

Cytokine-induced apoptosis inhibitor 1 inhibits the growth and proliferation of multiple myeloma

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Abstract. The present study investigated the differential expression of cytokine-induced apoptosis inhibitor 1 (CIAPIN1) in human multiple myeloma (MM) bone marrow tissue and adjacent healthy bone marrow tissue. In addition, the effect of a transduced CIAPIN1 gene on the growth of the RPMI-8226 human MM cell line was investigated. CIAPIN1 protein expression was detected in 32 samples of paraffin-embedded MM and adjacent healthy bone marrow tissue using immunohistochemistry. The CIAPIN1 gene (Ad-CIAPIN1, small interfering RNA) was inserted into a lentiviral vector and transfected into the RPMI-8226 human MM cell line. The expression of target proteins CIAPIN1 and insulin-like growth factor 1U (IGF-1), cell cycle-regulatory proteins and functional proteins was detected using western blotting. MTT and soft agar colony formation assays were conducted, and cellular tumorigenicity in nude mice was assessed, in order to investigate the proliferative capacity of cells *in vitro* and *in vivo*. Flow cytometry was applied in order to analyze changes in the cell cycle and cell apoptosis. CIAPIN1 expression was significantly reduced in cells from the 32 MM samples compared with those from healthy bone marrow ($P < 0.05$). Upregulation of CIAPIN1, following transduction by lentiviral vectors, caused cells to arrest in G1/S phase of the cell cycle and significantly inhibited the growth of the RPMI-8226 MM cell line *in vitro* and *in vivo*. CIAPIN1 was shown to inhibit cell growth. Specifically, it inhibited cyclin-dependent kinase 2, cyclin-dependent kinase 4 and insulin-like growth factor-1. Increased expression of CIAPIN1 also led to an increase in the levels of p27 and Rb, an effect that may have been achieved via regulation of cell cycle proteins and functional proteins. The results of the present study suggest that downregulation of the CIAPIN1 gene in MM cells may be associated with the development of this disease. CIAPIN1 transfection in

RPMI-8226 cells significantly inhibited the growth of tumor cells, suggesting that the CIAPIN1 gene is a potential tumor suppressor.

Introduction

Multiple myeloma (MM) is a malignant tumor derived from B cells. It is characterized by the uncontrolled proliferation of monoclonal plasma cells in the bone marrow and the presence of monoclonal immunoglobulin in the serum or urine. It is often accompanied by multiple osteolytic lesions, hypercalcemia, anemia, kidney damage and suppression of the immune system, and carries a poor prognosis. MM has a high incidence in middle-aged to elderly individuals and remains an incurable disease (1). Since the molecular etiology and mechanisms of drug resistance are not clear, there are currently no treatments that result in a complete cure. Therefore, further studies into the pathogenesis of MM are required, in order to develop clinical diagnostic and therapeutic approaches to this disease.

In humans, cytokine-induced apoptosis inhibitor 1 (CIAPIN1) is located on the long arm of chromosome 16. Shibayama *et al* (2,3) first identified CIAPIN1 as a regulatory molecule in the rat sarcoma signal transduction pathway, which is independent of apoptotic B cell lymphoma 2 and the cysteine-dependent aspartate-directed protease family. CIAPIN1 is widely distributed in healthy fetal and adult tissues, and the gene is expressed in differentiated tissue and metabolically active tissue (4). However, its expression has been shown to be inhibited in certain types of cancerous tissues, including gastric cancer and clear cell renal cell carcinoma (5,6). These findings suggest that the expression of the CIAPIN1 gene may be associated with the suppression of tumor development.

In the present study, the expression and effects of CIAPIN1 were investigated in multiple myeloma.

Materials and methods

Immunohistochemistry. Paraffin-embedded tissue samples from diseased and healthy bone marrow, were obtained from 32 patients with MM in whom the disease had been pathologically confirmed and healthy controls, respectively. The healthy controls ranged from 32-60 years old (23 males and 9 females) and the bone marrow examination did not reveal malignant blood disorders. Samples were obtained by Dr Xiaobo Wang from patients who underwent orthopedic surgery at The First

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Affiliated hospital of Sun Yan-sen University (Guangzhou, China) between July 2009- March 2013. The patients ranged from 37-69 years old (20 males and 12 females). The patients had not previously received radiotherapy, chemotherapy or biological response modifier therapy. A mouse anti-CIAPIN1 monoclonal antibody was produced by Abcam (ab154904; Cambridge, UK). All specimens were obtained from patients who had provided informed consent for research purposes. The protocols used in the present study were approved by the Hospital's Protection of Human Subjects Committee of Sun Yat-sen University (Guangzhou, China). The use of human tissue in this study was approved by the institutional review board of The First Affiliated hospital, Sun Yat-sen University and was conducted in accordance with international guidelines for the use of human tissue. All slides were treated with polylysine in preparation for immunohistochemistry. In order to recover antigens, slides were treated with boiling 0.01 mol/l citrate buffer (Jingyan Chemicals Co., Ltd, Shanghai, China) followed by incubation at 95°C for 5 min. Following natural cooling of the buffer to room temperature, slides were washed with phosphate-buffered saline (PBS; Jingyan Chemicals Co., Ltd) three times, every 2 min. The mouse monoclonal anti-CIAPIN1 primary antibody was diluted to 1:1,000 and the secondary antibody, biotinylated goat anti-mouse Immunoglobulin G (IgG; 33207ES60, Yeasen Biotechnology Co., Ltd., Shanghai, China), was diluted to 1:500. The positive control consisted of CIAPIN1-stained human SkHep1 xenograft tissue. For the negative control, 0.01 M PBS was used instead of the primary antibody. Criteria for positively stained tumor cells: Presence of brown-yellow staining of the tumor cells nuclei and/or irregular thickness and shades of granules in the cytoplasm (only tumor cells were assessed). Two types of CIAPIN1 cells were identified: CIAPIN1 (-), $\leq 25\%$ tumor nuclei containing brown staining or granules; and CIAPIN1 (+), $>25\%$ tumor nuclei containing brown staining or granules.

Cell culture. Exponentially growing RPMI-8226 cells and WIL2-S B lymphocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 complete media (Cyagen Biosciences, Guangzhou, China) containing 15% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Minsheng Chemical Co., Ltd., Beijing, China). 293T cells were cultured in high-glucose Dulbecco's modified Eagle's Medium (DMEM; Cyagen Biosciences) containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were cultured in a 37°C, 5% CO₂ incubator with saturated humidity. When cells had reached 80-90% confluence, they were passaged, and adherent 293T cells were removed using 0.25% trypsin (Cyagen Biosciences) digestion.

Extraction of bone marrow mononuclear cells. Bone marrow (3-5 ml) was extracted from the posterior iliac spines of patients and controls, and transpired into 0.5 ml anticoagulant heparin saline solution (1,200 U/ml; Minsheng Chemical Co., Ltd.). Subsequently, 2-4X volume of PBS was added to the solution. The diluted bone marrow solution was slowly added to a lymphocyte separation medium (bone marrow : lymphocyte separation medium = 1:1)

for gradient centrifugation at 100 x g for 20 min at 20°C. Following centrifugation, the solution was separated into four layers. The top layer consisted of the plasma containing platelets. The second layer consisted of a white film, which contained the mononuclear cells; the third layer consisted of the separation medium; and the fourth layer contained the granulocytes and erythrocytes. Mononuclear cells were aspirated into a new tube, mixed with 5 ml PBS and centrifuged at 60 x g for 10 min at 20°C. Cells were washed twice with PBS and resuspended in PBS for counting.

Construction of CIAPIN1-expressing lentiviral vector. The primers designed for CIAPIN1 gene amplification were based on the GenBank CIAPIN1 sequence. In order to facilitate the expression of the vector in eukaryotic cells, a CACC sequence was added to the primer's 5' end and the terminator sequence was removed from the primer's 3' end. The following primers were synthesized by GENEWIZ, Inc. (Suzhou, China): 5'CCG CTCGAGATGGCAGATTTTGGGATCTCTGC3' for ciapin1XhoIF and 5'ATAAGAATGCGGCCGCTAGGCATCAT GAAGATTGCTATC 3' for ciapin1NotIR. The CIAPIN1 gene was amplified using the high fidelity enzyme KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) using the full CAIPIN1 cDNA as a template, with the ciapin1XhoIF and ciapin1NotIR primers [2 mM deoxyribonucleotide mixture; 2.5 μ l 10X KOD buffer; 1.5 μ l (25 mM) MgSO₄; 0.3 μ l ciapin1XhoIF; 0.3 μ l ciapin1NotIR; 0.3 μ l KOD-Plus-Neo; and 17.1 μ l double distilled (dd)H₂O]. The cycling conditions were as follows: 94°C for 5 min and 30 cycles of 98°C for 30 sec; followed by 58°C for 30 sec; and then 68°C for 1 min and 68°C for 5 min. The amplified products were purified and mixed with pLVX-IRES-ZsGreen1 vector. Subsequently, they were digested with the restriction enzymes XhoI and NotI at 37 °C for 3 h and recovered using a DNA gel extraction kit (Dongsheng Biotech Ltd., Guangzhou, China). In brief, polymerase chain reaction (PCR) products were electrophoresed, sectioned and added to BD solution (Novoprotein Scientific, Inc., Shanghai, China) at 60°C for 10 min. PCR products were then transferred to a DNA purification column (held for 2 mins, centrifuged at 12,000 x g for 1 min; Novoprotein Scientific, Inc.) and the supernatant was removed, then centrifuged following resuspension in 500 μ l PE at 1,000 x g for 1 min and the supernatant was removed. Subsequently, the DNA was eluted at 60°C in 30 μ l ddH₂O and centrifuged at 400 x g for 1 min. PCR products were inserted into the vector using T4 DNA ligase (D2011A; Takara Biotechnology Co., Ltd., Dalian, China) at 16°C for 2 h. Competent DH5 α cells (Invitrogen Life Technologies) were transformed, plated on lysogeny broth (LB; Jingyan Chemicals Co., Ltd) medium containing ampicillin (Minsheng Chemical Co., Ltd.) and incubated at 37°C for 18 h. Colonies were selected and cultured in LB medium containing ampicillin at 37°C. Plasmids were then purified for sequence analysis in order to obtain the CIAPIN1-expressing lentiviral vector pLVX-IRES-ZsGreen1. In brief, 1.5 ml gel was centrifuged at 4°C for 30 sec at 12,000 x g and the supernatant was removed. The pellet was then resuspended in 120 ml lysis buffer STET (Rong Bio-Science Technology Co., Ltd., Shanghai, China) for 50 sec, 10 ml lysozyme (10 mg/ml) and boiling water for 50 sec, prior to further centrifugation at 4°C for 10 min at 12,000 x g to remove bacterial debris.

BLAST sequence analysis was then performed (NCBI/BLAST/blastnsuite-2sequences/).

Construction of CIAPIN1-small interfering RNA (si-RNA) lentiviral vector. The specific CIAPIN1 siRNA target was designed by Sigma-Aldrich (St. Louis, MO, USA). To facilitate cloning into the vector, the sequences CACC and AAAA were annealed to the 5' ends of the following complementary single-stranded DNA hairpins respectively: 5'GATCCGTGTTTCAGCCCTGACTCTTCTC-GAGAAGAGTCAGGGCTGAACACTTTTTTG3' for ciapin1siRNABamHIF and 5'AATTCAAAAAGTGTTCAGCCTGACTCTTCTCGAGAAGAGTCAGGGCTGAA CACG' for ciapin1siRNAEcoRIR. These single-stranded DNA hairpins were synthesized by GENEWIZ Inc. The pLVX-shRNA2 vector was mixed with the ciapin1siRNABamHIF and ciapin1siRNAEcoRIR annealed products and digested using the restriction enzymes *Bam*HI and *Eco*RI (Sigma-Aldrich) for 4 h at 37°C. The digestion products were recovered using a DNA gel extraction kit (Dongsheng Biotech Ltd.), and either the ciapin1siRNA (Sigma-Aldrich) or the unrelated control siRNA was inserted into the vector using T4 DNA ligase (D2011A; Takara Biotechnology Co., Ltd.) at 16°C for 2 h. Competent DH5 α cells (Invitrogen Life Technologies) were transformed, plated on an LB medium containing ampicillin and incubated at 37°C for 18 h. Colonies were selected and cultured, and plasmids were purified for sequence analysis in order to obtain the CIAPIN1-siRNA lentiviral vector and the unrelated control vector, pLVX-NC.

Lentiviral packaging. Recombinant lentiviral plasmids and the two packaging plasmids were extracted using a Plasmid Extraction kit (PD1212-01; Biomiga, Inc., San Diego, CA, USA) according to the manufacturer's instructions. These plasmids were endotoxin-free. After 8 h, the 293T cells were transfected and cultured in a complete media using Lipofectamine 2000[®] according to the manufacturer's instructions (Invitrogen Life Technologies). The cells were harvested at 24 h and 48 h, centrifuged at 200 x g for 5 min and filtered separately through a 0.45 μ m needle filter (Zhejiang Aijiren Technology Co., Ltd., Shanghai, China). The virus pellet was collected by centrifuging at 50,000 x g for 2 h at 4°C, re-suspended in 1 ml PBS, further filtered by a 0.22 μ m needle filter (Zhejiang Aijiren Technology Co., Ltd.) and stored at -80°C.

Virus infection and screening. RPMI-8226 cells were seeded at 2x10⁵ cells/well in 24-well plates and cultured at 37°C for 18 h. The CIAPIN1-expressing lentivirus, CIAPIN1-siRNA lentivirus and the unrelated control siRNA lentivirus were transfected into RPMI-8226 cells separately and polybrene (Jingyan Chemicals Co., Ltd) was added to obtain a final concentration of 6 μ g/ml. The multiplicity of infection (ratio of virus to cell number) was 10:1. Following culture for 6 h, the cells were replenished with fresh media and blasticidin (SelleckChem, Houston, TX, USA) was added to produce a final concentration of 5 μ g/ml for screening. Stably transduced cells were obtained after 2-3 weeks.

Reverse transcription-quantitative PCR (RT-qPCR). Using ABI's Primer Express software 3.3 and the glyceraldehyde

3-phosphate dehydrogenase (GAPDH) gene as an internal control, primers and probes were synthesized by Biosune Biotech Ltd Inc. (Nanjing, Jiangsu, China). Using a small RNA extraction kit (Dongsheng Biotech Ltd.), total RNA was extracted from CIAPIN1 overexpressing RPMI-8826 cells, CIAPIN1-siRNA-RPMI-8826 cells, unrelated siRNA-RPMI-8826 cells and control RPMI-8826 cells, and amplified using the ABI 7500 Fast & 7500 RT-PCR PCR system, according to the manufacturer's instructions (Applied Biosystems Life Technologies, Beijing, China). The gene copy number was calculated according to the corresponding cycle threshold value. The ratio of CIAPIN1 gene copy number to internal GAPDH gene copy number in the samples was interpreted as the relative expression level of the CIAPIN1 gene.

Western blotting. Cells (5x10⁵) were collected and sonicated. Protein content was measured using coomassie blue staining protocol (7) and the samples were run on a SDS-PAGE. The gels were electronically transferred to polyvinylidene difluoride (PVDF) membranes (Hangzhou Kaijie Membrane Separation Technology Co., Ltd., Hangzhou, China), followed by incubation in 5% non-fat milk (Nestle, York, PA, USA) at 4°C for 6 h. The membranes were incubated with mouse anti-human CIAPIN1 monoclonal IgG (cat. no. sc-271298) overnight at 4°C and further incubated with mouse anti-human (E030170; Earthox, Millbrae, CA, USA) or goat anti-human (PL03b-0297M; PL Laboratories, Port Moody, BC, Canada) horseradish peroxidase-conjugated secondary antibodies for 2 h. A chemiluminescence kit (cat. no. RPN2106; Amersham Pharmacia Biotech, Uppsala, Sweden) was used to detect levels of CIAPIN1, insulin-like growth factor-1U (IGF-1, dilution 1:500; cat. no. sc-1422; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse monoclonal cyclin-dependent kinase 2 (CDK2; cat. no. 610145), mouse monoclonal CDK4, p27 (dilution 1:500; cat. no. 559677; BD Biosciences, San Jose, CA, USA) and rabbit monoclonal retinoblastoma protein (Rb, dilution 1:500; cat. no. 8516; Cell Signaling Technology, Inc. Beverly, MA, USA) in the PVDF membranes.

MTT assay. Exponentially growing cells in each group were diluted into 4x10⁴/ml single-cell suspension and seeded in 96-well cell culture plates at 200 μ l/well. The cells were cultured at 37°C in 5% CO₂, for 24, 48, 72 and 96 h. Subsequently, the cells were cultured in 20 μ l of 5 mg/ml MTT for 4 h. The supernatants were discarded and 150 μ l dimethylsulfoxide (Jingyan Chemicals Co., Ltd) was added to each of the cell cultures, followed by vortexing for 10 min in order to completely dissolve the crystals. The optical density (OD) of 490 nm was measured in a microplate reader (iMARK; Bio-Rad, Hercules, CA, USA). A cell growth curve was created in Excel.

Cell cycle analysis. Synchronized cells in each group were collected at 1x10⁶ cells per sample. The cells were washed twice with PBS, and ice-cold ethanol (75%; Jingyan Chemicals Co., Ltd) was added for overnight fixation at 4°C. Fixed cells were washed once with PBS and 100 μ g/ml RNase was then added, followed by propidium iodide staining (Jingyan Chemicals Co., Ltd) in darkness for 30 min. Flow cytometric analysis (BD FACSCalibur; BD Biosciences) was used to

Table I. CIAPIN1 expression in multiple myeloma tissues and adjacent healthy tissues.

Group	Total cases	CIAPIN1 gene (n)		Positive %
		Positive cases	Negative cases	
Multiple myeloma	32	9	23	28.2 ^a
Healthy bone marrow	32	17	15	53.1

Percentage of positive CIAPIN1 expression in multiple myeloma tissues was significantly lower than that of adjacent normal bone marrow tissues. ^aP<0.05, compared with healthy bone marrow tissues. CIAPIN1, cytokine induced apoptosis inhibitor 1; n, number.

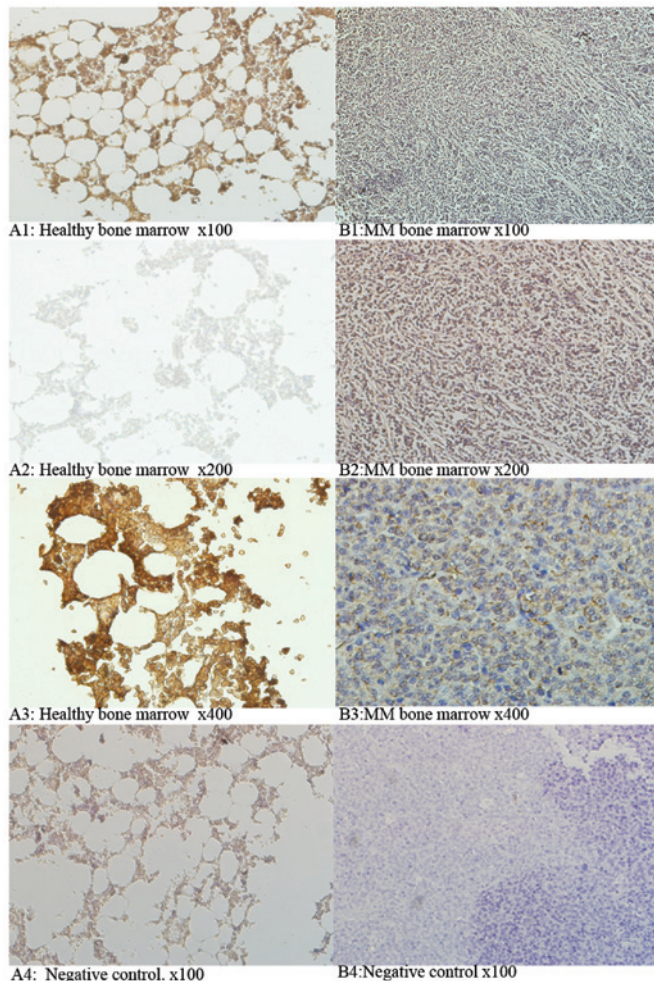


Figure 1. CIAPIN1 protein expression in MM tissues and adjacent paired normal bone marrow tissues. (A) Healthy bone marrow tissues and (B) MM bone marrow tissues. Immunohistochemical images were captured from MM tissues (32 cases) and adjacent tissues (32 cases) at different magnifications A1, x100; A2, x200; A3, x400; A4, negative control, x100; B1, x100; B2, x200; B3, x400; B4, negative control, x100. The percentage of CIAPIN1 (+) samples in MM was 28.2% (9/32). This was significantly lower than 53.1% (17/32) in healthy bone marrow tissues (P<0.05). CIAPIN1, cytokine induced apoptosis inhibitor 1; MM, multiple myeloma.

detect changes in cell cycle progression and ModFit 4.0 software (Verity Software House, Topsham, ME, USA) was used to analyze data in the FC files.

Soft agar colony formation assay. Each cell line was diluted in RPMI-1640 culture medium containing 20% FBS

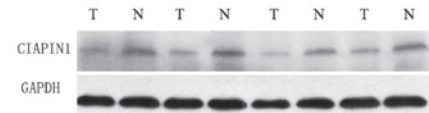


Figure 2. Representative western blot illustrating detection of CIAPIN1 expression in specimens of MM tissue and adjacent healthy bone marrow tissue. MM tissues exhibited a reduction in CIAPIN1 expression compared with their matched adjacent noncancerous tissues. T, MM tissue; N, healthy bone marrow tissue; MM, multiple myeloma; CIAPIN1, cytokine induced apoptosis inhibitor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

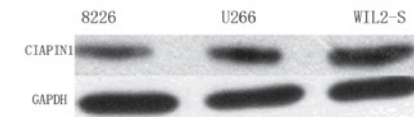


Figure 3. CIAPIN1 expression in MM cell lines and human WIL2-S B lymphocyte cell lines. MM derived cell lines exhibited a decrease in CIAPIN1 expression compared with the healthy B lymphocyte cell lines. MM, multiple myeloma; CIAPIN1, cytokine induced apoptosis inhibitor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(500 cells/ml). Each cell suspension sample (9 ml) was mixed with 1 ml of 3% low-melting point agarose solution (Jingyan Chemicals Co., Ltd) in order to produce the agarose suspension. The agarose suspension was seeded in 6-well cell culture plates at 3 ml/well (three copies for each cell line). Subsequently, the cells were solidified at 4°C for approximately 10 min prior to incubation. Following 18 days of culturing at 37°C and 5% CO₂, cells were stained with 0.05% crystal violet dye (Jingyan Chemicals Co., Ltd) and observed under a microscope (BX51; Olympus Corp., Tokyo, Japan).

Cellular tumorigenicity in nude mice. In total, 24 male BALB/c nude mice (age 4-6 weeks; weight, 18-20 g), were randomly divided into four groups with six mice per group. Cells (0.2 ml; 1x10⁷ cells/ml) in the exponential growth phase from each group were inoculated subcutaneously into the forelimb of nude mice to form a diameter of ~3 mm around the skin rash caused by the tumor cells, using a 1 ml sterile disposable syringe (Minsheng Chemical Co., Ltd.). At 20 days post-inoculation, the nude mice were sacrificed by cervical dislocation and tumors were removed and measured using a vernier caliper. Tumor volume was measured according to the following equation: $V = \frac{a \times b^2}{2}$ (long diameter) x (short diameter)/2. A portion of tumor tissue from each group was fixed in 10% neutral formalin (Jingyan Chemicals Co., Ltd), and stained

Table II. Cell cycle test results of RPMI-8226 cells in each experimental group.

Clones expressing CIAPIN1 protein	RPMI-8226		
	G1	S	G2
RPMI-8226	60.85	31.60	7.55
RPMI-8226-NC	59.41	32.82	7.77
RPMI-8226-siRNA	41.12 ^a	51.80	7.09
RPMI-8226-CIAPIN1	74.04 ^a	20.01	5.95 ^a

^aP<0.05 vs. RPMI-8226 group. Representative experiment of three with similar results. si, small interfering; NC, negative control; CIAPIN1, cytokine induced apoptosis inhibitor 1.

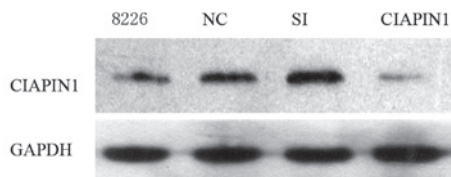


Figure 4. Upregulation and downregulation of lentivirus-mediated exogenous CIAPIN1 gene expression in RPMI-8226 cells. The expression of CIAPIN1 was evaluated using western blot analysis. GAPDH was used as an internal control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 8226, RPMI8226; NC, negative control; SI, small interfering RNA; CIAPIN1, cytokine induced apoptosis inhibitor 1.

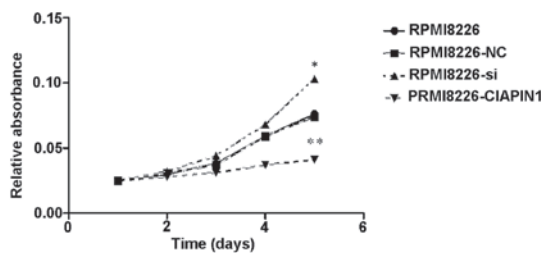


Figure 5. Effect of lentivirus-mediated CIAPIN1 expression on the proliferation of RPMI-8226 cells. Monolayer growth rates of cells were determined by MTT assays. Values represent the mean from at least three separate experiments. CIAPIN1, cytokine induced apoptosis inhibitor 1. *P<0.05 vs. control, **P<0.01 vs. control.

with hematoxylin and eosin (Jingyan Chemicals Co., Ltd) for pathological confirmation. The remaining tissue was stored at -80 °C.

Statistical analysis. Data were analyzed using SPSS 20.0 (IBM SPSS, Armonk, NY, USA) and the results are presented as the mean ± standard deviation. One-way analysis of variance and Student's t-tests were conducted. P<0.05 was considered to indicate a statistically significant difference.

Results

Reduced expression of CIAPIN1 in MM cells. The measurement of CIAPIN1 expression in 32 MM and adjacent healthy

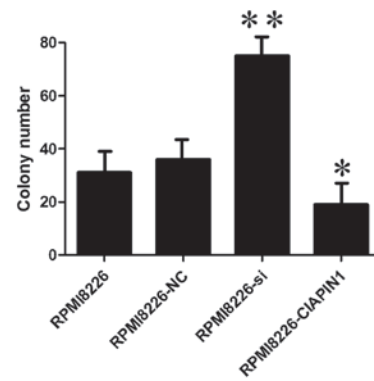


Figure 6. Effect of lentivirus-mediated CIAPIN1 expression on cell colony formation in RPMI-8226 cells. Cells were placed in media containing soft agar and cultured for 18 d. Values represent the mean from at least three separate experiments. Values are presented as the mean ± standard error of the mean for ≥3 independent experiments. *P<0.05 vs. control and **P<0.01 vs. control. CIAPIN1, cytokine induced apoptosis inhibitor 1; NC, negative control; si, small interfering RNA.

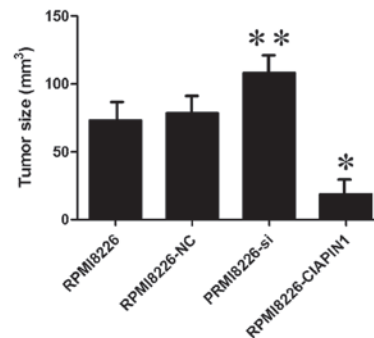


Figure 7. Inhibitory effect of CIAPIN1 on murine tumor sizes. The average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. These tumors were verified as being multiple myeloma by hematoxylin and eosin staining. Values are presented as the mean ± standard error of the mean for ≥3 independent experiments. *P<0.05 vs. control and **P<0.01 vs. control. CIAPIN1, cytokine induced apoptosis inhibitor 1; NC, negative control; si, small interfering RNA.

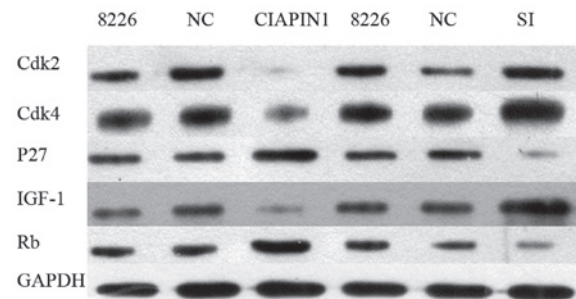


Figure 8. Expression of cell cycle-regulatory proteins and ICF-1. All gene expression levels were quantitatively analyzed and are expressed as the ratio of the levels of GAPDH. The expression of Cdk2, Cdk4, Rb, p27 and IGF-1 proteins were evaluated before and after infection using western blot. Cdk, cyclin-dependentkinase; CIAPIN1, cytokine induced apoptosis inhibitor 1; IGF-1, insulin-like growth factor-1; Rb, retinoblastoma protein; 8226, RPMI8226; NC, negative control; SI, small interfering RNA.

bone marrow tissue samples using immunohistochemistry, suggested that CIAPIN1 is primarily located in the cytoplasm and cell membranes of MM cells (Fig. 1). MM tissue samples

consisted of 28.2% (9/32) CIAPIN1 (+) cells, which was significantly lower than the proportion in the healthy bone marrow tissue of 53.1% (17/32; $P < 0.05$; Table I). Compared with adjacent healthy bone marrow tissue, there was a reduction in the expression of CIAPIN1 in the MM tissue (Fig. 2). CIAPIN1 expression was also lower in human MM cell lines than in human WIL2-S B lymphocyte cells (Fig. 3).

Overexpression of the CIAPIN1 protein inhibits growth and tumorigenicity of MM cells in vitro and in vivo. Transduction of the CIAPIN1 gene, Ad-CIAPIN1, in the RPMI-8226 cells, caused the CIAPIN1 protein levels to increase (Fig. 4), which significantly inhibited RPMI-8226 cell growth (Fig. 5, $P < 0.05$). However, the siRNA group exhibited the opposite effect (Fig. 4). In the colony formation assay, Ad-CIAPIN1-transfected tumor cells formed significantly fewer colonies compared with the control cells (Fig. 6, $P < 0.05$). Furthermore, in the *in vivo* subcutaneous tumor formation experiments, inoculation with RPMI-8226-CIAPIN1 cells produced tumors of markedly reduced sizes, compared with the inoculation of parental cells with empty vector-transfected cells (Fig. 7, $P < 0.05$). However, the siRNA cells exhibited the opposite effect. Thus, the results of *in vitro* and *in vivo* tests suggest that CIAPIN1 inhibits tumor cell proliferation.

CIAPIN1 proteins target cell cycle-regulatory genes, induce cell cycle arrest in the G1/S phase and inhibit IGF-1 secretion. Flow cytometric analyses showed that the level of expression of CIAPIN1 proteins had a significant effect on the cell cycle in RPMI-8226 cells (Table II). At 48 h following transduction, 74.04% of RPMI-8226-CIAPIN1 cells were in the G1 phase, whereas only 59.41% of RPMI-8226-NC cells were in the G1 phase (Table II, $P < 0.05$). No significant differences were observed in the percentage of cells in the G2 phase between groups. However, the RPMI-8226-siRNA group exhibited a substantial reduction in the proportion of cells in the G1 phase (Table II, $P < 0.05$). These results indicate that CIAPIN1 inhibits cell entry into the S phase. Therefore, CIAPIN1 directly acts on cells in order to inhibit cell cycle progression, which may in part explain the inhibitory effect of CIAPIN1 on the growth of MM cells. In order to investigate the molecular mechanisms underlying CIAPIN1-induced cell cycle arrest, the levels of cell cycle regulatory genes (CDK2, CDK4 and IGF-1) were analyzed. The results indicated that upregulation of CIAPIN1 protein reduces the expression of CDK2, CDK4 and IGF-1, but enhances that of p27 and Rb proteins. By contrast, the downregulation of CIAPIN1 by specific siRNA led to the opposite effect (Fig. 8). Therefore, CIAPIN1 appears to inhibit the growth of MM cells through the regulation of various proteins involved in the G1/S progression. Furthermore, CIAPIN1 may disrupt IGF-1 expression and reduce the growth, survival, adhesion, migration and drug resistance of MM cells.

Discussion

MM remains a clinical challenge due to its complex pathogenesis. Certain genes have been identified that are involved in the progression of MM. However, further research is required in order to identify diagnostic markers and therapeutic target

proteins in MM. The present study showed that CIAPIN1 gene expression was significantly lower in MM tissues, suggesting that CIAPIN1 expression may be correlated with the development of MM and thus act as a potential novel diagnostic marker.

CIAPIN1 expression was modified in MM cell lines by lentiviral transduction, and *in vitro* and *in vivo* cell proliferation was subsequently investigated. The results suggested that CIAPIN1 arrested the cell cycle at the G1/S phase and inhibited the growth of MM cells. In order to further explore how CIAPIN1 inhibits the growth of MM cells, the expression of molecules involved in cell cycle regulation (CDK and CDK inhibitor molecules) was investigated in primary MM and transfected cells. CDK2 and CDK4 are involved in G1/S transition (8,9). The expression of cyclin D and CDK4 promotes the phosphorylation of Rb during the early to mid-G1 phase of the cell cycle (10). At the mid-G1 point of the cell cycle and towards the end of the G1/S phase, the expression of CDK2 also enhances the phosphorylation of Rb (11). The p27 protein negatively regulates cell cycle progression and inhibits the activity of a variety of cyclins and CDKs. However, p27 primarily inhibits kinases that are involved in the G1 phase, such as the cyclin E-CDK2 and cyclin D-CDK4 kinase complexes, p27 therefore prevents cells from progressing to the S phase (12,13). The present study indicates that CIAPIN1 is involved in cell cycle progression, in part by inhibiting the expression of CDK4 and CDK2. In addition, it enhances the expression of p27, thereby inhibiting Rb phosphorylation. Furthermore, the proliferative effects of IGF-1 on the interleukin-6-dependent and non-dependent MM cell lines have previously been investigated (14). Following chemotherapy, patients with low levels of serum IGF-1 exhibited reduced utilization of IGF-1 in the bone marrow, a reduction in growth stimulated by paracrine signaling and a remission of malignant clone (15). The results of the present study suggest that the upregulation of CIAPIN1 expression suppresses the expression of IGF-1 and may inhibit IGF-1-induced proliferation, apoptosis, adhesion, migration and drug resistance in MM cells.

In conclusion, the present study suggests that CIAPIN1 may function as a tumor suppressor due to its reduced expression in MM cells. The results indicate that CIAPIN1 may suppress tumor growth through inhibition of CDKs and IGF-1. However, a more precise mechanism remains to be further elucidated.

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