cells per well and cultured overnight to 90% confluence prior to transfection. For luciferase assays, cotransfections were performed with one of these three pGL3-Basic/LUC/stap series: pGL3-Promotor/LUC, pGL3-Basic/LUC/

Table 2. Sequence of the primers used to generate the mutated binding sites in the candidate transcription factors involved in MLAA-34 regulation

Transcription Factor	Orientation	Sequence (5'-3')
E2F1	Forward	5'-AGCGCAGGTGCCCTCACTGTCATTCCTACTTCACCATTTAC-3'
	Reverse	5'-GTAAATGGTGAAGTAGGAATGACAGTGAGGGCACCTGCGCT-3'
MZF-1	Forward	5'-CCGCCCTGCCTCTACACTCTTTGAGGGAAGGCGTAA-3'
	Reverse	5'-TTACGCCTTCCCTCAAAGAGTGTAGAGGCAGGGCGG-3'
SP1	Forward	5'-ATCTCAACCACAACAACTGTCATTGCCCTCTACCGGGGATG-3'
	Reverse	5'-CATCCCCGGTAGAGGGCAATGACAGTTGTTGTGGTTGAGAT-3'
USF2	Forward	5'-AAACCTCCCGAGACTGTCATTGATCTGGCCGGGAAGTA-3'
	Reverse	5'-TACTTCCCGGCCAGATCAATGACAGTCTCGGGAGGTTT-3'

SV40 and Renilla luciferase vector. After 24 h of transfection, cells were washed twice with PBS and exposed to 200 μl of 1 × passive lysis buffer. Luciferase activity was assayed with 50 μl lysis supernatant and 50 μl luciferase assay reagent using a luminometer. Luciferase assays were performed as triplicates 3 independent experiments and its activity was measured using Dual-luciferase Reporter Assay System (Promega). Data were reported as the relative luciferase activity calculated as the ratio of firefly luciferase activity and Renilla luciferase activity.

Site-directed mutagenesis

Computer analysis (Gene-regulation, Transfac 6.0. and ALGGEN-PROMO) of the MLAA-34 core promoter region proximal 600 bp sequence that we predicted reveals a number of potentially important cis-regulatory sequences. The promoter was predictably bound by transcription factors: Signal transducer and activator of transcription 3 (STAT3), E2F Transcription Factor 1 (E2F1), Myeloid Zinc Finger 1 (MZF-1), Specificity Protein 1 (SP1), and Upstream Stimulatory Factor 2 (USF-2). The promoter regions of four targeted sequences were subjected to site-directed mutagenesis to study the function of each transcription factor as potential MLAA-34 gene regulators. Site-directed mutagenesis was performed using Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions and the primers are listed in **Table 2**. Four constructs (pGL3-Basic/LUC/sE2F1, pGL3-Basic/LUC/sMZF-1, pGL3-Basic/LUC/sSp1, pGL3-Basic/LUC/sUSF-2) containing mutated sequences in the transcription binding sites were generated to prevent each transcription factor from binding to the specific region of the MLAA-34 promoter region, then luciferase activity was tested in both HEK293 and U937 cells. Two constructs (pGL3-Basic/EGFP/E2F1, pGL3-Basic/EGFP/MZF-1) containing mutated sequences in the transcription binding sites were generated to prevent each transcription factor from binding to the specific region of the MLAA-34 promoter region using the primers listed in **Table 2**, then green fluorescent protein expression was tested in U937 cells.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from U937 and HEK293 cells were isolated using a Nuclear Extraction kit (Panomics). For the EMSA assay, E2F1 probe (5'-TCTACCGGGGATGAGGGAAGGCGTAACCTCCG-3') and MZF-1 probe (5'-CCGCCCTGCCTCTACCGGGGATGAGGGAAGGCG3') were labeled by combining 250 ng of the annealed oligonucleotide; 2 μl of 10 × T4 kinase buffer (Takara Bio); 1 μl each of 5 mM stock of dATP, dTTP, and dGTP; 8 μl of water; 1 μl of T4 polynucleotide kinase (Takara Bio) and 5 μCi of (32P)-dCTP (Amersham Biosciences) at 37°C for 1 h. The labeled probe was isolated using Microspin columns (Bio-Rad, Hercules, CA, USA). A typical reaction consisted of 10 ng of nuclear protein extracts, 250 μg/ml poly (deoxyinosinic-deoxycytidylic acid), and radiolabeled double-stranded oligonucleotides in a solution of 30 mM HEPES, 100 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.3 mM EDTA, and protease inhibitor (Sigma-Aldrich). Binding between oligonucleotides and E2F1 or MZF-1 was induced by incubating samples at 37°C for 20 min. Final binding complexes were loaded onto 6% polyacrylamide gels and the gel was dried,

and separated at 150 V for 2.5 h. Competition experiments were performed by adding a 100-fold excess of unlabeled oligonucleotides. The gel was exposed to Fuji film (Tokyo, Japan) at 25°C for 24 h and visualized by a FLA-7000 phosphoimager (Fuji Film Life Science, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed using ChIP Assay Kit (Upstate Biotechnology, Charlottesville, VA, USA) according to the manufacturer's instructions. Briefly, nuclear extracts was isolated from U937 cells using a Nuclear Extraction kit from Panomics (Santa Clara, CA, USA) and sonicated to produce 500-1000 bp chromatin fragments. The sonicated nuclear extract was incubated with E2F1 and MZF-1 antibody (Cell Signaling Technology, Danvers, MA, USA) for 90 min respectively. Mouse immunoglobulin G (IgG) (Sigma-Aldrich) was used as a control to monitor nonspecific interactions and anti-RNA polymerase II antibody (Sigma Aldrich) was used as a positive control. Antibody-E2F1-DNA complexes and Antibody-MZF-1-DNA were pulled down after extensive washing to reduce non-specific binding between proteins and antibodies. Reverse cross-linked of protein-DNA complexes by heating at 65°C for 4 h. Before immunoprecipitation, a small chromatin-protein sample was excluded and used as input sample for a positive control for the PCR reaction. DNA was extracted with phenol/chloroform, precipitated with ethanol and then used as templates for PCR amplification. The E2F1 binding region in the MLAA-34 promoter was amplified using a pair of primers: 5'-TTGGCAAGGCTGGGTTC-3' (forward) and 5'-GCGGGACTGTTGTGGTTG-3' (reverse). The MZF-1 binding region in the MLAA-34 promoter was amplified using a pair of primers: forward: 5'-AGCCTTCATCTCAACCACAAC-3'; reverse: 5'-CTCCCATGTAGTGATCGGTTT-3'. The final PCR products were visualized on a 1% agarose gel.

Reverse transcription polymerase chain reaction (RT-PCR)

Total U937 cellular RNA was extracted using TRIzol reagent (Invitrogen, USA) and 5 μg of RNA was used to synthesize cDNA using Superscript First-Strand Synthesis Kit (Promega, USA) according to the manufacturer's protocols. The following primers were used at the indicated annealing temperature: E2F1 forward 5'-CCG-GAATTC(EcoRI)-CGTGAGCGTCATGGCCTTGG-3' and reverse 5'-CGG-GGTACC (KpnI)-CCCTGGTCCCTCCAAGCCCTG-3' at 57°C for 30 cycles, a predicted band was 1345 bp; MZF-1 forward 5'-CCG-GAATTC(EcoRI)-AAGGGAGGAGAGGGTGTAGAAACGG-3' and reverse 5'-CGG-GGTACC(KpnI)-TGTAATCGCCAGCCTCACAATAACC-3' at 58°C for 30 cycles, a predicted band was 2480 bp; β-actin (X00351) gene was used as an internal standard and was amplified with primers, sense: 5'-CTACAATGAGCTGCGTG-3', and antisense: 5'-GGTCTCAAACATGATC-3' at 58°C for 30 cycles, a predicted band was 109 bp. Then RT-PCR products were electrophoresed through a 1.5% agarose gel with ethidium bromide 1.5 g/L for 0.5 hour. As a control, β-actin expression was used to normalize mRNA expression levels. The PCR products were then inserted into the EcoRI and KpnI site of pcDNA3.1vector, and named pcDNA3.1/E2F1, pcDNA3.1/MZF-1. All recombinant plasmids described above were finally verified by DNA sequencing.

Western blotting

The cells were harvested by suspension in RIPA buffer on ice for 20 min. Protein concentrations were measured with a bicinchoninic acid assay kit (Promega). Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was blocked with Tris-buffered saline plus 0.1% Tween-20 (TBST) containing 5% skimmed milk for 2 h at room temperature, then incubated with the appropriate primary antibodies in TBST containing 5% skimmed milk overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary immunoglobulin antibodies (Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. The membrane was probed with the primary antibody against each of the following proteins: E2F1, MZF-1, MLAA-34 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, the bands were visualized by chemiluminescence using a chemiluminescence kit (NENTM Life Science Products Inc, Boston, MA, USA) and the specific bands were recorded on X-ray film. β-actin was used to normalize protein levels.

Construction of siRNA expression vector for E2F1 and MZF-1

The sequence in E2F1 and MZF-1 gene cDNA was chosen as our target sites for design of siRNA using siRNA design software from the

internet (Ambion® technical resources). The siRNA designed towards a non-specific sequence was used as a control. BLAST search against EST libraries was performed to confirm that no other human gene was targeted.

siRNA sequence for E2F1: sense strand: 5'-CCUGGAGCAAGAAGCAGUAUUGCCA-3', antisense strand: 5'-UGGCAAUACUGCUUCUUGCUCCAGG-3'. siRNA sequence for MZF-1: sense strand: 5'-GAGGUCCUAUCAGAGAAGA-3', antisense strand: 5'-UCUUCUCUGAUAGGACCUC-3'. The shRNAs were subcloned into pSilencer4.1-CMVneo (Amibion, Austin, TX, USA) and named as pSilencer4.1-E2F1-siR and pSilencer 4.1-MZF1-siR. The recombinant vector was confirmed by the digestion analysis of restriction endonuclease and all inserted sequences were verified by DNA sequencing.

Statistical analysis

Data are shown as mean ± SD. One-way ANOVA was performed with SPSS software (version 19.0; SPSS, Chicago, IL, USA). Statistical significance was based on a value of $P<0.05$.

Results

Construction of MLAA-34 promoter-containing reporter clone and identification of the core region

The 2069 bp MLAA-34 upstream promoter region and segments of promoter region with different length were amplified by PCR from U937 cells genomic DNA successfully without non-specific amplification (**Figure 1A**). All constructs were also confirmed by restriction digestion (*Kpn* I and *Hind* III) (**Figure 1B**) and Sanger sequencing. Luciferase reporter assay confirmed that 2069 bp promoter region had promoter activity compared to pGL3-basic vector ($P<0.001$), although not as strong as plasmid control promoter (**Figure 1C**). The highest luciferase activity was observed in cells transfected with the pGL3-Basic/LUC/stap6 (see **Table 1**) construct and no significant different with plasmid control promoter ($P>0.05$). Promoter activity of other constructs were all significantly reduced (**Figure 1D**). These data suggested that the region of approximately 600 bp upstream of the translational start was critical for regulating the expression of the MLAA-34 gene.

Identification of transcriptional regulators that interact with the promoter core region

To identify transcriptional factors of MLAA-34 that may directly interact with the 600 bp core region, four prediction program (TESS, Generegulation Transfac6.0 ALGGEN-PROMO) were utilized. As a result, four candidate genes, E2F1, MZF-1, SP1 and USF2 were selected (**Figure 2A**). Additional constructs based on pGL3-Basic/LUC/stap6 were generated that contained mutated nucleotides in regions corresponding to the binding sites that interact with E2F1, MZF-1, SP1 and USF2 (Stat3 is used anddiscussed in our other paper). Promoter activities were examined in both HEK293 and U937 cells transfected with pGL3-Basic/LUC/stap6 (wild type control) or reporter constructs containing the specific mutation, namely pGL3-Basic/LUC/sE2F1, pGL3-Basic/LUC/sMZF-1, pGL3-Basic/LUC/sSp1, pGL3-Basic/LUC/sUSF-2. No significant change in luciferase activities was observed in cells transfected with pGL3-Basic/LUC/sSp1 and pGL3-Basic/LUC/sUSF-2 ($P>0.05$), whereas significantly increased luciferase activity was observed in cells transfected with pGL3-Basic/LUC/sE2F1 ($P<0.05$) and decreased luciferase activity was observed in cells transfected with pGL3-Basic/LUC/sMZF-1 compared to wild-type control ($P<0.01$) (**Figure 2B** and **2C**). Also significant increased GFP expression was observed in cells transfected with pGL3-Basic/EGFP/E2F1 compared to cells transfected with pGL3-Basic/EGFP/MZF-1 (**Figure 2D**). All our results above indicated that both E2F1 and MZF1 play a key role in regulating MLAA-34 expression. E2F-1 would downregulate MLAA-34 expression as a transcription repressor, whereas MZF-1 would upregulate MLAA-34 expression as a transcription activator.

Direct binding of E2F1 and MZF1 to the MLAA-34 promoter region

Direct interaction of E2F1 and MZF-1 protein with the MLAA-34 promoter region was studied using a ChIP assay and EMSA in U937 cells. First, a ChIP assay was performed to pull down complexes containing E2F1 or MZF-1 protein and E2F1 or MZF-1 binding site of the MLAA-34 promoter region using E2F1 or MZF-1-specific antibody and primers specific for the E2F1 or MZF-1 protein binding sequence. As shown in **Figure 3A**, anti-E2F1 or MZF-1 antibody had a

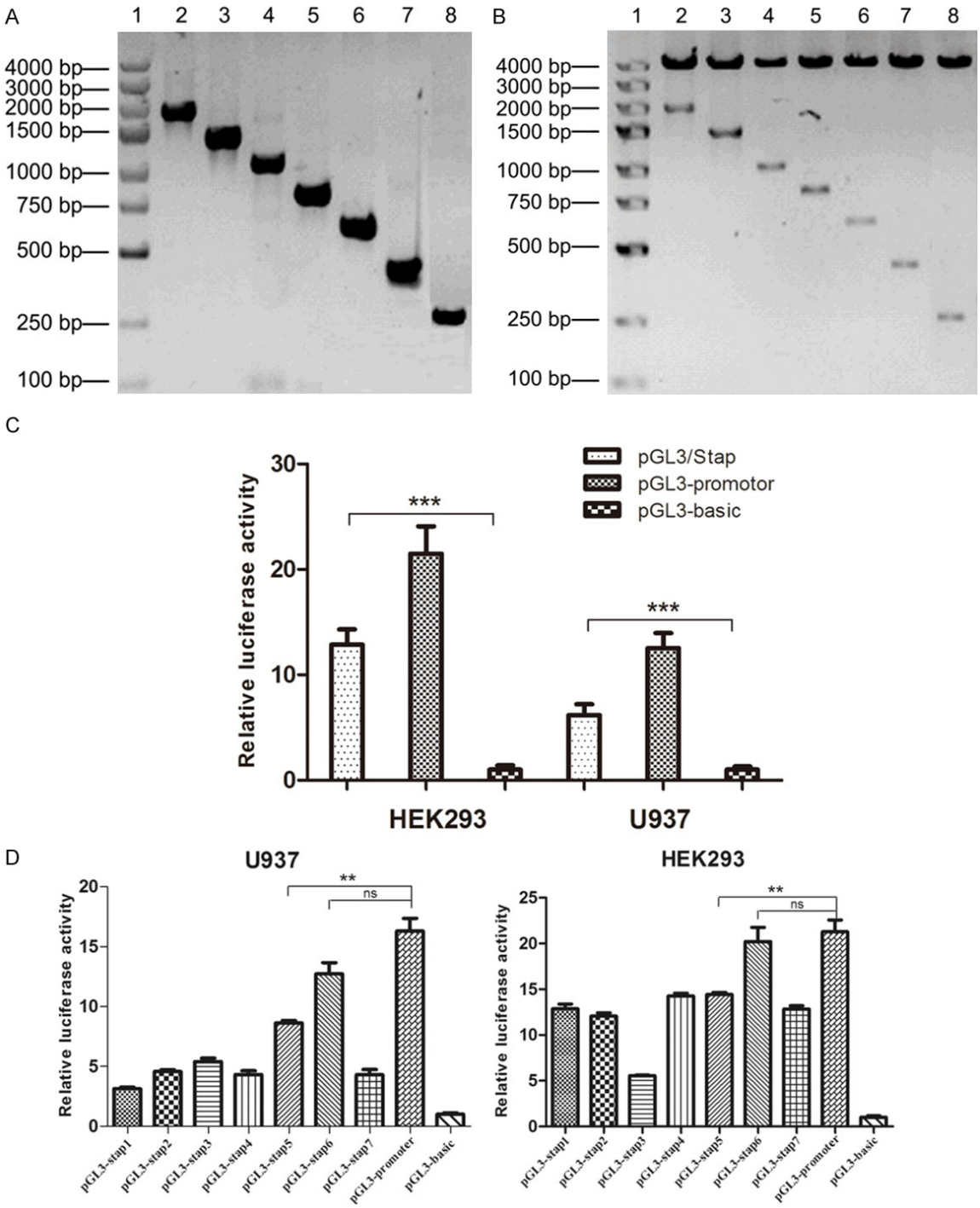


Figure 1. Identification of the MLLA-34 promoter region. A. PCR results for various portions of the 2069 bp MLAA-34 promoter region. Segments of promoter region lane number, name and length: 1. DNA marker, 2. Stap (2069 bp), 3. Start 2 (1500 bp), 4. Start 3 (1000 bp), 5. Start 4 (800 bp), 6. Start 5 (600 bp), 7. Start 6 (402 bp), 8. Start 7 (200 bp). B. A series of MLAA-34 promoter report constructs were digested by *Kpn I* and *Hind III*. C. HEK293 and U937 cells were transfected with pGL3-Basic/LUC/stap, pGL3-Promotor/LUC, and pGL3-Basic/LUC/SV40 respectively. The Renilla vector was used to normalize the transfection efficiency. Luciferase activity of the pGL3-Basic/LUC/SV40 empty vector was used as a control. D. U937 and HEK293 cells were transfected with a series of MLAA-34 promoter reporter constructs. The Renilla vector was used to normalize the transfection efficiency. Luciferase activity of the pGL3-Basic/LUC/SV40 empty vector was used as a control. **, P<0.01; ***P<0.001; ns, not significant.

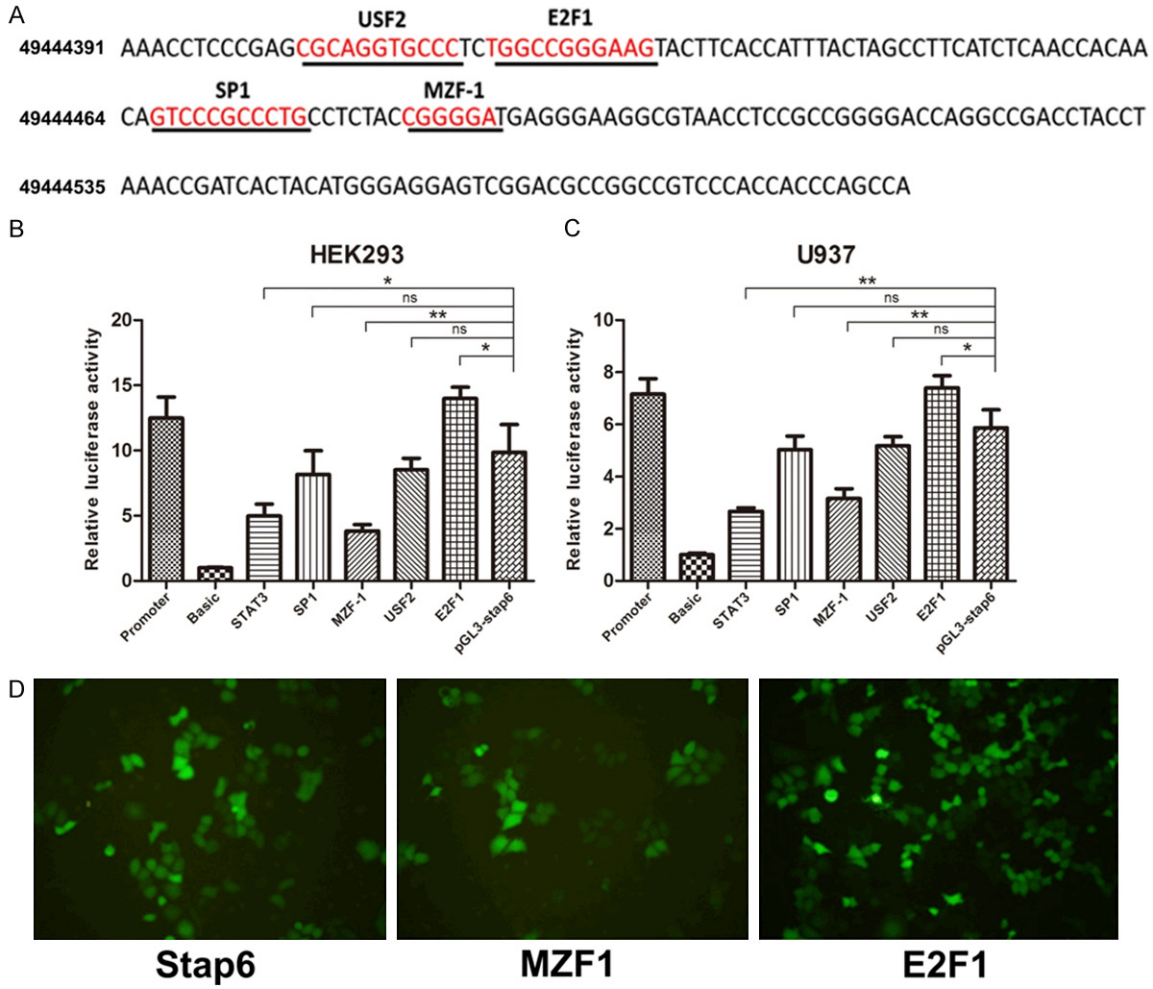


Figure 2. Identification of transcriptional regulators. A. The 600 bp region upstream of the MLAA-34 translation start site was analyzed using a promoter prediction program. B. The four binding sites of the predicted transcription factors were targeted for site-directed mutagenesis. Four mutational constructs were transfected into HEK293. The Renilla vector was co-transfected to normalize the transfection efficiency. C. The four binding sites of the predicted transcription factors were targeted for site-directed mutagenesis. Four mutational constructs were transfected into U937 cells. The Renilla vector was co-transfected to normalize the transfection efficiency. D. Expression of GFP in U937 cells by transfection of plasmid pGL3-Basic/LUC/stap6, pGL3-Basic/EGFP/E2F1 and pGL3-Basic/EGFP/MZF-1, respectively (200x). *P<0.05; **, P<0.01; ns, not significant.

higher affinity to the chromatin extracts of U937 cells than the mouse IgG antibody. This was confirmed by detecting DNA fragments containing the E2F1 or MZF-1-binding sequence in the MLAA-34 promoter region, which were the products of PCR using primers specific for the E2F1 or MZF-1-binding region and a template DNA eluted from the chromatin-protein complexes. Direct binding of the E2F1 or MZF-1 protein to the MLAA-34 promoter was further confirmed by an EMSA with lysates from U937 cells (**Figure 3B**). Nuclear extract from U937 cells was incubated with oligonucleotide probes containing either the wild type or mutated E2F1 and MZF-1 binding site in the MLAA-34 promoter region separately and were radiolabeled. Only samples containing radiolabeled wild type probe showed detectable binding affinity U937 cells (both lane 2 in **Figure 3B**). Binding affinity was reduced when nuclear extracts were incubated with additional excess non-radiolabeled probes (both lane 3 in **Figure 3B**) and mutant probes (both lane 4 in **Figure 3B**). Supershift with antibodies indicated a specific interaction transcription factor and binding sequence (both lane 5 in **Figure 3B**). Our results confirmed that there is a specific protein-DNA interaction between E2F1 or MZF-1 and the E2F1-

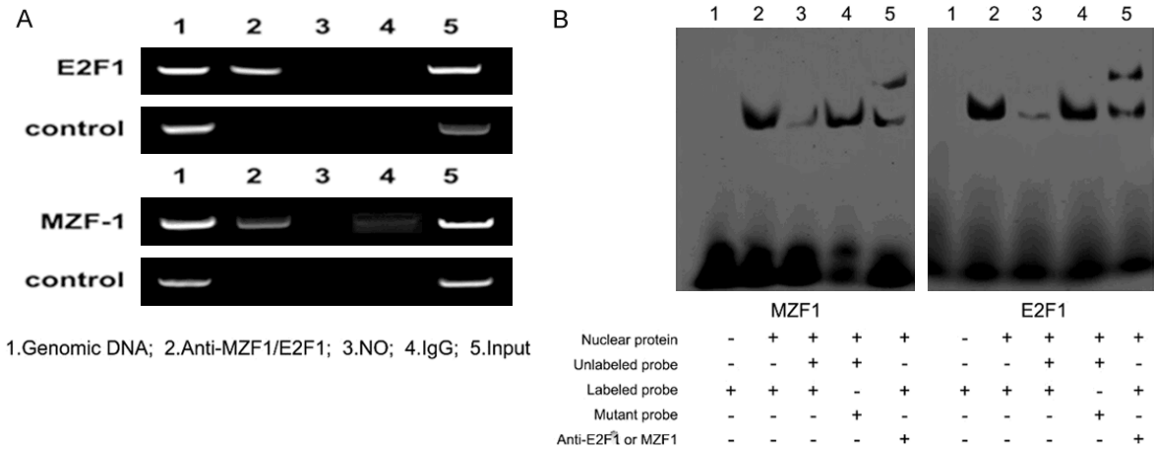


Figure 3. A. ChIP assay was performed in U937 cells using the antibodies and primer set described in Materials and methods. Binding affinity was measured by PCR. Binding affinity of mouse normal IgG was used as a negative control. B. EMSA was carried out using U937 cell nuclear extracts using radiolabeled oligonucleotides containing the wild type or mutated sequences corresponding to the E2F1 and MZF-1 binding site of the MLAA-34 promoter respectively. Unlabeled oligonucleotides containing either wild type or mutant binding sites were used as a specific competitor.

binding sequence (MZF-1-binding sequence) in the MLAA-34 promoter; that is E2F1 and MZF-1 protein could bind the MLAA-34 gene promoter region.

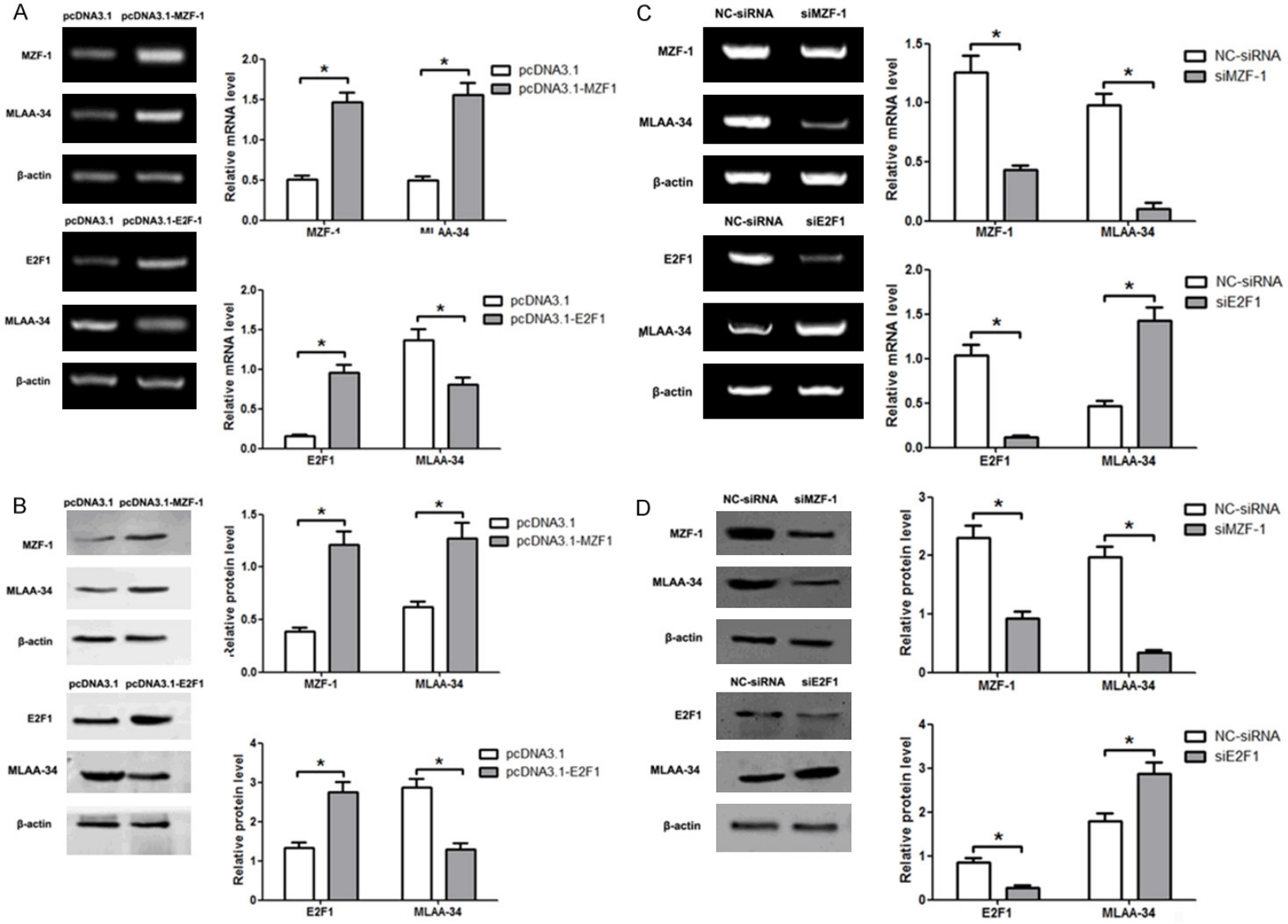
Regulatory effects of E2F1 and MZF-1 on MLAA-34 gene transcription and expression

The overexpression of MZF-1 gene by transfecting pcDNA3.1/MZF-1 into U937 cells increased MLAA-34 expression (**Figure 4A** and **4B**, upper part). The upregulated E2F1 expression by transfecting pcDNA3.1/E2F1 into U937 cells resulted in decreased MLAA-34 expression (**Figure 4A** and **4B**, lower part). To verify our data, small interfering RNA (siRNA) was used for as gene silencing study. As expected, the downregulated MZF-1 in U937 cells decreased MLAA-34 expression (**Figure 4C** and **4D**, upper part). Silencing E2F1 gene resulted in upregulated MLAA-34 expression (**Figure 4C** and **4D**, lower part). All the results above imply that MZF-1 may positively regulate MLAA-34 expression, while E2F1 may be a negative regulator of MLAA-34.

Discussion

The identification of biomarkers for prognosis, diagnosis, and therapy of human cancer is a long-standing challenge in cancer research. Much evidence shows that the humoral immune system of cancer patients recognizes tumor-related antigens [2, 4, 6, 8, 18, 20]. As in our previous studies, we have identified 15 distinct novel acute monocytic leukemia-associated antigens (MLAA) recognized exclusively by sera from allogeneic leukemia patients but not by normal donor sera using SEREX analysis [3]. MLAA-34 is one of these identified novel antigens. The complete nucleotide sequence of full-length of MLAA-34 amplified from U937 cells is 1,671 bp, containing 1,014 nucleotides within the putative coding region, flanked by 451 bp in the 5-untranslated region and 206 bp in the 3-untranslated region. It was proposed that MLAA-34 is a novel CAB39L's splice variant associated to acute monocytic leukemia [14]. Also, our previous studies showed that MLAA-34 over-expression is associated with unfavorable clinical features at diagnosis and a strong correlation between anti-apoptosis with the upregulation of MLAA-34 [16]. In addition, preliminary proteomic analysis suggests that a number of genes belonging to different signaling pathways are involved in apoptosis in U937 cells in association with MLAA-34, which would disclose a novel cross-link between MLAA-34 and the Ras, Wnt, calcium and chemokine signaling pathways (Zhang, et al., 2013). However, the regulation of MLAA-34 had not been studied before.

In the present study, it was found that E2F1 and MZF-1 are two transcription factors regulating


A
pcDNA3.1
pcDNA3.1-MZF-1
MZF-1
MLAA-34
β-actin
pcDNA3.1
pcDNA3.1-E2F-1
E2F1
MLAA-34
β-actin
Relative mRNA level
pcDNA3.1
pcDNA3.1-MZF1
MZF-1
MLAA-34
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E2F1
MLAA-34
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MZF-1
MLAA-34
β-actin
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pcDNA3.1-E2F1
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β-actin
NC-siRNA
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MLAA-34
β-actin
Relative protein level
NC-siRNA
siMZF-1
MZF-1
MLAA-34
Relative protein level
NC-siRNA
siE2F1
E2F1
MLAA-34

Figure 4. E2F1 and MZF-1 overexpression experiment was conducted. pcDNA3.1/E2F1 and pcDNA3.1/MZF-1 were transfected into U937 cells, respectively. A. The mRNA levels were detected by RT-PCR compared to samples treated with no siRNA. B. Protein levels were determined by western blot. E2F2 or MZF-1 siRNA were transfected into U937 cells. C. The mRNA levels were detected by RT-PCR compared to samples treated with no siRNA. D. Protein expression levels were determined by western blot. All expression levels were normalized to that of β-actin. *P<0.05.

MLAA-34 gene expression in U937 cells. Initial attempts to identify the core region of the MLAA-34 promoter were made by analyzing the promoter region starting 2 kb upstream of the translational start site. Our results showed that a region of approximately 600 bp upstream of the MLAA-34 gene was sufficient to control gene expression and have the highest transcription efficiency. Interestingly, the binding sites of numerous transcriptional factors including E2F1, MZF-1, SP1, and USF2 were found within this 600 bp region using the promoter prediction program (Gene-regulation, Transfac 6.0. and ALGGEN-PROMO). E2F1 and MZF-1 were selected for further investigation for the significant change in reporter gene expression after mutating the E2F1 and MZF-1 binding sites.

In order to clarify which transcription factor helps regulate MLAA-34 gene expression, a typical reporter gene assay was performed and promoter activities were measured in U937 that displayed a level of luciferase activity proportional to promoter activities. In addition, promoter activity assays were performed using reporter gene constructs containing mutated nucleotides located in the middle of the binding sites for each potential transcription factor. Promoter activity decreased when the luciferase reporter construct containing the point mutation site within binding site for E2F1 was expressed in U937. Promoter activity increased when the luciferase reporter construct containing the point mutation site within binding site for MZF-1 was expressed in U937, thereby indicating that E2F1 and MZF-1 are two MLAA-34 transcription factors. Additionally, MLAA-34 promoter activity was elevated in U937 cells in which E2F1 levels were reduced by siRNA and MLAA-34 promoter activity. It was decreased in U937 cells in which MZF-1 levels were reduced by siRNA. MLAA-34 expression inversely correlated with that of E2F1 and positively correlated with MZF-1. The direct binding of E2F1 and MZF-1 to the MLAA-34 promoter region was confirmed by performing a ChIP assay and EMSA. All of these data consistently indicated that E2F1 and MZF-1 may interact with a specific sequence in the MLAA-34 promoter region, and their binding could reduce and increase MALL-34 gene expression, respectively.

In summary, the regulation of MLAA-34 by E2F1 and MZF-1 has been demonstrated. This novel regulation mechanism of gene expression will lead to a better understanding of the mechanisms involved in M5, and MLAA-34 may serve as a potential novel marker for the early diagnosis and gene therapy of M5.

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Disclosure of conflict of interest

None.

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