Expression of LINC01606 in multiple myeloma and its effect on cell invasion and migration

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Abstract: Given the increasing incidence of multiple myeloma (MM) in recent years, a full understanding of its pathogenesis to find effective molecular markers carries huge implications for future clinical diagnosis and treatment of MM. As the research advances, accumulating studies have pointed out that long non-coding RNAs (LncRNAs) may be the key to the future diagnosis and treatment of neoplastic diseases. Objective: This study investigated the clinical implications of LncRNA LINC01606 in MM and its effects on the biological behavior of MM cells. Methods: In this prospective study, 72 patients with MM (group A) admitted between July 2014 and July 2016 and 68 healthy subjects (group B) who concurrently underwent physical examination in our hospital were included. The expression of LINC01606 in peripheral blood of patients in the two groups was detected to analyze its diagnostic and prognostic value in MM. In addition, MM cells were purchased and transfected with plasmids for mimics, inhibitors and negative control of LINC01606 and miR-579-3p respectively to detect the changes in cell proliferation, invasion and migration. Results: The expression of LINC01606 in group A was higher than that in group B (P<0.050). The sensitivity and specificity of peripheral blood LINC01606 in predicting MM were 85.29% and 72.39%, respectively (P<0.001). Prognostic follow-up analysis revealed higher LINC01606 levels in the dead than those in the survival. The predictive sensitivity of LINC01606 for the 3-year mortality of MM patients was 63.16%, and the specificity was 86.00% (P<0.001). Higher expression of LINC01606 indicated increased risk of 3-year mortality in patients with MM (P<0.001). Compared with LINC01606 overexpression and miR-579-3p inhibition, the proliferation, invasion and migration of cells decreased more significantly by LINC01606 inhibition and miR-579-3p overexpression (P<0.050). Dual luciferase reporter (DLR) assay confirmed the targeting relationship between LINC01606 and miR-579-3p. There was no significant difference in the activity of MM cells co-transfected with LINC01606-inhibitor and miR-579-3p-inhibitor plasmids compared with the blank group (P>0.050). Conclusions: LINC01606, with a high expression profile in MM, promotes the proliferation, migration and invasion of MM cells through targeted inhibition of miR-579-3p, which may be the key to future diagnosis and treatment of MM.

Keywords: LINC01606, multiple myeloma, miR-579-3p

Introduction

Multiple myeloma (MM) is a malignancy characterized by hyperplasia of plasma cells, which is mostly found in the elderly [1]. In the United States alone, there were approximately 80,000 new MM patients in 2011 [2]. In addition, evidence has shown that the incidence of MM is on the rise year by year [3]. However, due to non-specific symptoms of MM at the early stage, it is easy to be ignored or mishandled by patients. As a result, the disease has reached the advanced stages when diagnosed [4], increasing the treatment difficulty and worsening the prognosis of patients [5]. Therefore, a thorough understanding of the pathogenesis of MM to find effective molecular markers carries huge clinical implications for future diagnosis and treatment of MM. Statistics show that the mortality of patients with advanced MM is increasing [6]. At present, however, MM is mainly diagnosed through the combination of traditional tumor markers and imaging techniques and confirmed by pathological biopsy.

With the deepening of research, accumulating studies have pointed out that long non-coding RNAs (LncRNAs) may be the key for future diagnosis and treatment of neoplastic diseases [7, 8]. LncRNAs are essential in various life activi-
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ties, including dose compensation, epigenetic regulation, cell cycle control and cell differentiation regulation, and have been confirmed to be associated with carcinogenesis and progression [9, 10]. For instance, LncRNA LINC-01606 is shown to be closely related to triple-negative breast cancer [11]. However, its role in MM has not been clarified. In addition, research has identified that the Wnt/β-catenin signaling pathway plays a vital part in the progression of MM [12], and that LINC01606 participates in gastric carcinogenesis and progression through the Wnt/β-catenin signal pathway [13]. Hence, we speculated that LINC01606 may be associated with the occurrence and progression of MM. Reviewing previous studies, we found that both LINC01606 and miR-579-3p were abnormally expressed in gastric cancer [14]. Since miR-579-3p has been proved to play an important role in promoting bone diseases such as osteoporosis and rheumatoid arthritis [15, 16], we hypothesized that LINC01606 might affect MM through miR-579-3p. For verification, we analyzed the expression and mechanism of LINC01606 and miR-579-3p in MM, aiming to provide new ideas for future clinical diagnosis and treatment of MM.

Materials and methods

Main reagents

TRIzol (TRIzol™ LS Reagent, Invitrogen, USA, 10296010); Reverse transcription kit (TaKaRa, Japan, PrimeScript™ RT reagent Kit, RR036A); TB Green® Fast qPCR Mix (TaKaRa, Japan, RR430A); Cell-counting kit-8 (CCK-8; Biosharp, China, BS350B); Transwell Chamber (Corning, USA, 3402).

General information

This prospective study comprised 72 patients with MM (group A) and 68 healthy controls (group B) who were enrolled between July 2014 and July 2016. Inclusion criteria: Patients (age range: 30-60 years) who were pathologically diagnosed with MM were enrolled. Exclusion criteria: Patients with prior chemoradiotherapy, severe infectious diseases, or poor treatment compliance due to mental disorders, as well as those with a life expectancy <1 month and referred patients, were excluded. This study was approved by the Ethics Committee of the Third Hospital of Hebei Medical University (Approval No.: HMUTH-058-2014), and all the enrolled participants signed the informed consent.

Sample collection

After admission, 4 mL of fasting venous blood was collected from each participant in the early morning, left to coagulate at room temperature for 30 min, and centrifuged for 10 min (400 × g) to obtain upper serum for subsequent detection. There was no significant difference in general information such as age, gender, and body mass index (BMI) between the two groups (P>0.050).

Follow-up

During the 3-year follow-up, patients with MM were reviewed in the hospital to record their prognosis and survival.

Predictive value

Receiver Operating Characteristic Curve (ROC) was used to analyze the predictive value of LINC01606 in patients with MM.

Cell data

Human MM cell lines (RPMI8226, LP1, KMS26 and U266) and normal plasma cell line (nPCs) were all purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO₂ and saturated humidity. miR-579-3p mimics, miR-579-3p inhibitors and negative control (NC) were purchased from GenePharma. Small interfering RNA (si-LINC01606) and NC of LINC01606 were also obtained from Gene-Pharma. The full-length LINC01606 was amplified and inserted into the pcDNA3.1 vector (Invitrogen) to construct the LINC01606 over-expression plasmid (pcDNA-3.1-LINC01606). Cell transfection was performed using the Lipofectamine 2000 Reagent, strictly following the kit instructions.

PCR detection

Total RNA was extracted from cells or serum using Trizol, and its purity, concentration and integrity were detected by ultraviolet (UV) spectrophotometer and agarose gel electrophore-
sis. The total RNA was then reverse-transcribed into cDNA according to the reverse transcription kit instructions. Reaction system: RNase inhibitor: 1 μL, 5 × Reaction buffer: 5 μL, deoxyribonucleoside triphosphates (dNTPs; 10 mmol/L): 1.25 μL, Reverse transcriptase: 1 μL, 25 mmol/L MgCl₂: 4 μL, Oligo (dT) 15 primer: 1 μL, Random primer: 1 μL, and nuclease-free double distilled water, in a final volume of 25 μL. The expression profiles of RNAs were measured by the SYBR Green Realtime PCR Master Mix kit according to the instructions, with a reaction system of 20 μL. Reaction conditions (40 cycles): 95°C, 30 s; 60°C, 30 s; 70°C, 10 s. The primer sequences of genes (internal reference: β-actin) are as follows: LINC01606 upstream: 5'-GCTGGACATTTC-TCCCTTCA-3'; downstream: 5'-GAGTCCTCTGCT-TCCTCCT-3'; β-actin upstream: 5'-GCAAGG-AGTATGACGAG-3'; downstream: 5'-GCAA-TAAA-GCCATGCAATC-3'. Three replicate wells were set up for each sample, and the expression was calculated using the 2-ΔΔCt method.

CCK-8 assay

After digestion with trypsin to form a single cell suspension, cells were inoculated into 96-well cell culture plates (100 μL/well) at a density of 1 × 10⁴/well. Then, at specific time points (24, 48, 72 and 96 h after culture), 10 μL CCK-8 solution (Beyotime Corporation) was added into each well for another 1 h of incubation. The absorbance of each well was determined by a microplate reader at the wavelength of 450 nm.

Transwell invasion assay

The transfected MM cells were prepared into a single cell suspension in serum-free medium. Then, 3 × 10⁴ cells were inoculated on the surface of the Transwell upper chamber, and 500 μL complete medium containing 10% FBS was added into the lower chamber. The upper surface of the Transwell bottom membrane was coated with Matrigel gel and cultured at 37°C for 24 h. Then the chamber was fixed with formaldehyde and stained with 0.2% crystal violet solution for 10 min. Finally, 10 visual fields of the lower chamber were randomly examined under the microscope (200 ×) to calculate the number of invaded cells.

Wound-healing assay

The transfected MM cells were cultured in 12-well plates. Then, a straight line was created with a 20 μL sterile pipette tips when a cell confluence of 95-100% was observed. Under the microscope, the healing was observed immediately and 48 h after the scratch. Scratch healing rate = (immediate scratch area - scratch area 48 h later)/immediate scratch area × 100%. The ability of cell migration is proportional to the rate of scratch healing.

DLR assay

The Starbase (http://starbase.sysu.edu.cn/) was used to predict the binding sites between LINC01606 and miR-579-3p. cDNA fragments containing wild-type (LINC01606-WT) or mutant LINC01606 (LINC01606-mut) fragments were subcloned into the downstream luciferase gene in the psi-CHECK2 luciferase reporter vector. Then, miR-579-3p-mimics or miR-579-3p-inhibitor was co-transfected with LINC01606-WT or LINC01606-mut reporter vectors using transfection reagents (Invitogen, USA). Forty-eight hours after transfection, the firefly and renin luciferase activities in cell lysates were continuously measured using DLR kits (Promega, USA).

Statistical methods

The experimental data were analyzed and visualized by SPSS22.0 and Graphpad 8.0 respectively. The counting data were recorded in the form of % and compared by the Chi-square test. The measurement data were expressed as mean ± standard deviation; inter-group comparisons were performed using the independent samples t test, multi-group comparisons were conducted by one-way ANOVA and LSD post-hoc test, and multi-time point comparisons were carried out by repeated ANOVA and Bonferroni post-hoc test. The predictive value of LINC01606 was analyzed by ROC. The survival rate was calculated by the Kaplan-Meier method and compared by the log-rank test. The significance level was set at P<0.050.

Results

Expression of LINC01606 and miR-579-3p in MM

qPCR detection revealed that the expression of LINC01606 was higher and the expression of miR-579-3p was lower in group A compared with group B (P<0.050; Figure 1).
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Within the 3-year follow-up, 69 out of the 72 patients in group A were successfully followed up, with a success rate of 95.83%. A total of 19 patients died within 3 years, and the overall 3-year survival rate was 72.46%. The dead and the survival patients were further divided into death group and survival group respectively for analysis. It was found that the expression of LINC01606 was higher in the death group ($P<0.050$). When the cut-off value was 3.495, the sensitivity, specificity, AUC and 95% CI of LINC01606 in predicting the 3-year mortality of MM patients were 63.16%, 86.00%, 0.820 and 0.710-0.930 respectively ($P<0.001$). With the cut-off value as the boundary, the patients were further subdivided into high (LINC01606 ≥ 3.495, n=19) and low (LINC01606 < 3.495, n=50) LINC01606 groups. The comparison of prognosis and survival revealed that the 3-year survival rate of the high LINC01606 group was significantly lower than that of the low LINC01606 group ($P<0.001$; Figure 3).

Comparison of LINC01606 expression in MM cells and nPCs cells

The expression of LINC01606 in MM cell lines RPMI8226, LP1, KMS26 and U266 was higher than that in the normal plasma cell line nPCs ($P<0.050$). Among them, RPMI8226 and U266 exhibited the highest LINC01606 levels ($P<0.050$), so the two were selected for follow-up experiments. We detected the biological behavior of RPMI8226 and U266 after transfection of si-LINC01606. It was found that the proliferation, invasion, and migration of RPMI8226 and U266 were remarkably reduced compared with those of the NC group ($P<0.050$; Figure 4).

Effect of miR-579-3p on MM cells

The proliferation, invasion, and migration of RPMI8226 and U266 were decreased after trans-
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We predicted through Starbase (http://starbase.sysu.edu.cn/) that there were potential binding sites between LINC01606 and miR-579-3p. DLR assay revealed that the fluorescence activity of LINC01606-WT was inhibited by miR-579-3p-mimics (P<0.050). The expression of miR-579-3p in MM cells after LINC01606 transfection was detected. It was found that the expression of miR-579-3p was the highest in the si-LINC01606 group, followed in descending order by the NC group and the pcDNA-3.1-LINC01606 group (P<0.050; Figure 6).

Effect of co-transfection of si-LINC01606 and miR-579-3p-inhibition on MM cells

RPMI8226 and U266 were transfected with plasmids for LINC01606 inhibitor+miR-579-3p inhibitor (Co-transfection group), LINC01606 inhibitor, and NC respectively to detect the changes in biological behavior. There was no significant difference in biological behavior between the Co-transfection group and the NC group (P>0.050), while the proliferation, invasion, and migration in the si-LINC01606 group were lower than those in the other two groups (P<0.050; Figure 7).

Discussion

The current clinical screening of early tumors mainly relies on tumor markers such as CEA and CA199. Although these markers are sensitive to the occurrence of neoplastic diseases, they cannot accurately identify the exact tumor type [17]. Recently, CA199 has been confirmed to be highly expressed in cerebral infarct tissues [18]. In the face of the increasingly high incidence of MM, it is urgent to find new effective markers for early diagnosis and targeted therapy of MM [19, 20]. By exploring the effect of LINC01606 on MM, this study may carry huge clinical implications for future diagnosis and treatment of MM.

First, we compared the clinical data of patients and found no statistical difference between the two groups, indicating the feasibility for further research. Then we analyzed the expression of LINC01606 in MM cases and controls. The results showed that the expression of peripheral blood LINC01606 was high in patients with MM, suggesting that LINC01606 is involved in the occurrence and progression of MM. Sathipati et al. [21] also revealed the highly expressed LINC01606 in neuroblastoma, which is in line with our experimental results. ROC analysis revealed that the predictive sensitivity
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Figure 4. Effect of LINC01606 on MM cells. A: LINC01606 expression in MM cells and nPCs. *P<0.050 vs. RPMI8226; #P<0.050 vs. LP1; &P<0.050 vs. KMS26; @P<0.050 vs. U266. B: Proliferation of RPMI8226. C: Proliferation of U266. D: Invasion of RPMI8226 and U266. E: Migration of RPMI8226 and U266. *P<0.050.

and specificity of LINC01606 for the occurrence of MM were 85.29% and 72.39% respectively, indicating that LINC01606 can be used as an effective diagnostic marker as well as a feasible clinical screening tool for MM in the future. Compared with the commonly used clinical tumor markers such as CEA and CA199, LINC01606 yielded a more significant specificity, which can aid in clinical screening of MM. What’s more, a sensitivity as high as 85.29% indicates that LINC01606 has a significant response to MM, which can greatly improve the early diagnosis rate of MM. Yang et al. [11] proposed that LINC01606 was a potential core LncRNA in triple negative breast cancer, which further illustrates the huge application prospect of LINC01606 as a tumor marker in clinical practice. In the present study, patients were followed up for 3 years to record their survival. The results showed that the expression of LINC01606 in the dead was significantly higher than that in the survival, suggesting that LINC01606 also has a favorable predictive value for the 3-year mortality in patients. After grouping patients according to the cut-off value, we found that the higher the expression of LINC01606, the greater the risk of death in the patient, demonstrating that LINC01606 partici-
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Figure 5. Effect of miR-579-3p on MM cells. A: Proliferation of RPMI8226. B: Proliferation of U266. C: Invasion of RPMI8226 and U266. D: Migration of RPMI8226 and U266. *P<0.050.

Figure 6. Relationship between LINC01606 and miR-579-3p. A: The online website predicted the binding sites of LINC01606 and miR-579-3p. *P<0.050. B: Dual luciferase reporter assay confirmed the relationship between LINC01606 and miR-579-3p. *P<0.050. C: The expression of miR-579-3p in MM cells after transfection of LINC01606. *P<0.050 vs. the pcDNA-3.1-LINC01606; #P<0.050 vs. the si-LINC01606 group.

LINC01606 participates in the occurrence of MM and is closely related to the prognosis of patients with MM. Hence, we argue that LINC01606 may be a prognostic rehabilitation evaluation index for MM in the future.

The above experiments have initially evaluated the clinical application of LINC01606 in MM, but the underlying mechanism remains elusive. Therefore, the expression of LINC01606 in MM cell lines and normal plasma cell line nPCs was detected. The results determined higher LINC01606 expression in the MM cell lines, which is consistent with the results of previous clinical findings. Then, we inhibited the expression of LINC01606 in MM cells to test the changes in biological behavior of cells. It was found that the proliferation, invasion and migration of MM cells were significantly reduced, indicating that LINC01606 acts as an oncoprotein in MM. Literature has shown that LINC01606 can also accelerate gastric carcinogenesis and progression [22], which is similar to our findings. Yu et al. [23] also proposed
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that LINC01606 promoted gastric carcinogenesis through sponge adsorption of miR-423-5p. Whereas, the underlying mechanism of LINC01606 in MM has not been elucidated. Further, we transfected miR-579-3p-mimics into MM cells to test the changes in biological behavior. It was found that the proliferation, invasion, and migration of MM cells were significantly reduced after miR-579-3p overexpression, which confirms the close relationship between miR-579-3p and MM. Later, we found the potential binding sites between LINC01606 and miR-579-3p through the online prediction website Starbase. DLR assay revealed that the fluorescence activity of LINC01606-WT was inhibited by miR-579-3p-mimics, and the level of miR-579-3p was decreased after the transfection of LINC01606-mimics, indicating that LINC01606 inhibits miR-579-3p in a targeted manner. Evidence has shown that miR-579-3p has a similar mechanism of action in lung cancer [24], which also supports our findings. After simultaneous inhibition of LINC01606 and miR-579-3p in MM cells, we observed that cell activity was not statistically different from that of the NC group, but was higher than that of the si-LINC01606 group, suggesting that LINC01606 is involved in the biological behavior of MM cells by targeting miR-579-3p.

This study mainly explored the clinical implications and underlying mechanism of LINC01606 in MM, but there are still deficiencies due to limited experimental conditions. First, further investigation is warranted to explore LINC01606 targeted therapy for MM. Second, due to the absence of Western blot and nude mouse tumorigenicity assay, we were unable to evaluate the signaling pathway through which LINC01606 affects MM, which will be the focus of our future research. Third, the short prognostic follow-up time prevented us from evaluating the effect of LINC01606 on long-term prognosis of patients with MM. We will carry out more comprehensive experimental analyses to address the above shortcomings to obtain more accurate results for clinical reference.

In summary, LINC01606 is highly expressed in MM and can promote the proliferation, migration, and invasion of MM cells through targeted inhibition of miR-579-3p, which may be a therapeutic target for MM in the future.

Disclosure of conflict of interest

None.

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