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ORIGINAL ARTICLE

Identification and functional characterization of the novel acute monocytic leukemia associated antigen MLAA-34

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Abstract We have previously applied the method of serologic analysis of recombinant cDNA expression library (SEREX) on acute monocytic leukemia to identify monocytic leukemia-associated antigens. Using this approach, we identified a novel gene, MLAA-34, which exclusively reacted with sera from allogeneic leukemia patients but not with normal donor sera. Here, we further characterized its gene structure and explored the function. We first determined both 5′ and 3′ end by RLM-RACE and cloned full-length cDNA of MLAA-34 in U937 cell line. Analysis of full cDNA sequence showed that MLAA-34 is highly homologous to known human gene CAB39L, but differs from two transcript splice variants of CAB39L. Thus, we propose that MLAA-34 is a novel CAB39L's splice variant associated with acute monocytic leukemia. Because the functions of MLAA-34 and CAB39L are both very unclear, then we investigated the role of MLAA-34 in U937 cell line using RNA interference technology. The results showed that the downregulation of MLAA-34 expression significantly suppressed the proliferation of U937 cells in vitro, and increased the spontaneous apoptosis of these leukemia cells. All these data indicated that MLAA-34 may be a novel anti-apoptotic factor related closely to carcinogenesis or progression of acute monocytic leukemia. The anti-apoptotic pathways of MLAA-34 remain further exploration. This study warrants further investigations to verify MLAA-34 as a promising antigen and a molecular target for therapeutic applications in acute monocytic leukemia.

Keywords MLAA-34 · Acute monocytic leukemia associated antigen · Identification · CAB39L · Splice variant · Anti-apoptotic factor

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Introduction

Although the frequency of complete remission of acute monocytic leukemia has increased, the median duration of remission is only about 6 months, even when remission is achieved by treatment with conventional cytotoxic antileukemic drugs [1]. Allogeneic hematopoietic stem cell transplantation has proved to be the most effective therapeutic method for acute leukemia, but its application so far has been much limited. These situations clearly call for novel and more effective therapy.

In last two decades, the immunogenic vaccine, involving peptide, protein, or DNA, has brought new perspectives for tumor immunotherapy. Various peptides derived from leukemia-associated antigens are under clinical investigation for AML patients in current peptide vaccination trials: (1) the proteinase 3-derived peptide, PR1 [2]; (2) WT1-derived peptides [3–5]; (3) the RHAMM/CD168-derived peptide, R3 [6]. Vaccination strategies using peptides in patients with hematologic malignancies were shown to be safe, and specific immune responses could be detected. In 1995, we have developed a leukemia vaccine strategy by using inactivated autologous leukemia cells combined with IL-2, GM-CSF and IL-6, which showed evident effect on patients with relapsed or refractory acute leukemia, especially acute monocytic leukemia [7]. The premise for the application of

vaccine-based approaches to cancer therapy is the recognition of tumor-specific and tumor-associated antigens by the immune system. In order to enhance the effectiveness and elucidate the mechanism of our leukemia vaccine, we have applied the method of serologic analysis of recombinant cDNA expression library (SEREX) on acute monocytic leukemia to identify monocytic leukemia-associated antigens (MLAA).Thirty-five distinct novel antigens were identified by SEREX analysis through reaction with the sera from acute monocytic leukemia patient. The nucleotide sequence data of these genes have been deposited in GenBank databases. The detection of antigen-specific antibodies in allogeneic sera indicated that fifteen of the 35 novel antigens were recognized exclusively by sera from allogeneic leukemia patients but not by normal donor sera [8].

MLAA-34 (GenBank no: AY288977) is one of the novel fifteen antigens reacting exclusively with sera from allogeneic leukemia patients but not with normal donor sera. These results suggest that it is a novel monocytic leukemia-associated antigen. As a novel leukemia-associated antigen, it is necessary to further explore its gene structure and the role in carcinogenesis. In present study, we cloned the full-length cDNA sequence of MLAA-34, and applied RNA interference technology to investigate its function in U937 cell line.

Materials and methods

Cell and cell culture

U937 (human lymphoma monocyte) cells were obtained from the Institute for Cancer research, the School of Life Science and Technology, Xi'an Jiaotong University (China). Cells were cultured in RPMI-1640 media (pH 7.4) containing 2 mM L-glutamine, 10 mM HEPES, 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, cells were used in the log-growth phase.

Characterization of MLAA-34 transcript by 5′- and 3′-RACE

For characterization of the intact MLAA-34 transcript, 5′- and 3′-RACE were conducted using the RNA-ligase-mediated RACE (RLM-RACE) Kit (Takara, DaLian, China) as described by the manufacturer. We used existing MLAA-34 sequences as a guide for primer design. For 5′-RACE reaction, the two corresponding MLAA-34-specific reverse primers were: GSP1, 5′-CCTCTTCTGAAGCCTTGTCTG TCT-3′; GSP2, 5′-TTCTGCTGGATTTTTGTGTGATTT AC-3′. Likewise, for 3′-RACE reaction, the two corresponding MLAA-34-specific forward primers were: GSP3, 5′-GCCAGTCCTCACAAAACACAGCCT-3′; GSP4, 5′-ACTCATTGAGTTTCTGAGCA -3′. For both 5′- and 3′-RACE, the nested PCR reactions began with the primary PCR consisting of 20 cycles of 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 2 min, followed by the secondary PCR consisting of 25 cycles of 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 2 min. The 5′- and 3′-RACE PCR products were electrophoresed on a 1% agarose gel in 1× TAE buffer. The resultant band was eluted from the gel using the TaKaRa Agarose Gel DNA Purification Kit (Takara, DaLian, China), and then directly cloned into the pGEM-T easy vector (Promega, MI). Three clones from each band were sequenced. The sequencing reactions were performed by the DNA Sequencing Service at Invitrogen Corporation (China) using ABI PRISM 377 automated sequencers.

RT-PCR for isolation and sequencing of transcript of MLAA-34

Sequences of 5′- and 3′-RACE products of MLAA-34 were used to design primers for isolating the full-length transcript of MLAA-34. RT-PCR was carried out by using High Fidelity PrimeScriptTM RT-PCR Kit (Takara, DaLian, China). The MLAA-34 forward primer was: 5′-AA GAAAGAATATCAGGAGCA-3′, and the reverse primer: 5′-ACAGCAGAAAAAATAGGCAC-3′. The RT-PCR product was electrophoresed on a 1% agarose gel in 1× TAE buffer. The resultant band was cloned and several clones from the band were sequenced as described above.

Bioinformatics

Based on the alignment of the transcripts to the latest genome sequence (human build 36.3), we used BLAST searches of GenBank at NCBI to align our newly cloned full-length sequences to genomic sequence to determine gene location and organization and splicing patterns. The putative coding sequences were predicated using Open Reading Frame (ORF) Finder of NCBI. Derived protein sequences were analyzed using various bioinformatics tools: SignalP for signal peptide prediction [9], PSORT and TargetP for cellular localization prediction [10, 11], ScanProsite for potential specific motif scanning [12], and ClustalX for multiple sequence alignment (http://us.expasy.org/) [13].

Vector-based shRNA plasmid constructs and stably transfected tumor cells

pSUPER vector (Oligoengine) contained a polymerase-III H1-RNA gene promoter and a puromycin resistance gene to enable antibiotic selection in mammalian cells. Two

pairs of complementary oligonucleotides (shRNA-H and shRNA-L) were designed by siRNA Selection Program of Whitehead Institute for Biomedical Research [14] and synthesized, targeting MLAA-34 cDNA (GenBank Accession No.AY288977.2) at nucleotide 961–979 and 896–914, respectively (Table 1). The synthesized shRNA cassette was annealed and ligated into the pSUPER vector according to the manufacturer's instruction. The target sequences were submitted to a BLAST search against the human genome sequence to ensure that only the MLAA-34 gene was targeted. The scrambled control plasmid (shRNA-non) was a circular plasmid encoding a shRNA which had the sequence not present in the human genome databases [15]. The RNAi plasmid DNAs for MLAA-34 and the scrambled control were then prepared for cell transfection. U937 cells were cultured in RPMI 1640 supplemented with 10% FCS. Cells were transfected with the above constructs using Effectene® Transfection Reagent (Qiagen) according to its protocol. After 48 h, cells were transferred to the selection medium, which contained 1 μg/ml puromycin (Sigma) in RPMI 1640 with 10% FBS. Several single cell clones were selected and propagated in selective medium after 7 days of incubation.

Quantitative Real-time PCR for validation of RNAi

RNA isolation and Real-time PCR were performed as previously described [16]. Total RNA was isolated from U937 cells using an acid guanidinium-phenol-chlorogorm method (Trizol, Invitrogen), and cDNAs were synthesized using a PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara, DaLian, China). The relative amount of mRNA was determined using a SYBR®Premix Ex TaqTM (Takara, DaLian, China) with gene-specific primers for either MLAA-34 or β-actin. All the analysis steps were carried out according to the manufacturer's instructions. PCRs were performed in a 96-well format in the BioRad iQ5 and MyiQ™ Real Time PCR Detection System (BioRad Laboratories). Three independent PCR were performed from the same RT sample. Primer sequences used for amplification were as follows: MLAA-34, the forward primer: 5′-CCAC AGATTGCCTTACGTTG-3′, the reverse primer: 5′-AA GAAGTCTGCTACCAACAC-3′; β-actin, the forward primer: 5′-CCTGTACGCCAACACAGTGC-3′, the reverse primer: 5′-ATACTCCTGCTT GCTGATCC-3′. The expression of MLAA-34 mRNA within each sample was normalized against β-actin and the expression level was calculated using the ΔΔCT (deltadelta threshold cycle) method.

MTT assay

MTT assay was used to determine cell proliferative rate. Cells were seeded at a concentration of 1×10^3 per well in 96-well plates. In all experiments, triplicates of cells were allowed to grow for 7 days in medium supplemented with 10% FCS at 37°C and 5% CO_2. Aliquots of 20 μl of 5 mg/ml MTT (Sigma) in PBS were added to each well, and the cells were incubated for another 4 h, followed by the addition of 150 μl DMSO. Absorbance values were determined on the enzyme linked immunosorbent assay (ELISA) reader (DASIT, Milan, Italy) at 492 nm.

Analysis of cell cycle and apoptosis

Cells were seeded into a six-well tissue culture plate. After 48 h when the cells were 70–80% confluent, cells were harvested and washed in cold sterile phosphate buffered saline (PBS). The cell cycle analysis was performed as described [17]. Annexin V (Ann-V) and propidium iodide (PI) staining were done using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), according to manufacture's protocol, followed by flow cytometric analysis of cells.

Morphological study of shRNA transfected U937 cells

After seeded into six-well plates and cultured 48 h, the cell morphology changes were observed by light microscope (Nikon, Japan). Meanwhile, cells were harvested and fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, China). Image capture and slide evaluations were performed using a Nikon 80i fluorescence microscope equipped with ACT-2U Imaging Software (Nikon, Japan). Apoptotic cells were defined by the condensation of nuclear chromatin, fragmentation, or margination to the nuclear membrane.

Table 1 Hairpin siRNA insert sequence

	Sequence	Target nucleotide sequence on MLAA-34 cDNA
shRNA-1	**GATCCCCgtacgtggagttgtcaacaTTCAAGAGAtgttgacaactccacgtacTTTTTA**	**gtacgtggagttgtcaaca**
shRNA-2	**GATCCCCtgtattcgacatgaaccacTTCAAGAGAgtggttcatgtcgaatacaTTTTTA**	**tgtattcgacatgaaccac**
shRNA-non	**GATCCCCactaccgttgttataggtgTTCAAGAGAcacctataacaacggtagtTTTTTA**	**actaccgttgttataggtg**

Bases underlined can form shRNA

Statistical analysis

Statistical significance was assessed by comparing mean (± SD) values with Student's *t*-test for independent groups. $P < 0.05$ was considered statistically significant.

Results

Identification of MLAA-34

Because the MLAA-34 sequence was obtained from cDNA libraries, it is possible that the sequence is not full-length or mature transcripts. Thus we determined both 5′ and 3′ end by RLM-RACE in U937 cell line. After 5′- and 3′-RACE using primer sequence based on the previous existing MLAA-34 sequence (GenBank no: AY288977.1), we obtained a single product. The 5′-RACE primers amplified a fragment of 0.6 kb and the 3′-RACE primers amplified a fragment of 0.3 kb. Three clones of 5′- and 3′-RACE were sequenced, respectively. The sequence analysis revealed that the 5′-RACE sequence extended the previous MLAA-34 cDNA sequence 290 bp further 5′, but the 3′-RACE sequence was identical to it. The previous MLAA-34 cDNA sequence is 1,381 bp in length, and then we propose that the full-length transcript of MLAA-34 is 1,671 bp in U937 cell line. To confirm this new sequence, we carried out RT-PCR to clone the MLAA-34 full-length cDNA sequence using the primers designed from the 5′- and 3′-RACE sequence obtained above in U937 cell line. The RT-PCR product was a single band of 1.5 kb, and the sequence analysis confirmed this sequence. In addition, we have not been able to detect any other splice variants of MLAA-34 in U937 cells. Thus, by assembling all the RACE clone and RT-PCR clone, we identified a full-length mRNA of 1,671 bp for MLAA-34 in U937 cells. We have submitted this revision of MLAA-34 to GenBank database, and the new sequence has been released on 22-FEB-2008 and the accession number is AY288977.2.

Chromosomal localization and bioinformatics analysis of MLAA-34

We cloned full-length cDNA sequence of MLAA-34 from human lymphoma monocyte cell line U937. Comparison of the MLAA-34 sequence with the newly available working version of the human genome allowed the assignment of MLAA-34 to chromosome 13q14.2-q14.3, with at least two chromosome 13 clones showing sequence identity to MLAA-34 (GenBank no: AL136218 and AL138875). One human sequence tagged site (STS) sequence within MLAA-34 (UniSTS name: RH103315) also confirm that MLAA-34 resides on human chromosome 13. The corresponding gene has been assigned the Official Human Gene Nomenclature database symbol CAB39L (calcium binding protein 39-like) and given Gene ID #81617.

A comparison of MLAA-34 cDNA and genomic sequence permitted the definition of exon-intron organization. MLAA-34 gene contains 12 exons and 11 introns. Analysis of MLAA-34's location on chromosome 13q14.2-q14.3 shows two previously sequenced full-length transcripts from cDNA libraries, and GenBank accession number BC010993 and BX647518 list 1,482 and 2,371 bp (long) sequences cloned from human cDNA libraries of choriocarcinoma of placenta and cerebellum, respectively. Those two previously full-length transcripts constitute of CAB39L's two splice variants in NCBI Gene database. Other sequences are available through GenBank, but are comprised of overlapping expressed sequence tags (ESTs) and the composite sequences may or may not represent true transcripts.

The clone BX647518 (long) contains 11 exons, and the clone BC010993 has nine exons. Compared with the MLAA-34 sequence and the clone BX647518 (long), MLAA-34 cDNA sequence is highly homologous to it (99%). However, an exon of 1,37 bp (exon 3, nucleotide sequences: 283–419 bp) was found to be present in the MLAA-34 cDNA but not in the clone BX647518. In addition, the 3′ end of MLAA-34 exon 12 excluding the poly (A) tail decurtate 841 bp compared with corresponding 3′ end of the clone BX647518 exon 11. The computational model of MLAA-34 (GenBank no: AY288977.2) and CAB39L-raleted sequences was shown in Fig. 1.

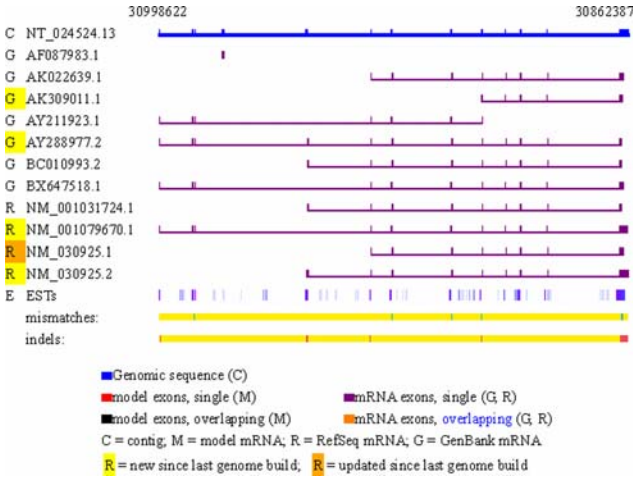

Fig. 1 The computational model of MLAA-34 and CAB39L related sequences. The display was produced using the Evidence Viewer of NCBI. The numbers just above the *terminatio bar* are the coordinates of the 5′ and 3′extremes of the gene on the contig. There are about 12 exons belonging to MLAA-34 (AY288977.2). The computational model generated from selected GenBank entry of IMAGE clone and all available ESTs aligning in this region was shown. The mismatches and indel lines indicate that the positions of any mismatches, insertion/deletions (indels), or NM/mRNA sequence gaps in any of the NM/mRNA alignments for a given exon

Taken together, these results suggest that MLAA-34 is a novel CAB39L-related splice variant, which is specially associated with acute monocytic leukemia. To date, we have not been able to access any publication regarding the functional information of the CAB39L gene and its related sequence.

The complete nucleotide sequence of full-length cDNA of MLAA-34 cloned from U937 cells is 1,671 bp in length, containing 1,014 nucleotides in the putative coding region, flanked by 451 bp in the 5′-untranslated region and 206 bp in the 3′-untranslated region. The open reading frame indicates that putative MLAA-34 protein consists of 337 amino acid residues with a molecular weight of 39,087 Da and a PI of 8.49 (Fig. 2). The amino acid sequence analysis of the encoded protein using the PSORT II and TargetP programs predicts this protein is probably a cytoplasmic protein. In addition, MLAA-34 contains potential sites for protein kinase c phsophorylation (10–12: ShK; 35–37: TdK; 186–188: TfK; 224–226: TKR; 228–230: SlK), N-myristoylation site (77–82: GLlVTL), amidation site (92–95: eGKK), casein kinase II phosphorylation site (175–178: StfD; 186–189: TfKD; 208–211: TifE; 250–253: SkPE; 313–316: TddE), cAMP- and cGMP- dependent protein kinase phosphorylation site (225–228: KRqS) according to the Prosite database. Because the function of MLAA-34 protein is unknown, we investigate its role in carcinogenesis using RNA interference technology.

MLAA-34 knockdown by siRNA-expressing vector

A DNA based siRNA method was used to genetically knockdown MLAA-34 expression in the U937 cell line [18]. For the stable transfection of three siRNA vectors, puromycin-resistant cells were collected and resistant mono clones were subjected to Real-time RT-PCR to analyze the MLAA-34 expression. When compared with the scrambled control cell line shRNA-non and parental U937 cells, MLAA-34 transcripts were reduced 1.45-fold and 1.01-fold in two shRNA-H stable transfection mono clones, H3 and H4 clones (Fig. 3), respectively. However, the several stable transfection mono clones of shRNA-L siRNA-expressing vector showed no apparent suppression in MLAA-34 expression (data not shown), stressing the critical importance of the dsRNA design for a silencing experiment. Then these H3 and H4 mono clones were selected for the ulterior experiments. The results above suggested that siRNAs were capable of downregulating expression of MLAA-34.

MLAA-34 downregulation inhibited the proliferation of U937 cells

To explore the role of MLAA-34 in the regulation of proliferation of U937 cells, we applied two shRNA-H stable transfectants: H3 and H4 clones, in which MLAA-34 was downregulated, to detect the cell growth using the MTT assay. As shown in Fig. 4, MLAA-34 silencing by siRNA

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452 atgaaaaaaatgccttttgtttagtaaatcacacaaaaatccagca
    M  K  K  M  P  L  F  S  K  S  H  K  N  P  A
497 gaaattgtgaaaatcctgaaagacaatttggccatttttggaaaag
    E  I  V  K  I  L  K  D  N  L  A  I  L  E  K
542 caagacaaaaagacagacaaggcttcagaagaagtgtctaaatca
    Q  D  K  K  T  D  K  A  S  E  E  V  S  K  S
587 ctgcaagcaatgaaagaaattctgtgtggtacaaacgagaaagaa
    L  Q  A  M  K  E  I  L  C  G  T  N  E  K  E
632 ccccgacagaagcagtggctcagctagcacaagaactctacagc
    P  P  T  E  A  V  A  Q  L  A  Q  E  L  Y  S
677 agtggcctgctggtgacactcatagctgacctgcagctgatagac
    S  G  L  L  V  T  L  I  A  D  L  Q  L  I  D
722 tttgagggaaaaaaagatgtgacccagatatttaacaacatcttg
    F  E  G  K  K  D  V  T  Q  I  F  N  N  I  L
767 agaagacagataggcactcggagtcctactgtggagtatattagt
    R  R  Q  I  G  T  R  S  P  T  V  E  Y  I  S
812 gctcatcctcatatcctgtttatgctcctcaaaggatatgaagcc
    A  H  P  H  I  L  F  M  L  L  K  G  Y  E  A
857 ccacagattgccttacgttgtgggattatgctgagagaatgtatt
    P  Q  I  A  L  R  C  G  I  M  L  R  E  C  I
902 cgacatgaaccacttgccaaaatcatcctctttttctaatcaattc
    R  H  E  P  L  A  K  I  I  L  F  S  N  Q  F
947 agagatttctttaagtacgtggagttgtcaacatttgatattgct
    R  D  F  F  K  Y  V  E  L  S  T  F  D  I  A
992 tcagatgcctttgctactttcaaggatttactaaccagacataaa
    S  D  A  F  A  T  F  K  D  L  L  T  R  H  K
1037 gtgttggtagcagacttcttagaacaaaattacgacactattttt
     V  L  V  A  D  F  L  E  Q  N  Y  D  T  I  F
1082 gaagactatgagaaattgcttcagtctgagaatttatgttactaag
     E  D  Y  E  K  L  L  Q  S  E  N  Y  V  T  K
1127 agacagtctttaaagctgctaggggagctgatcctggaccgtcac
     R  Q  S  L  K  L  L  G  E  L  I  L  D  R  H
1172 aactttgccatcatgacaaagtatatcagcaagccggagaacctg
     N  F  A  I  M  T  K  Y  I  S  K  P  E  N  L
1217 aaactcatgatgaacctccttcgggataaaagtcccaacatccag
     K  L  M  M  N  L  L  R  D  K  S  P  N  I  Q
1262 tttgaagcctttcatgtttttaaggtgtttgtggccagtcctcac
     F  E  A  F  H  V  F  K  V  F  V  A  S  P  H
1307 aaaacacagcctattgtggagatcctgttaaaaaatcagcccaaa
     K  T  Q  P  I  V  E  I  L  L  K  N  Q  P  K
1352 ctcattgagtttctgagcagcttccaaaaagaaaggacggatgat
     L  I  E  F  L  S  S  F  Q  K  E  R  T  D  D
1397 gagcagttcgctgacgagaagaactacttgattaaacagatccga
     E  Q  F  A  D  E  K  N  Y  L  I  K  Q  I  R
1442 gacttgaagaaaacggccccttga 1465
     D  L  K  K  T  A  P  *
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Fig. 2 The complete coding sequence of MLAA-34 and putative amino acid sequence. The open reading frame of MLAA-34 and putative amino acid sequence were predicted using ORF Finder of NCBI. The complete coding sequence (CDS) is about 666 nucleotides which codes a putative protein consists of 337 amino acid residues with a molecular weight of 39,087 Da and a PI of 8.49. The translational start codon and stop codon are indicated in *bold*

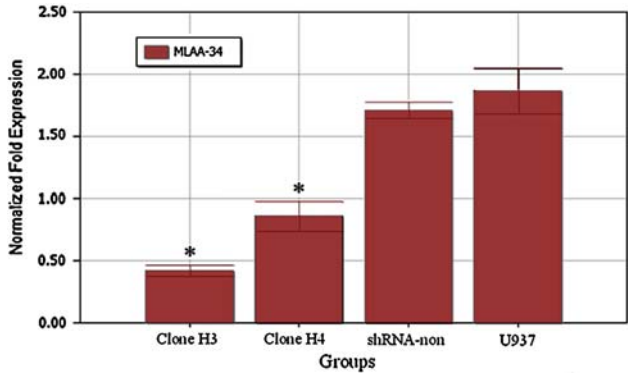

Fig. 3 Real time PCR quantification of the MLAA-34 mRNA level. MLAA-34 expression levels were normalized using β-actin. *Asterisk* represents highly significant difference between experimental group and U937 control group ($P < 0.05$). Data represent mean ± sd of three independent experiments. Control is the parental U937 cells

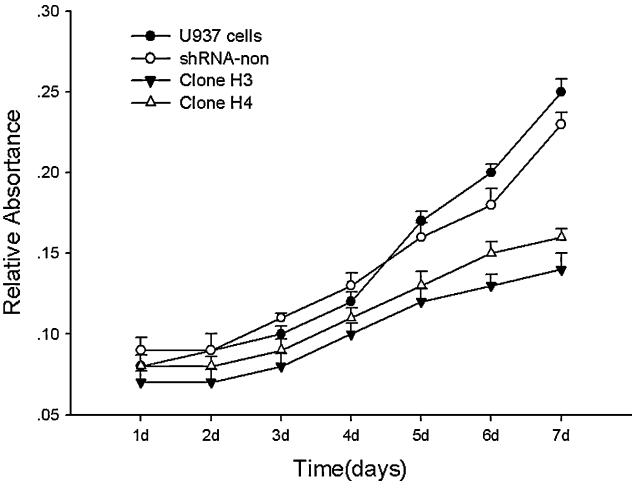

Fig. 4 Interference of MLAA-34 expression inhibits U937 cells proliferation. Growth rates of cells were determined by MTT assays. Data represent mean ± sd was from at least three separate experiments

constructs significantly inhibited the proliferation of U937 cells, suggesting that downregulation of MLAA-34 expression exerted a negative effect in proliferation.

MLAA-34 downregulation increased the apoptosis of U937 cells

The data we showed above demonstrated that downregulation expression of MLAA-34 in U937 cells could significantly inhibit tumor cells growth in vitro. We sought to determine the mechanism responsible for the MLAA-34 RNAi mediated inhibition of U937 cells growth. In the initial experiment, we performed FACS analysis for cell cycle phase determination to study whether downregulation expression of MLAA-34 could potentially induce change in cell cycle distribution in U937 cells. The results showed that downregulation expression of MLAA-34 induced minimal

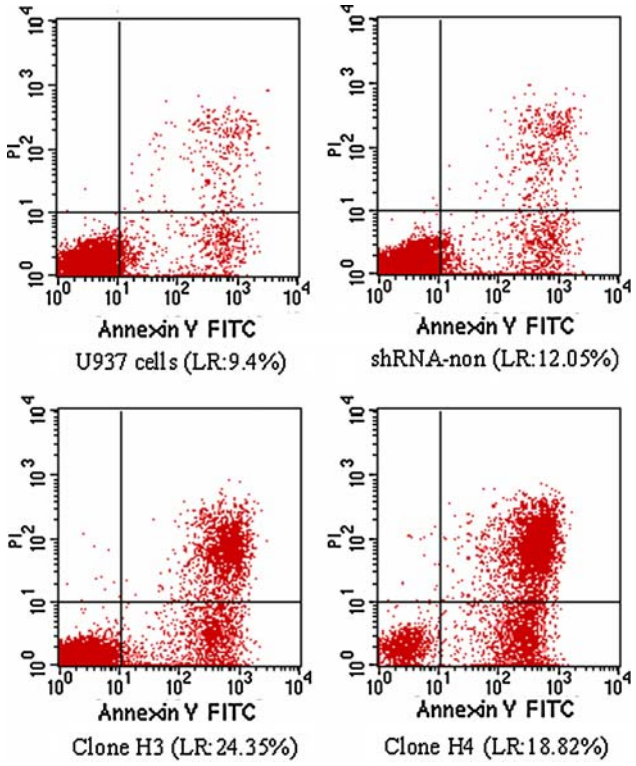

Fig. 5 Flow cytometry analysis of apoptosis by Annexin V (*x*-axis) and propidium iodide (*y*-axis) staining

changes in cell cycle distribution including G1, S and G2/M in U937 cells (data not shown). Unexpectedly, downregulation expression of MLAA-34 cells showed increased population of sub-G1 region (a hypodiploid DNA peak), suggesting that downregulation expression of MLAA-34 induced apoptosis in U937 cells during the in vitro culture, since cells undergoing apoptosis were often recognized as an accumulated cell population in sub-G1 region [19].

To further confirm that observed increase in sub-G1 population induced by downregulation expression of MLAA-34 in U937 cells was due to apoptosis, we examined the percentage of apoptotic cells that expressed stable siRNA against MLAA-34 versus cells transfected with the scrambled control plasmid (shRNA-non), using simultaneous Annexin V fluorescein and propidium iodide (PI) staining. Cells that exist in early apoptosis are Annexin V-FITC positive and PI negative. The number of apoptotic cells increased in both two shRNA-H stable transfectants, H3 and H4, as compared with cells transfected with the controls. Apoptotic cells were 24.35 ± 3.82% for H3 transfectants, and 18.82 ± 2.29% for H4 transfectants; the corresponding values in the shRNA-non control cells and the parental U937 cells were 12.05 ± 2.03% and 9.40 ± 1.92%, respectively (Fig. 5). All the results suggested that siRNA targeted to MLAA-34 promotes the acceleration of apoptosis through enhancing spontaneous apoptotic

probability, and confirmed the apoptotic contribution to the decrease in cell growth.

Observation of morphological changes

To observe the morphological changes of two shRNA-H stable transfected U937 cells, we performed morphological examination of cells by light microscopy and fluorescence microscope. Under light microscopy, the stable transfected U937 cells showed morphological features such as cell shrinkage, chromatin compaction, condensation of cytoplasm and nuclear fragmentation (Fig. 6). Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin (Fig. 7). In the control groups, the nuclei were stained a less bright blue and the color was homogeneous. However, condensed chromatin could be observed amidst the two shRNA-H stable transfected cells and some of them formed the structure of apoptotic bodies, which is one of the classic characteristics of apoptotic cells. Those features of transfected U937 cells confirmed that downregulation expression of MLAA-34 induced the apoptosis of U937 cells.

Discussion

The identification of biomarkers for diagnosis, prognosis, and therapy of human cancer has been a long-standing challenge in cancer research. Convincing evidence now exists that the humoral immune system of cancer patients recognizes tumor-related antigens [20–24]. The structural definition of the antigenic targets recognized by serum antibody in cancer patients has been revolutionized by the development of an expression cloning strategy termed SEREX. SEREX is an acronym that stands for the serological analysis of autologous tumor antigens by recombinant cDNA expression cloning. It allows an unbiased search for an antibody response and direct molecular definition of immunogenic tumor proteins based on their reactivity with autologous patient sera [25]. The overall algorithm of SEREX analysis is to define the complete spectrum of immunogenic gene produces in cancer. To date, more than 2,000 different immunogenic tumor products have been defined by SEREX analysis of a wide range of tumor types (see SEREX database [26]). Of these SEREX-identified genes, approximately two-thirds represent previously known genes, whereas one-thirds is novel gene products. One of the key questions related to SEREX-defined antigens is what role do (if any) these antigens play in carcinogenesis, especially for the novel gene antigens.

As in 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 [8]. MLAA-34 is one of these novel antigens. The investigation of novel antigens mRNA expression level in peripheral blood mononuclear cells of 40 cases of newly diagnosed AML patients and 30 cases healthy donor also showed that MLAA-34 mRNA specially overexpressed in AML patients compared with normal donor

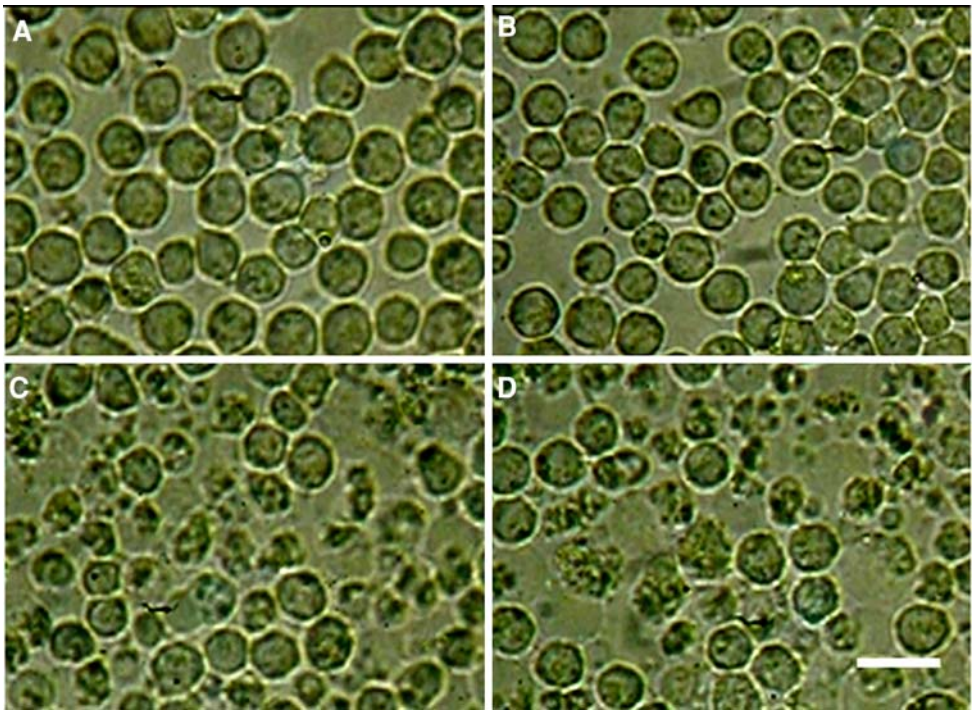

Fig. 6 Morphology of U937 cells (original magnification 400×). **a** U937 cells, **b** shRNA-non, **c** clone H3, **d** clone H4. *Scale bar* 20 μm

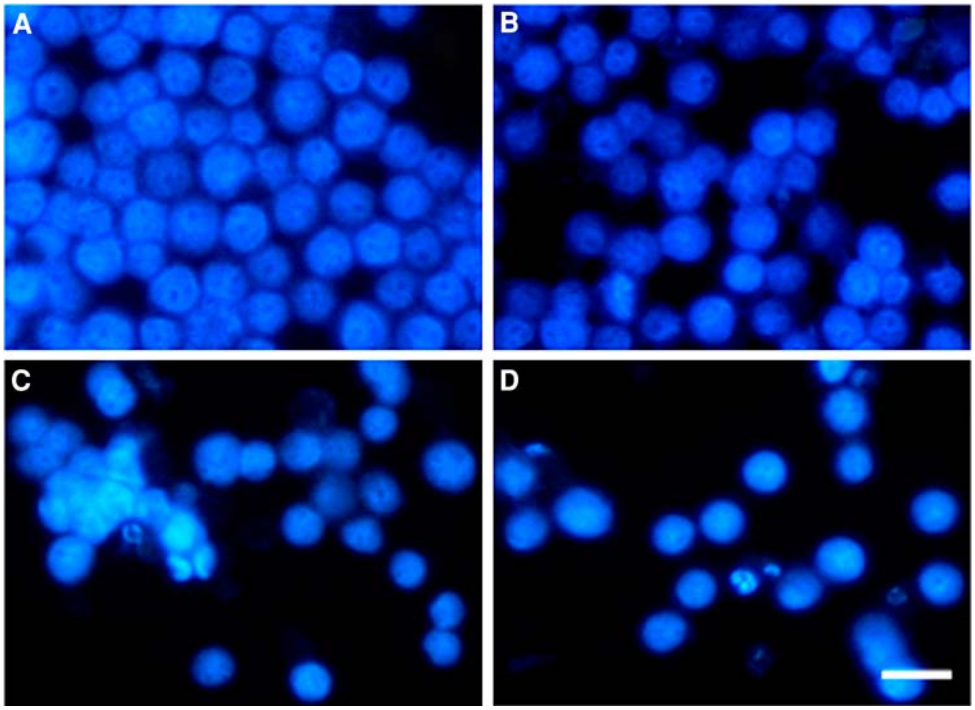

Fig. 7 Hoechst staining of U937 cells (original magnification 200×). **a** U937 cells, **b** shRNA-non, **c** clone H3, **d** clone H4. *Scale bar* 20 μm

(data not published). These results suggested that MLAA-34 is a novel acute monocytic leukemia-associated antigen. The previous works have proved that SEREX analysis has the potential of revealing a number of proteins that may be involved in cellular functions related to carcinogenesis [27–29]. For example, the receptor for hyaluronan acid-mediated motility (RHAMM) involved in cell growth and metastasis was identified as a leukemia-associated antigen using SEREX analysis [29]. These results point out the need to further study the gene structure and function of MLAA-34.

In this study, we first determined both the 5′ and 3′ end of MLAA-34 cDNA sequence in U937 cells by RLM-RACE. As such, we identified a novel 290 bp transcribed region upstream of the previously reported sequence, and then the novel 5′ region was verified by the full-length clone of MLAA-34 cDNA sequence by RT-PCR and sequencing. MLAA-34 cDNA sequence is highly homologous to human CAB39L, located on chromosome 13q14. CAB39L, previously known as the hypothetical protein FLJ12577, was first identified by the Nedo Full-length cDNA sequencing Project in Japan [30]. The two full-length transcripts of CAB39L (GenBank no: BC010993 and BX647518) were cloned from human cDNA libraries of choriocarcinoma of placenta [31] and cerebellum, respectively. The CAB39L's two splice variants in NCBI Gene database result from these two full-length transcripts. Interesting, the sarcoma antigen NY-SAR-79 identified from human sarcoma by SEREX analysis was also related to CAB39L, although NY-SAR-79 sequence only include partial coding sequence [32]. These results implied that CAB39L-related sequences is closely involved in the carcinogenesis of tumor. To date, no functional information is available for the CAB39L-related sequences. Compared with the CAB39L-related sequences in human, MLAA-34 shows apparent alternative splicing. As the Fig. 1 shown, MLAA-34 is a novel CAB39L's splice variant associated with acute monocytic leukemia. In addition, we did not find any other splice variants of CAB39L in U937 cell line.

The double-stranded RNA-mediated interference has emerged as a powerful reverse genetic tool to silence gene expression [18, 33–36]. Here we adopted pSUPER vector system to elucidate the functions of MLAA-34 in U937 cells. The vector transcribes a shRNA from the H1 promoter, which is then spliced into 21–23nt siRNA, overcoming the limitations of transient and non-renewable nature of small interference RNA (siRNA). In this study, we have successfully selected an efficient target sequence, and found that the pSUPER construct of MLAA-34-targeted shRNA specifically and effectively downregulated MLAA-34 mRNA in U937 cells. We first showed that the downregulation of MLAA-34 expression significantly suppressed the proliferation of U937 cells in vitro, and increased the spontaneous apoptosis of these leukemia cells. Apoptosis is an important mechanism regulating survival of acute myeloid leukemia cells. Loss of the inhibition of apoptosis is important in leukemogenesis and may influence the prognosis [37]. Cancellation of this process with increased resistance to cell death is a common feature of malignant cells and represents a significant obstacle to therapy of human

cancers [38, 39]. All these data indicated that MLAA-34 may be a novel anti-apoptotic factor related closely to carcinogenesis or progression of acute monocytic leukemia.

The critical event of apoptosis is activation of caspases via death receptor (external) and mitochondrial (intrinsic) pathways, what initiate DNA fragmentation, cleavage of several critical proteins and, finally, cell disintegration. The mammalian apoptotic caspases have been classified as either initiator caspases (caspases-2, -8, -9, and -10) or effector caspases (caspases-3, -6, and -7) based on their structure and function. It is absolutely critical to the development and survival of an organism that the caspases are tightly regulated, as the inappropriate activation of these enzymes can have severe consequences with respect to the development of various disease conditions. In normal cells, limited activation of the caspase cascade may be essential in cellular differentiation and/or replication [40], thus making the preservation of caspase control essential; however, in cancer cells it is often observed that caspase activation pathways are impaired or absent, thereby preventing cells from dying when they should [41]. Moreover, caspase-independent mode of cell death as triggered by mitochondrial apoptosis inducing factor (AIF) has been also found [42]. Apoptosis is controlled at multiple steps, each of which is influenced by pro- and anti-apoptotic proteins. A delicate balance between pro- and anti-apoptotic mechanisms determines whether a cell death signal can activate the execution of the apoptotic cell death program. Several proteins play critical roles in maintaining an adequate balance between too much and too little apoptosis. One of the mechanisms through which tumor cells are believed to acquire resistance to apoptosis is by overexpression of inhibitor of apoptosis proteins, which inhibit apoptosis by binding to specific caspases and possibly by other mechanisms. In U937 cells, the downregulation of MLAA-34 expression significantly increased the spontaneous apoptosis of these leukemia cells. Based on these facts, we will first study the interaction of MLAA-34 and caspases to investigate the anti-apoptotic pathways of MLAA-34.

In conclusion, we first cloned the full-length cDNA sequence of MLAA-34 in U937 cells, and proposed that MLAA-34 is a novel CAB39L's splice variant associated to acute monocytic leukemia. For the first time, we show that MLAA-34 may be a novel anti-apoptotic factor related closely to carcinogenesis or progression of acute monocytic leukemia. This study warrants further investigations to verify MLAA-34 as a promising antigen and a molecular target for therapeutic applications in acute monocytic leukemia.

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