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Original Research

Long noncoding RNA H19 promotes vincristine resistance in multiple myeloma by targeting Akt

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Abstract: Multiple myeloma is a malignant proliferation of plasma cells that results from a single clone. Manifestations include bone pain or fractures, kidney failure, susceptibility to infection, anemia, and hypercalcemia. To investigate the relationship between vincristine (VCR) resistance and Long noncoding RNA H19 (Lnc-RNA H19) in multiple myeloma (MM) this experiment was set up. For this aim, RT-PCR was used to detect the expression of lnc-RNA H19 in 60 MM patients from No.215 Hospital of Shaanxi Nuclear Industry and 50 healthy controls, and further detected the expression of related genes in myeloma cell lines and VCR myeloma resistant strains. MTT assay, flow cytometry assay, western-blotting assay and luciferase assay were used to analyze the growth, apoptosis and protein phosphorylation levels of drug-resistant RPMI 8226/VCR and RPMI 8226 cells after VCR treatment and plasmid transprinting. Results showed that the relative expression of lnc-RNA H19 was significantly increased in MM patients and drug-resistant strains RPMI 8226-VCR (****p<0.0001), while apoptosis of various MM cell lines increased after VCR treatment, while apoptosis of RPMI 8226-VCR was significantly decreased (***p<0.001). Lnc-RNA H19 overexpression plasmid pcdna3.1-h19 and Akt overexpression plasmid pcdna3.1-akt decreased apoptosis in RPMI 8226 cell lines without VCR resistance (***p<0.001), while the recombination of siRNA-h19 and siRNA-akt plasmid increased apoptosis in RPMI 8226-VCR (***p<0.001). The lnc-RNA H19/Akt pathway is closely related to the occurrence of VCR resistance in MM cells, and the down-regulation of H19 can significantly improve the sensitivity of VCR in MM.

Key words: Multiple myeloma; Vincristine; Lnc-RNA H19; RT-PCR; Multiple myeloma cell line.

Introduction

Multiple myeloma (MM) is a serological malignant tumor featured by the abnormal monoclonal plasmacytosis in the marrow. The disease can affect the bone marrow of all bones, but the most common sites of conflict are the thighs, back, pelvis, or upper arms. It is more common in men aged 70-50 years. Multiple myeloma is more common in farmers and people with wood and leather and petroleum derivatives. The risk of developing the disease increases with age (1-3). The monoclonal immunoglobulin secreted by these plasma cells that are usually detected in serum and/or urine brings about the damage to the organs, with manifestations like anemia, hypercalcemia, renal insufficiency and osteopathy (4-6).

LncRNA H19 is kind of non-coding RNA sequence transcribed from H19/igf2 in human chromosome 11p5.5 (7-10) that acts as either the oncogene or the tumor suppressor gene, which depends on the tumor types. As reported, H19 is rich in a variety of tumor tissues in human beings, suggesting that the high expression of H19 may facilitate tumorigenesis (11-14).

In this study, we aimed to clarify the potential effect of lncRNA H19 on the VCR resistance in the treatment of MM and found that lncRNA H19 is highly expressed in VCR-resistant RPMI 8226/VCR cells, while this upregulation may associate with the poor prognosis of MM. Further work revealed that in the development of resistance to VCR, the phosphorylation level of Akt is increased, representing the activation of Akt, which results in the enhancement of the VCR resistance. In addition, we also noted that the downregulation of lncRNA H19 would enhance the sensitivity to the VCR, while overexpression results in the opposite changes. Thus, these findings suggest that lncRNA H19 is a potential target in the molecular treatment of MM.

Materials and Methods

Subjects

Between January 2018 and January 2019, we selected 60 MM patients that were confirmed by the pathological examination and 50 healthy controls from No.215 Hospital of Shaanxi Nuclear Industry. Prior to the study, all patients were informed of the content of this study and agreed to participate in this study with the written informed consent, and this study was reviewed and approved by the Ethical Committee of the No.215 Hospital of Shaanxi Nuclear Industry.

Cell culture

HEK-293T and MM cell lines (JJN-3, U266, ANBL6 and RPMI 8226) and the VCR-resistant cell line (RPMI 8226-VCR) (Shanghai Cell Bank of Chinese Academy of Sciences) were cultured in the RPMI medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% streptomycin-penicillin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. Then, the normal human marrow $CD138^+$ plasma cells were selected by magnetive beads (EasyStep™, Stem Cell, Canada).

RT-PCR

Serum RNA was extracted by the use of the extraction kit (Life Technologies, USA) according to the instructions. cDNA was prepared by use of the Reverse transcription kit (Thermo Fisher Scientific, USA) in the reverse transcription system consisting of the 10 μL RNA extraction, 4 μL 5 x reaction buffer, 2 μL 10 mM dNTP, 1 μL primers, 1 μL Revert Aid RT, 1 μL RNase inhibitor and 1 μL RNase-free water. Following incubation at 42°C for 60 min and 70°C for 5 min, the resultant cDNA was preserved -80°C for later use. All reactions were detected in the Roche LightCycler 480 (Roche, Switzerland) under the following conditions: 95°C for 30 s and 45 cycles of 5 s at 95°C and 30 s at 60°C. The magnification of the production was ascertained by the melting curve. H19 primer sequences are shown as follows: Forward, 5′-GCGGGTCTGTTTCTTTACTTC-3′, and reverse, 5′-TTTCATGTTGTGGGTTCTGG-3′; Akt: Forward, 5′-GGCCCAGATCACCATCAC-3′, and reverse, 5 ′-CTATCGTCCAGCGCAGTCCA-3′; primers used to amplify 18S: Forward, 5′-GTAACCCGTTGAACCCCATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′. The result with a CT value > 40 was deemed negative, and the relative expression of the target lncRNAs was calculated by $2^{-\Delta\Delta Ct}$.

Flow cytometry

Cell apoptosis was evaluated by the double staining of Annexin V-FITC and PI for 48 h and the flow cytometry (FACScan, BD Biosciences, Shanghai, China). Cells were categorized into live cells, dead cells, early-apoptotic cells and apoptotic cells. A comparison was carried out in the percentage of the early-apoptotic cells among different groups. Measurement of the cell apoptosis was performed according to the standard protocols (Roche Molecular Biochemicals, Indianapolis, IN).

MTT assay

The inhibitory effect of VCR on the MM cell stain was evaluated by the *in vitro* MTT assay. Cells cultured in 96-well plate at 37°C for 24, 48 and 72 h were incubated with the 20 μg/mL MTT (Promega) for 4 hours to detect the vitality of cells in each well. Then, the medium in each well was replaced by a 30 μL MTT solution, followed by incubation at 37°C and 5% CO_2 for 15 min. Thereafter, 200 μL DMSO was added into each well, followed by incubation on a shaker for 10 min. Results were reflected by the optical density at 490 nm that was normalized to that at 620 nm.

Western blotting

Protein samples of MM cells were placed at 25 μg/lane into the SDS-PAGE for electrophoresis to isolate the proteins. Then, proteins in the gel were transferred into the PVDF membrane for incubation with 5% non-fat milk in TBST for 1 h at room temperature. Then, proteins on the membrane were probed by incubating with the anti-Akt and -pAkt antibodies (Abcam, UK) at 4°C overnight, followed by washing the membrane for 15 min. Then, the resulting immunoblots were incubated with the horseradish peroxidase-conjugated rabbit anti-goat IgG (Amersham) for 1 h at room temperature. The final immunoblots were visualized by incubating with the enhanced chemiluminescence plus (Amersham).

Statistical analysis

GraphPad Prism was utilized for statistical analysis. Normal distribution of data was tested by the D'agostino-Pearson test. For data in the normal distribution, Student's *t*-test was performed for comparison between two groups, or One-Way ANOVA in combination with Tukey's *post hoc test* for the comparison among groups. For those not conforming to the normal distribution, the Mann-Whitney U test was adopted for comparison between two groups, or the Kruskal-Wallis test and Dunn multiple comparison test for comparison among groups. $P < 0.05$ suggested that the difference had statistical significance.

Results

H19 is increased in MM and correlates with the poor prognosis of MM

To explore the effect and mechanism of H19 in MM, we firstly detected the expression profile of H19 in MM, and as shown in Figure 1A, we found that H19 was upregulated in MM ($P < 0.0001$). Subsequently, we detected the expressions of H19 in the human MM cell lines (JJN-3, U266, ANBL6, RPMI 8226 and RPMI 8226-VCR), and found that H19 was upregulated in RPMI 8226-VCR cell lines, instead of other cell lines ($P < 0.0001$; Figure 1B).

To further validate the correlation of H19 expression with the clinical-pathological features of MM, we divi-

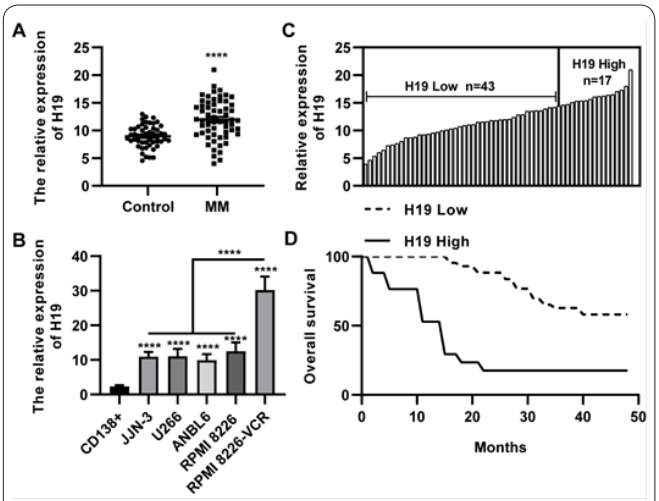

Figure 1. Correlation between expression of lncRNA H19 and prognosis of MM patients. A: High expression of lncRNA H19 in MM patients; B: High expression of lncRNA H19 in MM cells; C: Youden index of lncRNA H19 in 60 MM patients; D: correlation between the survival of patients and lncRNA H19 expression in Kaplan Meier survival curve.

ded the correlation between H19 expression and Youden indexes into the weak (n=43) and strong (n = 17) (Figure 1C). Then, the overall survival (OS) curve was prepared for the H19 expression by use of the Kaplan Meier method to explore the correlation between the H19 expression and prognosis of MM patients (Figure 1D) and found the significant correlation between the high expression of H19 and poor OS.

High expression of H19 promotes the VCR-resistance of MM cells

To explore the potential correlation between H19 expression and VCR resistance in MM, we subsequently detected the resistance of RPMI 8226 and RPMI 8226-VCR to the VCR in 10 nM. According to the analysis, we found that RPMI 8226-VCR showed stronger resistance to VCR then RPMI 8226 cells ($P < 0.001$). To rule out the effect of other factors, we transfected the RPMI 8226 cells with pcDNA3.1-H19 overexpression plasmid and RPMI 8226-VCR cells with siRNA-H19. According to the results of flow cytometry and MTT assay, we found that in RPMI 8226 cells, H19 overexpression enhanced their resistance to VCR and proliferation (Figure 2C and D, $P < 0.001$). On the contrary, H19 inhibition in RPMI 8226-VCR cells increased their sensitivity to the VCR (Figure 2E and F, $P < 0.001$). Thus, H19 is the primary factor regulating VCR resistance.

H19 regulates VCR resistance via the Akt pathway

To find out the target gene of H19 in regulating VCR resistance, we performed the Western blotting for RPMI 8226 cells transfected with the pcDNA3.1-H19 overexpression plasmids and found that the phosphorylation level of Akt in these cells was increased evidently in comparison with those with no transfection ($P < 0.05$; Figure 3A). Meanwhile, RT-PCR results revealed the significant increase in the relative mRNA expression of Akt in the transfected cells ($P < 0.001$; Figure 3B), and similar change was observed in the RPMI 8226-VCR cells ($P < 0.001$; Figure 3C). Thus, H19 may affect the VCR resistance via the Akt signal pathway.

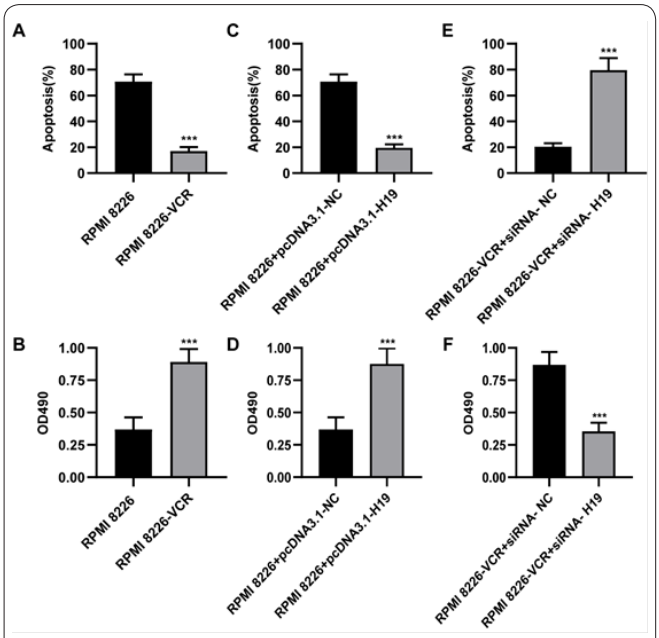

Figure 2. lncRNA H19 enhances the resistance of MM cells to VCR. A and B: Apoptosis and proliferation of RPMI 8226 and RPMI 8226-VCR in treatment of 10 nM VCR by flow cytometry and MTT assay; C and D, Apoptosis and proliferation of pcDNA3.1-H19-transfected RPMI 8226 in treatment of 10 nM VCR by flow cytometry and MTT assay; E and F, Apoptosis and proliferation of siRNA-transfected RPMI 8226-VCR in treatment of 10 nM VCR by flow cytometry and MTT assay.

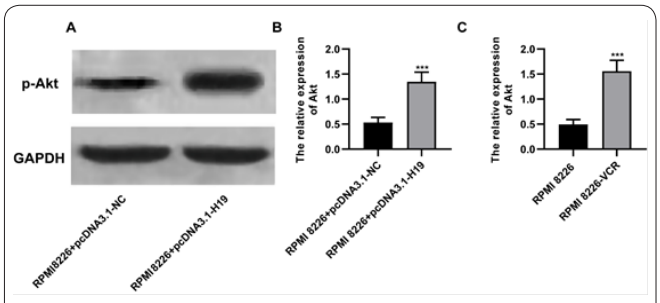

Figure 3. LncRNA H10 targets Akt to promote the resistance of MM cells to VCR. A: Phosphorylation of Akt is increased in pcDNA3.1-H19-transfected RPMI 8226 cells detected by RT-PCR; B: mRNA expression of Akt is increased in pcDNA3.1-H19-transfected RPMI 8226 cells detected by RT-PCR; C: mRNA expression of Akt is increased in RPMI 8226-VCR cells.

Akt pathway alters the H19-mediated VCR resistance

To verify the role of the Akt pathway in lncRNA H19-mediated VCR resistance, we carried out the flow cytometry and MTT assay. As a result, in RPMI 8226 cells, we found that the transfection of pcDNA3.1-Akt plasmid increased the resistance of cells to VCR, while the upregulation of Akt showed no effect on H19 expression (Figure 4A, B and C). In RPMI 8226-VCR cells, those transfected by siRNA-H19 decreased the resistance, which, however, was rescued by the upregulation of Akt (Figure 4D and E). Moreover, the trans-

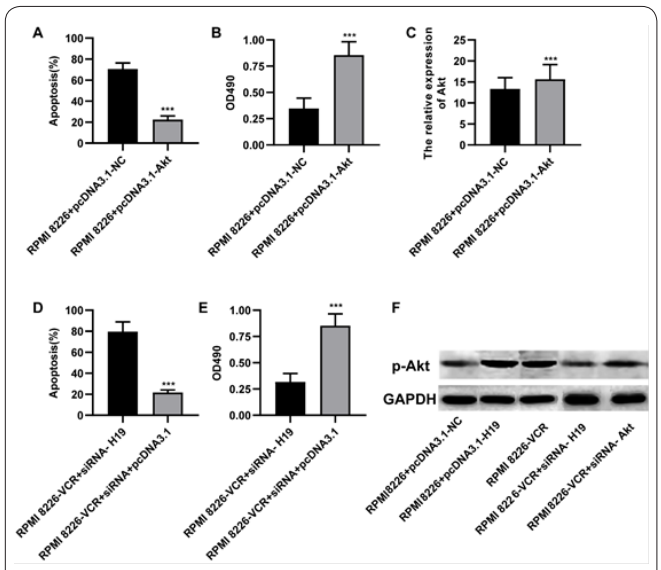

Figure 4. LncRNA H19 regulates the expression of Akt to alter the resistance of MM cells to VCR. A and B: Apoptosis and proliferation of pcDNA3.1-H19-transfected RPMI 8226 in treatment of 10 nM VCR by flow cytometry and MTT assay; C: Akt in high expression does not alter the expression of H19; D and E: Effect of H19 downregulation or Akt upregulation in RPMI 8226/VCR on resistance to VCR detected by flow cytometry and MTT assay; F: Co-transfection of siRNA-H19 and siRNA-Akt decreases the phosphorylation of Akt.

fection of pcDNA3.1-Akt plasmid also enhanced the phosphorylation of Akt in RPMI 8226 cells, while in RPMI 8226-VCR cells, transfection of siRNA-H19 and siRNA-Akt plasmids curbed the phosphorylation of Akt (Figure 4F). Thus, lncRNA H19 could regulate the resistance of MM cells to VCR by regulating the Akt pathway.

Discussion

Recently, continuous development in the lncRNA has deepened the understanding of the functional diversity in a variety of literatures, including the regulation of transcription (15-19). In MM, lncRNA has also been widely investigated, especially the involvement in the dysfunction in MM (20). However, little is known about the molecular mechanism of lncRNA in MM (20).

In this study, we found that lncRNA H19 is highly expressed in RPMI 8226-VCR cells and the correlation with the poor prognosis. Moreover, we also validated that lncRNA H19, as an endogenous lncRNA, facilitates the expression of Akt, by which it can regulate the resistance to VCR. These results provide new insights into the development of a treatment strategy.

Akt, as a kind of serine/threonine kinase activated in a phosphatidylinositol3 kinase-dependent manner (22,23), is the central regulator involved in many cellular processes, including proliferation, differentiation, survival and metabolism of cells (21,22). As reported, blocking the Akt signal pathway can inhibit the growth and facilitate the apoptosis in tumor cells, while its correlation with the sensitivity of tumor cells to the chemotherapeutics is still under investigation (24). In the work of Chakraborty *et al*., the Akt pathway is activated in the retinoblastoma (25).

In many studies, RNA study has been instrumental in discovering the relationships between traits, the cause of diseases, and sometimes their treatment (26-31). This feature can be considered more in the future (32-33).

In our work, we found that inhibition of Akt activity contributes to the decrease in the resistance of RPMI 8226-VCR to VCR, thereby exhibiting the resistance to that in the control group and that Akt activation is regulated by the expression of lncRNA H19. Co-transfection of siRNA-H19 and pcDNA3.1-Akt plasmids hardly alters the resistance of RPMI 8226-VCR cells to VCR, which was also confirmed by the Western blotting and RT-PCR. Thus, lncRNA H19 potentially regulates the VCR resistance in MM cells via targeting Akt.

To be concluded, high expression of lncRNA H19 may represent the poor prognosis of MM, but for the limitation in the sample size, this conclusion should be validated in large sample-size trials. Through the traditional analysis, we reveal that lncRNA H19 may promote the progression of MM by upregulating Akt, and the potential correlation between lncRNA H19 and Akt signal pathway. These findings suggest that lncRNA H19 is a key molecular marker that is able to predict the prognosis of MM and block the resistance of MM cells to VCR as the molecular target.

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