Lower expression of the *RASSF10* gene induces myeloma cell proliferation due to hypermethylation of the gene promoter in multiple myeloma

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Abstract

Background: Multiple myeloma (MM) is an incurable malignant neoplasm of plasma cells, in which genetic defects, epigenetic aberrations and bone marrow microenvironment are involved in the pathogenesis. *RASSF10* acts as a tumor suppressor gene by methylation in glioma and several other cancers, but its role in MM remains unknown.

Methods: In order to explore the role of *RASSF10*, mRNA expression was detected in MM patients and analyzed with overall survival.

Results: Expression of the *RASSF10* gene significantly decreased in newly diagnosed MM patients, and was positively correlated with overall survival. RPMI-8226 and OPM-2 cell lines with lower *RASSF10* expression were selected for further study. Overexpression of *RASSF10* in these two cell lines inhibited proliferation and induced apoptosis. The *RASSF10* gene promoter in MM cell lines was hypermethylated, and downregulated after decitabine treatment. Meanwhile, expression of the *RASSF10* gene was upregulated. MM cells with overexpression of *RASSF10* were injected into nude mice and exerted anti-MM activity *in vivo*.

Conclusions: Low expression of *RASSF10* contributed to the proliferation of myeloma cells by hypermethylation of its promoter.

Background

Multiple myeloma (MM) is a malignant neoplasm of plasma cells that accumulate in the bone marrow (BM) and is characterized by end-organ damage (CRAB) caused by secretion of a monoclonal protein (M-protein) (1). Over the last 10 years, therapy of MM has improved using novel agents, including proteasome inhibitors and immunomodulatory drugs, in combination with autologous stem cell transplantation, alkylating agents and/or glucocorticoids. Unfortunately, MM remains an incurable disorder and all patients ultimately relapse due to the development or drug resistance. Therefore, further breakthroughs are still needed to improve patient outcome (2). Apart from the central role of genetic defects, an increasing number of studies has shown that epigenetic aberrations are also involved in the pathogenesis of MM. DNA methylation and post-translational histone modifications are the most common epigenetic mechanisms known to disturb normal gene expression (3-6).

The *RASSF* family encodes distinct tumor suppressors, which consists of six classical members (*RASSF1–6*) and four N-terminal members (*RASSF7–10*). The former group contains both the *RA* domain and the *SARAH* domain, and the latter contains an *RA* domain within its extreme N termini but lacks the *SARAH* domain. *RASSF* functions shown epigenetic silencing, *RAS* signaling, microtubule stability, cell cycle apoptosis, and immune system and nuclear transport (7,8). *RASSF10*, as the newest member, has a CpG island of 2254 bp with 209 CpGs and acts as a tumor suppressor gene by methylation of the gene promoter in glioma and several other cancers, such as thyroid cancer, prostate carcinoma, and leukemia (9-12). Recent research shows that *RASSF10* is hypermethylated in B-acute lymphocytic leukemia (B-ALL)

and chronic Lymphocytic Leukemia (CLL), and associated with prognostic parameters (13). MM is a B-cell malignant disease, which has been investigated for treatment with DNA methyltransferase (DNMT) inhibitors (decitabine or 5-azacytidine) (14). However, the role of *RASSF10* in MM remains unknown.

Materials And Methods

Patients

Thirty newly diagnosed MM patients, 17 remission patients and 19 normal controls were enrolled in this study. All the patients were inpatients in the Department of Hematology, Tianjin Medical University General Hospital from June 2016 to August 2017. All MM patients were diagnosed according to the Guidelines for the Diagnosis and Management of Multiple Myeloma in China (2017 revision) (15). The clinical features of all MM patients are shown in Table 1. The remission patients who received bortezomib-basic regimens for at least four cycles were defined as very good partial response, complete response or stringent complete responses. Meanwhile, supportive therapies were given in these patients, including blood transfusion and anti-infective agents. Ten milliliters of bone marrow (BM) was taken from the patients and normal controls. This study was approved by the Ethical Committee of the Tianjin Medical University (IRB2020-WZ-075 and IRB2020-DW-04). Written informed consent was obtained from the patients for publication.

Cell lines

Human MM cell lines RPMI-8226, OPM-2 and U266 were obtained from the Cell Culture Center, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. All cell lines were cultured in 90% RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C with 5% CO_2 and 95% humidity.

Magnetic-activated cell sorting (MACS)

BM mononuclear cells (BMMCs) were isolated from heparin-anticoagulated BM of MM and normal controls using Ficoll-Hypaque density gradient centrifugation. $CD138^+$ cells were purified using the anti-CD138 mAb-conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Ten million cells were resuspended in 80 ml buffer. Then, 20 ml CD138 MicroBeads (Miltenyi Biotec) were added and incubated at 4°C in the dark for 15 min. After washing with 2 ml buffer, the cells were centrifuged at 300 g for 5 min. The cells were resuspended up in 500 ml buffer. The MS column was placed in the magnetic field of a suitable MACS separator (Miltenyi Biotec). After preparing the column by rinsing with 3´ 500 ml buffer, the cells were applied to the column. The column was washed with 1 ml buffer and all flow-through containing unlabeled cells was collected. Magnetically labeled cells were immediately flushed out by firmly pushing the plunger into the column and collected. The purity of enriched $CD138^+$ cells isolated was evaluated by flow cytometry and was generally >90%.

RNA expression analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, California, USA). First-strand cDNA was generated using the FastQuant RT Kit (Tiangen, Beijing, China). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate with SYBR Green (Tiangen) using IQ5 PCR instrumentation (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR primers were as follows: 5'-GGCTCAACACGGACCTAGAG-3'(F), 5'-GTCAGCTCCAAAGTGTGCAA-3'(R). β-Actin was used as an internal control.

DNA isolation and methylation analysis by bisulfite sequencing

DNA was extracted from MM cell lines and normal controls with a TIANamp Genomic DNA kit (Tiangen), and concentrations of DNA were determined by a micro-ultraviolet spectrophotometer (Bio-Rad). Bisulfite sequencing was performed by Genechem (Shanghai, China). The experiment was repeated three times and the values were averaged.

Lentivirus transfection

RASSF10 lentivirus was purchased from Genechem, and lentivirus was transfected into MM cell lines and cells. The MOI=100, the cells were not selected using antibiotics, and Con238 (Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin) was used as a control. The efficiency of transfection was measured by flow cytometry and inverted microscopy.

Cell proliferation

MM cell lines and cells at 72 h after transfection were analyzed for proliferation and apoptosis. Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8) (Engreen, Beijing, China). Absorbance at 450 nm was read at 1, 2, 3 and 4 days using a 96-well microplate reader (BioTek, Winooski, VT, USA). The experiment was repeated three times and the values were averaged.

Flow cytometry

The proportion of cells undergoing apoptosis was measured using the Apoptosis Detection kit (BD Bioscience, San Diego, CA, USA). Cells were stained with fluorescein isothiocyanate–annexin V and propidium iodide (PI) and analyzed with a flow cytometer (FACScan; BD Biosciences, Mountain View, CA, USA). All assays were conducted in triplicate. The experiment was repeated three times and the values were averaged.

Western blotting

Cellular proteins were extracted in radio-immunoprecipitation assay buffer (Beyotime, Shanghai, China) and protein concentrations were determined using a BCA assay kit (Beyotime). Cell extracts (30 mg) were boiled with equal amounts of loading dye for 10 min and separated by 12% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Hybond-ECL; Thermo Fisher Scientific, Shanghai, China). Membranes were blocked in Phosphate buffered saline (PBS) with 0.1%

Tween 20 (PBS-T) containing 5% non-fat milk for 1 h, and incubated with primary and secondary antibodies in PBS-T containing 5% non-fat milk. The following primary antibodies were used: RASSF10 (diluted 1:1000) (Abcam, Cambridge, UK), bcl-2 (diluted 1:1000), caspase3 (diluted 1:500), GAPDH (diluted 1:1000) (Cell Signaling Technology, Danvers, MA, USA). Primary antibody incubation was carried out overnight at 4°C. The membranes were washed with wash buffer (1×PBS and 0.01% Tween-20) and incubated with anti-rabbit or anti-mouse secondary antibody. The experiment was repeated three times and the values were averaged.

In vivo tumor growth in nude mice

Female BALB/c-nu nude mice aged 4–5 weeks were purchased from Beijing Hua Fukang Bioscience Company and were housed and monitored in a pathogen-free environment. RPMI-8226 MM cell line and transfected cells ($n=10^7$) were prepared in 100 ml serum-free RPMI-1640 medium and injected subcutaneously into the right dorsal flank of nude mice (n=3 each group). Measurement of tumor volume and tumor quality was performed after 21 days, and tumor volume (V) was calculated using the formula: $V=0.5\times a\times b^2$, where a and b represent the longer and shorter tumor diameters, respectively. At the end of each study, animals were killed and tumors were collected and fixed in formalin for hematoxylin and eosin (HE) staining and immunohistochemical staining of anti-CD138 or anti-RASSF10 antibody to assess tumor growth. CD138 and *RASFF10* staining was quantified by Image J software. All procedures were approved by the Animal Ethics Committee of the Tianjin Medical University General Hospital.

Statistical analysis

Student's *t*-test was conducted for two-group comparisons. For many-group comparisons, one-way ANOVA (if the data were normally distributed) or Kruskal–Wallis test (if the data were not normally distributed) was used. The data are expressed as the mean ± SEM or median. Kaplan–Meier survival curves were constructed, and difference in survival rates was tested by log-rank test. Statistical analyses were performed using SPSS version 21.0. A value of $p<0.05$ was considered significant.

Results

Expression of the *RASSF10* gene significantly decreased in newly diagnosed MM patients and MM cell lines, and was positively correlated with overall survival

The purity of $CD138^+$ cells sorted from BMMCs was >90%. Expression of *RASSF10* mRNA in the newly diagnosed MM (NDMM) group (0.20±0.29) was significantly lower than that in the remission group (0.64±0.61) and normal control group (0.62±0.61) (both $p<0.01$), while there was no significant difference between the latter two groups (Fig. 1A). Expression of *RASSF10* mRNA in MM cell lines RPMI-8226, OPM-2 and U266 was significantly more decreased than NCI-H929 and LP-1 (Fig. 1B). Protein expression of the *RASSF10* gene was downregulated in the newly diagnosed MM group and MM cell lines RPMI-8226, OPM-2 and U266 (Fig. 1C).

The median follow-up time of NDMM patients was 18 months (95% CI 15–22 months). The overall survival of patients with higher expression of *RASSF10* mRNA (median selected as the cut-off value = 0.1) was 21 months (95% CI 18–25 months), which was significantly longer than for patients with lower expression of *RASSF10* mRNA (13 months, 95% CI 9–17 months) (p<0.05) (Fig. 1D).

Overexpression of *RASSF10* in RPMI-8226 and OPM-2 cells inhibited proliferation and induced apoptosis

The transfection efficiency rates of the *RASSF10* gene in MM cell lines RPMI-8226, OPM-2 and U266 was 69.3%, 58.67% and 34.7%, respectively. RPMI-8226 and OPM-2 cells were chosen for further experiments. After transfection, expression of the *RASSF10* gene was significantly upregulated in RPMI-8226 and OPM-2 cells (p=0.0007 and p=0.0185, respectively) (Fig. 2A).

Proliferation of RPMI-8226/RASSF10 cells by CCK-8 was significantly decreased compared with RPMI-8226/control at 48, 72 and 96 h (p<0.001, p<0.001 and p<0.001, respectively), while there was no significant difference at 24 h (Table 2 and 3), (Fig. 2B1,2). The apoptosis rate of RPMI-8226/RASSF10 cells was elevated significantly at 24, 72 and 96 h (p<0.05, p<0.05 and p<0.01, respectively). Similar results were found for OPM-2/RASSF10 cells (Table 2, Fig. 2C1,2; D1,2). Furthermore, apoptosis-related proteins were detected by western blotting. Protein expression of the *RASSF10* gene was upregulated. The expression of apoptosis-related protein the cleaved caspase-3 was up-regulated, while bcl-2, an apoptosis inhibitor, was down-regulated at the protein level. (Fig. 2E).

The *RASSF10* gene promoter in MM cell lines was hypermethylated and expression of the *RASSF10* gene was upregulated after decitabine treatment

Methylation of the *RASSF10* gene promoter (CpG island divided into Pairs 1–6) in MM cell lines RPMI-8226 and OPM-2 was detected by bisulfite sequencing. The methylation levels of Pair 2 in RPMI-8226 and Pairs 2 and 5 in OPM-2 cells were significantly higher compared with those in normal controls (p<0.001, p<0.05 and p<0.05, respectively), while other levels were low (Fig. 3A).

We treated these MM cell lines with decitabine (0, 0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 mM) at 24, 48 and 72 h. Proliferation of RPMI-8226 cells significantly reduced from 0.9640 (0 mM) to 0.8606 (1.25 mM), 0.8567 (2.5 mM), 0.8430 (5 mM), 0.8103 (10 mM) and 0.8263 (20 mM) at 48 h. The results were better at 72 h, from 1.1340 (0 mM) to 0.5297 (20 mM) (Fig. 3B1). Apoptosis of RPMI-8226 cells at 72 h significantly increased to 22% (1.25 mM), 28% (2.5 mM), 39% (5 mM), 43% (10 mM) and 44% (20 mM) (Fig. 3B2). In OPM-2 cells, there were significant differences from 1.25μM DAC at 72h, which are less effective than RPMI-8226 (Fig 3C1,2).

We further detected the methylation of the *RASSF10* gene promoter in RPMI-8226 cells after decitabine treatment. The methylation level was partly downregulated in Pair 2 after 2.5 mM decitabine treatment at 72 h (p<0.05) (Fig 3D). At the same time, the level of *RASSF10* mRNA in RPMI-8226 cells significantly increased from ($1.57 \times 10^{-5} \pm 2.02 \times 10^{-6}$) to ($1.44 \times 10^{-3} \pm 7.79 \times 10^{-4}$) after decitabine treatment (2.5 mM) at

48 h (p<0.05) (Fig 3E). The results indicated that the upregulated *RASSF10* gene level was due to demethylation by decitabine.

Overexpressed *RASSF10* exerts anti-MM activity *in vivo*

Nude mice (BALB/c-nu) were injected subcutaneously with RPMI-8226 and OPM-2 cells in the right scapular region. The RPMI-8226 nude mice had subcutaneous tumor formation. The lymph nodes, liver and spleen were significantly enlarged, and myeloma cells infiltrated the liver. After the recovery of *RASSF10* expression (RPMI-8226/RASSF10 cells), the subcutaneous tumor formation volume was reduced, and myeloma cells did not infiltrate the liver. The tumor volume and mass of the RPMI-8226 control group were 96.97±15.22 mm^3 and 87.57±19.75 mg at 21 days, respectively. The tumor volume and mass of the RPMI-8226/RASSF10 group were significantly reduced to 16.56±3.15 mm^3 and 19.90±4.60 mg, respectively (both p<0.001) (Fig. 4A, B).

The tumor tissue formed subcutaneously in nude mice was stained with HE and showed a large number of irregular cells, short spindle or cubic, with irregular nuclei, coarse staining, and obvious nucleoli. Immunohistochemistry showed $CD138^+$ cells (40–50%) in the RPMI-8226/control group, which became weaker (~30%) in the RPMI-8226/RASSF10 group. *RASSF10* gene expression was upregulated from 10–20% in the RPMI-8226/control group to 50–60% in the RPMI-8226/RASSF10 group (Fig. 4C).

Discussion

RASSF10 acts as a tumor suppressor gene in some cancers, including non-hematological and hematological malignancies, such as ALL and CLL. However, the role of *RASSF10* remains unknown in MM. In our study, we found that *RASSF10* mRNA expression was significantly lower in newly diagnosed MM patients compared with normal controls, and upregulated after remission, which was associated with survival. Furthermore, overexpression of the *RASSF10* gene inhibited proliferation of MM cell lines *in vitro*. After injection of RPMI-8226/RASSF10 cells into mice, we found that myeloma mass decreased. These results indicate that the *RASSF10* gene may contribute to the pathogenesis of MM. However, the mechanism of this gene remains unclear in MM.

Wei et al. (16) studied the function of *RASSF10* in gastric cancer, and showed that the *RASSF10* gene was silenced in 75% of cell lines and totally methylations and partly methylations in promotor at six cell lines by Methylation-Specific PCR (MSP). Furthermore, that study indicated that the *RASSF10* gene inhibited proliferation of cancer cells by Wnt/β-catenin signaling *in vivo*. Jin et al. (17) showed similar results in hepatocellular carcinoma, in which *RASSF10* suppressed hepatocellular carcinoma growth by activating p53 signaling. *RASSF10* induced blockage of the G2/M phase and made cancer cells sensitive to docetaxel, which indicates that *RASSF10* is a resistance marker. Western blotting showed upregulation of p53 and p21 and downregulation of MDM2 and bcl-2 after overexpression of *RASSF10*. In our study, we treated MM cell lines with decitabine, a demethylation agent, to observe the level of *RASSF10* gene

expression. As we expected, methylation of the *RASSF10* promoter was downregulated and *RASSF10* gene expression upregulated after decitabine treatment.

Epigenetic aberrations play an important role in the mechanism of MM, especially DNA methylation. Hypermethylation of genes seems to be associated with the progression of monoclonal gammopathy of undetermined significance to MM and to plasma cell leukemia (18). Many studies have revealed hypermethylation of specific loci (19) and some of them are associated with poor prognosis of MM patients, including *SPARC*, *BNIP3*, *DAPK*, *RARβ*, *EGLN3*, *DCC*, *TGFβR2*, *CD9*, *RASD1* and p16 (20-26). These findings stress the importance of hypermethylated genes in MM. Therefore, DNMT inhibitors can target this aberrant DNA methylation in MM, such as 5-azacytidine (AZA) and decitabine. Lavelle et al. revealed that decitabine restored the expression of p16 by DNA demethylation in MM cell lines. In addition, decitabine induced G0/G1- and G2/M-phase arrest linked with p21 or p38, respectively (27). Another study showed that decitabine has potent anti-myeloma activity *in vitro* by depleting myeloid-derived suppressor cells in BM (28). However, use of AZA or decitabine in MM is limited because of induction of DNA damage. Recent research showed that decitabine enhanced the effect of bortezomib in an MM cell line (29). Decitabine, combined with quisinostat, a histone deacetylase inhibitor, showed increased anti-myeloma effects and altered immune cell constitution, such as increased dendritic cells and naive T cells, in a mouse myeloma model (30). Another study indicated that decitabine-mediated apoptosis in MM can be enhanced by combination with histone deacetylase inhibitor (31). In our study, decitabine alone reduced apoptosis by 50% *in vitro*, which indicates decitabine may be used in combination with other drugs.

Conclusion

The *RASSF10* gene is significantly downregulated in newly diagnosed MM patients and positively correlated with overall survival. The *RASSF10* gene inhibits the proliferation of myeloma cells *in vitro* and *in vivo*. We demonstrated the hypermethylation of the promoter of *RASSF10*, which can be modified by decitabine. To our knowledge, this is the first study to reveal the role of *RASSF10* in MM and explore the effect of decitabine on this gene. However, the effect of decitabine alone in MM is not satisfactory, and combination with other types of drugs should be tried in the future.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University (IRB2020-WZ-075 and IRB2020-DW-04). Written informed consent was obtained for all patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RF designed the research plan and revised the manuscript. JC and HL performed the experiments, analysed the data and wrote the manuscript. ZL, QS, FJ and SY contributed to the experimental work. LL, JS and KD recorded the clinical characteristics of the patients with MM. All authors read and approved the final version of the manuscript.

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Abbreviations

MM: Multiple myeloma

BM: bone marrow

M-protein: monoclonal protein

B-ALL: B-acute lymphocytic leukemia

CLL: chronic Lymphocytic Leukemia

DNMT: DNA methyltransferase

FBS: fetal bovine serum

MACS: Magnetic-activated cell sorting

BMMCs: BM mononuclear cells

PCR: polymerase chain reaction

PBS: Phosphate buffered saline

MOI: multiplicity of infection

HE staining: hematoxylin and eosin staining

NDMM: newly diagnosed MM

AZA: 5-azacytidine

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Tables

Table1. The clinical characteristics the patients of newly diagnosed and remission MM

Clinical features	Newly diagnosed MM	Remission MM	Controls
N	30	17	19
Median age（range）	66(44-81)	60(45-75)	37(19-69)
Gender（N, %）			
Male	17(56.7)	7(41.2)	9(47.4)
Female	13(43.3)	10(58.8)	10(52.6)
Subtpye			
Light chain MM (N, %)	7(23.3)	2(11.8)	
IgG κ (N, %)	10(33.3)	8(47.1)	
IgG λ(N, %)	7(23.3)	1(5.88)	
IgA κ(N, %)	2(6.67)	1(5.88)	
IgA λ (N, %)	4(13.3)	1(5.88)	
Non secreting type(N, %)	0	4(23.5)	
ISS stage (N, %)			
	3(10.0)	7(41.2)	
	15(50.0)	5(29.4)	
	12(40.0)	5(29.4)	
High-risk Fish(N, %)	11(36.7)	5(29.4)	

MM: multiple myeloma; N: number;

Table 2. The cell proliferation rates of MM cell lines after overexpression of RASSF10.

	Cell proliferation (OD value)			
	24h	48h	72h	96h
RPMI-8266/Cnt	0.94±0.01	1.67±0.06	1.81±0.06	2.17±0.08
RPMI-8266/RASSF10	0.98±0.03	1.27±0.08*	1.28±0.05*	1.35±0.02*
OPM-2/Cnt	1.34±0.03	1.68±0.10	1.84±0.03	2.38±0.05
OPM-2/RASSF10	1.34±0.03	1.50±0.04*	1.62±0.08*	1.99±0.01*

*compared with Cnt group, $p<0.01$.

Table 3. The cell apoptosis rates of MM cell lines after overexpression of RASSF10.

	Apoptosis rate (%)			
	24h	48h	72h	96h
RPMI-8266/Cnt	27.37±2.67	39.42±3.38	40.97±1.73	47.99±2.62
RPMI-8266/RASSF10	41.02±4.63*	44.76±5.07	52.31±1.23*	63.96±8.19*
OPM-2/Cnt	17.60±0.99	19.12±2.23	20.25±1.14	24.70±0.78
OPM-2/RASSF10	32.47±2.55*	35.26±7.03*	33.38±1.72*	44.04±1.72*

*compared with Cnt group, $p<0.01$.

Figures

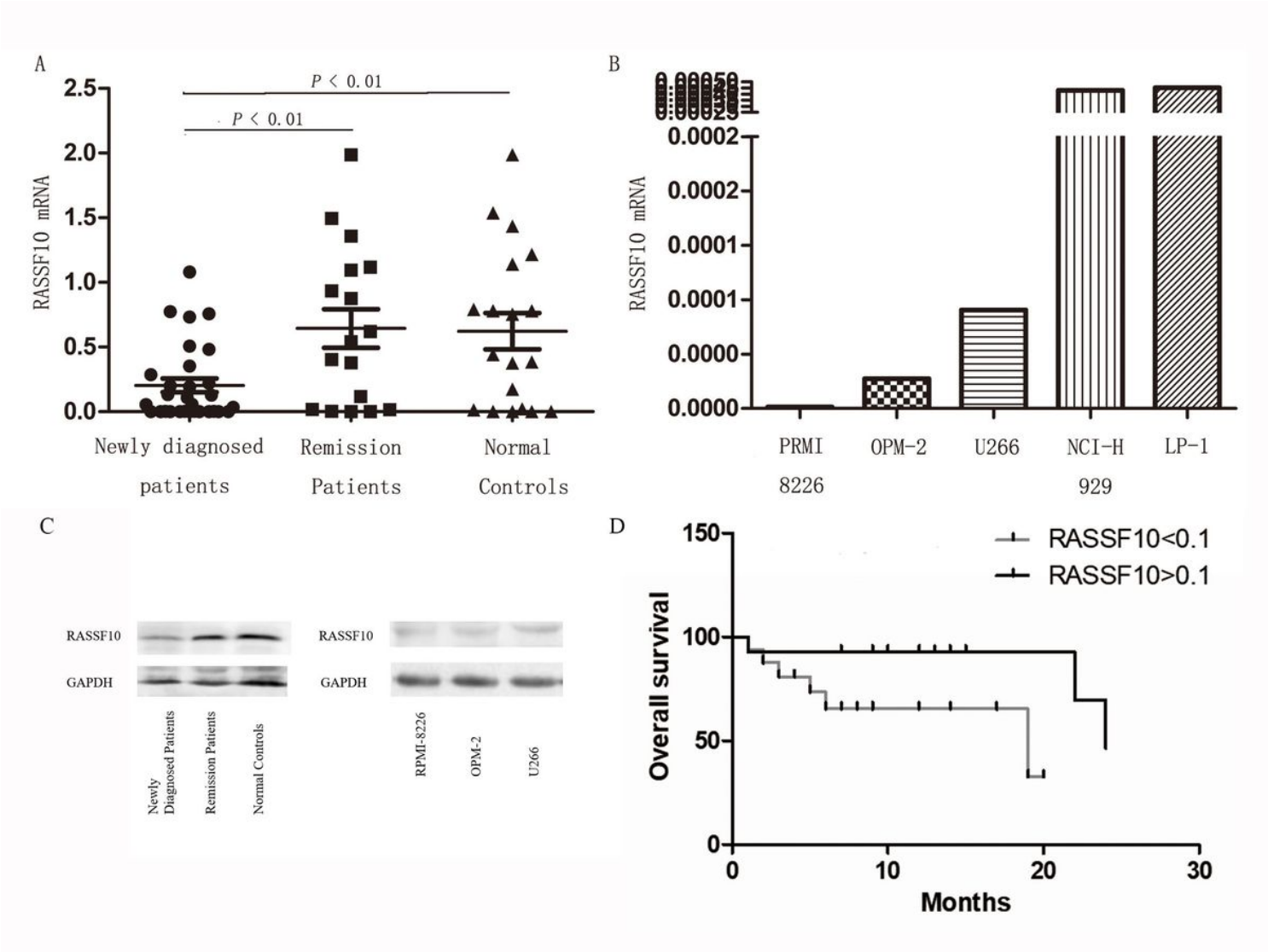

Figure 1

Expression of RASSF10 mRNA in MM patients and cell lines. A. RASSF10 mRNA expression was significantly lower in newly diagnosed MM patients. B. RASSF10 mRNA expression in different MM cell lines. C. Protein expression of the RASSF10, MM patients, normal control and MM cell lines RPMI-8226, OPM-2 and U266 as detected by western blotting. D. Overall survival of newly diagnosed MM patients with different levels of RASSF10 expression.

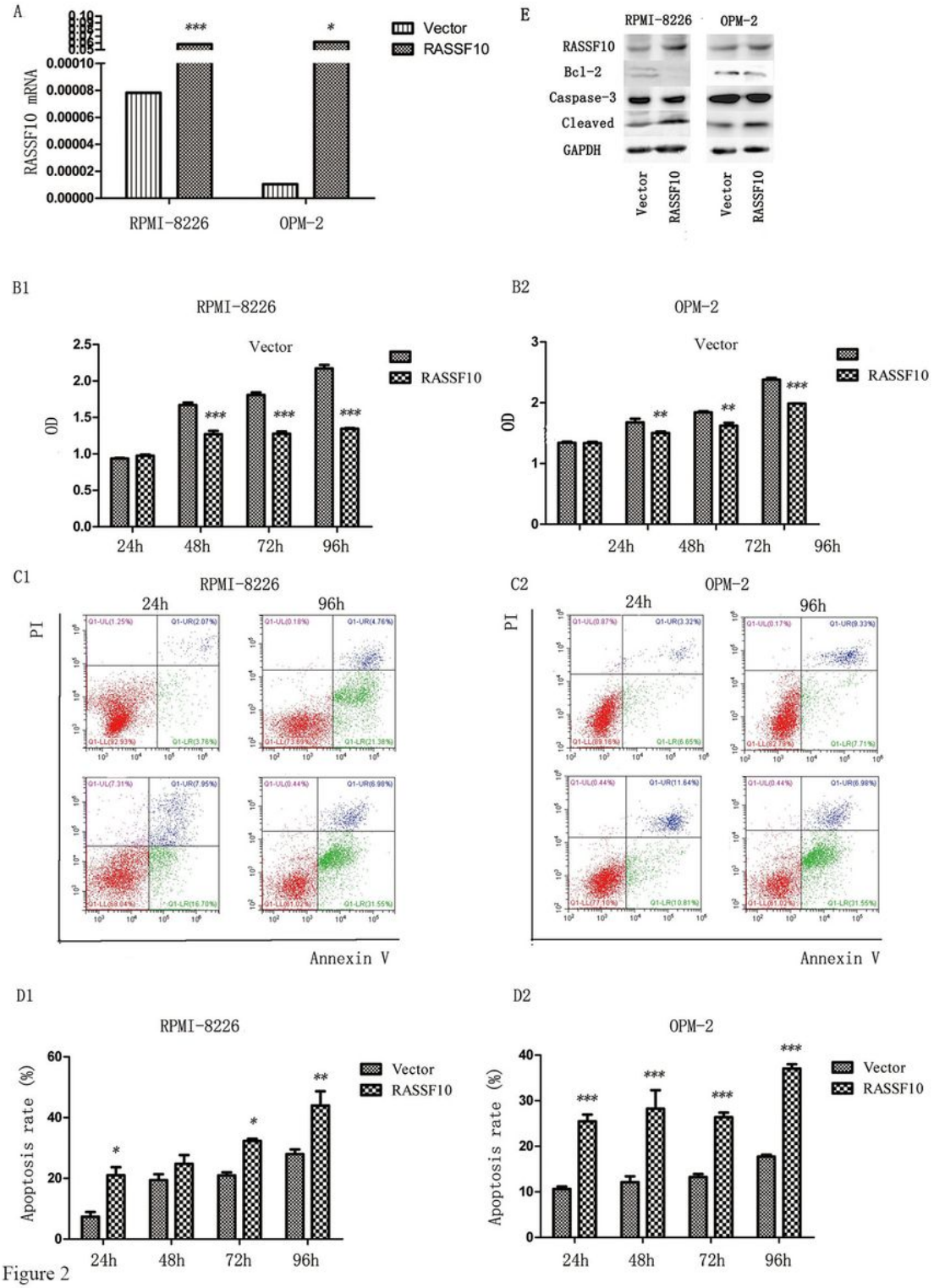

Figure 2

Figure 2

Proliferation of MM cell lines was inhibited after RASSF10 overexpression. A. RASSF10 expression upregulated after transfection in RPMI-8226 and OPM-2 cells. B. Cell survival was inhibited after RASSF10 overexpression in RPMI-8226 (B1) and OPM-2 (B2) cells. C. Apoptosis was detected by flow cytometry using annexin V and PI in RASSF10 overexpression in RPMI-8226 (C1) and OPM-2 (C2) cells. D. Apoptosis of cell lines increased after RASSF10 overexpression in RPMI-8226 (D1) and OPM-2 (D2) cells.

E. Protein expression of RASSF10, apoptosis-related protein the cleaved caspase-3 and apoptosis-related protein bcl-2 as detected by western blotting. *p<0.05, **p<0.01, ***p<0.001.

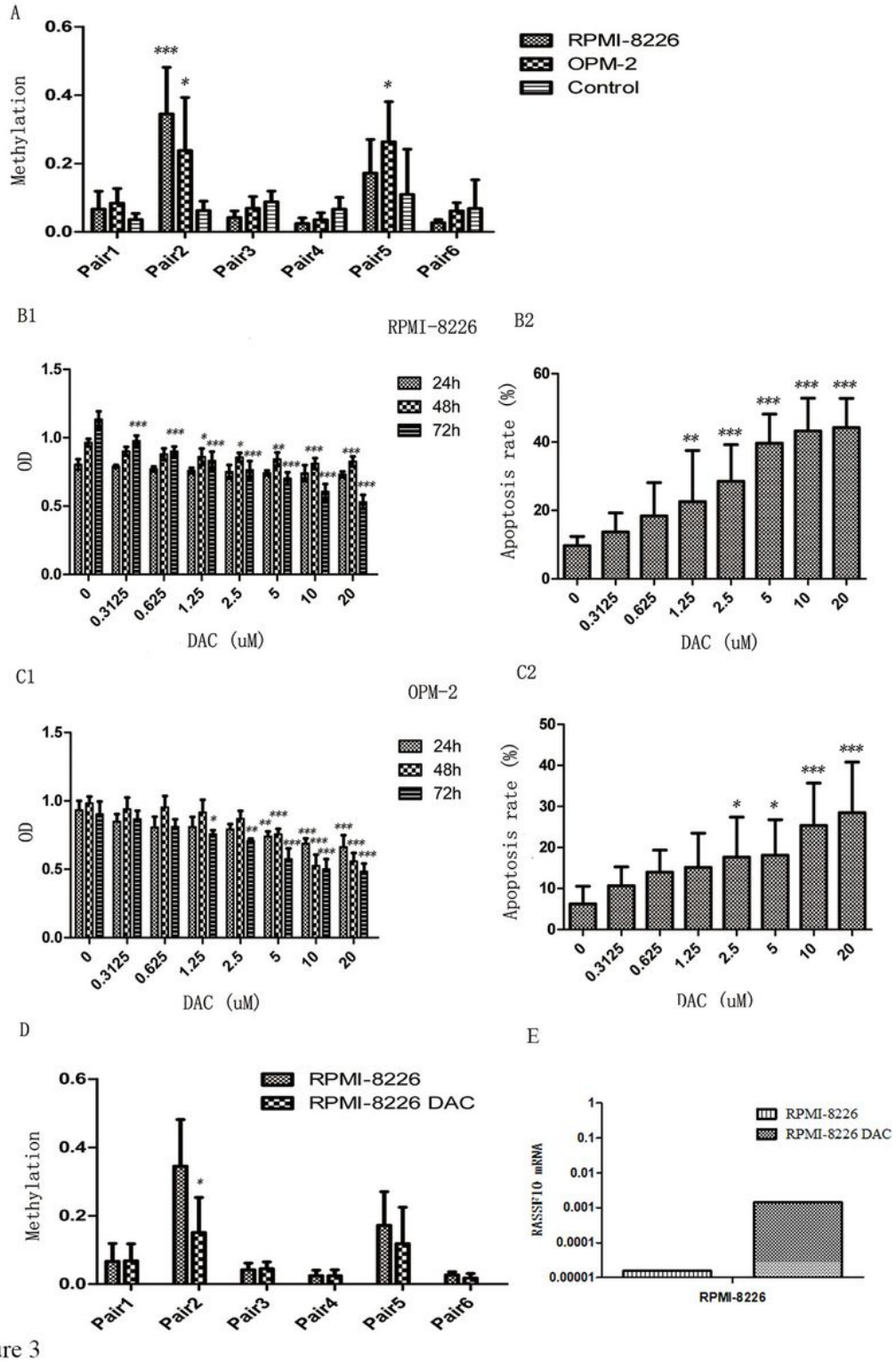

Figure 3

Figure 3

MM cell lines treated with decitabine and methylation of RASSF10 promoter downregulated after decitabine treatment. A. Methylation of the RASSF10 gene promoter in RPMI-8226 and OPM-2 cells detected by bisulfite sequencing. B. Survival of RPMI-8226 cells after decitabine treatment for 24, 48 and

72 h (B1). Apoptosis of RPMI-8226 cells after decitabine treatment for 72 h (B2). C. Survival of OPM-2 cells after decitabine treatment for 24, 48 and 72 h (C1). Apoptosis of OPM-2 cells after decitabine treatment for 72 h (C2). D. Methylation of the RASSF10 gene promoter in RPMI-8226 cells detected after decitabine treatment (2.5 □M) for 72 h. *p<0.05, **p<0.01, ***p<0.001.

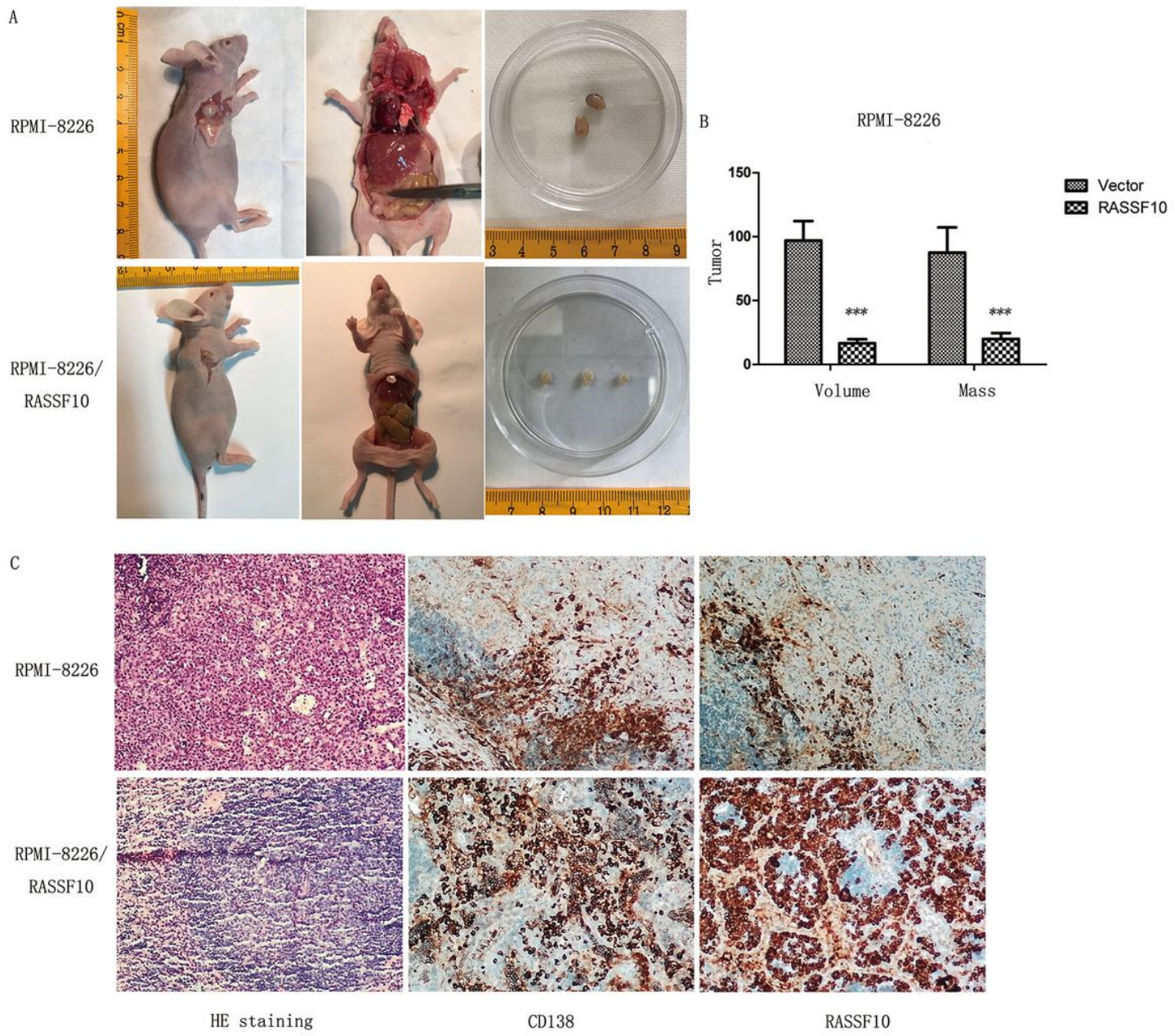

Figure 4

Figure 4

Role of RASSF10 gene overexpression in MM animal model. A. Subcutaneous tumor, lymph nodes, liver and spleen were observed at 21 days after RPMI-8226/Vector and RPMI-8226/RASSF10 injection. B. Tumor volume and mass of RPMI-8226/RASSF10 were significantly reduced compared with RPMI-8226/Vector. C. HE staining, CD138 and RASSF10 staining of subcutaneous tumor in RPMI-8226/Vector and RPMI-8226/RASSF10 mice. ***p<0.001.