Original Article

mir-19a-3p Functions as an Oncogene by Regulating FBXO32 Expression in Multiple Myeloma

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Background: Multiple myeloma (MM) remains a virtually incurable hematologic malignancy, which is featured with the aberrant growth of malignant plasma cells.
Aims: To elucidate the functions of miR-19a-3p in MM.
Study design: experimental study.
Methods: CCK-8 assay was performed to detect cell viability. Flow cytometry was conducted to detect cell apoptosis. Bioinformatics analysis predicted miR-19a-3p associated biological function, pathway, core regulatory network, and target genes. Luciferase reporter assay verified the target sequence of miR-19a-3p regulating FBXO32.
Results: miR-19a-3p is up-regulated in MM cells ($P< 0.01$) and patients ($P< 0.001$). Overexpressed miR-19a-3p significantly increased cell viability ($P< 0.05$) and inhibited cell apoptosis ($P< 0.01$). FBXO32 is a target gene of miR-19a-3p ($P< 0.01$). Besides, FBXO32 is downregulated in MM and significantly decreased cell viability ($P< 0.05$) and promoted cell apoptosis ($P< 0.01$). FBXO32 significantly rescued the influence of miR-19a-3p inhibiting cell apoptosis ($P< 0.05$).
Conclusion: miR-19a-3p promoted cell proliferation and inhibited cell apoptosis by degrading target FBXO32 mRNA in MM.
Keywords: FBXO32, miR-19a-3p, multiple myeloma, oncogene

Multiple myeloma (MM), accounting for about 13% of haematological malignancy, is featured with the aberrant growth of malignant plasma cells(1). Previous research achievements have greatly increased life expectancy and

quality of life. However, MM remains a virtually incurable hematologic malignancy(2). Multiple myeloma pathogenesis is involved in a series of biological processes, such as gene mutations, chromosomal abnormalities, epigenetic modifications, cell proliferation, tumour-microenvironment, and evolution of drug-resistant tumour cells(3, 4). Identifying essential genes in disease progression is of the most significant importance for identifying new therapeutic approaches and prolonging the prognosis in patients (5, 6).

MicroRNAs (miRNAs), 18~24 nucleotides in length, are a kind of non-coding RNA. MiRNAs regulate post-transcription gene expression by degrading or repressing target mRNAs (7, 8). miRNAs mediate various biological function, such as differentiation, proliferation, apoptosis, and migration (9, 10). A series of cancer-associated miRNAs has recently been profiled in MM using microarray analysis(10). Previous findings demonstrated that miR-19a-3p acts as a novel poor prognostic indicator in MM (11). However, the mechanism of miR-19a-3p regulating cellular function in myeloma cells has not been elucidated.

FBXO32 (atrogin-1) belongs to the F-box protein family(12). Recent findings have reported that FBXO32 is downregulated in cancers and may function as a tumour suppressor(13-17). Besides, emerging studies suggest that FBXO32 is a novel apoptosis regulator(18). However, the upstream regulatory factor of FBXO32 in tumorigenesis remains unclear.

This study showed that miR-19a-3p is significantly overexpressed and significantly suppressed cell apoptosis in MM cells. FBXO32 was negatively regulated by miR-19a-3p. Besides, our results demonstrated that FBXO32 was a miR-19a-3p target gene in MM cells. FBXO32 rescued the function of miR-19a-3p inhibiting cell apoptosis in MM cells. These results suggested that miR-19a-3p promoted MM cells development by regulating FBXO32 expression.

Methods and Materials

2.1 Cell lines and clinical specimens

Cells (MM.1S, U266, RPMI8266, IM9, H929, and W63) were purchased from the American Type Culture Collection (ATCC, MD, USA). Bone marrow (BM) specimens were provided by 8 healthy donors (5 males, 3 females; age range, 25-55 years) and 8 MM patients (4 males, 4 females; age range, 30-56 years) from (for blind review). Clinical samples were purified with CD138 MicroBeads according to the Miltenyi-Biotec protocol. Ethical approval was obtained from (for blind review). Cells were grown in the RPMI 1640 medium, containing 10% fetal bovine serum (FBS), penicillin (100U/ml), and streptomycin (100μg/ml).

2.2 Quantitative real-time PCR

We extracted total RNAs (cells and BM specimens) using trizol reagent (Invitrogen, MD, USA) following the instructions. The SYBR Green primers are listed in table1. Reverse transcription of complementary DNA (cDNA) and quantitative PCR were performed using Takara reagent (Takara, Shiga, Japan). GAPDH and U6 were conducted as the standard for normalization. The $2^{-\Delta\Delta CT}$ method was used to determine mRNA expression levels.

2.3 Transfection

Invitrogen (Thermo Fisher Scientific, USA) was responsible for synthesizing the miR-19a-3p mimics (cat. no. MIMAT0000073) and inhibitors (cat. no. MIMAT0021837). FBXO32 overexpression vector was synthesized by Sangon Biotech (Shanghai, China). Vectors were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, USA).

2.4 CCK-8 assays

Cell counting kit-8 (DOJINDO, Japan) reagent was conducted to test cell viability. U266 and H929 cells (5×10^3/well) were seeded on plates and incubated 24h, 48h, or 72h. The medium was replaced with 10μl CCK-8 reagent and 100μl fresh medium. After 1h at 37℃, the wavelength of optical density (OD) values was 450 nm.

2.5 Flow cytometry

The FITC/PI reagent (Keygen, Nanjing, China) was performed to detect cell apoptosis. FlowJo_V10 software was used to analyze the results. The upper right quadrant (UR) represented late apoptotic cells. The right lower quadrant (UR) represented viable apoptotic cell.

2.6 Bioinformatics analyses

The biological function of miR-19a-3p and FBXO32 in cancer was explored in Cancer Hallmarks Analytics Tool (CHAT)(19). Targets of miR-19a-3p were analyzed by TargetScan(20), miRDB(21) and DIANA-microT(22) database. Online Venn tools (http://bioinformatics.psb.ugent.be/webtools/Venn/) visualized the overlapping target genes of these databases. Enrichment analysis (gene ontology and pathway) was performed using Enrichr analysis tool(23). The protein interaction network of miR-19a-3p target genes was analyzed at STRING analysis tool(24) and visualized by Cytoscape software. The Cytoscape-MCODE algorithm further extracted the core subnetwork.

2.7 Dual-luciferase reporter assays

The estimated binding site of miR-19a-3p or mutant 3'-UTR sequences were cloned in the XhoI and NotI (Promega, Madison, USA) restriction sites to the psiCHECK2 vector. The cells were co-transfected with the vectors of wild-type (WT) or mutated (Mut) FBXO32 3'-UTR, and the miR-19a-3p NC/mimics/inhibitors. Luciferase assay reagent (Promega, Madison, USA) was conducted to detect the luciferase activity after 48 hours.

2.8 Western blotting

Cleaved caspase 3 and BCL-2 (Abcam, San Francisco, USA) expression levels were detected by western blotting using following instructions. Cells were lysed with Lysis Buffer (CST, Danvers, USA). The supernatant protein was collected and normalized, and equivalent protein (30μg) was electrophoresed and then transferred onto the polyvinylidene difluoride (PVDF) membranes. A blocking buffer was used to block proteins on the PVDF membranes at 4□ overnight. Then, The membranes incubated with the specific antibody, and were washed in washing buffer (0.1% Tween 20 in PBS). Proteins were then visualized by ECL reagents (KeyGen, Nanjing, China). GAPDH (ImmunoWay, TX, USA) expression levels were used as the standard for normalization.

2.9 Statistical analyses

We analyzed the results using SPSS 21.0 software (SPSS, Chicago, USA). Normality distribution of variables was tested by the Shapiro Wilk test. Mean± standard deviation (SD) was used as descriptive statistics for normal distributed data. Median (Min-Max) was used as descriptive statistics for non-normal distributed data. The t-test and one-way ANOVA tested normal distributed results. Multiple comparisons were made using LSD and SNK tests. Either $\chi 2$ tests or Fisher's exact tests were used to analyze non-normal distributed data. All experiments presented here were conducted at least three independent times. *P* values <0.05 was regarded as signification.

Results

3.1 Basic expression levels of miR-19a-3p in MM cells and patients

Using Cancer Hallmarks Analytics Tool (CHAT), we revealed that miR-19a-3p is associated with inducing angiogenesis and sustaining proliferative signaling (Figure 1A). RT-qPCR assays revealed that miR-19a-3p was increased in MM cells and patients ($P< 0.01$) (Figure 1B, C).

3.2 miR-19a-3p regulated cell viability and apoptosis in MM cells

To further investigate the biological function of miR-19a-3p in MM, we overexpressed or knocked down miR-19a-3p in U266 and H929 cells ($P< 0.01$) (Figure 1D, E). CCK-8 assays revealed that overexpressed miR-19a-3p significantly promotes cell viability ($P< 0.05$). Besides, miR-19a-3p knockdown suppressed cell viability ($P< 0.05$) (Figure 2A, B). Flow cytometry was conducted to detect cell apoptosis in U266 and H929 cells, which

showed that miR-19a-3p overexpression significantly inhibits cell apoptosis and miR-19a-3p knockdown increased cell apoptosis ($P< 0.01$) (Figure 2C).

3.3 Predicting miR-19a-3p target genes

miR-19a-3p target genes were estimated through three bioinformatics databases, including TargetScan, miRDB, and DIANA-microT. A total of 692 genes were overlapped in three bioinformatics databases (Figure 3A). Enrichr analysis tool was used to analyze the correlated biological function and pathway of these overlapping target genes, which results revealed that these target genes are correlated with multiple biological function and pathway, such as negative regulation of cytoplasmic translation, protein kinase activity, FoxO signaling pathway, etc. ($P< 0.05$) (Figure 3B-E). Besides, we analyzed the protein interaction network of these overlapping genes through the STRING analysis tool (Figure S1) and extract core subnetworks using the cytoscape-MCODE analysis tool. Finally, we obtained 5 significant core subnetworks and found that FBXO32 is a core gene in the core subnetworks ($P< 0.05$) (Figure 3F).

3.4 FBXO32 was a target gene of miR-19a-3p in MM cells

In CHAT, FBXO32 is also associated with sustaining proliferative signaling (Figure 4A). Then, we found that FBXO32 3'-UTR sequences matched the "seed sequence" of miR-19a-3p, which indicated FBXO32 is related to the regulation of MM (Figure 4B). Luciferase report system revealed that miR-19a-3p mimics significantly inhibits the activity of FBXO32-wt 3'UTR among H929 and U266 cells ($P< 0.05$). miR-19a-3p inhibitors significantly promoted the activity of FBXO32-wt 3'UTR among H929 and U266 cells ($P< 0.01$) (Figure 4C, D). miR-19a-3p does not regulate the activity of FBXO32-mt 3'UTR among H929 and U266 cells (Figure 4C, D). Besides, RT-qPCR assays revealed that FBXO32 is significantly downregulated in MM patients ($P< 0.001$) (Figure 4E) and cells ($P< 0.05$) (Figure 4F). miR-19a-3p mimics significantly inhibited FBXO32 mRNA expression ($P< 0.05$) (Figure 4G). miR-19a-3p inhibitors significantly promoted FBXO32 mRNA expression in U266 and H929 cells ($P< 0.001$) (Figure 4H).

3.5 miR-19a-3p regulated cell viability and apoptosis through FBXO32 in MM cells

FBXO32 was significantly overexpressed in U266 and H929 cells ($P< 0.01$) (Figure 5A). CCK-8 assays revealed that FBXO32 significantly inhibits cell viability ($P< 0.05$) (Figure 5B). Flow cytometry showed that FBOX32 significantly promotes cell apoptosis ($P< 0.01$) (Figure 5C, D). Further, we found that FBXO32 significantly reverses the function of miR-19a-3p promoting cell viability ($P< 0.05$) (Figure 5E). Also, FBXO32 significantly reversed the function of miR-19a-3p inhibiting cell apoptosis ($P< 0.05$) (Figure 5F, G). WB revealed that miR-19a-3p significantly inhibits cleaved caspase 3 expression and increases Bcl-2 expression, which was also reversed by FBXO32 overexpression ($P< 0.05$) (Figure 5H).

Discussion

MM remains an incurable haematological malignancy given the clinical use of chemotherapeutics, glucocorticoids, and novel treatments (25). Hence, it is essential to identify new biomarkers in tumorigenesis and progression, which contribute to realizing the pathogenesis and find novel treatment for MM. Recently, studies have demonstrated aberrant miRNA expression in carcinoma tissues and cells, which may indicate significant molecular and clinical implications of miRNA in tumorigenesis and progression(26).

Previous studies reported that miR-19a-3p was increased in many cancers (including MM, hepatocellular carcinoma, breast cancer, etc.)(5, 27, 28). Currently, our results presented that miR-19a-3p was overexpressed in MM cells and patients (Figure 1B, C). Interestingly, the significance level in MM patients ($P<0.001$) was higher than in MM cells ($P<0.01$). Although the significance level does not account for the difference in expression, it is an interesting situation to consider. On the one hand, it may be caused by the lack of clinical samples. The miRNA expression levels of different individuals were significantly different. On the other hand, these cell lines are derived from multiple myeloma patients, but screened. Cell lines are not equivalent to primary cells. Further

studies revealed that miR-19a-3p suppressed cell apoptosis and promoted cell viability in U266 and H929 cells (Figure 2A, C). These results showed that miR-19a-3p is an oncogene in MM cells. miRNAs alter gene expression by degrading or repressing target mRNAs. Subsequently, we predicted miR-19a-3p target genes in MM cells. Bioinformatics analysis ultimately extracted five core subnetworks (Figure 3F), which provides the research direction. Our previous studies screened these potential regulatory molecules. Eventually, we found that FBXO32 may be regulated by miR-19a-3p.

FBXO32 is a novel E3 ligase, which is one of the four subunits of the ligase complex of ubiquitin-proteins (13). Recent evidence revealed the function of FBXO32 in tumorigenesis (29). FBXO32 was reported to be decreased in cancers, and FBXO32 induced apoptosis and increased cisplatin chemosensitivity (14-16, 29). Our results demonstrated that FBXO32 is down-regulated in MM cells and tissues (Figure 4E, F). Besides, miR-19a-3p negatively regulated FBXO32 mRNA expression (Figure 4G, H) in U266 and H929 cells. Luciferase assay verified that miR-19a-3p regulates gene expression by degrading FBXO32 mRNA (Figure 4C, D).

Next, FBXO32 was overexpressed in U266 and H929 cells, and the results revealed that FBXO32 significantly suppressed cell viability and increased cell apoptosis (Figure 5B, C). In order to verify that miR-19a-3p actually regulates a variety of biological functions by regulating FBXO32 expression in multiple myeloma, both mimics and FBXO32 overexpression plasmid were transfected in U266 cells, so as to detect cell viability, cell apoptosis, and apoptosis-associated protein expression. Our findings demonstrated that FBXO32 overexpression reverses the functions of miR-19a-3p increasing cell viability and suppressing apoptosis (Figure 5E, F). Also, FBXO32 overexpression (Figure 5H) abolishes the influences of miR-19a-3p suppressing cleaved caspase 3 and increasing BCL-2.

Based on the above results, we preliminarily revealed that miR-19a-3p directly regulates FBXO32. It would be significant to discover detailed mechanism in MM in the future. More studies are still necessary to confirm these findings, which will provide strong evidence supporting the function of miR-19a-3p and FBXO32 in MM occurrence and development.

In conclusion, our current work confirmed the expression levels of miR-19a-3p in MM cells and patients. miR-19a-3p significantly promoted cell viability and suppressed cell apoptosis. Moreover, FBXO32 was a target gene of miR-19a-3p. FBXO32 was downregulated and significantly suppressed cell viability and promoted cell apoptosis in MM cells. Rescue assay further suggested that miR-19a-3p plays a role of oncogene by regulating FBXO32 expression in MM cells.

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Figure Legends

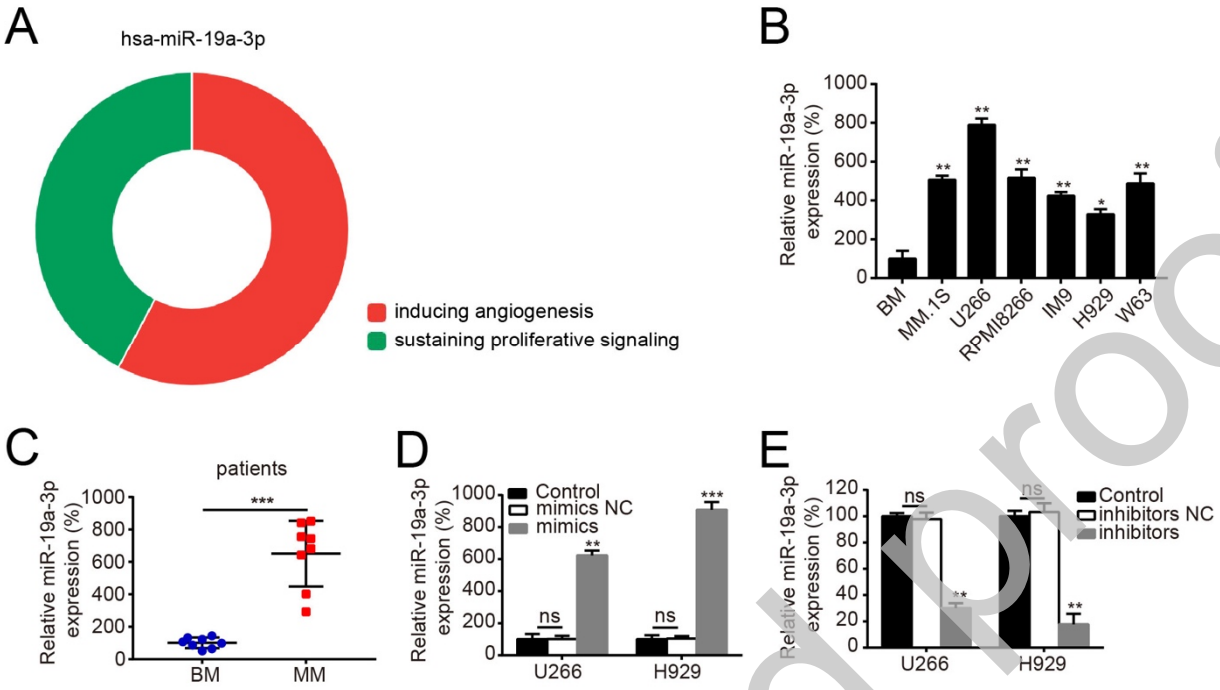

FIG. 1. Expression of miR-19a-3p in multiple myeloma cell lines and tissues (A) Association between miR-19a-3p and hallmarks of cancer from Cancer Hallmarks Analytics Tool (CHAT), which can be accessed at http://chat.lionproject.net/. (B) Relative miR-19a-3p expression levels between normal bone marrow cells (BM) and multiple myeloma cell lines. (C) Relative miR-19a-3p expression levels between normal bone marrow cells (BM) and multiple myeloma patients (MM). (D) miR-19a-3p mimics increased miR-19a-3p expression in U266 and H929 cells. (E) miR-19a-3p inhibitors suppressed miR-19a-3p expression in U266 and H929 cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; each bar represented Means ± SD from three independent experiments.

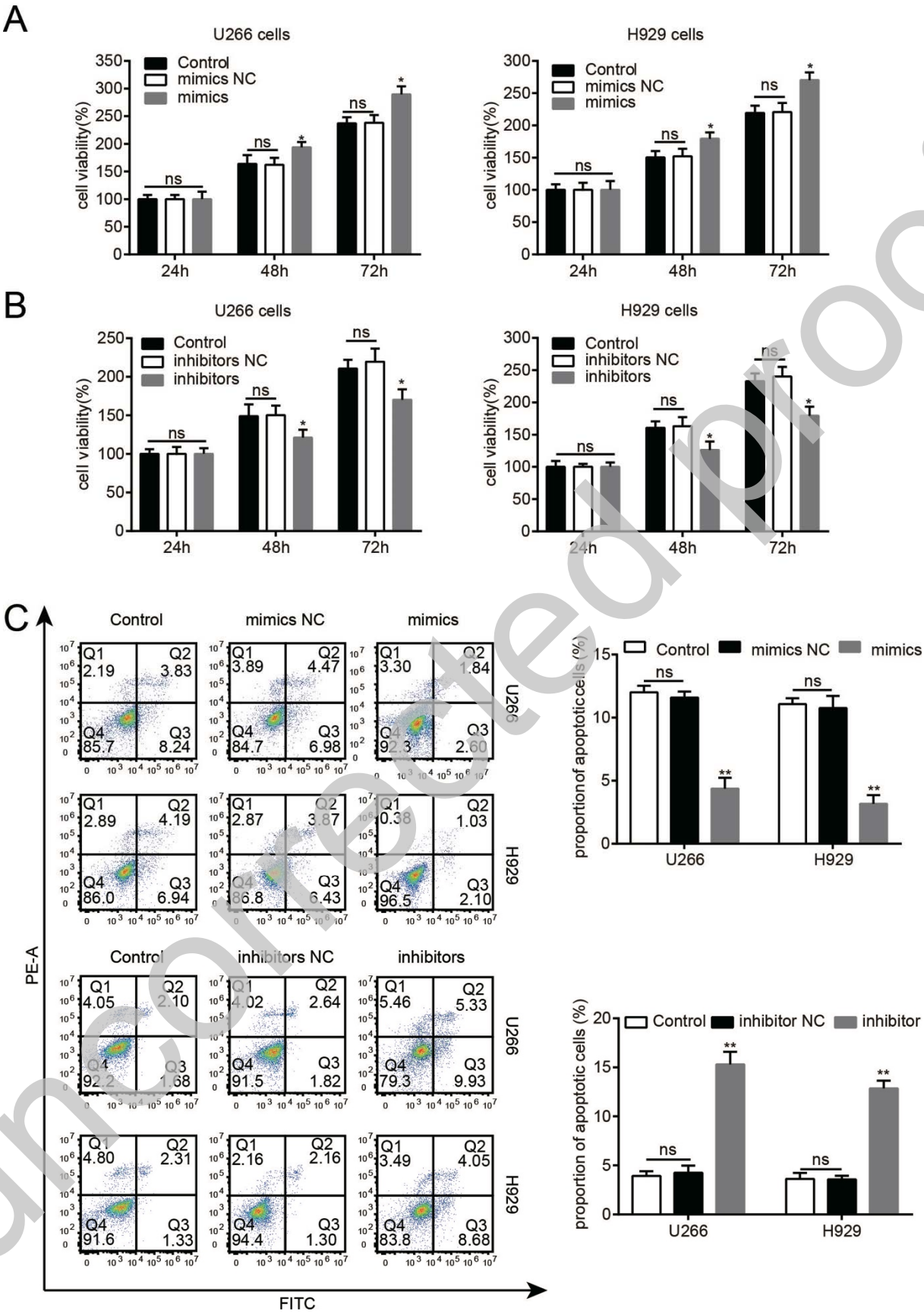

FIG. 2. miR-19a-3p promoted cell viability and inhibited cell apoptosis in MM cell lines (A) miR-19a-3p overexpression promoted cell viability in U266 cells and H929 cells. (B) miR-19a-3p inhibitors decreased cell

viability in U266 cells and H929 cells. (C) miR-19a-3p overexpression inhibited cell apoptosis, and miR-19a-3p inhibitors promoted cell apoptosis in U266 cells and H929 cells. *, $P<0.05$; **, $P<0.01$; each bar represented Means ± SD from three independent experiments.

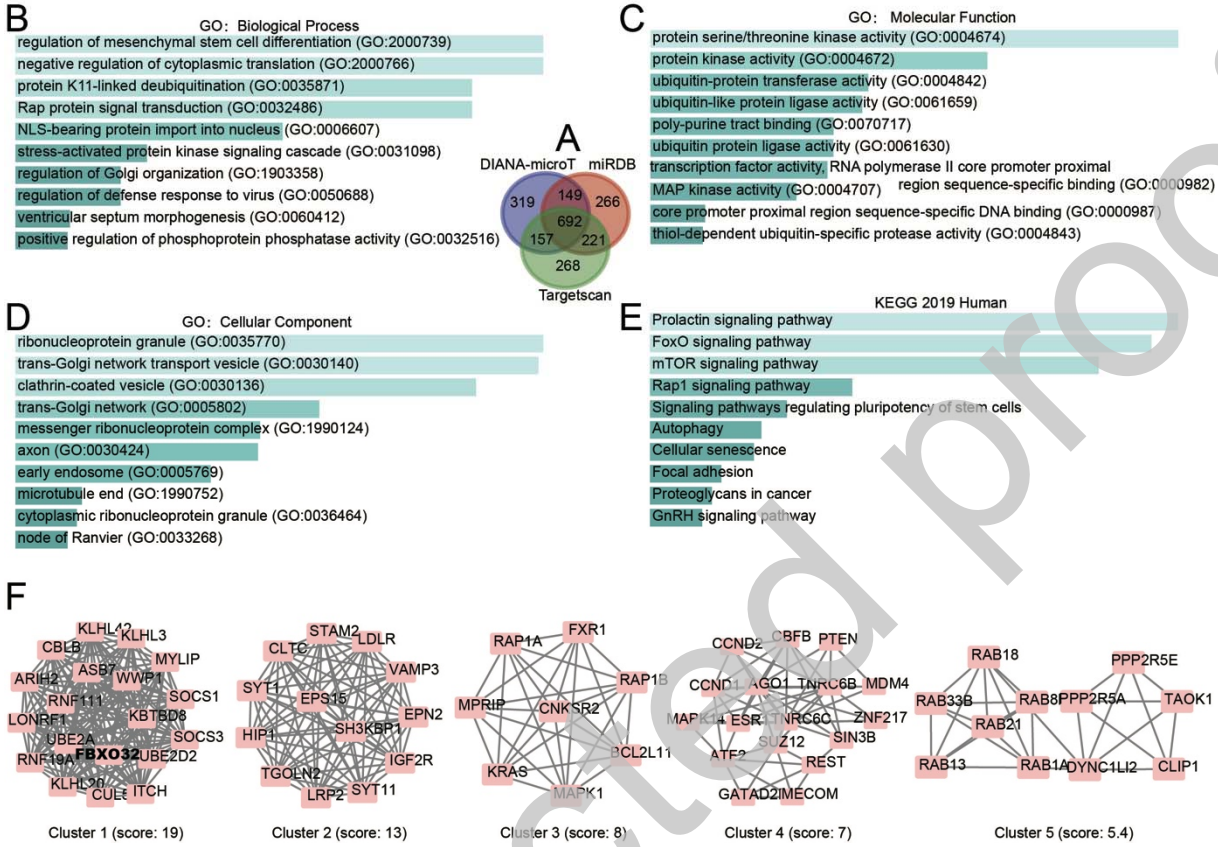

FIG. 3. Predicting the target genes of miR-19a-3p by TargetScan, miRDB and DIANA-microT and enrichment analysis (A) Venn of 692 overlapping 1317 target genes (DIANA), 1328 target genes (miRDB) and 1338 target genes (Targetscan). (B-E) GO enrichment analysis, and KEGG enrichment analysis revealed miR-19a-3p target genes correlated with multiple biological functions and pathways at Enrichr analysis tool (http://amp.pharm.mssm.edu/Enrichr/). (F) Cytoscape software was used to analyze core subnetworks in miR-19a-3p target genes.

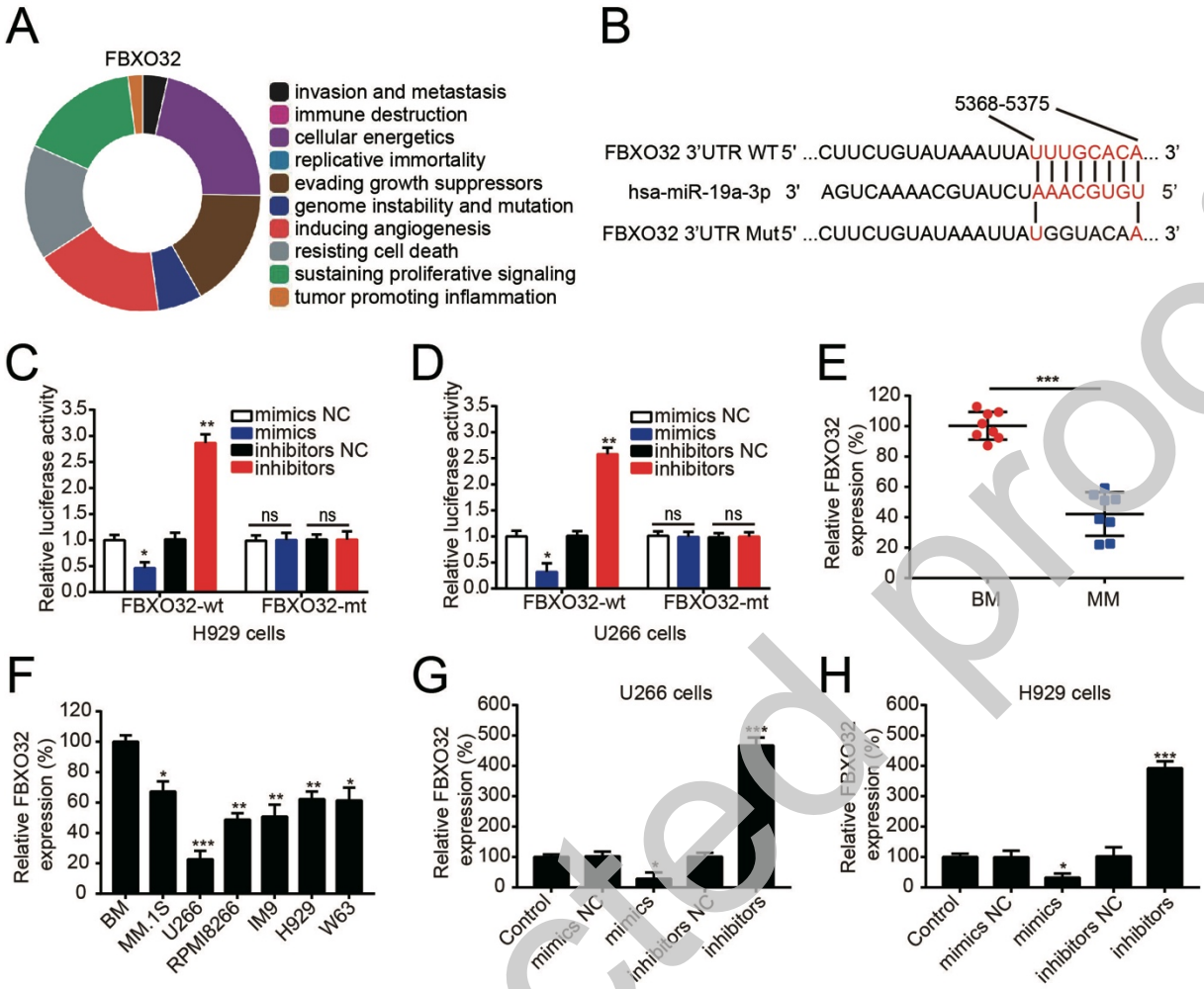

FIG. 4. FBXO32 acted as a direct target of miR-19a-3p in MM cell lines (A) Association between FBXO32 and hallmarks of cancer from Cancer Hallmarks Analytics Tool (CHAT). (B) miR-19a-3p and its predicted binding sequences in the 3'-UTRs of FBXO32 Using Targetscan analysis tool. (C, D) The dual-luciferase report system was used to detect the effect of miR-19a-3p on FBXO32 in H929 cells and U266 cells. (E) Relative FBXO32 expression levels between BM and MM. (F) Relative FBXO32 expression levels between BM and multiple myeloma cell lines. (G, H) miR-19a-3p negatively regulated the expression of FBXO32. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; each bar represented Means ± SD from three independent experiments.

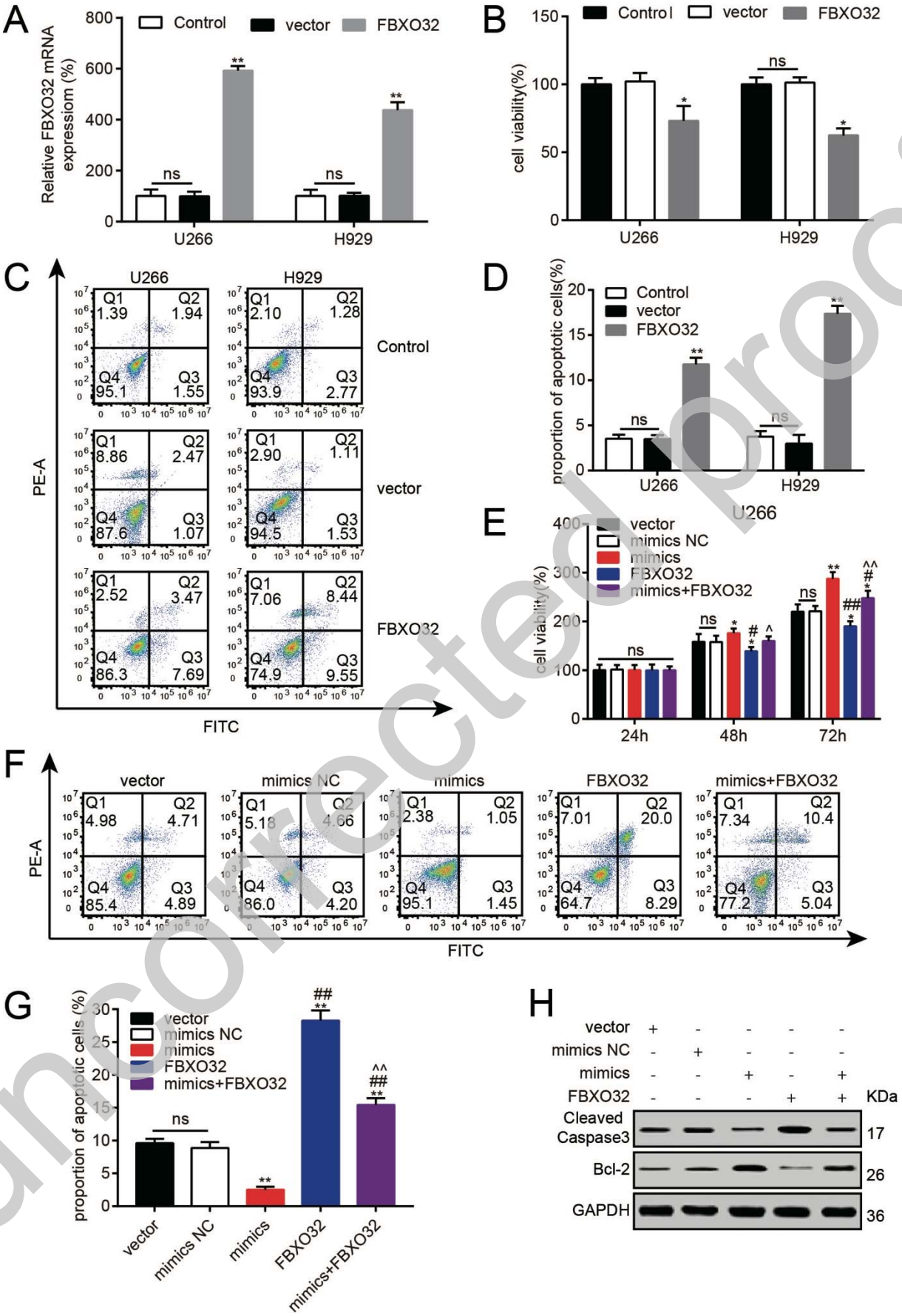

FIG. 5. miR-19a-3p regulates cell viability and cell apoptosis via FBXO32 in MM cell lines (A) FBXO32

was overexpressed in U266 and H929 cells. (B) Overexpressed FBXO32 significantly inhibited cell viability in U266 and H929 cells. (C, D) Overexpressed FBXO32 significantly promoted cell apoptosis in U266 and H929 cells. (E) FBXO32 rescued the effect of miR-19a-3p promoting cell viability. (F, G) FBXO32 saved the result of miR-19a-3p inhibiting cell apoptosis. (H) WB detected the expression of cleaved caspase 3 and BCL-2. *P<0.05 vs vector group and mimics group, #P<0.05 vs mimics group, ^ P<0.05 vs FBXO3 group; each bar represented Means ± SD from three independent experiments.

Fig. S1 Protein interaction network of miR-19a-3p target genes was analyzed at STRING analysis tools and visualized by Cytoscape software.