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The expression and functional characterization associated with cell apoptosis and proteomic analysis of the novel gene MLAA-34 in U937 cells

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Abstract. MLAA-34 is a novel acute monocytic leukemia (M5)-associated antigen (MLAA) that plays a role in the apoptosis of U937 cells. However, the expression and molecular mechanism of MLAA-34 in U937 cells remain largely unclear. Here, we utilized three strategies to gain insight into the expression and molecular functions of MLAA-34 and to identify its interacting proteins and pathways involved in the fine-tuning of the MLAA-34 response. Western blot analysis was performed to assess the expression of MLAA-34 in 41 cell lines and five mixed cell types, which revealed that MLAA-34 is most strongly expressed in U937 cells. Immunostaining indicated that MLAA-34 is localized in the cytoplasm and cell membrane. Furthermore, lentivirus-mediated overexpression of MLAA-34 in the U937 cell line led to significant suppression of apoptosis and increased the potential of tumorigenicity. Co-immunoprecipitation (Co-IP), shotgun and bioinformatic analysis identified 256 proteins and 225 of them were annotated by gene ontology categories. This analysis revealed 71 proteins involved in cell apoptosis or proliferation of biological processes and signaling pathways. Moreover, the effect of MLAA-34 apoptosis may be through interaction with the Ras, Wnt, calcium and chemokine signaling pathways and thirteen of the annotated proteins may interact with MLAA-34 and participate in carcinogenesis directly. This study provides a basis for a better understanding of the molecular mechanism and proteomics in the inhibition of apoptosis by MLAA-34 in U937 cells and indicates that MLAA-34 may be a potential candidate for the early diagnosis and therapeutic application of M5.

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Introduction

Leukemia is the leading cause of mortality worldwide in patients with malignant tumors under the age of 35 years. Patients with acute myeloid leukemia (AML) who have relapsed or are refractory to conventional chemotherapy have a poorer prognosis and response to chemotherapy than those with *de novo* AML, which remains a formidable therapeutic challenge even with the introduction of several new therapeutic strategies (1-3). M5 is largely incurable with high relapse rates, infiltration and a median remission duration of only six months, approximately (4). Moreover, M5 has been reported to have a worse prognosis than other subtypes of AML (5). Thus, a vaccine or a new drug against M5 is required as a strategic tool for the control of this disease, but none are currently available for practical use.

The MLAA-34 gene (GenBank no. AY288977.2) has been confirmed to be a novel splice variant of CAB39L (calcium binding protein 39-like). MLAA-34 was first discovered in M5 in an effort to identify monocytic leukemia-associated antigens by serologic analysis of a recombinant cDNA expression library (SEREX) that reacted exclusively with sera from allogeneic leukemia patients but not with normal donor sera (6,7). The 1671 kb gene is located on 13q14.2 and was initially cloned in our laboratory from U937 cells (7). CAB39L has three alternative transcripts and has been predicted to encode a 337 aa protein. The three alternative transcripts of CAB39L have been recognized to encode the same protein, differing only in their 5' untranslated regions [GenBank nos. BC010993 (1482 bp), BX647518 (2371 bp) and AY288977.2].

In our previous study, MLAA-34 and CAB39L were identified with RNA interference (RNAi) in the U937 cell line as novel anti-apoptotic factors that are closely related to carcinogenesis or progression of M5 (7). Clinical research has shown that MLAA-34 mRNA expression is upregulated in refractory/relapsed M5 patients compared with newly diagnosed, healthy donors and AML patients in complete remission; high expression of MLAA-34 is more prominent in the M5 subtype than in other AML patients; MLAA-34 overexpression has been found to be associated with unfavorable clinical features at diagnosis and has been shown to be an independent prognostic factor (8).

However, for MLAA-34, there are no exact reports regarding its cellular localization and expression in manifold cell lines; the anti-apoptotic mechanism of MLAA-34 remains unclear.

The purpose of this study was to conduct an in-depth search for the expression and anti-apoptotic mechanism of MLAA-34 through the lentivirus-mediated overexpression in the U937 cell line, and to then apply proteomics to identify its correlated proteins or pathways that might perform functions important for the apoptosis and proliferation of U937 cells.

Materials and methods

Cell culture. U937, HL60, K562, RPMI-8226, HepG2, Hep3B, MHCC97-H, RC-K8, SGC-7901, Eca109, BGC823, MKN45, GES-1, BxPC-3, A375, T24, HUVEC, BMSCs, LO2, HeLa, 293T, 293, RD, RT4, 5637, EJ, UM-UC-3, 2537, J82, Tsu-Prl, MAH, LiBr, Hut-78, HCT116$^+$, FBL-3, C6, astrocyte, 3T3-L1, NIH3T3, Vero and MDCK cell lines were all maintained in our laboratory and cultured in RPMI-1640 or DMEM supplemented with 10% fetal calf serum. The medium for cell lines expressing the neomycin resistance gene was supplemented with 0.5 mg/ml G418. Human epithelial tissue, normal human peripheral blood mononuclear cells (PBMCs), M5 patient and non-M5 acute leukemia patient PBMCs were all obtained from over 30 cases of patients or healthy young individuals. Mouse splenocytes were obtained from 30 mice.

Antibodies and reagents. CAB39L and MLAA-34 share the same open reading frame (ORF), the CAB39L antibody was used in this report. Antibodies specific for CAB39L (sc-100390), β-catenin (sc-133240), Rab-3D (sc-26559), Rap-1B (sc-1481) and PGK1 (sc-130335) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A monoclonal mouse antibody against β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The SAP kit and AP-Red kit were provided by Zhongshan Co. Beijing, China (SAP-9102, ZLI-9042). The lentivirus packaging system and enhanced infection solution (ENi.S) were purchased from GeneChem Limited Company (Shanghai, China). The SYBR Green PCR kit and SYBR Master Mixture were purchased from Takara Bio, Inc. (Dalian, China). The Endo-free Plasmid Mini kit was purchased from Qiagen, USA (12163). M-PER® Mammalian Protein Extraction Reagent was purchased from Pierce, Rockford, IL, USA (78503).

Western blot analysis. Cells were collected at a concentration of 2x10^7/ml. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene fluoride membranes, which were incubated with the primary antibody CAB39L (1:200). Western blot analyses were performed according to standard methods. The protein bands were visualized by applying SuperSignal West Pico Chemiluminescent Substrate (34079; Pierce). The exposed film was then analyzed using a densitometer.

Immunohistochemistry and immunofluorescence. For analysis of the subcellular localization of MLAA-34, U937 cells were washed with ice-cold PBS, blocked with 10% normal goat serum and incubated with a primary antibody against CAB39L at a dilution of 1:50 for 2 h at 37˚C. Next, the cells were washed again and incubated with the appropriate biotinylated secondary antibody (goat anti-mouse IgG antibody) for 20 min at 37˚C. Incubation with serum alkaline phosphatase (SAP; ALP) was then performed at 37˚C for 20 min, and the immunolabeling was visualized with a mixture of AP-Red solution. Counterstaining with hematoxylin was performed. For immunofluorescence, the cell samples were incubated with the monoclonal antibody CAB39L (diluted 1:50) and fluorescein isothiocyanate (FITC)-labeled or rhodamine-labeled goat anti-mouse IgG as the primary and secondary antibodies, respectively. The mounted cells were visualized with a fluorescent microscope.

Construction and identification of the MLAA-34 lentivirus vector and upregulated MLAA-34 stably transfected cell line. The full-length MLAA-34 cDNA sequence was assembled by searching the NCBI database and amplified by RT-PCR from U937 cells. First-strand cDNA synthesis was performed using a commercial kit (Boehringer Mannheim, Milan, Italy). The restriction enzyme site for *Age*I (ACCGGT) was introduced into the 5' and 3' PCR primers. To generate cDNA coding for full-length MLAA-34 by PCR, the following primers were designed using plasmid MLAA-34 as the template: MLAA-34-Age, I-F, GAGGATCCCCGGGTACCGGTCGCCACCATGAAAAAA ATGCCTTTG and MLAA-34-Age, I-R, TCACCATGGTGGC GACCGGAGGGGCCGTTTTCTTCAAG. The PCR conditions consisted of 30 cycles, and the cycle parameters were: 94˚C for 5 min, then 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 68˚C for 1 min, followed by a final extension of 68˚C for 10 min. The PCR product was purified using an Agarose Gel DNA Purification kit (Takara Bio, Inc.). The two recovered products were ligated using an In-Fusion kit (631774; Becton, Dickinson and Co., USA). To confirm that the ligation was correct, MLAA-34-SEQF, GACAGATAGGCACTCGGAG; Ubi-F, GGGTCAATATGTAATTTTCAGTG; and EGFP-N-R, CGTCGCCGTCCAGCTCGACCAG primers were designed. The cycle parameters were: 30 cycles of 94˚C for 30 sec, 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 50 sec, followed by a final extension of 72˚C for 6 min. For detection of MLAA-34 expressed by recombinant lentivirus *in vitro*, purified pGC-FU-MLAA-34 vectors were transfected into 293T cells using Lipofectamine 2000 reagent (11668-019; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. This vector was termed MLAA-34-Lentivirus, and the vector without MLAA-34 cDNA was pGC-FU-GFP-LV. The titer of the recombinant lentivirus was determined by real-time qPCR on 293T cells. For identification of the recombinant MLAA-34 lentivirus vector, the virus was added to targeted U937 cells at multiplicity of infections (MOIs) of 10, 20, 50, 80, 100, 120 and 200 with ENi.S and 5 μg/ml polybrene. MLAA-34-Lentivirus and pGC-FU-GFP-LV transfected U937 cells were used as the test, and non-transfected cells were used as the control. The expression level of MLAA-34 was detected by western blot analysis and RT-PCR. The best MOI was chosen.

Cells were grown in selective media (containing G418) for two weeks, expanded and grown as independent clones for at least two weeks. Resistant colonies were counted, and the expression of GFP was confirmed by fluorescence microscopy, RT-PCR and western blot analysis.

Fluorescence microscopy, MTT, flow cytometry and DNA ladder. To determine the effect of upregulation of MLAA-34 by the MLAA-34-Lentivirus, non-transfected cells and cells transfected with pGC-FU-GFP-LV and MLAA-34-Lentivirus were examined. Cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. Cellular proliferation was measured once per day during a seven-day period. In brief, 20 μl of sterile MTT (Sigma) dye (5 mg/ml) was added to the cells, which were then incubated for another 4 h at 37˚C. Then, 150 μl of dimethylsulfoxide was added to each well. The spectrophotometric absorbance was measured at a wavelength of 490 nm on an enzyme immunoassay analyzer.

Fixed cells were stained with 2.5 g/ml of DAPI (4',6-diamidino-2-phenylindole) solution to detect apoptotic nuclei. Quantification of apoptosis was determined by counting the number of apoptotic cells. The cells were stained using an Annexin V-PE/7-AAD apoptosis detection kit (KGA1015; Nanjing KeyGen Biotech. Co., Ltd.) according to the manufacturer's instructions and were analyzed by flow cytometry using a Beckman Coulter flow cytometer.

For cell cycle analysis, the cells were fixed in 70% ethanol and stained with propidium iodide (PI; Biosea Biotechnology Co., Beijing, China) at a final concentration of 20 μg/ml in Triton X-100 containing 10 mg/ml RNase. Following incubation, the samples were analyzed on a flow cytometer.

Fragmented DNA was isolated using a DNA extraction kit (C0008; Beyotime) according to the manufacturer's instructions. The eluants containing DNA pellets were electrophoresed on a 1% agarose gel at 80 V for 1.5 h. The gel was examined and photographed using an ultraviolet gel documentation system.

Co-immunoprecipitation (Co-IP) and SDS-PAGE. Co-IP was performed using a Profound™ Mammalian Co-IP kit (23605; Pierce). Transfected U937 cells ($2x10^7$/ml) were washed, centrifuged and resuspended in lysis buffer for incubation. The cell lysates were centrifuged to remove the supernatant material, and the CAB39L antibody was cross-linked to the antibody coupling resin. The lysed cell sample was then applied to the antibody support to form immune complexes. Then, unbound proteins were washed away three times. The samples were then eluted, and coupling buffer was added to obtain the immunoprecipitated protein. Finally, the Co-IP protein concentrations were determined using a BCA Protein Assay kit (23225; Pierce). The proteins were analyzed by SDS-PAGE, and the gel was stained with Coomassie Blue.

Mass spectrometry analysis (MS, shotgun) and protein identification. After separation by SDS-PAGE, discrete bands were excised from and subjected to in-gel tryptic digestion. The extracted peptides were analyzed using shotgun HPLC-ESI-MS proteomics approach (LTQ; Thermo Finnigan, San Jose, CA, USA). High-performance liquid chromatography (HPLC) separation was performed with a capillary LC pump. The mobile phases used for the reverse phase were i) 0.1% formic acid in water, pH 3.0; ii) 0.1% formic acid in ACN. The collision energy was set automatically by the LTQ system. Following acquisition of full scan mass spectrum, three MS/MS scans were acquired for the next three most intense ions using dynamic exclusion. Peptides and proteins were identified using

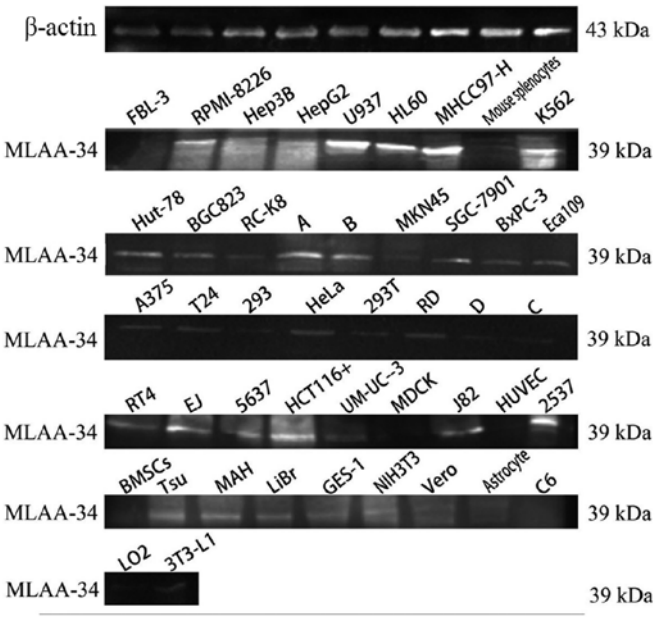

Figure 1. Expression of MLAA-34 by western blot analysis. A, commixture of M5 patient PBMCs; B, commixture of non-M5 acute leukemia patient PBMCs; C, normal human PBMCs; D, human epithelial tissue.

Bioworks Browser 3.1 software (Thermo Finnigan), which uses the MS and MS/MS spectra of peptide ions to search against the NCBI human protein database. The protein identification criteria that we used were based on Delta CN (≥0.1) and Xcorr (one charge ≥1.9, two charges ≥2.2, three charges ≥3.75). The protein identification results were extracted from the SEQUEST out file with in-house software (BuildSummary). The cellular localization, molecular function and biologic process were determined using the gene ontology annotation DAVID (http://david.abcc.ncifcrf.gov/). For pathway analysis, the KEGG database was searched. To identify the corresponding proteins in mixed protein obtained by Co-IP, western blot analysis was performed as previously described.

Statistical analysis. The RT-PCR results were analyzed by the self-contained software of iQ5 (Bio-Rad Co.). Statistical analyses were performed using an analysis of variance (ANOVA). All results are expressed as the means ± standard deviations from at least three experiments. $P<0.05$ was considered to indicate statistically significant differences.

Results

Expression of human MLAA-34 protein. With western blot analysis, a strong specific band of ~39 kDa was observed in U937 and MHCC97-H cells, and reduced expression was observed in other leukemia or lymphoma cell lines and PBMCs from leukemia patients. Much fainter bands were observed in solid tumor cell lines, and no expression was detected in normal human cell lines or primary animal cells (Fig. 1).

Identification and cellular localization of MLAA-34. Immunohistochemical staining confirmed the presence of MLAA-34 in U937 cells and the subcellular localization was detected primarily in the cytomembrane and cytoplasm (Fig. 2).

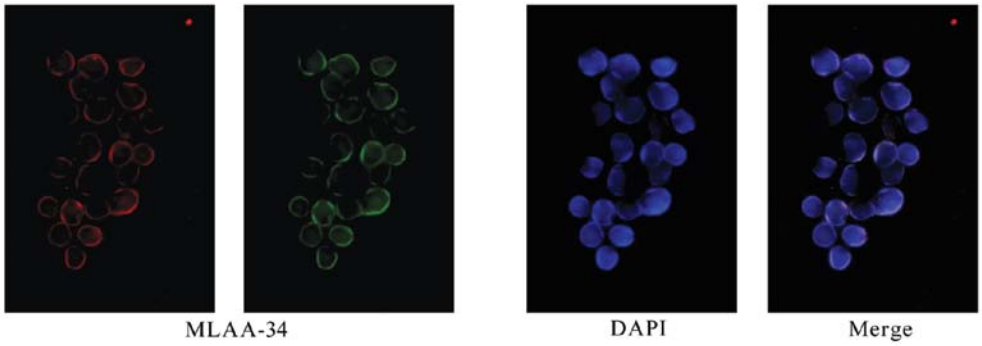

Figure 2. The subcellular localization of MLAA-34 protein in U937 cells (x40). The MLAA-34 protein was distributed predominantly in the cytomembrane and cytoplasm (red or green). The nuclei were stained by DAPI (x40).

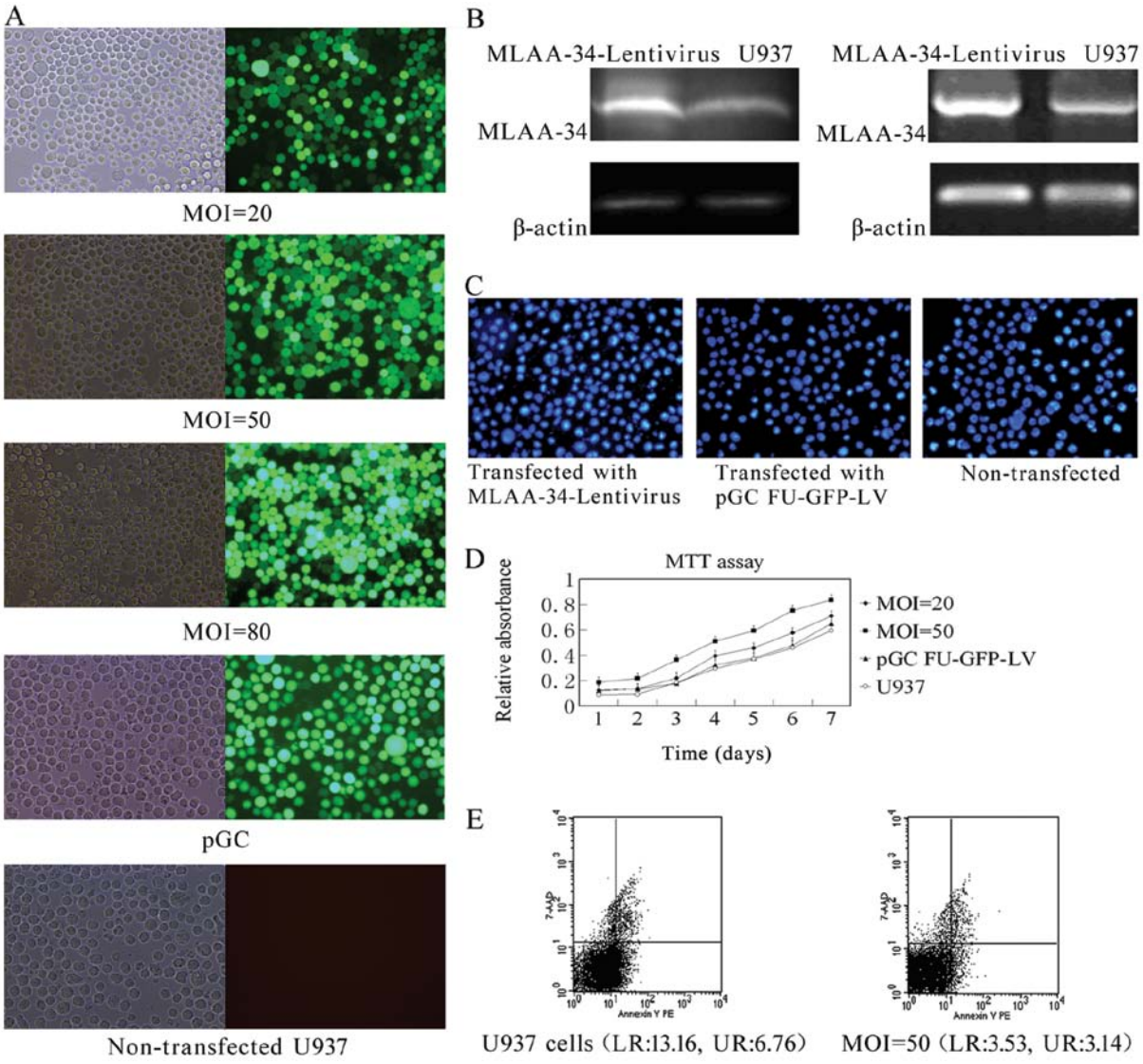

Figure 3. Lentivirus-mediated gene transfer system for overexpression of MLAA-34 was constructed successfully and markedly decreases apoptosis and promotes proliferation in U937 cells. (A) Morphological observation of MLAA-34-Lentivirus transfected U937 cells at different MOIs. The transfection efficiency was higher at MOI=50 and 80, but the growth condition was the best at MOI=50. (B) MLAA-34-Lentivirus upregulated the expression of MLAA-34 in U937 cells, estimated by western blot analysis (left) and RT-PCR (right). (C) Morphological changes in the morphology of the cell nucleus were observed by DAPI staining (blue). (D) Cell viability was measured by MTT assays. (E) The cells were stained with Annexin V-PE and 7-AAD for flow cytometry analysis.

MLAA-34 is upregulated by the lentiviral vector. A human MLAA-34 lentivirus gene transfer vector encoding the green fluorescent protein (GFP) sequence was constructed. The pGC-FU-MLAA-34-GFP plasmid has an insert of ~771 bp, which is in accord with the MLAA-34 cDNA [identities, 1009/1012 (99%)]. The pilot experiments showed that 293T cells could

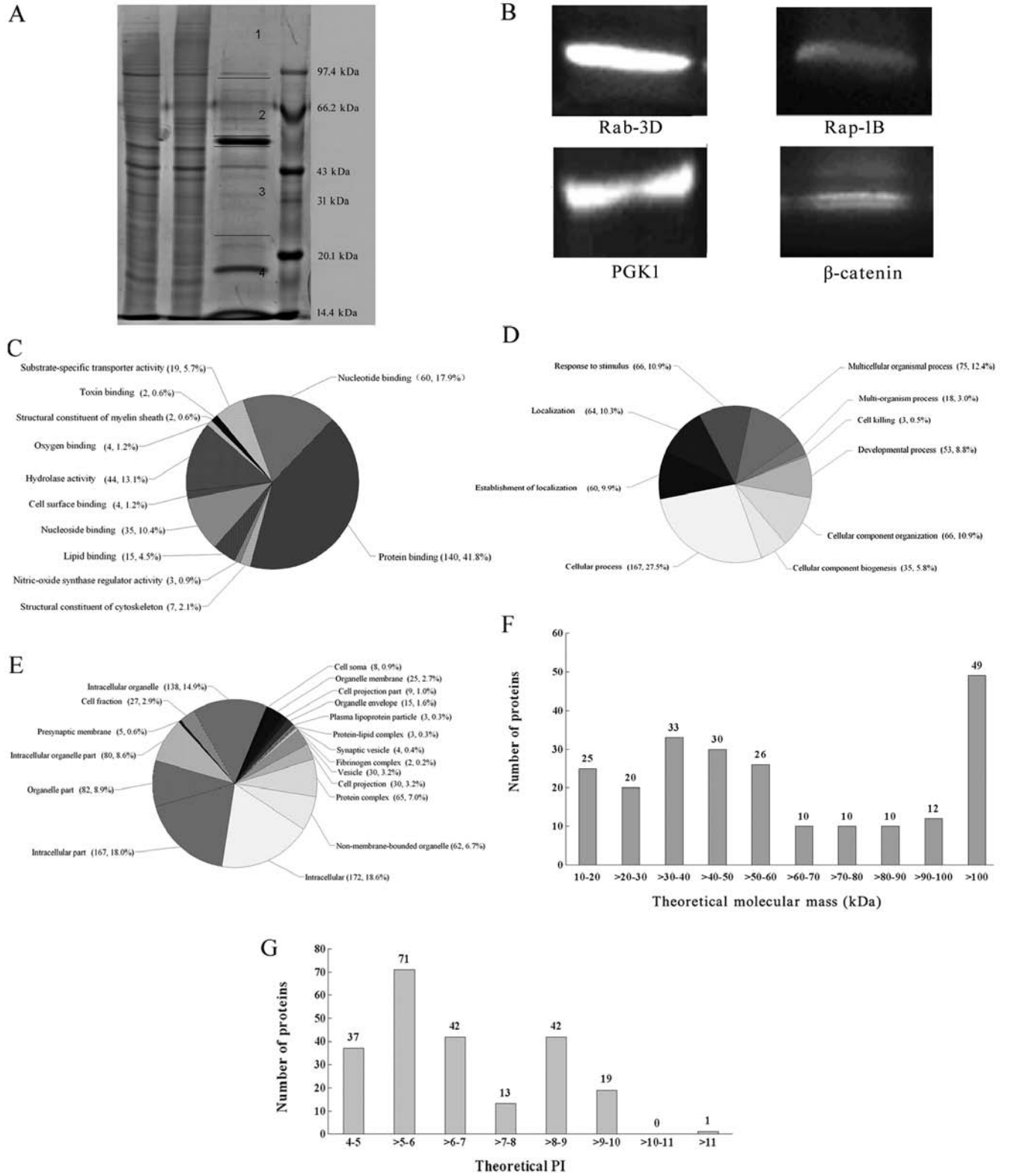

Figure 4. Proteomics analysis of MLAA-34 in stably transfected U937 cells. (A) SDS-PAGE gel images after Co-IP and the cutting sites in the gel band. Four spaced sections were excised. From left to right were cell lysate, elutriant after Co-IP, sediment after Co-IP and marker. (B) Western blot analysis. The major bands that migrated at ~21, 25, 92 and 45 kDa corresponding to Rap-1B, Rab-3D, β-catenin and PGK1. (C-E) Numbers and percentages of the annotated proteins with molecular function, biological process and cellular localization. (F and G) Distributions of theoretical molecular mass and PI for all of the annotated proteins.

be successfully infected by the packaged virus; the virus titer reached higher than $2x10^8$ TU/ml, indicating that a high-titer lentiviral packaging platform was preliminarily established. The pGC-FU-MLAA-34-GFP plasmid was confirmed by western blot analysis. MLAA-34-Lentivirus and control pGC-FU-GFP-LV virus were produced. After obtaining ideal U937 cells, we transfected the cells with the MLAA-34-Lentivirus and pGC-FU-GFP-LV viruses at different MOIs. The transfec-

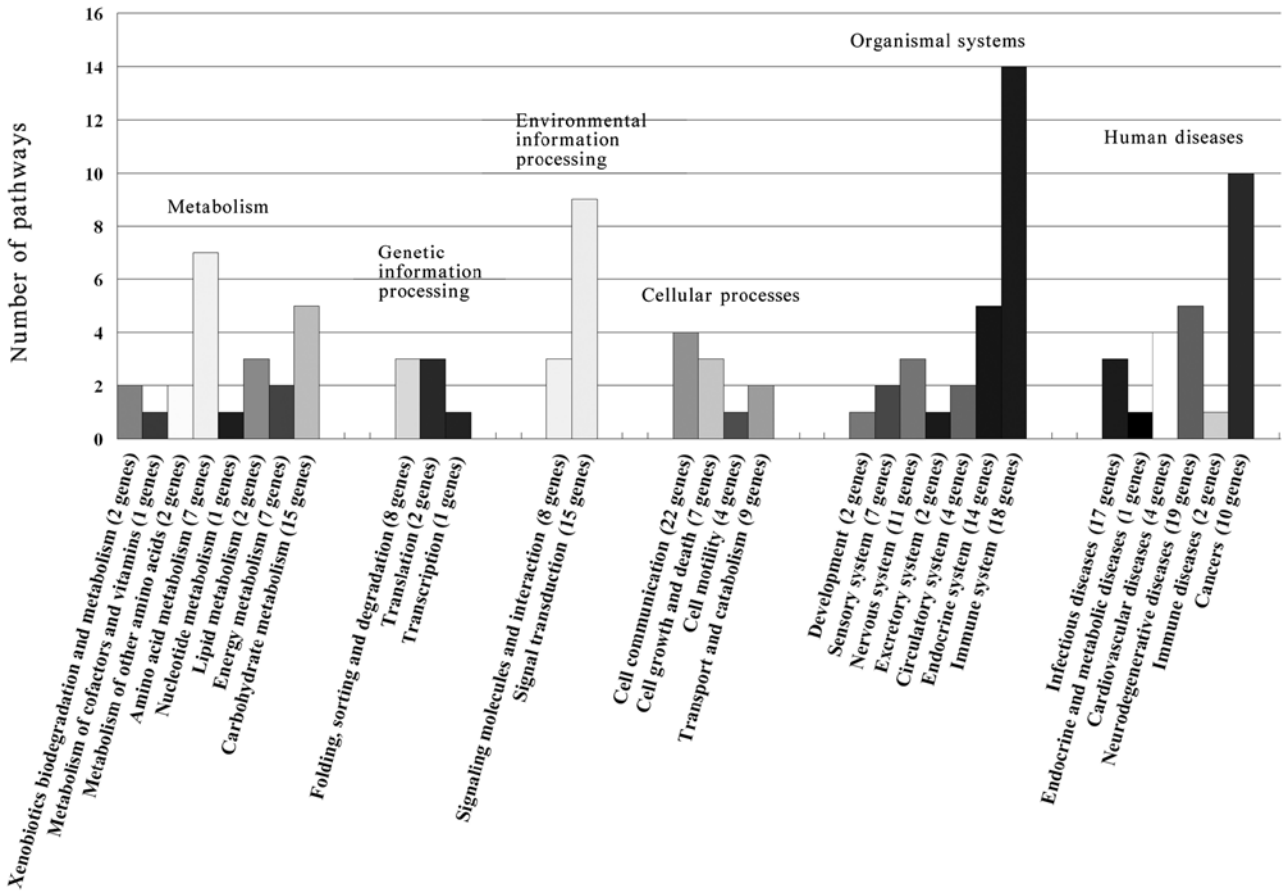

Figure 5. Categories of related pathways in 225 annotated proteins according to KEGG pathway taxonomy.

tion efficiency was ~95% or higher on Day 5 or later at the MOI of 50 (Fig. 3A). Five days after transfection, the recombinant MLAA-34-Lentivirus caused a pronounced increase in the expression of MLAA-34 compared with non-transfected U937 cells (Fig. 3B).

Establishment of U937 cell line stably overexpressing MLAA-34. In preliminary studies, 400 μg/ml of G418 were found to maintain adequate selection pressure. The expression of GFP and MLAA-34 were observed. After the cells had been frozen in liquid nitrogen for six months and revived monthly, the U937 cells expressed higher levels of MLAA-34 in ~400 μg/ml of G418, and ~95% of the lentivirus-transfected U937 cells overexpressed MLAA-34. These results suggested that the stably transfected U937 cell line was successfully established by lentivirus and that the expression of MLAA-34 can be long lasting even after passage.

Effect of upregulating MLAA-34 on apoptosis and growth of U937 cells. Observations of morphology revealed increasing cell shrinkage, nuclear condensation and fragmentation in non-transfected and pGC-FU-GFP-LV transfected cells. By contrast, cells transfected with MLAA-34-Lentivirus predominantly appeared uniformly stained without condensation (Fig. 3C). These results further support the findings that anti-apoptotic changes in the cell and nuclear morphology are induced by MLAA-34 overexpression. MTT assays suggested that the lentiviral overexpression of MLAA-34 induces anti-apoptotic effects that result in a promotion effect on U937 cells; these data suggest that MLAA-34 might accelerate cell proliferation (Fig. 3D). In agreement with the anti-apoptotic effects of MLAA-34, cells overexpressing MLAA-34 accumulated in the S-phase (~67.63% compared with ~49.6% of cells in the S-phase in the control) and showed a corresponding increase in cell numbers in the G2/M phase. The percentages of early (lower right) and late apoptotic (upper right) cells were markedly reduced in U937 cells after transfection with MLAA-34-Lentivirus (Fig. 3E). These results are in agreement with the DNA ladder assay and are even more evident at the MOI=50, in which the cells transfected with MLAA-34-Lentivirus showed a further increase. All of these results suggest that MLAA-34 inhibits apoptosis in U937 cells.

Co-IP, shotgun and western blot analysis. Protein extracts with Co-IP were separated by SDS-PAGE and the gel was cut into four pieces for shotgun ESI-MS analysis (Fig. 4A). A total of 256 proteins were identified by the LC ESI-MS analysis and BIOWORKS in the NCBI HUMAN protein databases, of which 225 (87.9%) proteins were annotated by DAVID and the remaining 31 (12.1%) proteins have no DAVID terms (Table I). The expression of Rap-1B, Rab-3D, β-catenin and PGK1 was verified by western blot analysis (Fig. 4B).

Classification of the 225 annotated proteins in terms of molecular function, biological process and cellular localization was performed according to the DAVID. Molecular function was clustered and the protein binding (140, 41.8%) and nucleotide binding (60, 17.9%) groups were the majority (Fig. 4C).

Table I. The 225 annotated proteins identified by the shotgun approach.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
1	IPI00022434	ALB albumin	Apoptosis	
2	IPI00023598	TUBB4 tubulin β-4 chain		
3	IPI00013475	TUBB2A tubulin β-2A chain		
4	IPI00180675	TUBA1A tubulin α-1A chain		
5	IPI00387144	TUBA1B tubulin α-1B chain		
6	IPI00013683	TUBB3 tubulin β-3 chain		
7	IPI00007752	TUBB2C tubulin β-2C chain	Apoptosis	
8	IPI00218343	TUBA1C tubulin α-1C chain		
9	IPI00021439	ACTB actin, cytoplasmic 1		
10	IPI00646909	TUBA8 tubulin α-8 chain		
11	IPI00646779	TUBB6 tubulin β-6 chain		
12	IPI00257508	DPYSL2 dihydropyrimidinase-related protein 2		
13	IPI00908469	cDNA FLJ52712, highly similar to Tubulin β-6 chain		
14	IPI00410714	HBA1; HBA2 hemoglobin, α-2; hemoglobin, α-1		
15	IPI00021428	ACTA1 actin, α skeletal muscle		
16	IPI00026268	GNB1 guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β-1	Cell proliferation, Ras protein signal transduction	Chemokine signaling pathway, Attenuation of GPCR signaling, Erk1/Erk2 MAPK signaling pathway, CXCR4 signaling pathway
17	IPI00220281	GNAO1 isoform A-1 of Guanine nucleotide-binding protein G(o) subunit α	Regulation of calcium ion transport, G-protein coupled receptor protein signaling pathway	
18	IPI00021907	MBP myelin basic protein		
19	IPI00024067	CLTC clathrin heavy chain 1		
20	IPI00216171	ENO2 enolase 2 (γ, neuronal)		
21	IPI00465248	ENO1 isoform α-enolase of A-enolase		
22	IPI00220706	HBG1 hemoglobin subunit γ-1		
23	IPI00219018	GAPDH glyceraldehyde-3-phosphate dehydrogenase		
24	IPI00398700	GNAO1 isoform A-2 of Guanine nucleotide-binding protein G(o) subunit α	Regulation of calcium ion transport, G-protein coupled receptor protein signaling pathway	
25	IPI00025363	GFAP glial fibrillary acidic protein		
26	IPI00303476	ATP5B ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide		
27	IPI00022977	CKB creatine kinase B-type		
28	IPI00413140	DNM1 dynamin 1		
29	IPI00154742	IGL λ protein		
30	IPI00022463	TF serotransferrin		
31	IPI00220737	NCAM1 neural cell adhesion molecule 1	Regulation of calcium-mediated signaling	
32	IPI00022891	SLC25A4 ADP/ATP translocase 1		Calcium signaling pathway
33	IPI00007188	SLC25A5 ADP/ATP translocase 2		Calcium signaling pathway
34	IPI00009532	ABAT 4-aminobutyrate aminotransferase, mitochondrial		
35	IPI00012451	GNB4 guanine nucleotide-binding protein subunit β-4		Chemokine signaling pathway
36	IPI00291006	MDH2 malate dehydrogenase 2, NAD (mitochondrial)		

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
37	IPI00298497	FGB fibrinogen β chain		
38	IPI00220993	CNP 2',3'-cyclic nucleotide 3' phosphodiesterase		
39	IPI00027547	DCD dermcidin		
40	IPI00219446	PEBP1 phosphatidylethanolamine-binding protein 1	Regulation of cAMP-mediated signaling, regulation of MAPKKK cascade	Calcium signaling pathway
41	IPI00414123	CRMP1 collapsin response mediator protein 1		
42	IPI00217507	NEFM neurofilament, medium polypeptide		
43	IPI00465439	ALDOA aldolase A, fructose-bisphosphate		
44	IPI00029111	DPYSL3 dihydropyrimidinase-like 3		
45	IPI00219813	RTN1 reticulon-1		
46	IPI00001453	INA α-internexin		
47	IPI00237671	NEFL neurofilament light polypeptide	Apoptosis	
48	IPI00743576	ATP6V0A1 ATPase, H^+ transporting, lysosomal V0 subunit a1		
49	IPI00418262	ALDOC fructose-bisphosphate aldolase C	Apoptosis	
50	IPI00029751	CNTN1 contactin-1	Notch signaling pathway	
51	IPI00549543	NCDN neurochondrin		
52	IPI00024975	KIF15 kinesin family member 15		
53	IPI00027497	GPI glucose-6-phosphate isomerase		
54	IPI00010154	GDI1 GDP dissociation inhibitor 1	Small GTPase mediated signal transduction	
55	IPI00554752	PRKAR2B protein kinase, cAMP-dependent, regulatory, type II, β		Apoptosis, Insulin signaling pathway
56	IPI00028888	HNRNPD heterogeneous nuclear ribonucleoprotein D0		
57	IPI00033025	SEPT7 septin 7		
58	IPI00784156	AP2B1 adaptor-related protein complex 2, β1 subunit		
59	IPI00026272	HIST1H2AB; HIST1H2AE histone cluster 1, H2ae; histone cluster 1, H2ab		
60	IPI00219661	PLP1 proteolipid protein 1		
61	IPI00015671	TUBAL3 tubulin α chain-like 3		
62	IPI00216298	TXN thioredoxin	Cell proliferation	
63	IPI00215715	CAMK2A calcium/calmodulin-dependent protein kinase II α	Regulation of NF-κB transcription factor activity	ErbB signaling pathway, Calcium signaling pathway, Wnt signaling pathway
64	IPI00020926	HOXA4 homeobox A4		
65	IPI00022314	SOD2 superoxide dismutase 2, mitochondrial	Cell proliferation, apoptosis	
66	IPI00024266	MGST3 microsomal glutathione S-transferase 3		
67	IPI00382470	HSP90AA1 heat shock protein 90 kDa α (cytosolic), class A member 1 isoform 1		NOD-like receptor signaling pathway, pathways in cancer, Ahr signal transduction pathway, AKT signaling pathway
68	IPI00019971	STXBP2 syntaxin-binding protein 2		
69	IPI00289861	ZCCHC11 zinc finger CCHC domain-containing protein 11		
70	IPI00013508	ACTN1 α-actinin-1		
71	IPI00007682	ATP6V1A V-type proton ATPase catalytic subunit A		
72	IPI00003925	PDHB pyruvate dehydrogenase E1 component subunit β, mitochondrial		
73	IPI00910290	Aryl hydrocarbon receptor nuclear translocator	Cell proliferation	Pathways in cancer, Ahr signal transduction pathway

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
74	IPI00022488	HPX hemopexin	Regulation of protein kinase cascade, interferon-γ-mediated signaling pathway, regulation of JAK-STAT cascade	
75	IPI00023302	SYN2 synapsin-2		
76	IPI00902614	USP24 ubiquitin carboxyl-terminal hydrolase 24		
77	IPI00220644	PKM2 pyruvate kinase isozymes M1/M2		
78	IPI00414676	HSP90AB1 heat shock protein HSP 90-β	Interferon-γ-mediated signaling pathway, type I interferon-mediated signaling pathway	NOD-like receptor signaling pathway, pathways in cancer
79	IPI00647704	IGHA1 immunoglobulin heavy constant α 1		
80	IPI00215747	FABP7 fatty acid-binding protein, brain	Cell proliferation	PPAR signaling pathway
81	IPI00026053	CLDN11 claudin-11		
82	IPI00025447	EEF1A1 elongation factor 1-α		
83	IPI00182944	CAMK2B calcium/calmodulin-dependent protein kinase type II β chain		ErbB signaling pathway, Calcium signaling pathway, Wnt signaling pathway
84	IPI00411486	OPALIN opalin		
85	IPI00299608	PSMD1 proteasome (prosome, macropain) 26S subunit, non-ATPase, 1		
86	IPI00299399	S100B protein S100-B	Cell proliferation	Calcium signaling pathway
87	IPI00175169	ARFGAP1 ADP-ribosylation factor GTPase-activating protein 1	Small GTPase mediated signal transduction, Ras protein signal transduction	
88	IPI00005614	SPTBN1 spectrin β chain, brain 1		
89	IPI00017597	MAPRE3 microtubule-associated protein RP/EB family member 3		
90	IPI00175092	RNF149 ring finger protein 149		
91	IPI00293613	TBK1 TANK-binding kinase 1	Regulation of protein kinase cascade, regulation of I-κB kinase/NF-κB cascade	Toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway
92	IPI00015029	PTGES3 prostaglandin E synthase 3		
93	IPI00169383	PGK1 phosphoglycerate kinase 1		
94	IPI00015148	RAP1B ras-related protein Rap-1b	Cell proliferation, small GTPase mediated signal transduction	MAPK signaling pathway, Chemokine signaling pathway
95	IPI00028946	RTN3 reticulon-3	Apoptosis	
96	IPI00163849	EPS15L1 epidermal growth factor receptor substrate 15-like 1	Calcium ion binding	
97	IPI00645078	UBA1 ubiquitin-like modifier-activating enzyme 1		Ubiquitin mediated proteolysis
98	IPI00005705	PPP1CC γ-1 of serine/threonine-protein phosphatase PP1-γ catalytic subunit		Insulin signaling pathway
99	IPI00159927	NCAN neurocan core protein	Calcium ion binding	
100	IPI00003420	MAPRE2 microtubule-associated protein, RP/EB family, member 2	Cell proliferation	
101	IPI00017566	FBXL7 F-box/LRR-repeat protein 7		
102	IPI00027252	PHB2 prohibitin-2		
103	IPI00015141	CKMT2 creatine kinase, sarcomeric mitochondrial		
104	IPI00027770	SYP synaptophysin		
105	IPI00290035	PCDH15 protocadherin-15	Calcium ion binding	
106	IPI00027462	S100A9 S100 calcium binding protein A9	Calcium ion binding	

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
107	IPI00022462	TFRC transferrin receptor protein 1		
108	IPI00300020	SLC1A2 excitatory amino acid transporter 2		
109	IPI00019884	ACTN2 α-actinin-2	Apoptosis	
110	IPI00000875	EEF1G cDNA FLJ56389, highly similar to Elongation factor 1-γ		
111	IPI00435928	RASGRF1 Ras protein-specific guanine nucleotide-releasing factor 1		Ras signaling pathway
112	IPI00790581	MPRIP protein		
113	IPI00383660	ZNF530 zinc finger protein 530		
114	IPI00218896	ADH1A alcohol dehydrogenase 1A		
115	IPI00300341	TCEB1 transcription elongation factor B polypeptide 1		Ubiquitin mediated proteolysis,
116	IPI00021891	FGG Γ-B of Fibrinogen γ chain		pathways in cancer
117	IPI00747180	WDR52 WD repeat protein 52		
118	IPI00642126	KIAA1618 isoform 1 of protein ALO17		
119	IPI00164441	UNC13A unc-13 homolog A		
120	IPI00027820	ESPN isoform 1 of Espin		
121	IPI00185659	CCDC62 isoform 2 of Coiled-coil domain-containing protein 60		
122	IPI00000816	YWHAE 14-3-3 protein epsilon	Apoptosis	
123	IPI00160552	TNR isoform 1 of Tenascin-R		
124	IPI00164347	CNGB1 cyclic nucleotide gated channel β 1 isoform b		
125	IPI00166979	KIAA1239 Leucine-rich repeat and WD repeat-containing protein KIAA1239		
126	IPI00217240	WDR75 WD repeat-containing protein 75		
127	IPI00017704	COTL1 coactosin-like protein		
128	IPI00008305	HPCAL4 hippocalcin-like protein 4		
129	IPI00440493	ATP5A1 ATP synthase subunit α, mitochondrial	Cell proliferation	
130	IPI00024547	C2orf25 chromosome 2 open reading frame 25		
131	IPI00074962	ANK2 isoform 4 of Ankyrin-2		
132	IPI00395663	ANKS1A ankyrin repeat and SAM domain-containing protein 1A		
133	IPI00029769	HCK isoform p59-HCK of Tyrosine-protein kinase HCK		Chemokine signaling pathway, GPCR signaling
134	IPI00216592	HNRNPC isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2		
135	IPI00000792	CRYZ quinone oxidoreductase		
136	IPI00219806	S100A7 S100 calcium binding protein A7	S100/CaBP-9k-type, calcium binding	
137	IPI00216856	ANKMY2 ankyrin repeat and MYND domain-containing protein 2		
138	IPI00027434	RHOC rho-related GTP-binding protein RhoC	Small GTPase mediated signal transduction, regulation of I-κB kinase/NF-κB cascade	Ras signaling pathway
139	IPI00396341	C2orf67 chromosome 2 open reading frame 67		
140	IPI00015785	CRB1 crumbs homolog 1	Calcium ion binding	
141	IPI00893234	OBSL1 obscurin-like 1		
142	IPI00028277	FTO isoform 1 of Protein fto		
143	IPI00060800	LOC124220 uncharacterized protein UNQ773/PRO1567		
144	IPI00007765	HSPA9 stress-70 protein, mitochondrial	Anti-apoptosis	
145	IPI00852669	ZNF516 zinc finger protein 516		
146	IPI00024994	TULP4 tubby-related protein 4		

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
147	IPI00010466	PRKCB isoform B-I of protein kinase C β type		MAPK signaling pathway, ErbB signaling pathway, Calcium signaling pathway, Chemokine signaling pathway, Phosphatidylinositol signaling system, Wnt signaling pathway, VEGF signaling pathway, pathways in cancer
148	IPI00815811	ZNF235 zinc finger protein 235		
149	IPI00163187	FSCN1 fascin homolog 1	Cell proliferation	
150	IPI00793780	TMCO5B transmembrane and coiled-coil domain-containing protein 5B		
151	IPI00011088	CLDN12 claudin-12		
152	IPI00011986	C5orf42 chromosome 5 open reading frame 42		
153	IPI00061780	ITCH itchy E3 ubiquitin protein ligase homolog	Cell proliferation	Ubiquitin mediated proteolysis
154	IPI00152653	DNAH5 dynein heavy chain 5, axonemal		
155	IPI00175416	PLCH11-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-1	Calcium ion binding	
156	IPI00218352	ESR1 estrogen receptor1	Estrogen receptor signaling pathway	
157	IPI00791536	MCF.2 cell line derived transforming sequence-like 2	Regulation of Rho protein signal transduction, regulation of Ras protein signal transduction, regulation of small GTPase mediated signal transduction	
158	IPI00009619	CADM3 isoform 2 of cell adhesion molecule 3		
159	IPI00179330	UBC; RPS27A; UBB ubiquitin and ribosomal protein S27a precursor	Apoptosis	
160	IPI00217776	ICK intestinal cell (MAK-like) kinase		
161	IPI00009439	SYT1 synaptotagmin-1	Calcium ion binding	
162	IPI00784869	DNAH10 isoform 1 of Dynein heavy chain 10, axonemal		
163	IPI00020265	ANKRD20A1 ankyrin repeat domain-containing protein 20A1		
164	IPI00007189	CDC42 isoform 1 of cell division control protein 42 homolog		MAPK signaling pathway, Chemokine signaling pathway, VEGF signaling pathway, Pathways in cancer, Ras signaling pathway
165	IPI00032325	CSTA cystatin-A		
166	IPI00032808	RAB3D ras-related protein Rab-3D	Small GTPase mediated signal transduction	Ras signaling pathway
167	IPI00216308	VDAC1 voltage-dependent anion-selective channel protein 1	Apoptosis	Calcium signaling pathway
168	IPI00021841	APOA1 apolipoprotein A-I	Cell proliferation, small GTPase mediated signal transduction, Ras protein signal transduction, Rho protein signal transduction, Cdc42 protein signal transduction, G-protein coupled receptor protein signaling pathway	PPAR signaling pathway
169	IPI00645906	CXorf39 isoform 1 of uncharacterized protein CXorf39		
170	IPI00220032	CTNND2 isoform 2 of Catenin δ-2		
171	IPI00002459	ANXA6 Annexin VI isoform 2	Calcium ion transport	
172	IPI00184119	DNAJC6 isoform 2 of putative tyrosine-protein phosphatase auxilin		

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
173	IPI00152949	TMEM168 transmembrane protein 168		
174	IPI00032402	ATP8A1 isoform long of probable phospholipid-transporting ATPase IA		
175	IPI00008380	PPP2CA serine/threonine-protein phosphatase 2A catalytic subunit α isoform	Apoptosis, MAPKKK cascade, second-messenger-mediated signaling, regulation of JAK-STAT cascade	Wnt signaling pathway, TGF-β signaling pathway, AKT signaling pathway, Erk1/Erk2 MAPK signaling pathway
176	IPI00219217	LDHB L-lactate dehydrogenase B chain		
177	IPI00394855	C12orf63 chromosome 12 open reading frame 63		
178	IPI00025366	CS citrate synthase, mitochondrial		
179	IPI00218570	PGAM2 phosphoglycerate mutase 2		
180	IPI00303484	OR52K2 olfactory receptor 52K2	G-protein coupled receptor protein signaling pathway	
181	IPI00005565	DGKQ diacylglycerol kinase θ	G-protein coupled receptor protein signaling pathway, activation of protein kinase C activity by G-protein coupled receptor protein signaling pathway	Phosphatidylinositol signaling system
182	IPI00168218	DOK7 isoform 2 of protein Dok-7		
183	IPI00154645	TBC1D15 isoform 1 of TBC1 domain family member 15	Rab protein signal transduction, Ras protein signal transduction, small GTPase mediated signal transduction	
184	IPI00386494	SPPL2B isoform 1 of signal peptide peptidase-like 2B		
185	IPI00465436	CAT catalase	Apoptosis, regulation of protein kinase cascade, regulation of phosphoinositide 3-kinase cascade, regulation of NF-κB transcription factor activity	
186	IPI00007612	KCNJ1 isoform 1 of ATP-sensitive inward rectifier potassium channel 1		
187	IPI00020153	BSN protein bassoon		
188	IPI00298547	PARK7 protein DJ-1	Small GTPase mediated signal transduction, Ras protein signal transduction	
189	IPI00456969	DYNC1H1 cytoplasmic dynein 1 heavy chain 1		
190	IPI00030144	PPIAL4C; PPIAL4A; PPIAL4G; PPIAL4B Peptidylprolyl cis-trans isomerase A-like 4B		
191	IPI00376119	PRKACB isoform 2 of cAMP-dependent protein kinase catalytic subunit β	G-protein coupled receptor protein signaling pathway, second-messenger-mediated signaling, cAMP-mediated signaling	MAPK signaling pathway, Calcium signaling pathway, Chemokine signaling pathway, Apoptosis, Wnt signaling pathway, Hedgehog signaling pathway, Insulin signaling pathway
192	IPI00025753	DSG1 desmoglein-1	Calcium ion binding	
193	IPI00292934	USP53 inactive ubiquitin carboxyl-terminal hydrolase 53		
194	IPI00024684	MX2 interferon-induced GTP-binding protein Mx2		
195	IPI00384998	NFASC isoform 7 of Neurofascin		
196	IPI00217494	SMG7 smg-7 homolog, nonsense mediated mRNA decay factor (*C. elegans*)		

Table I. Continued.

No.	Accession no.	Protein description	Biological processes (partly)	KEGG pathways (partly)
197	IPI00171594	DACT1 dapper homolog 1	Wnt receptor signaling pathway	
198	IPI00006612	SNAP91 isoform 1 of Clathrin coat assembly protein AP180		
199	IPI00291922	PSMA5 proteasome subunit α type-5		
200	IPI00056040	NRN1L neuritin-like protein		
201	IPI00013421	GPM6B isoform 1 of neuronal membrane glycoprotein M6-b		
202	IPI00738216	KIAA0947 isoform 1 of uncharacterized protein KIAA0947		
203	IPI00022133	EPB41L2 4.1G protein		
204	IPI00853219	RAPGEF2 rap guanine nucleotide exchange factor 2	Small GTPase mediated signal transduction, cAMP-mediated signaling	MAPK signaling pathway
205	IPI00375609	JAKMIP3 janus kinase and microtubule-interacting protein 3		
206	IPI00178185	BICD2 isoform 1 of protein bicaudal D homolog 2		
207	IPI00006146	SAA2 serum amyloid A2		
208	IPI00014843	LRRC16A isoform 1 of Leucine-rich repeat-containing protein 16A		
209	IPI00183368	SMG1 phosphatidylinositol 3-kinase-related kinase (*C. elegans*)		
210	IPI00019038	LYZ lysozyme C		
211	IPI00397801	FLG2 filaggrin-2	Calcium ion binding	
212	IPI00186290	EEF2 elongation factor 2		
213	IPI00783097	GARS glycyl-tRNA synthetase		
214	IPI00029468	ACTR1A α-centractin		
215	IPI00010845	NDUFS8 NADH dehydrogenase (ubiquinone) iron-sulfur protein 8, mitochondrial		
216	IPI00256861	MACF1 microtubule-actin crosslinking factor 1	Wnt receptor signaling pathway, calcium ion binding	
217	IPI00017292	CTNNB1 Catenin β-1	Apoptosis, cell proliferation, regulation of MAPKKK cascade	Wnt signaling pathway, pathways in cancer
218	IPI00005966	NDUFA12 13 kDa differentiation-associated protein variant (Fragment)		
219	IPI00022229	APOB apolipoprotein B-100		
220	IPI00307259	DNAJC13 dnaJ homolog subfamily C member 13		
221	IPI00216085	COX6B1 cytochrome *c* oxidase subunit VIb isoform 1		
222	IPI00022774	VCP valosin-containing protein	Apoptosis, ER-nuclear signaling pathway	
223	IPI00479640	C1orf113 chromosome 1 open reading frame 113		
224	IPI00033019	KCNB1 potassium voltage-gated channel subfamily B member 1		
225	IPI00455876	RING1 isoform 2 of E3 ubiquitin-protein ligase RING1		

Seventy-one of the proteins are correlated with cell proliferation or apoptosis according to biological processes and KEGG pathways (in the right two tiers). PEBP1, GNB1, ARFGAP1, RAP1B, RASGRF1, RHOC, PRKCB, MCF.2, CDC42, RAB3D, APOA1, PPP2CA, TBC1D15, PARK7, PRKACB, RAPGEF2 and CTNNB1 are concerned with the Ras signaling pathway; CAMK2A, CAMK2B, PRKCB, PPP2CA, PRKACB, CTNNB1, MACF1 and DACT1 are concerned with the Wnt signaling pathway; SLC25A4, SLC25A5, PEBP1, CAMK2A, CAMK2B, S100B, PRKCB, VDAC1 and PRKACB participate in the Calcium signaling pathway; GNB1, GNB4, RAP1B, HCK, PRKCB, CDC42 and PRKACB are involved in the Chemokine signaling pathway; 15 proteins are concerned with calcium-mediated biological processes; 10 proteins are concerned with small GTPase mediated signal transduction and 8 proteins are concerned with the G-protein coupled receptor protein signaling pathway.

For biological processes, annotated proteins are particularly involved in the cell process (167, 27.5%) and the multicellular organismal process (75, 12.4%) (Fig. 4D). Most (172, 18.6%) of the annotated proteins were localized in the intracellular (Fig. 4E). Distribution of molecular mass and isoelectric points (PI) of the annotated proteins was analyzed. Molecular mass ranged between 10.19 and 620.42 kDa in size, most of them were between 10 and 60 kDa (Fig. 4F). PI of the proteins ranged between 4.35 and 11.05 with the most PIs between four and ten (Fig. 4G). To uncover the signaling pathways of the 225 annotated proteins, the protein sequences were searched against the KEGG reference pathway database. The pathways were ascribed to metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases (Fig. 5). Among them, the immune system, cancer and signal transduction were more than others. On the other hand, the specific expressed proteins related pathways displayed more differences and 71 proteins were involved in cell apoptosis or proliferation biological processes and KEGG pathways (Table I).

Discussion

In this study, to evaluate the function of MLAA-34 in M5 cells, we used the well-characterized cell line U937. In our previous research, we reported that the MLAA-34 protein is probably a cytoplasmic protein predicted by the amino acid sequence analysis of the encoded protein (7). Here, we verified that MLAA-34 is localized in the cytoplasm and cell membrane. Western blot analysis showed that the expression of MLAA-34 differed between different cell types and was observed to be stronger in U937. Although U937 cells are generally difficult to transfect, the U937 cells were transfected with MLAA-34-Lentivirus and pGC-FU-GFP-LV. A stably transfected U937 cell line was successfully established and expressed MLAA-34 at a high level, which aided in the study exploring the effect of MLAA-34 on M5 and will be critical for further research using U937 cells and animal models. In addition, an analysis of the cell morphology, apoptosis, proliferation and cell cycle revealed that the overexpression of MLAA-34 markedly inhibited apoptosis of U937 cells. These results suggested that MLAA-34 maybe a novel anti-apoptotic factor of M5, which is consistent with the RNAi in our previous study.

The proteins that interact with MLAA-34 or CAB39L remain unclear. To analyze complex mixtures of proteins, shotgun is considered the most powerful (9,10). Using the MLAA-34 protein as bait, 256 proteins were identified and 225 of them have DAVID terms. Among these proteins, 71 proteins correlated with cell apoptosis or proliferation biological processes and KEGG pathways. Twenty-eight proteins are involved in cell apoptosis or proliferation; nine proteins are associated with the calcium signaling pathway and seven proteins participate in the chemokine signaling pathway; 17 proteins are concerned with the Ras signaling transduction pathway and 8 proteins are concerned with Wnt signaling pathway. The Ras, Wnt, calcium and chemokine signaling pathways may be involved in anti-apoptosis with MLAA-34 in U937 cells. As is known, the Ras family plays an important role in the molecular pathogenesis of myeloid leukemia, and Ras mutations have been preferentially associated with monocytic subtypes in AML (11). The Ras and Wnt signaling pathways are known to be key anti-apoptosis pathways in AML-M5 (12). Understanding the molecular genetics of leukemia has led to an appreciation that particular molecular abnormalities give rise to specific subtypes of the disease. For example, in myeloid leukemogenesis, PML-RAR-α and BCR-ABL are defining features of acute promyelocytic leukemia and chronic myeloid leukemia, respectively (13). In this case, MLAA-34 may either play an important role in leukemogenesis or play a dual role in subsequent differentiation, as in the case of PML/RAR. The results suggest that MLAA-34 might be an important agent for subtype diagnosis in AML. However, an understanding of how these identified proteins or pathways interact with MLAA-34 requires further study.

In addition to the typical pathways such as pathways in cancer and apoptosis, there were several notable pathways such as the GPCR signaling, the insulin signaling pathway, the ErbB signaling pathway, the NOD-like receptor signaling pathway, the Ahr signal transduction pathway, the AKT signaling pathway, the Toll-like receptor signaling pathway, the RIG-I-like receptor signaling pathway, the ubiquitin mediated proteolysis, the hedgehog signaling pathway, the phosphatidylinositol signaling system, the PPAR signaling pathway, the VEGF signaling pathway and the TGF-β signaling pathway worthy of further validation (Table I). Otherwise, there are some proteins mainly involved in tumorigenesis concerned with MLAA-34 as discussed below. PGK1 is secreted by tumor cells and may play a role in inhibiting tumor angiogenesis (14). GAPDH has been shown to be upregulated in several types of cancer and downregulated by chemotherapeutic drugs, and could be considered a potential target to observe the effects of bisphosphonates on cancer cells (15). In addition, GAPDH was the best control gene in the apoptosis pattern on the myeloid cell lines (16). CRMP1 is a suppressor of tumor cell invasion of the local stroma and might be a functional modulator of the Wnt signaling pathway *in vivo* (17,18). As the trigger of TBK-1 pathway, TBK1 is important for tumor angiogenesis and tumor-associated microvascular inflammation and expressed at significant levels in many solid tumors (19,20). A recent study has demonstrated that SEPT7 could function in gliomagenesis and in the suppression of glioma cell proliferation (21).

Markedly, some p53 or caspase-related proteins were also identified, such as CLTC, PPP2CA, SOD2, PARK7, HSPA9, TXN, ESR1 and YWHAE. CLTC associates with p53 not only in nuclei but also in cytosol, and co-localizes with p53 at the plasma membrane in human cancer cells (22). CLTC expression enhances p53-dependent transactivation (23). As a downstream mediator of the antiproliferative effects of PPP2CA, p53 plays an important role in PPP2CA-directed cell cycle arrest and apoptosis (24). The SOD2 growth-retarding functions are at least partially due to triggering of a p53-dependent cellular senescence program (25). DJ-1 (PARK7) bound to p53 *in vitro* and *in vivo* and they were found colocalized. DJ-1 positively regulates p53 through Topors-mediated sumoylation (26). Previous studies indicated that HSPA9 could bind to p53 and sequesters it in the cytoplasm, thus providing a mechanism of inactivation of wild-type p53 and contributing to human carcinogenesis (27,28). Additional studies have shown that TXN induces p53 DNA binding activity *in vitro* and enhances p53-dependent expression of its target gene p21 and DNA repair

genes (29). Additional studies also indicated that caspases could be activated by TXN due to its disulfide reducing properties (30). ESR1 might activate caspases-8, -9 and -3 and induce tumor cell apoptosis, it also showed the downregulation of β-catenin signaling implicating the suppression of proliferation and metastasis of tumor cells (31,32). The cleavage of YWHAE by caspase-3 during apoptosis might contribute to cell death by preventing the association of YWHAE with Bad (33). The key event during apoptosis that is common to all pathways is the activation of caspases. P53 is a well-known tumor suppressor gene, and mutational inactivation of p53 function or deletion of the gene increases susceptibility to cancer (34-37). On the basis of these findings, we will further study the interaction between MLAA-34 and caspases or p53 to investigate the anti-apoptotic mechanisms of MLAA-34 in U937 cells.

To our knowledge, this is the first report showing the cellular localization and expression of MLAA-34 in U937 cells. We have demonstrated for the first time that the overexpression of MLAA-34 by lentivirus can significantly suppress the apoptosis of U937 cells, and a cell line stably overexpressing MLAA-34 was successfully established. Another key finding of this study is the information from proteomics evidence that MLAA-34 may be a tumor-correlated gene, and this is the first time it is revealed that the preliminary framework of proteins and pathways interlink with MLAA-34 in U937. Furthermore, it will be essential to integrate data from many different sources to obtain an accurate understanding of MLAA-34 protein networks.

Gene therapy remains the most promising, if not the only, approach to treating genetic diseases. An example of this is the use of rituximab for the treatment of lymphoma and other types of cancer. Rituximab is a mouse/human chimeric IgG(1)-κ monoclonal antibody that targets the CD20 antigen found on the surface of malignant and normal B lymphocytes (38). Most cellular processes are performed by multiprotein complexes. The identification and analysis of their components provides insight into how the ensemble of expressed proteins (the proteome) is organized into functional units (39). Nevertheless, for a viable clinical approach, extensive research is needed in the future to regulate the expression of the target gene and improve its safety.

In conclusion, our current results provide new evidence that MLAA-34 may be a novel anti-apoptotic factor *in vitro*, and the data presented here show a strong correlation between anti-apoptosis with the upregulation of MLAA-34. In addition, preliminary proteomic analysis suggests that a number of genes belonging to different signaling pathways may be 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. Findings of the present study 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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