Serum miR-30d as a novel biomarker for multiple myeloma and its antitumor role in U266 cells through the targeting of the MTDH/PI3K/Akt signaling pathway

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Abstract. Multiple myeloma (MM) is a hematological tumor and is characterized by the infiltration of malignant clonal plasma cells (PCs) in bone marrow. MicroRNAs (miRNAs or miRs) have been reported to play an important role in the genesis and progression of MM. However, little is known about the clinical diagnostic value and biological functions of miR-30d in MM. In this study, to investigate the role of miR-30d in MM, we used reverse transcription-quantitative polymerase chain reaction quantitative (RT-qPCR) to detect the relative expression level of miR-30d in the serum of 81 patients with primary MM and 78 healthy donors (HDs). The biological functions of miR-30d were then assessed by CCK-8 assay, flow cytometric analysis of apoptosis and western blot (WB) analysis in U266 cells. Moreover, the confirmation of the target gene of miR-30d was conducted by luciferase reporter assay. Our results indicated that miR-30d expression was significantly downregulated in the serum of patients with primary MM compared with that of the HDs and that it was significantly associated with several clinical indicators of MM. Further cell functional analyses using the U266 cells revealed that miR-30d functions as a tumor suppressor gene in MM by inhibiting cell viability and promoting cell apoptosis. Moreover, miR-30d was confirmed to directly bind to the 3'UTR of its target gene, metadherin (*MTDH*) and inhibit the activation of the downstream PI3K/Akt signaling pathway. On the whole, the findings of this study indicate that the serum expression level of miR-30d is of great significance to the diagnosis and treatment monitoring of patients with MM. Moreover, miR-30d carries out its antitumor role in U266 cells through the inhibition of the activation of the PI3K/Akt signaling pathway by negatively regulating *MTDH*, which reveals its potential for use as a therapeutic target for MM.

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Introduction

Multiple myeloma (MM) is a type of heterogeneous malignancy with the biological characteristics of malignant plasma cell (PC) proliferation in the bone marrow and complex genetic alterations (1). Eventually, the abnormal malignant proliferation of PCs may result in the abnormal secretion of monoclonal immunoglobulin (M-Ig or M protein). The M protein may cause several complications in patients with MM, such as hyperviscosity syndrome, bone injury and renal impairment. Some studies have indicated that the classification of M protein may be an important indicator for the diagnosis of patients with MM. Moreover, the precise quantification of the types and levels of M protein is critical to monitoring the response of patients to therapy (2,3). However, M protein classification cannot cover all patients with MM and this disease has been found to have a great heterogeneity at the genetic level. Therefore, researchers have focused on the field of genetic molecular research as regards MM in the hope of identifying novel individualized biomarkers and treatment for this disease (4,5).

Currently, numerous researchers have demonstrate that microRNAs (miRNAs or miRs) can play crucial roles in the progression of a number of types of cancer, including MM (6,7). miRNAs are a class of small non-coding RNAs with a length of approximately 22 nucleotides. It has been extensively demonstrated that miRNAs can play a significant role in the regulation of cell functions by directly binding to the 3' untranslated region (3'UTR) of their target genes, eventually leading to the degradation of these tumor-associated genes (8). Certain studies have found that the dysregulated expression of miRNAs is closely related to the genesis, progression, tumor metastasis and drug resistance in MM (4,9,10). Furthermore, miRNAs have been proven to participate in the activation of several MM-associated signaling pathways, such as the nuclear factor (NF)-κB signaling pathway, the Wnt/β-catenin

signaling pathway, the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway and the PI3K/Akt signaling pathway (11,12). These results indicate that the knockdown of oncomiRNAs and the restoration of tumor suppressor miRNAs may lead to the development of novel treatment strategies for MM.

In recent years, an increasing number of researchers have paid attention to the study of circulating biomarkers due to their detectability and accessibility (13). Notably, the expression level of circulating miRNAs can reflect the pathological state and prognosis of patients accurately (14). Moreover, miRNAs in human body fluids are highly stable and easy to detect, and these advantages display their potential for use as biomarkers for various diseases (15). Therefore, seeking effective early biomarkers for diagnosis and targets for therapy is essential for the diagnosis and treatment of MM.

The majority of studies on miR-30d in solid tumors have indicated that it possesses tumor suppressor functions (16,17). However, research on the functions of miR-30d in MM and on its expression levels in serum is limited (18). In the present study, we found that the expression level of miR-30d was significantly downregulated in the serum of patients with MM compared with the serum of healthy donors (HDs) by reverse transcription-quantitative polymerase chain reaction quantitative (RT-qPCR). We then examined the association of the expression level of this miRNA with the patient clinicopathological data, and the results revealed that the expression level of miR-30d was significantly associated with the concentration of hemoglobin (HGB), platelet (PLT), creatinine (Cr) and β_2-microglobulin (β_2M), and bone marrow plasma cells (BMPC) infiltration in patients with MM. Following 2 periods of treatment, the serum miR-30d expression level in patients with primary MM was significantly increased, suggesting that miR-30d had a great potential for use as a diagnostic biomarker of MM. Furthermore, we examined the biological functions of miR-30d in U266 cells by CCK-8 assay and apoptosis by flow cytometric analysis. The results revealed that miR-30d inhibited cell proliferation and promoted cell apoptosis *in vitro*.

In order to conduct a more in-depth study of the mechanisms of action of miR-30d in MM cells, we began to search for its target gene. By utilizing bioinformatics software, we selected metadherin (*MTDH*) as a putative target of miR-30d. This regulatory association was also confirmed in a previous study (19). *MTDH*, also known as astrocyte elevated gene-1 (*AEG-1*) or *LYRIC*, is a novel oncogene that plays a crucial role in various human malignancies, including prostate carcinoma (20), breast carcinoma (21), non-small cell lung cancer (22) and cervical cancer (23). Abundant functional investigations *in vitro* and *in vivo* have indicated that MTDH is a valuable tumor biomarker and a potential therapeutic target in cancer. However, studies on the role of MTDH in MM are limited (24). Firstly, in this study, we found that *MTDH* promoted cell proliferation and inhibited cell apoptosis *in vitro*. Furthermore, results western blot (WB) analysis revealed that miR-30d inhibited the activation of the downstream PI3K/Akt signaling pathway by directly binding to its target gene, *MTDH*, which suggested that miR-30d plays an important anti-carcinogenic role in the pathological process of MM by targeting the MTDH/PI3K/Akt signaling pathway.

Materials and methods

Patient samples. In the present study, we enrolled 81 patients with primary MM and 78 samples from HDs at the Affiliated Hospital of Nantong University (Nantong, China) from July, 2015 to December, 2016. All patients with MM were definitively diagnosed from the results of a bone marrow biopsy. Moreover, all patients were divided into different stages and subtypes according to their clinical characteristics. Serum samples of patients were collected at the time of their first diagnosis without any treatment and stored in RNase-free tubes at -80˚C immediately until use. Normal PCs from three HDs as controls of MM cell lines were purified from their bone marrow specimen using $CD138^+$ magnetic bead separation technology (Miltenyi Biotec Corp., Gladbach, Germany) as previously described (25). All the protocols were approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University.

Detection of clinical parameters of patients. The serum hemoglobin (HGB) concentration was measured using an automatic blood cell analyzer Sysmex HST-N 302 (Sysmex Corp., Kobe, Japan) and determined by the colorimetry. The serum platelet (PLT) concentration was measured using an automatic blood cell analyzer Sysmex XE-2100 (Sysmex Corp.) and determined by the principle of electrical impedance. The serum calcium ion (Ca^{2+}), albumin (ALB), creatinine (Cr), β_2M and lactate dehydrogenase (LDH) concentrations were measured using an automatic biochemical analyzer ADVIA2400 (Siemens Corp., Berlin, Germany) with the corresponding kit (FUJIFILM Wako Pure Chemical Corp., Japan). The serum concentrations of light chain λ and κ were measured using an IMMAGE specific protein analyzer (Beckman Coulter Corp., Brea, CA, USA) with immune turbidimetry. The BMPC infiltration percentage in the bone marrow of patients with MM was determined by searching abnormal plasma cells under a microscope after staining the bone marrow smears (Wright-Giemsa Stain; Solarbio Corp., Beijing, China).

Extraction of RNA and cDNA synthesis. Serum total RNA was extracted from 400 μl serum samples using the Ambion mirVana PARIS kit (Life Technologies/Thermo Fisher Scientific Corp., Waltham, MA, USA) according to the manufacturer's instructions. Total RNA from the cell lines was extracted using TRIzol reagent (Invitrogen/Thermo Fisher Scientific Corp.) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were measured using a nanophotometer (Implen Corp., Munich, Germany). Subsequently, 10 μg total RNA were reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Corp.). The reaction condition for the mix was at 42˚C for 60 min and then at 70˚C for 5 min. Reverse transcription products were stored at -20˚C until use.

Quantitative (real-time) polymerase chain reaction (qPCR). To examine the expression level of serum miRNAs, qPCR was performed using the Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific Corp.). The reaction system included 3.0 μl cDNA (with three

technical replicates) and 10.0 μl FastStart Universal SYBR-Green Master Mix (Roche Corp., Mannheim, Germany). The PCR reaction condition consisted of incubation at 95˚C for 10 min, followed by denaturation at 95˚C for 10 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 30 sec, and repeating the cycle for 45 times. In serum, the expression level of miR-30d was normalized to its internal control U6 and the relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method as previously described (26) and using mixed normal serum as control. In U266 cells, the expression level of MTDH was normalized to its internal control, β-actin, and the relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method using normal PCs as a control. The sequences of the primers used are listed as follows: miR-30d forward, 5'-GCGTGTAAACATCCCCGAC-3' and reverse, 5'-CAGCCA CAAAAGAGCACAAT-3'; U6 forward, 5'-GCTTCGGCAGCA CATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTT GCGTGTCAT-3'; *MTDH* forward, 5'-TAAAACAAAACTG CGGACAC-3' and reverse, 5'-AGGGCACTGTTGTTAAA CCA-3'; and β-actin forward, 5'-AGCGAGCATCCCCCAAA GTT-3' and reverse, 5'-GGCACGAAGGCTCATCATT-3'.

Culture and transfection of MM cell lines. The U266, H929, RPMI-8226 cell lines were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% antibiotics and 1% glutamine (both from Gibco/Thermo Fisher Scientific Corp.) in a humidified atmosphere of 5% CO_2 at 37˚C. miR-30d mimics (M), its negative control (M-), miR-30d inhibitors (I) and its negative control (I-) were synthesized by Guangzhou RiboBio Corp. (Guangzhou, China). *MTDH*-pcDNA and its negative control were synthesized by GeneCopoeia (GeneCopoeia Corp., Guangzhou, China). These were transfected into MM cells using Lipofectamine 3000 (Invitrogen/Thermo Fisher Scientific Corp.) according to the manufacturer's instructions. After 48-72 h, the transfected cells were harvested for cell function experiments and WB analysis.

Cell proliferation assay. To detect the proliferation of MM cells, a Cell Counting kit-8 (CCK-8; Roche Corp., Basel, Switzerland) cell proliferation assay was performed. The MM cells were seeded at 5,000 cells per well in 96-well plates with 7 replicates. Subsequently, 100 μl CCK-8 reagent were added to each well and maintained in an incubator for 2 h at 37˚C. OD values were detected using a microplate reader (DeTie Corp., Nanjing, China) at 0, 24, 48 and 72 h, respectively. Finally, all data were collected for statistical analysis.

Cell apoptosis assay. To evaluate cell apoptosis, the 7AAD-Annexin V Apoptosis Detection kit (BD Biosciences Corp., San Jose, CA, USA) was applied according to the manufacturer's instructions. Briefly, the MM cells, which were transfected and after 48 h were collected into tubes, and then washed with cold PBS twice. Subsequently, 5 μl 7AAD and 5 μl Annexin V reagents were added to the tubes and mixed gently, then incubated together for 15 min at 25˚C in the dark. The apoptosis of the MM cells was analyzed using a FACSCalibur flow cytometer (BD Biosciences Corp.).

WB analysis. Total proteins were extracted from the MM cells using RIPA buffer (high) (Solarbio Corp.) and WB analysis was performed as previously described (27). Generally, the concentrations of proteins in MM cells were determined using the bicinchoninic acid (BCA) assay (Beyotime Institute of Biotechnology, Beijing, China). A total of 20 μg protein per lane were loaded and separated using 12% SDS-PAGE, then transferred onto nitrocellulose membranes (Merck Millipore Corp., Darmstadt, Germany), and blocked in PBS/Tween-20 containing 5% bovine serum albumin blocking buffer (Solarbio Corp.) for 2 h at at 25˚C. Subsequently, the membranes with proteins were incubated overnight at 4˚C with corresponding primary antibodies. The primary antibodies used in this study and their dilution rates are listed as follows: anti-MTDH (1:5,000; ab124789, anti-PI3K-p85 (1:500; ab182651), anti-phospho-Akt-Ser473 (1:1,000; ab81283) (all from Abcam, Cambridge, MA, USA), anti-total Akt (1:1,000; #9272) and anti-β-actin) (1:1,000; #4970) (all from Cell Signaling Technology Corp., Danvers, MA, USA). The membranes were then incubated with the secondary antibody (anti-rabbit IgG; 1:2,000; #14708; Cell Signaling Technology Corp.) for 1 h at 25˚C. Following washing 3 times with TBST buffer, the enhanced chemiluminescent (ECL) kit (BioVision Corp., Milpitas, CA, USA) was used to visualize the protein strips. The densitometry of protein strips was calculated using ImageJ software version 1.6.0 and normalized to β-actin.

Dual Luciferase reporter assay. Bioinformatics software, including TargetScan (http://www.targetscan.org), miRanda (http://www.microRNA.org) and PicTar (http://pictar.mdc-berlin.de/) were used to predict the target genes of miRNAs and the results indicated that *MTDH* may be the target gene of miR-30d. The wild-type (wt) *MTDH* 3'UTR and mutated (mut) *MTDH* 3'UTR were then bound to psiCHECK-2 vectors (Promega Corp., Madison, WI, USA), respectively. The final products were cloned and amplified into *Escherichia coli* (General Microbiological Culture Collection Center, Beijing, China). The U266 cells were then cultured in 6-well plates, and each well was co-transfected with wt/mut-*MTDH*-psiCHECK-2 and miR-30d mimics/miR-control using Lipofectamine 3000 for 48 h. Following the lysis of the cells, luciferase activity was measured and the fluorescence activity of Firefly luciferase and sea kidney luciferase (as an internal reference) was detected. The final results were calculated as the luciferase activity/sea kidney luciferase activity.

Statistical analysis. Data were analyzed using SPSS statistical analysis software, version 20.0 and figures were drawn using Graphpad Prism 5 software. Patient data were described by median and interquartile as they were not in accordance with Gaussian distribution. The Mann-Whitney U test was used to compare data differences between 2 groups. The Kruskal-Wallis H with the Tamhane's T2 post hoc test was used to compare differences among multiple groups. Paired data were analyzed using the Wilcoxon paired test. The correlation between the expression level of miR-30d with the patient clinicopathological characteristics was examined using Spearman's correlation coefficient analysis. Statistically significant differences between categorical variables were determined by the Chi-square test. Receiver operating characteristic (ROC)

curves and area under the ROC curve (AUC) were used to assess the diagnostic value of using miR-30d for MM. In all statistical analyses, a value of P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 159 serum samples were used in this study, which included 81 samples from patients with primary MM and 78 samples from HDs. The median age of the patients was 61 years of age (range, 38-88 years), while the median age of the HDs was 59 years of age (range, 45-82 years). There were no significant differences between the patients with MM and the HDs as regards the composition of age and sex (P>0.05). Among these newly diagnosed patients with MM, 38 received bortezomib-based treatment (arm A) and 43 received thalidomide-based treatment (arm B). According to the ISS staging system, 26 patients had stage I, 19 patients had stage II and 36 patients had stage III of the disease. According to the Durie-Salmon (DS) staging system, 3 patients had stage I, 39 patients had stage II and 39 patients had stage III of the disease. According to the monoclonal component, patients were divided into an IgG type of 28 cases, an IgA type of 17 cases, an IgM type of 1 case, a light chain type of 33 cases and a non-secretory type of 2 cases (Table I).

Table I. Basic characteristics of the patients with primary multiple myeloma and healthy donors.

	Patients (n)	Healthy donors (n)	P-value
Total	81	78	
Sex			0.629
Male	46	48	
Female	35	30	
Range of age (years)	38-88	45-82	0.063
ISS			
Stage I	26	ND	ND
Stage II	19	ND	ND
Stage III	36	ND	ND
DS			
Stage I	3	ND	ND
Stage II	39	ND	ND
Stage III	39	ND	ND
Type of monoclonal component			
IgG	28	ND	ND
IgA	17	ND	ND
IgM	1	ND	ND
Light chain	33	ND	ND
Non-secretory	2	ND	ND
Therapeutic strategy			
Arm A	38	ND	ND
Arm B	43	ND	ND

ND, not determined; ISS, international staging system; DS, Durie-Salmon; arm A, bortezomib-based treatment; arm B, thalidomide-based treatment. Statistically significant differences in sex between patients and healthy donors were determined by the Chi square test. Statistically significant differences in age between patients and healthy donors were determined by the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Expression levels and diagnostic accuracy of serum miR-30d levels in MM. The serum expression levels of miR-30d in 81 patients with MM and 78 HDs were detected by RT-qPCR. The results revealed that the serum expression level of miR-30d in the patients with MM was significantly downregulated compared with that in the HDs (P<0.0001) (Fig. 1A). No significant differences were observed in the serum miR-30d expression level between the patients in arm A and arm B (P>0.05) (Fig. 1B). Moreover, we made a short-term (2 periods of treatment) follow-up visit to 24 patients and collected their serum samples both at the time of diagnosis and following treatment. We concluded that the serum expression levels of miR-30d in patients receiving both arm A and arm B treatment were significantly improved (P=0.0360 and P=0.0304, respectively) (Fig. 1C and D). Subsequently, ROC curve analysis was conducted to assess the diagnostic accuracy of miR-30d. The results revealed that the serum level of miR-30d could differentiate patients with MM from HDs with areas under the ROC curve (AUC) of 0.800 (95% CI, 0.733-0.868; P<0.0001) (Fig. 1E). At the cut-off value of 2.908 for miR-30d, the sensitivity was 88.5% and the specificity was 63.0%.

Associations between serum levels of miR-30d and clinical parameters of the patients with MM. We assessed the associations of the miR-30d expression levels and the clinical parameters of patients with MM. Firstly, we stratified the patients with MM according to the international staging system (ISS) and Durie-Salmon (DS) staging to investigate the association between the serum miR-30d expression levels and the stages of MM. The results indicated that the miR-30d expression levels differed significantly between ISS stage I and II (P<0.01), and stage I and III (P<0.001), while no differences were observed between stage II and III (Fig. 2A). We also found that the miR-30d expression levels differed significantly between DS stage II and III (P<0.05), while miR-30d was not found to be associated with DS stage I and II, or I and III (Fig. 2B). Moreover, we failed to associate the expression levels of miR-30d with the types of monoclonal components of patients with MM (P=0.364) (Fig. 2C). Furthermore, we divided all patients into 2 groups (miR-30d low expression group and miR-30d high expression group) according to the median of the serum miR-30d expression levels. The results demonstrated that the concentrations of serum HGB, PLT, Cr and β_2M, and BMPC infiltration in patients with MM differed significantly between these 2 groups (P<0.05). Generally, the serum concentrations of HGB and PLT in patients with MM in the miR-30d high expression group were increased compared with those in the miR-30d low expression group, while the serum concentrations of Cr and β_2M, and BMPC infiltration in patients with MM in the miR-30d high expression group were decreased compared with those in the miR-30d low expression group. However, no significant differences were observed

Table II. Comparison of clinical parameters between the miR-30d low expression group and miR-30d high expression group in patients with multiple myeloma.

Parameters	Median (interquartile range)		P-value
	miR-30d low expression	miR-30d high expression	
HGB (g/l)	85 (77.5-104.5)	106 (83-130.5)	***0.0084***
PLT ($x10^9$)	127 (93-196.5)	179 (123-237.5)	***0.0123***
Ca^{2+} (mmol/l)	2.14 (2.02-2.235)	2.1 (1.98-2.195)	0.2557
ALB (g/l)	32.2 (29.1-36.2)	35.20 (29.45-38.1)	0.5684
Cr (μmol/l)	76 (49-121.5)	67 (47.5-75)	***0.0340***
β_2M (mg/l)	6.4 (3-8.8)	2.9 (1.82-4.8)	***0.0003***
LDH (ukat/l)	172 (126-231.5)	156 (126.5-209.5)	0.3758
Light chain λ (mg/dl)	345 (122-932.5)	364 (192-870)	0.9963
Light chain κ (mg/dl)	753 (384.5-2680)	520 (400.5-1855)	0.5938
BMPCs infiltration (%)	11.5 (2.875-37.5)	2.5 (0.5-11.5)	***0.0019***

HGB, hemoglobin; PLT, platelet; Ca^{2+}, calcium ion; ALB, albumin; Cr, creatinine; β_2M, β_2-microglobulin; LDH, lactate dehydrogenase; BMPCs, bone marrow plasma cells. Measurement data are presented as the median and interquartile. Statistically significant differences were determined by the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference. Statistically significant data are indicated in bold and italic font.

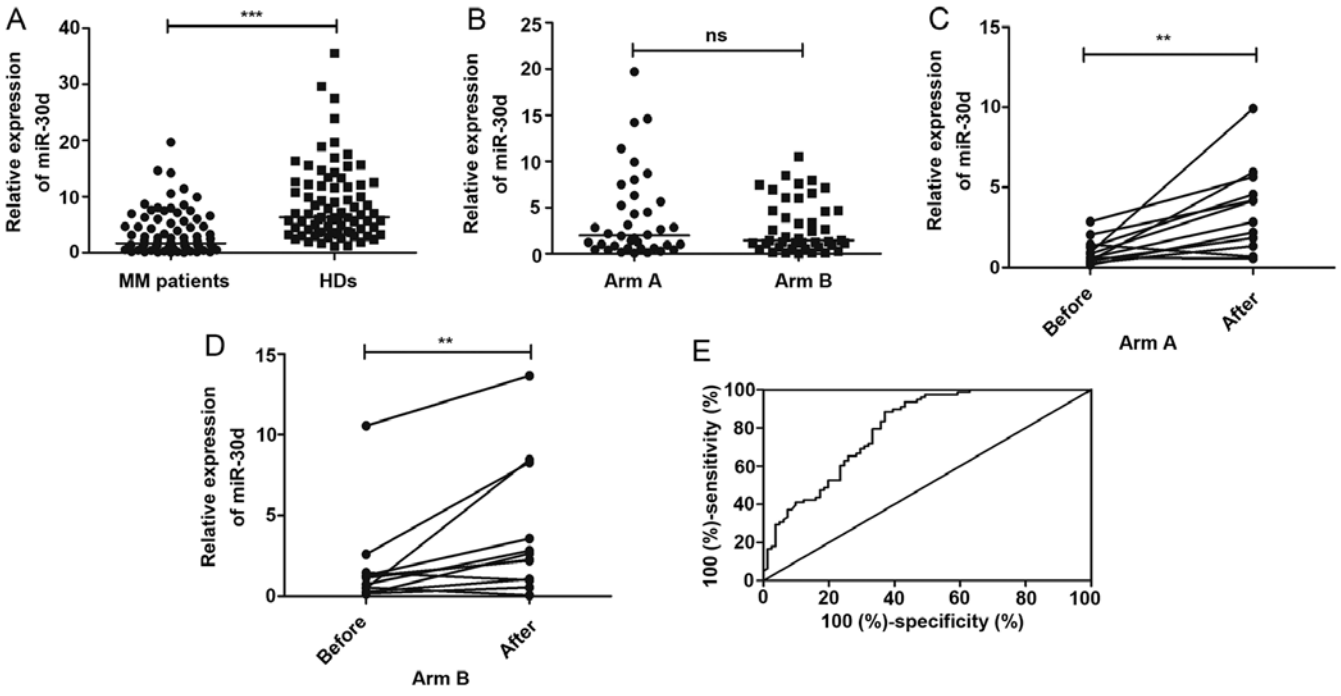

Figure 1. Expression levels and diagnostic accuracy of serum miR-30d in multiple myeloma (MM). (A) Serum expression levels of miR-30d in patients with MM and healthy donors (HDs) were measured by RT-qPCR. Measurement data were described by the median value and interquartile range, statistically significant differences were determined by Mann-Whitney U test. (B) Serum expression levels of miR-30d between arm A and arm B. Measurement data were described by the median value and interquartile range, statistically significant differences were determined by Mann-Whitney U test. (C) Dynamic changes in serum miR-30d expression levels in patients with MM before and after 2 periods of treatment in arm A; measurement data were described by the median value and interquartile range, and statistically significant differences were determined by the Wilcoxon matched paired test. (D) Dynamic changes in serum miR-30d expression levels in patients with MM before and after 2 periods of treatment in arm B; measurement data were described by the median value and interquartile range and statistically significant differences were determined by the Wilcoxon matched paired test. (E) ROC curve analysis of miR-30d revealed ane AUC of 0.664 (P<0.0001), a sensitivity of 88.5% and a specificity of 63.0%. P<0.05 was considered to indicate a statistically significant difference. **P<0.01, ***P<0.001; ns, no statistical significance.

in the other clinical indicators, including the concentrations of serum Ca^{2+}, ALB, LDH, light chain λ and light chain κ (P>0.05) (Table II).

Follow-up study of the association between the serum levels of miR-30d and the improved clinical parameters of the patients with MM. We collected the complete clinical indicators of

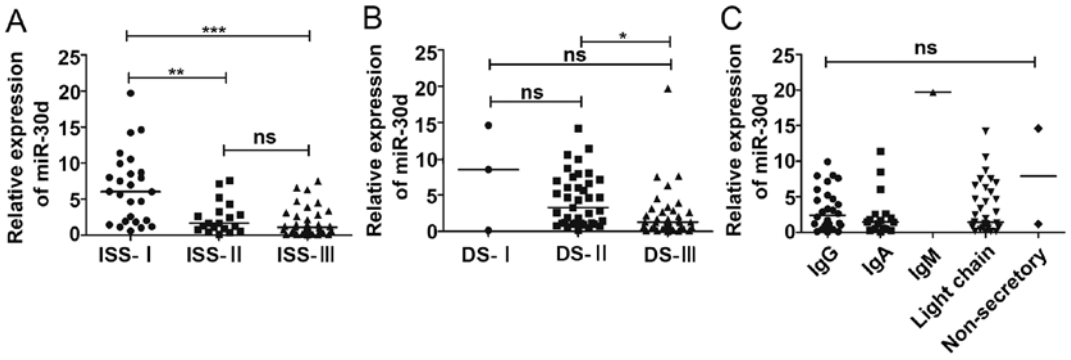

Figure 2. Associations between serum levels of miR-30d and patient clinical parameters. (A) The association between the serum expression levels of miR-30d and ISS stages. (B) The association between the serum expression levels of miR-30d and Durie-Salmon (DS) stages. (C) The association between the serum expression levels of miR-30d and monoclonal components. Measurement data were described by the median value and interquartile range, statistically significant differences were determined by Kruskal-Wallis test, and comparisons between 2 groups was performed by the Tamhane's T2 test. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01, ***P<0.001; ns, no statistical significance.

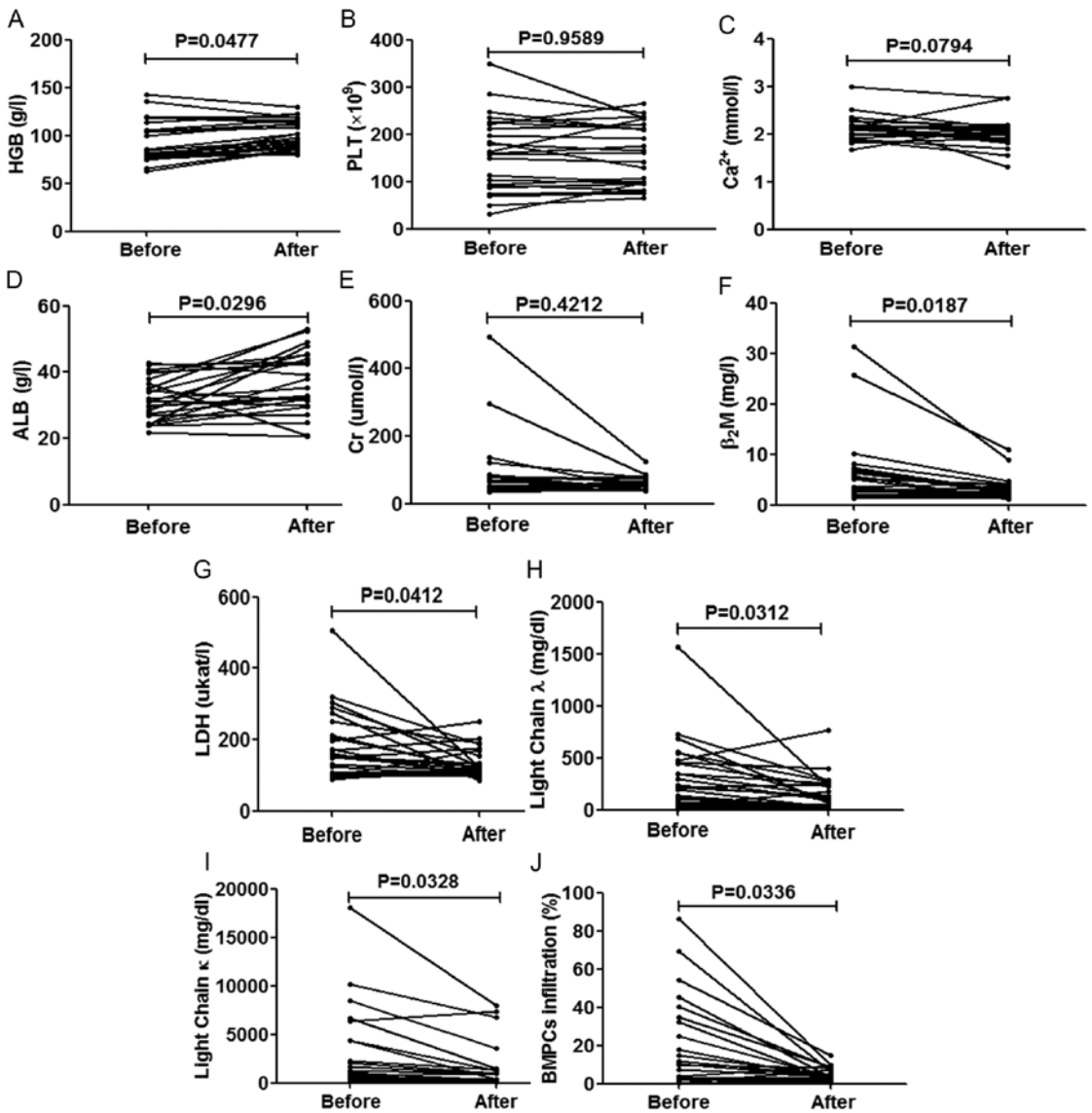

Figure 3. Follow-up study of the clinical parameters before and after treatment. (A) Comparison of the hemoglobin (HGB) concentration before and after treatment. (B) Comparison of the platelet (PLT) concentration before and after treatment. (C) Comparison of the serum calcium ion (Ca^{2+}) concentration before and after treatment. (D) Comparison of the albumin (ALB) concentration before and after treatment. (E) Comparison of the creatinine (Cr) concentration before and after treatment. (F) Comparison of β_2-microglobulin (β_2M) concentration before and after treatment. (G) Comparison of the lactate dehydrogenase (LDH) concentration before and after treatment. (H) Comparison of light chain λ concentration before and after treatment. (I) Comparison of light chain κ concentration before and after treatment. (J) Comparison of the bone marrow plasma cell (BMPC) infiltration rate before and after treatment. Statistically significant differences were determined by the Wilcoxon matched paired test. P<0.05 was considered to indicate a statistically significant difference.

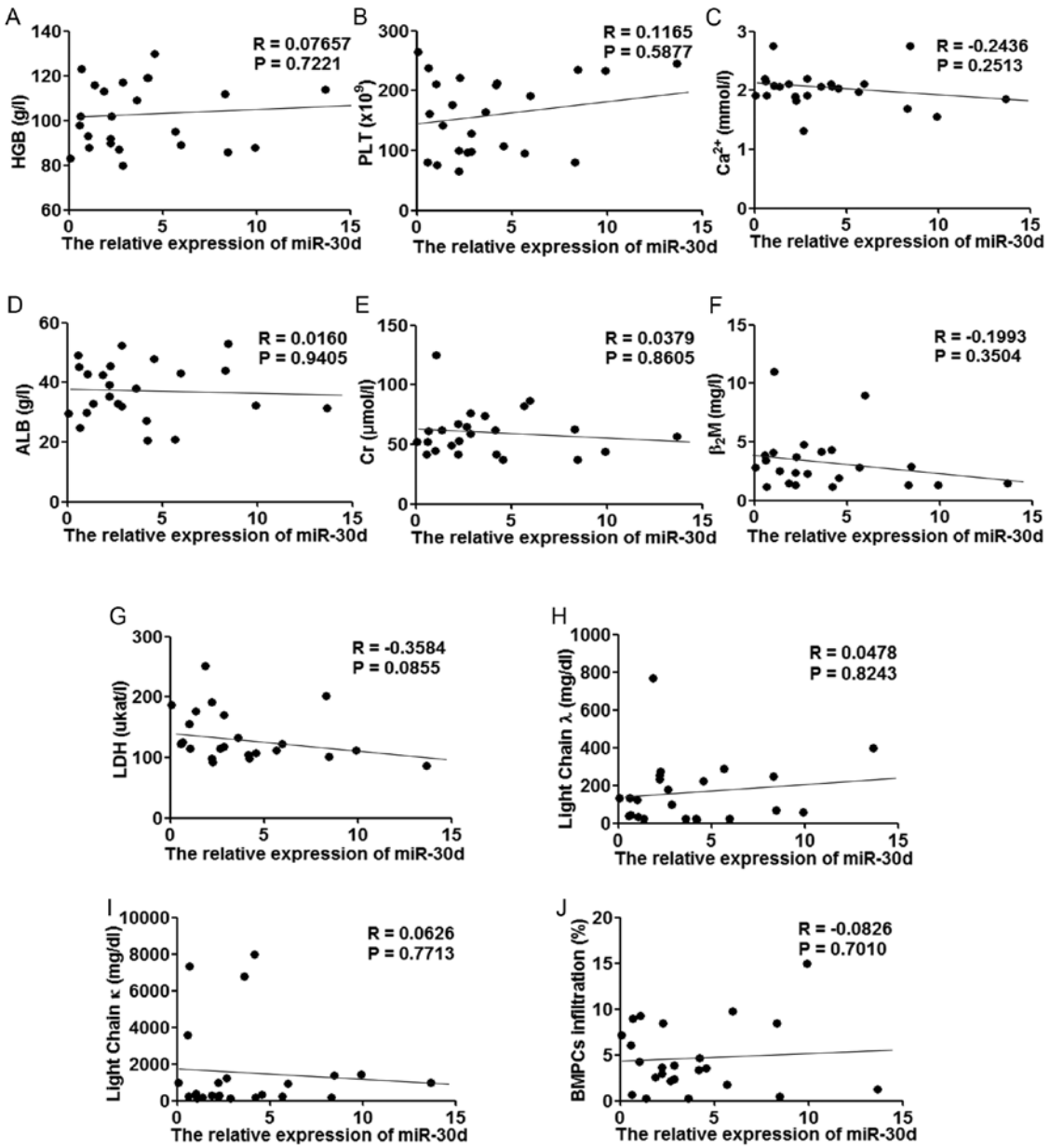

Figure 4. Follow-up study of the correlation between serum levels of mir-30d and the improved clinical parameters. (A) Correlation between the hemoglobin (HGB) concentration and miR-30d after treatment. (B) Correlation between the platelet (PLT) concentration and miR-30d after treatment. (C) Correlation between the serum calcium ion (Ca^{2+}) concentration and miR-30d after treatment. (D) Correlation between the albumin (ALB) concentration and miR-30d after treatment. (E) Correlation between the creatinine (Cr) concentration and miR-30d after treatment. (F) Correlation between the β_2-microglobulin (β_2M) concentration and miR-30d after treatment. (G) Correlation between LDH concentration and miR-30d after treatment. (H) Correlation between the light chain λ concentration and miR-30d after treatment. (I) Correlation between the light chain κ concentration and miR-30d after treatment. (J) Correlation between the bone marrow plasma cell (BMPC) infiltration rate and miR-30d after treatment. P<0.05 was considered to indicate a statistically significant difference. Statistically significant correlations were determined by Spearman's correlation coefficient analysis. P<0.05 was considered to indicate a statistically significant correlation.

24 patients who were followed up. Following 2 periods of treatment, the majority of the parameters of the patients significantly improved, as was expected (P<0.05), apart from the expression levels of PLT (P=0.9589), Cr (P=0.4212) (this condition may be due to the side-effects of chemotherapy) and Ca^{2+} (P=0.0794) (Fig. 3). We then compared the increased miR-30d levels with the improved clinical parameters; however, we failed to find any significant correlations between them (Fig. 4).

miR-30d expression is downregulated in MM cell lines compared with normal PCs. To investigate the role of miR-30d *in vitro*, we detected its expression level in MM cell lines by RT-qPCR analysis. The results revealed that the expression level of miR-30d was significantly decreased in MM cell lines (8226, H929 and U266 cells) compared with the normal $CD138^+$ purified PCs (PCs) from HDs. Moreover, the lowest miR-30d expression level was observed in the U266 cells; thus, we conducted further cell function experiments using this cell line (Fig. 5).

miR-30d inhibits the proliferation and promotes the apoptosis of U266 cells. To examine the effects of miR-30d on the viability of U266 cells, miR-30d mimics (M), mimic negative control (M^-),

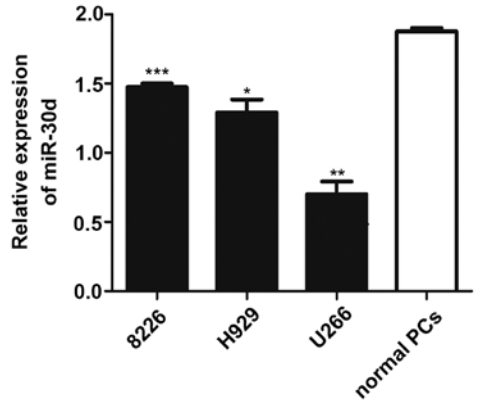

Figure 5. miR-30d expression is downregulated in multiple myeloma (MM) cell lines compared with normal plasma cells (PCs). Data are presented as the means ± SD. Statistically significant differences between groups were determined by the Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, **P<0.01, ***P<0.001.

miR-30d inhibitor (I) and inhibitor negative control (I^-) were transfected into the U266 cells for 48 h according to the manufacturer's instructions, respectively. The high transfection efficiency was confirmed by RT-qPCR (Fig. 6A). The viability of the U266 cells was then evaluated by CCK-8 assay. OD values were measured at 0, 24, 48 and 72 h, respectively. The results indicated that the cells in the M group displayed a significantly lower cell viability rate than those of the control group (P=0.029, 0.007, 0.003, respectively); equally, cells in the I^- group displayed a significantly higher cell viability rate than those in the control group (P=0.024, 0.009, 0.015, respectively). Therefore, miR-30d significantly inhibited the proliferation of the U266 cells (Fig. 6B and C). Moreover, at 48 h following transfection, the apoptotic rate of these 4 groups was detected by flow cytometry. The results revealed that the cells in the M group exhibited a higher apoptotic rate than those in the mimic negative control (M^-) group (P=0.013); the cells in the

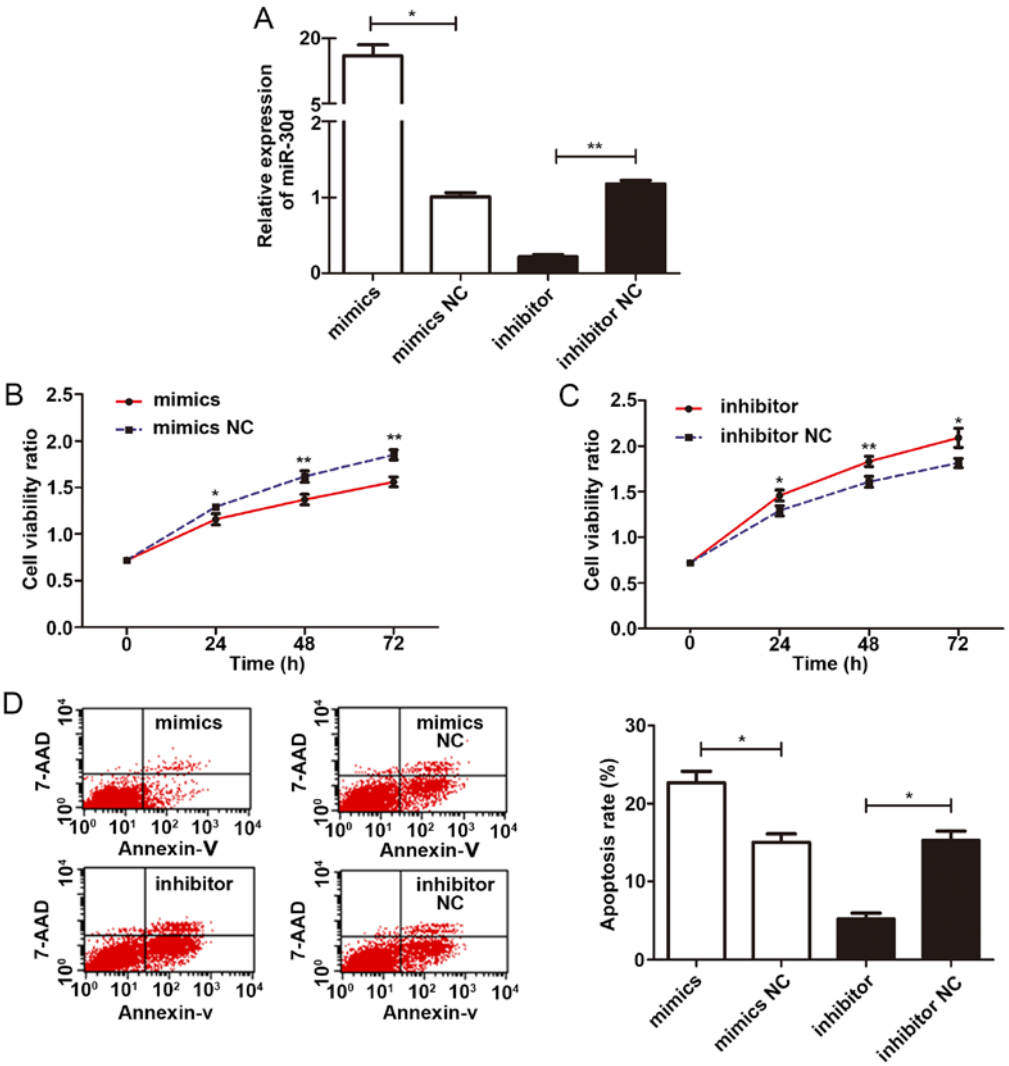

Figure 6. miR-30d inhibits the proliferation and promotes the apoptosis of U266 cells. (A) Transfection of miR-30d mimics significantly increased the expression level of miR-30d, whereas miR-30d inhibitor significantly decreased the expression level of miR-30d in U266 cells. (B) CCK-8 cell proliferation assay demonstrated that miR-30d mimics significantly decreased the viability rate of U266 cells compared to its negative control at 24, 48 and 72 h (P=0.029, 0.007, 0.003, respectively). (C) CCK-8 cell proliferation assay demonstrated that the miR-30d inhibitor increased the viability rate of U266 cells compared to its negative control at 24, 48 and 72 h (P=0.024, 0.009, 0.015, respectively). (D) Flow cytometric analysis demonstrated an increased apoptotic rate of U266 cells in the miR-30d mimics group compared to the mimics NC group (P=0.013) and a decreased apoptotic rate of U266 cells in the miR-30d inhibitor group compared to the inhibitor NC group (P=0.020). All experiments were carried out in triplicate. Statistically significant differences between groups were determined by the Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, **P<0.01.

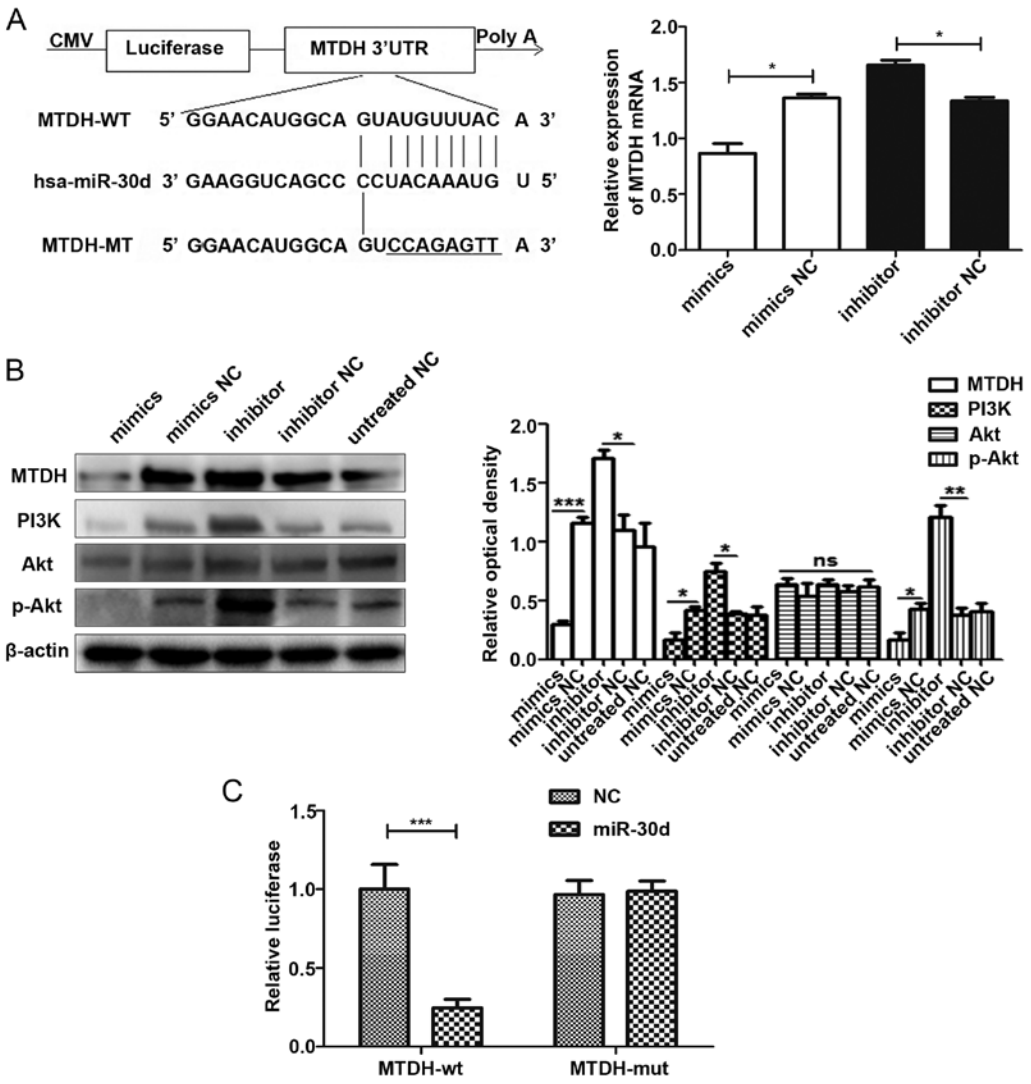

Figure 7. miR-30d binds to the 3'UTR of metadherin (*MTDH*) directly and acts as an inhibitor of the PI3K/Akt signaling pathway. (A) miR-30d was predicted to have a binding site in the 3'UTR of *MTDH*. miR-30d mimics decreased the mRNA expression level of MTDH, while the inhibitors exerted the opposite effect. (B) Results of western blot analysis revealed that miR-30d inhibited the protein expression levels of MTDH, p-Akt and PI3K with normalization to β-actin. The right panel shows the results calculated after gray scale scanning. (C) Luciferase reporter assays revealed that the overexpression of miR-30d could only significantly suppress the luciferase activity of the *MTDH*-wt group in U266 cells, while it had no effect on *MTDH*-mut group, thus indicating that miR-30d could bind to the 3'UTR of *MTDH* directly. All experiments were carried out in triplicate. p-Akt, phosphorylated Akt. Statistically significant differences between groups were determined by the Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, **P<0.01, ***P<0.001; ns, no statistical significance.

I group exhibited lower apoptotic rates than those in the I^{-} group (P=0.020). These findings confirmed that miR-30d promoted the apoptosis of U266 cells (Fig. 6D).

miR-30d binds to the 3'UTR of MTDH directly and acts as an inhibitor of the PI3K/Akt signaling pathway. Bioinformatics software was used to predict the target genes of miR-30d and *MTDH* was selected as the putative target of miR-30d. Moreover, we used RT-qPCR to preliminarily verify the negative association between miR-30d and the mRNA expression of *MTDH* (Fig. 7A). The results of WB analysis then proved that miR-30d mimics suppressed the expression of MTDH, while the miR-30d inhibitor promoted the expression of MTDH. At the same time, we also detected the protein expression levels of p-Akt and PI3K in the PI3K/Akt signaling pathway. The results indicated that miR-30d inhibited the expression of these 2 proteins and acted as an inhibitor of the PI3K/Akt signaling pathway (Fig. 7B). Furthermore, we conducted a luciferase reporter assay to confirm the direct binding association between miR-30d and *MTDH*. The final results indicated that miR-30d could bind to the 3'UTR of *MTDH* directly (Fig. 7C).

MTDH promotes the proliferation and inhibited the apoptosis of U266 cells. To determine the effects of *MTDH* on the viability of U266 cells, pcDNA of *MTDH* and its negative control were transfected into the U266 cells for 48 h according to the manufacturer's instructions. OD values were measured by CCK-8 cell proliferation assay at 0, 24, 48 and 72 h, respectively. The results indicated that the cells in the *MTDH* pcDNA group displayed a significantly higher cell viability rate than those in the vector control group (P=0.031, 0.017, 0.013, respectively). Therefore, pcDNA of *MTDH* significantly promoted the proliferation of U266 cells (Fig. 8A). Furthermore, at 48 h following transfection, the apoptotic rates

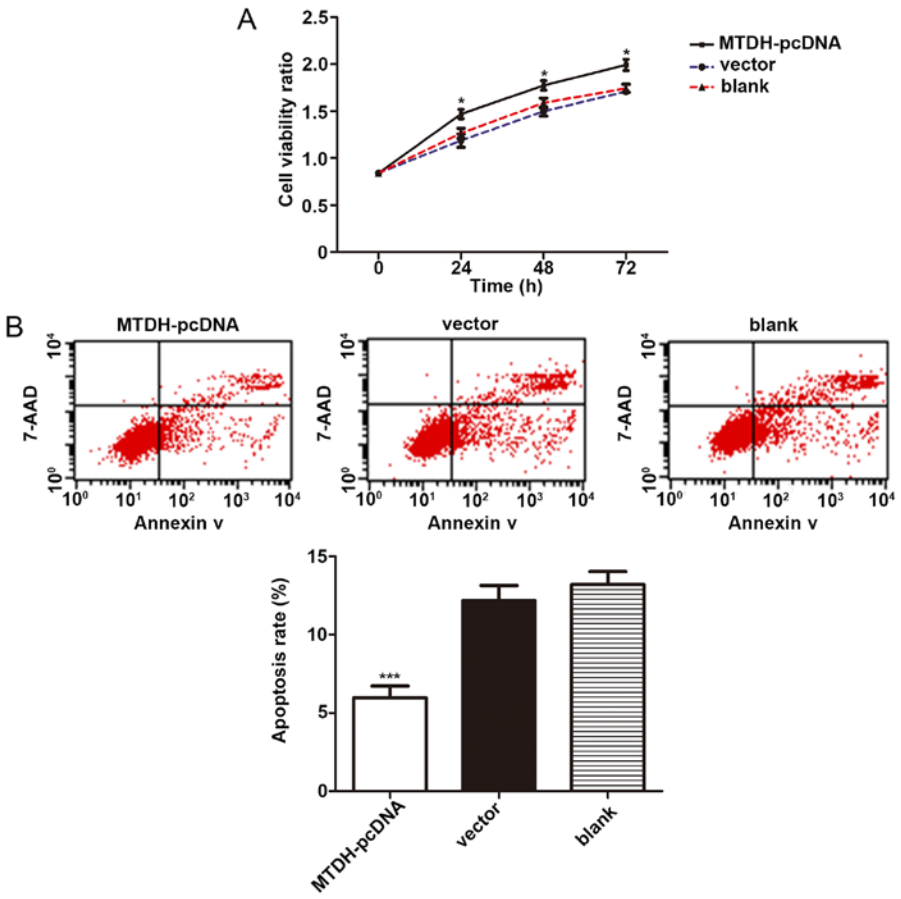

Figure 8. Metadherin (*MTDH*) promotes the proliferation and inhibits the apoptosis of U266 cells. (A) Transfection of *MTDH* pcDNA significantly increased the viability rate of U266 cells compared to its vector control at 24, 48 and 72 h (P=0.031, 0.017, 0.013, respectively). (B) Flow cytometric analysis demonstrated an decreased apoptotic rate of U266 cells in the *MTDH* pcDNA group compared to the vector group (P<0.001). All experiments were carried out in triplicate. Statistically significant differences between groups were determined by Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, ***P<0.001.

of the cells in these 3 groups were detected by flow cytometry. The results revealed that the *MTDH* pcDNA group had a lower apoptotic rate than the vector group (P<0.001) and confirmed that *MTDH* inhibited the apoptosis of the U266 cells (Fig. 8B). These results revealed the carcinogenic role of *MTDH* in U266 cells.

MTDH induces the activation of the PI3K/Akt signaling pathway. To investigate the role of *MTDH* in the pathogenesis of MM, *MTDH*-pcDNA and its negative control were transfected into the U266 cells. The expression levels of MTDH and the downstream PI3K/Akt signaling pathway-related proteins, including PI3K, Akt, p-Akt were then detected by WB analysis. The results revealed that the overexpression of MTDH induced the expression of PI3K and p-Akt to activate the PI3K/Akt signaling pathway (Fig. 9).

miR-30d suppresses the PI3K/Akt signaling pathway by targeting MTDH. To investigate the mechanisms through which miR-30d functions in MM cells by targeting *MTDH*, we co-transfected miR-30d mimics and *MTDH*-pcDNA together into U266 cells. We then measured the expression level of p-Akt following transfection at 72 h, and the results demonstrated that MTDH induced the expression level of p-Akt, while miR-30d mimics partially alleviated this carcinogenic effect by inhibiting the expression level of MTDH. These findings suggested that miR-30d exerts an inhibitory effect on the PI3K/Akt signaling pathway by targeting *MTDH* (Fig. 10).

Discussion

Numerous studies have indicated that genetic abnormalities, including genomic alterations, post-transcriptional regulation, epigenetic alterations, RNA editing and miRNA binding site sequence variation can affect the expression levels of miRNAs (28,29). In MM, certain pathological factors can also lead to the dysregulation of miRNA expression. One study using unsupervised cluster analysis revealed that $CD138^{+}$ cells isolated from patients with MM and monoclonal gammopathy of undetermined significance (MGUS) had a distinct miRNA expression profiling compared with the normal controls (30). In general, that study suggested that the aberrant expression level of miRNAs was associated with the pathogenesis, diagnosis and prognosis of MM. However, few studies to date have mentioned the specific role of miR-30d in MM (31). Therefore, this study focused on the exploration of the clinical value of this molecule in MM and its in-depth mechanisms of action in MM cells.

miRNAs can mediate interactions between cells, and exist stably in the circulating fluid. To a certain extent, some of them can reflect the pathological condition of diseases (14). In the present study, we collected serum of 81 newly-diagnosed

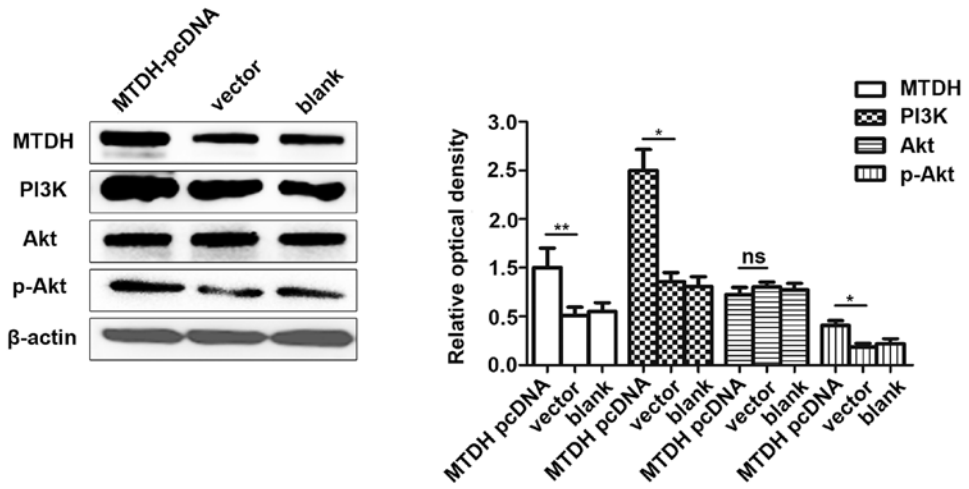

Figure 9. Metadherin (*MTDH*) induces the activation of the PI3K/Akt signaling pathway. Result of western blot analysis showing that *MTDH* induced the protein expression levels of p-Akt and PI3K to activate the PI3K/Akt signaling pathway. Relative protein expression was calculated after gray scale scanning. All experiments were carried out in triplicate. Statistically significant differences between groups were determined by the Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, **P<0.01; ns, no statistical significance.

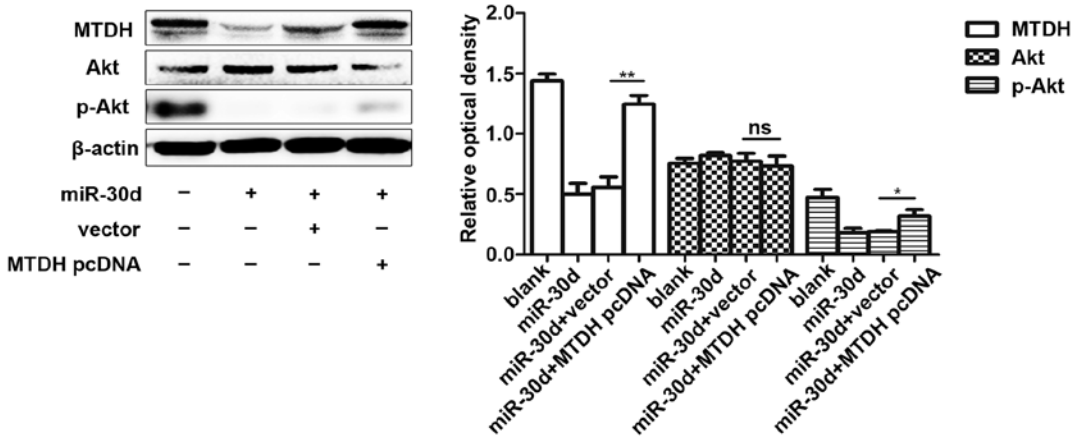

Figure 10. miR-30d supresses the activation of the PI3K/Akt signaling pathway by targeting metadherin (*MTDH*). miR-30d inhibited the activation of the PI3K/Akt signaling pathway by negatively regulating the expression level of *MTDH*. Relative protein expression was calculated after gray scale scanning. All experiments were carried out in triplicate. Statistically significant differences between groups were determined by the Kruskal-Wallis test, followed by the Tamhane's T2 test as a post hoc test. *P<0.05, **P<0.01; ns, no statistical significance

patients with MM and 78 HDs, and then detected the relative expression of miR-30d in serum by RT-qPCR. We found that miR-30d expression was significantly downregulated in the serum of patients with MM compared with HDs (P<0.0001). Moreover, the diagnostic value of miR-30d in serum was assessed by ROC curve analysis, and we found that when the cut-off value was 2.9082, sensitivity was 88.5%, specificity was 63%, and the AUC was of 0.800. Through the follow-up analyses of 24 patients with primary MM, we found that following 2 periods of treatment, the majority of the clinical parameters of the patients had improved (P<0.05) and the expression levels of miR-30d were increased (P<0.0001), indicating that miR-30d can reflect the progression of MM and thus has potential to monitor this disease. However, we failed to find any significant correlation between miR-30d and the improved clinical parameters of the patients with MM. Of note, it was also found that the expression level of miR-30d in serum was significantly associated with the percentage of BMPC infiltration, the value of PLT, β_2M, Cr, HGB and the stage of MM. These results suggest that the miR-30d level in serum of patients with MM possesses a certain diagnostic value in distinguishing patients with MM from healthy controls and is mainly associated with MM progression.

In related studies on MM, accumulating conclusions have confirmed that circulating miRNAs are significantly associated with the pathological processes and prognosis of diseases. miRNAs have high clinical values in the diagnosis and prognosis of diseases (32,33). Moreover, due to the advantages of their stable expression in fluids, easy to collect from specimens and high sensitivity for detection, miRNAs are expected to replace some traditional indicators. In recent years, the rapid development of high-throughput gene chip technology has aided researchers in the screening of a series of differentially expressed miRNAs in peripheral blood, and they then can select target miRNAs for further study. Yyusnita *et al* (34) analyzed differential expression profiles of miRNAs in the peripheral blood of 14 patients newly-diagnosed with MM, 24 follow-up patients with MM and 7 HDs by miRNA microarray technology.

Ultimately, they discovered 10 miRNAs differentially expressed both in the primary and follow-up patients with MM compared with the HDs. In addition, they also selected 3 miRNAs only differentially expressed in patients with primary MM and 8 miRNAs only differentially expressed in the follow-up patients with MM. These results demonstrated that miRNAs are widely involved in the different pathological processes of MM and may reflect the different states of MM. Importantly, this difference can be reflected by the expression levels of miRNAs in peripheral blood. However, summing up some results of microarray data (14,35), we found that profiling results were not consistent in different laboratories. These differences may be due to the different patient groups, methods of sample collections, microarray platforms used and statistical methods used, etc. In order to obtain more accurate results, it may be necessary to standardize the whole process of the microarray analysis.

miR-30d, a member of the miR-30 family (including miR-30a/b/c/d/e), is located on the human chromosome 8q24.22. In recent years, a number of studies have demonstrated that this molecule is involved in the development of many tumors (36). Xuan *et al* (37) found that the expression level of miR-30d was downregulated in prostate cancer. Further studies on cell function revealed that miR-30d inhibited the proliferation of prostate cancer cells and this inhibitory effect may be caused by the targeting of the Bim-1 gene. Moreover, another study on ovarian cancer indicated that miR-30d was not only closely related to cell proliferation, but was involved in the process of epithelial-mesenchymal transition (EMT) in ovarian cancer cell (17). Wu *et al* (19) also found that miR-30d functioned as a tumor suppressor in renal cell carcinoma and the mechanism was partly due to the fact that miR-30d induced apoptosis and was regulated by the Akt/FOXO pathway in renal cell carcinoma cells. By summarizing these discoveries, it has been identified that miR-30d often presents a low expression level in a number of types of cancers and its low expression probably promotes the occurrence and development of cancers. However, studies on the role of miR-30d in MM are limited. In this study, to clarify the role of miR-30d in MM, we performed cell function experiments and found that miR-30d inhibited cell proliferation and induced cell apoptosis. These results revealed that miR-30d functions as a tumor suppressor gene in U266 cells, which is consistent with its role in studies on other solid tumors (19,38,39). In order to examine the mechanisms of action of miR-30d, we predicted the target genes of miR-30d using bioinformatics software. We found that miR-30d had a direct binding site in the 3'UTR of *MTDH*. Through preliminary WB analysis experiments, we found that the miR-30d mimics decreased the protein expression of MTDH, while its inhibitor had the opposite effect. Subsequenlty, luciferase reporter gene experiments were carried out to confirm that miR-30d could bind to the 3'UTR of *MTDH* directly.

MTDH is a recognized oncogene in cancers and it has been demonstratd that it plays a role in carcinogenesis through the PI3K/Akt signaling pathway (40). Recent studies have also indicated that some oncogenic molecules can inhibit the apoptosis of MM cells and contribute to the progression of MM; thus, targeting these molecules could induce the apoptosis of MM cells and may provide a novel therapeutic strategy for MM (41,42). After investigating the available literature, we found that *MTDH* has been proven to function as an oncogene in some solid cancers (43). However, whether *MTDH* can affect the apoptosis of MM cells remains unknown. Thus, we aim to perform further experiments in the future to prove the association of *MTDH* with the apoptosis of the U266 cell line. In this study, we found that *MTDH* did have a similar mode of action in U266 cells, as the overexpression of *MTDH* induce the proliferation and inhibited the apoptosis of the U266 cell line. Subsequently, following the overexpression of *MTDH*, downstream p-Akt expression was induced. In addition, miR-30d and *MTDH* pcDNA were co-transfected into U266 cells, and the results of WB analysis revealed that miR-30d attenuated the promoting effect of *MTDH* on the expression of p-Akt. Therefore, miR-30d can partially reverse the carcinogenic effects of *MTDH*, suggesting that miR-30d may prove to be a novel potential target for use in the treatment of MM.

However, our study still has some shortcomings. We need to include a greater number of serum samples in the future to obtain a more accurate verification. In addition, we lacked follow-up data for the long-term survival analysis of miR-30d, and in future studies, we aim to examine its prognostic role in MM. Furthermore, as a serum biomarker, a single miRNA may not have sufficient diagnostic effectiveness; the expression of a panel of miRNAs may yield greater significance to the clinical application. In future studies, we aim to combine miR-30d with other important MM-associated miRNAs to evaluate the clinical diagnostic value. Our preliminary experiments were performed only on the U266 cell line and this may not represent the responses of all cells. Thus, in the future, we also aim to perform the same experiments using other cell lines to validate our findings.

In conclusion, the results of this study indicate that the serum expression level of miR-30d is significantly downregulated in patients with MM and it has a considerable diagnostic value. Moreover, miR-30d exerts a significant antitumor effect on U266 cells. The underlying mechanisms involve the binding of this miRNA to its target gene, *MTDH*, and this result in the decreased expression of this gene. The activation of *MTDH* downstream the PI3K/Akt signaling pathway is then inhibited, leading to a decrease in cell proliferation and the induction of cell apoptosis. Our preliminary results revealed that miR-30d has great potential for use as a novel serological marker and therapeutic target for MM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BZ and HCh wrote the initial draft, designed and conceived the study. HCo, SJ and XW designed and conceived the study and revised the manuscript. RJ, LS designed and performed the experiments. XZ, YP and CJ contributed to the acquisition and analysis of data for the work. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by Ethics Committee of the Hospital Affiliated to Nantong University and the committee's reference number was 2015-007. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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