hsa-miR-216a-3p regulates cell proliferation in oral cancer via the Wnt3a/β-catenin pathway

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Abstract. Oral cancer is one of the leading causes of death worldwide, with a reported 5-year survival rate of ~50% after treatment. The treatment measures for oral cancer are very expensive and affordability is low. Thus, it is necessary to develop more effective therapies to treat oral cancer. A number of studies have found that miRNAs are invasive biomarkers and have therapeutic potential in a variety of cancers. The present study included 30 oral patients and 30 healthy controls. Clinicopathological characteristic and miR-216a-3p/β-catenin expression level of 30 oral cancer patients were analyzed. In addition, two oral cancer cell lines (HSC-6 and CAL-27) were used for mechanism-of-action study. The expression level of miR-216a-3p was higher in oral cancer patients compared with healthy controls and positively associated with tumor stage. Inhibition of miR-216a-3p potently suppressed cell viability and induced apoptosis of oral cancer cells. It was found that effects of miR-216a-3p on oral cancer were through Wnt3a signaling. It was also found that the expression level of β-catenin was higher in oral cancer patients compared with healthy controls and positively associated with tumor stage; the effects of miR-216a-3p on oral cancer were through β-catenin. In conclusion, miR-216a-3p and the Wnt-β-catenin signaling pathway may be interesting candidates to develop effective therapies for oral cancers.

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Introduction

Oral cancer is one of the leading causes of mortality worldwide, with a reported 5-year survival rate of ~50% after treatment (1). Of the total oral malignancies ~90% are squamous cell carcinomas and the etiological basis of oral cancer is tobacco intake, smoking, smokeless tobacco (snuff or chewing tobacco), alcohol and areca nut intake, excessive sunlight exposure, passive smoking and human papillomavirus (HPV) (2). The management of oral cancer is a multidisciplinary endeavor, as each patient presents with a unique set of challenges, the management of which influences both overall survival and quality of life (2). The treatment measures for oral cancer are expensive and affordability is therefore low. Thus, it is necessary to develop more effective therapies to treat this difficult disease.

MicroRNAs (miRNAs) have emerged as a stimulating area of basic and translational biomedical study, owing to their influence on gene expression, robust presence in bodily tissues and fluids and their potential usefulness as disease biomarkers (3). A number of cancer studies have found that miRNAs are invasive biomarkers and have therapeutic potential for a variety of cancers. For example, miR106a was reported to promote the growth of breast cancer and suppress the sensitivity of transplanted tumors to cisplatin (4). miR-126 was found to regulate angiogenesis in breast cancer by targeting VEGF-A-mRNA, which was proposed as a novel therapeutic approach in breast cancer treatment (5). Therefore, it is worthwhile to investigate the effects of miRNAs for the development of novel biomarkers and anticancer drugs.

miRNAs are found to regulate different signaling pathways. As one of the important signaling pathways controlling a variety of cellular activities, the phosphoinositide-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway serves an important role in various aspects of cancer initiation and progression, including proliferation, apoptosis, metastasis, angiogenesis and drug resistance interacting with different miRNAs (6). The Notch signaling pathway serves an essential role in differentiation and development and is found to cross-regulate with miRNAs in cancer initiation/progression via regulating the expression of multiple

oncogenes and tumor suppressor genes (7). Wnt signaling pathway is well-known to be involved in numerous fundamental processes essential for embryonic development and normal adult homeostasis (8). Dysfunctional Wnt signaling has been related to the evolution of and maintenance of leukemic stem cells as well as a number of other different cancers (9). In chronic lymphocytic leukemia, it was found that miRNAs and signaling cascades of Wnt pathway had strong interaction (10). Therefore, it is worthwhile to explore the interaction of miRNAs and Wnt signaling and its effects on oral cancer.

As one member of miRNAs, miR-216a-3p has been explored in various diseases. Wang *et al* (11) found that miR-216a-3p serves an important role in Parkinson's disease. Chang and Kan (12) found that mesenchymal stem cell originated exosomal circular RNA circFBXW7 could obviously reduce cell proliferation, migration and inflammation via interacting miR-216a-3p in rheumatoid arthritis. Regarding tumors, a previous study found that miR-216a-3p markedly inhibited the proliferation and invasion of cervical cancer via suppressing ACTL6A-mediated YAP signaling (13). Moreover, Wang *et al* (14) found that miR-216a-3p significantly suppressed the proliferation of colorectal cancer cell proliferation via regulating genes including COX-2 and ALOX5. Thus, it is attractive to explore the effects of miR-216a-3p on oral cancer.

In the present study, two oral cancer lines, HSC-6 and CAL-27, were used to investigate the effects of rapamycin on oral cancer growth. It was found that expression level of miR-216a-3p was higher in oral cancer patients and positively correlated with tumor stages and inhibition of miR-216a-3p potently suppressed cell viability and induced apoptosis of oral cancer cells. In principle, it was found that effects of miR-216a-3p on oral cancer were through Wnt3a. It was also found that the expression level of β-catenin was higher in oral cancer patients and positively correlated to tumor stages and effects of miR-216a-3p on oral cancer were through β-catenin. The findings of the present study may provide important insight for treating oral cancers.

Materials and methods

Ethical statement. All patients agreed to participate in the study and provided written informed consent. The present study was approved by the ethical board of The Fourth Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 2022KY392).

Patients' basic information and peripheral blood mononuclear cells (PBMC) isolation. PBMCs were isolated from whole blood that was obtained from the blood biobank of Fourth Affiliated Hospital, Hebei Medical University. In total, there were 30 patients with oral cancer and 30 healthy controls included in the study. Blood (~5 ml) was obtained from patients before they received any intervention. The clinicopathological characteristics of the patients with oral cancer are listed in Table SI.

Cell culture. The two oral cancer cell lines, HSC-6 and CAL-27, were purchased from Tianjin IB Biology (https://www.innovationbiotechnology.com.cn/; Tianjin, China). The two cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. D6429-500ML; MilliporeSigma) supplemented with 10% fetal bovine serum (FBS; cat. no. MFCD00132239; MilliporeSigma), 1% L-glutamine (cat. no. 25030081; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (cat. no. V900929-100ML; MilliporeSigma) at 37˚C in a 5% CO_2 incubator. The cells were sub-cultured when they were 80% confluent. The HSC-6 cell line and CAL-27 cell line were regularly tested for *Mycoplasma* by using the MycoAlert Plus kit (Lonza Group, Ltd.) to make sure they were mycoplasma negative.

Western blot analysis. RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) was used to lyse the cells using SDS/PAGE sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol and 1 mM dithiothreitol]. Five microliters of the lysates were removed for protein concentration measurement (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Inc.). Samples were boiled at 95˚C for denature for 10 min. Subsequently, total protein (30 μg per lane) was separated by 10% SDS-PAGE. Post transferring, PVDF membranes (Beyotime Institute of Biotechnology) were blocked by 5% non-fat milk TBST buffer containing 0.5 ml/l TWEEN 20 for 45 min at room temperature, followed by incubation with primary antibodies (1:1,000 dilution), including anti-pro-caspase-3 (cat. no. ab32499; Abcam), anti-Caspase-3 (cat. no. ab2302; Abcam), anti-Wnt3a antibody [EPR21889] (cat. no. ab219412; Abcam), anti-β catenin antibody (IGX4794R-3; cat. no. ab223075; Abcam) and anti-β actin antibody (cat. no. mAbcam 8226; Abcam) at 4˚C overnight. After incubation with horseradish-peroxidase-coupled secondary antibodies (1:5,000 dilution; HRP-labeled goat anti-rabbit IgG H+L; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 1 h, the immunoblots were visualized using BeyoECL Plus (cat. no. P0018S; Beyotime Institute of Biotechnology). Densitometry was measured using ImageJ software (version: ImageJ bundled with 64-bit Java 8; National Institutes of Health).

Flow cytometric analysis of Annexin V/propidium iodide (PI) staining. HSC-6 or CAL-27 cells were seeded in 6 well plates (Costar; Corning, Inc.; 150,000 cells/well). When the confluence reached 60-70%, cells were treated with miR-216a-30 inhibitor 1 for 24 h in the cell culture incubator (37˚C, 5% CO_2). After the treatment, cells were harvested with trypsin/EDTA and stained for 15 min at room temperature using FITC Annexin V apoptosis Detection kit I (cat. no. 556547; BD Pharmingen). Results were analyzed by a FACSCanto II (BD Biosciences). As illustrated in the Results section below, PI^+ Quadrants Q1 and Q2 respectively represent necrosis and late-stage apoptosis/secondary necrosis; Quadrant Q4 represents viability ($AnnV^-/PI^-$) and Quadrant Q3 ($AnnV^+/PI^-$) represents early-stage apoptosis.

RNA isolation and reverse transcription-quantitative (RT-q)PCR. TRIzol (Beyotime Institute of Biotechnology) was used for total RNA isolation from both cell lines (seeded on 96-well plates, seeding density of 6,000 cells per

Table I. Primers of reverse transcription-quantitative PCR used in the present study.

Gene	Primer	Sequence	Product length (nt)
Caspase 3	Sense	TGAGGCGGTTGTAGAAGAGTTTCG	153
	Anti-sense	TTATTAACGAAAACCAGAGCGCC	
Wnt3A	Sense	ATAGGCTCCTTCCTGTGGGT	188
	Anti-sense	GAACCTTACAGGGGGTTGGG	
β-catenin	Sense	CTGAGGAGCAGCTTCAGTCC	161
	Anti-sense	CCATCAAATCAGCTTGAGTAGCC	
miR-216	Sense	CTCAGCTGGCAACTGTG	
	Anti-sense	GAACATGTCTGCGTATCTC	
GAPDH	Sense	AATGGGCAGCCGTTAGGAAA	168
	Anti-sense	GCGCCCAATACGACCAAATC	

miR, microRNA.

well) following the manufacturer's protocol. The BeyoRT First Strand cDNA Synthesis kit (cat. no. D7166; Beyotime Institute of Biotechnology) was used for cDNA synthesis from total RNA. RT-qPCR was performed using BeyoFast SYBR Green qPCR Mix (2X; cat. no. D7260-25 ml; Beyotime Institute of Biotechnology) on a 7500 Fast Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed at 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 1 min and extension at 72˚C for 1 min, and a final extension step at 72˚C for 10 min. GAPDH was used as an internal control. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (15). Primers used in the study are listed in Table I. This experiment was repeated three times.

Small interfering (si)RNA-based knockdown assay. Gene knockdown was performed using the siRNA approach. The sequence of siRNAs against mTOR was designed using siRNA-Target-Finder (GeneScript), followed by being synthesized and purchased from Synbio Technologies. The sequence of the empty vector siRNA-negative control (NC) was as 5'-UUCUCCGAACGUGUCACGU-3', that of siRNA-WNT3a was 5'-GCTTCTGCAGGAACTACGTGGAGAT-3' and the sequence of siRNA-β-catenin was 5'-CAGTTATGGTCCATC AGCTTTCTAA-3'. The sequences of Wnt3a and β-catenin primers, respectively, were as follows: Forward, 5'-AAGACA TGCTGGTGGTCGCAA-3' and reverse, 5'-AACCGCCAC AACAACGAGGCT-3'; and forward, 5'-AAGTAGCTGATA TTGATGGAC-3' and reverse, 5'-AAGCTCATCATACTG GCTAGT-3'.

The siRNAs (non-targeting Control siRNA and target siRNA) were transiently transfected into the HSC-6 and CAL-27 cell lines and 3D spheroids using FuGENE HD Transfection Reagent (cat. no. E2311; Promega Corporation) according to the manufacturer's instructions in the cell culture incubator (37˚C, 5% CO_2). The time of transfection of siRNA was 24 h before the subsequent experiments. The knockdown efficiency was evaluated using RT-qPCR and western blot assay following protocols described in this study.

Immunohistochemistry (IHC). The β-catenin protein expression levels in paraffin-embedded hepatocellular carcinoma tissues were examined by IHC as described previously (16). Briefly, IHC was performed on 4-μm sections. Following deparaffinization and rehydration, the endogenous peroxidase activity was blocked using 3% H_2O_2 (reagent A; UltraSensitive SP IHC kit; Maxim Biotech Inc.). Next, antigen retrieval was performed and normal serum (reagent B; UltraSensitive SP IHC kit; Maxim Biotech Inc.) was applied to the sections to block non-specific binding. Sections were then incubated at 4˚C overnight with the primary antibodies, including an anti-β-catenin antibody (E247)-ChIP grade (1:300 dilution; cat. no. ab32572; Abcam). Subsequently, the sections were incubated with the secondary antibody (reagent C; UltraSensitive SP IHC kit; Maxim Biotech Inc.) for 15 min, followed by incubation with streptavidin-peroxidase (reagent D, UltraSensitive SP IHC kit; Maxim Biotech Inc.) and 3,3-diaminobenzidine (DAB) was used to stain the sections for 30 sec at room temperature. Finally, sections were counterstained with hematoxylin for 5 min at room temperature and mounted. Sections of oral cancer tissue showing strong staining with the respective proteins during antibody optimization served as the positive controls.

Measurement of cytotoxicity using Cell Counting Kit-8 (CCK8) assay. The Cell Counting Kit-8 (cat no. C0037; Beyotime Institute of Biotechnology) was used to measure cytotoxicity of both cell lines, according to the manufacturer's instructions. Briefly, the HSC-6 cell line and CAL-27 cell line were seeded into 96-well plates at a cell density of $5x10^4$/ml) overnight. After 24 h, the cell culture medium was replaced by indicated concentrations of chemicals and the treatment was continued for 48 h. CCK8 solution (0.5 mg/ml; 100 μl) was added into each well and incubated for 3 h at 37˚C, followed by detection of optical density (OD) values at 450 nm using an Infinite M200 PRO Multimode Microplate Reader (Tecan Group, Ltd.). The percentage of live cells was calculated relative to the control.

Target genes of miRNA analysis using TargetScan. Target genes of miRNA analysis were evaluated using an online bioinformatics tool, i.e. TargetScan (https://www.targetscan.org/vert_80/).

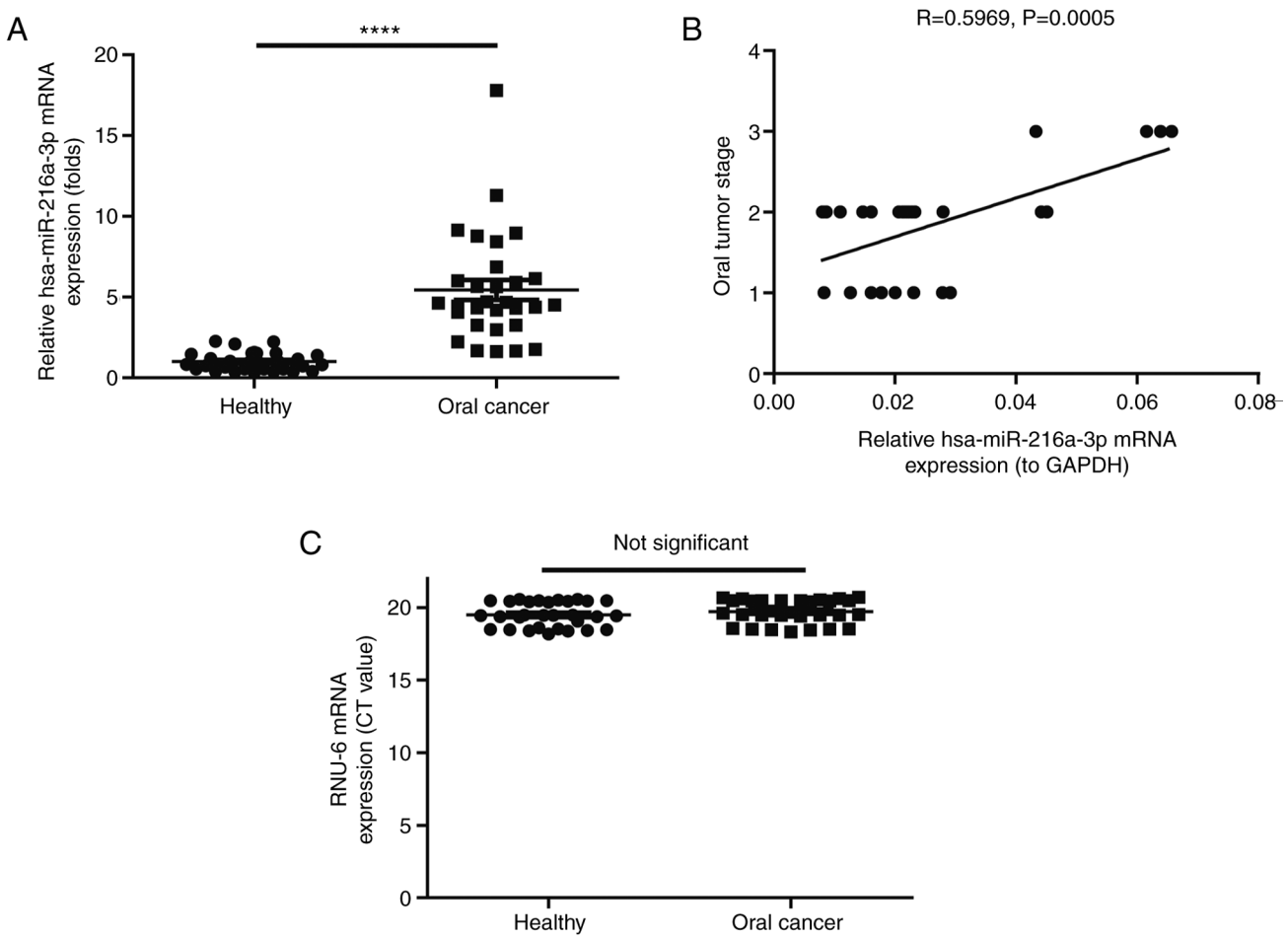

Figure 1. Expression level of miR-216a-3p is higher in oral cancer patients and positively correlated to tumor stages. (A) Expression level of miR-216a-3p in oral cancer patients was much higher than in healthy controls. (B) Expression level of miR-216a-3p was positively correlated with oral cancer stage (correlation coefficient R=0.5969; P=0.0005). (C) No significant difference was found between healthy controls and patients with oral cancer regarding the expression level of RNU-6. ****P<0.0001. miR, microRNA.

Statistical analysis. All data were presented as mean ± standard error of the mean. One-way ANOVA and Tukey's post-hoc test were used for statistical analysis of continuous variables and categorical variables were analyzed by Fisher's exact tests. Correlation analysis (Pearson) and statistical analysis were performed with GraphPad Prism 5.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression level of miR-216a-3p is higher in oral cancer patients and positively associated with tumor stage. To evaluate the effects of miR-216a-3p on oral cancer, the expression level of miR-216a-3p in oral cancer patients and healthy controls was measured using RT-qPCR. It was found that the expression level of miR-216a-3p in oral cancer patients was much higher than in healthy controls (P<0.0001; Fig. 1A). Of note, the expression level of miR-216a-3p was positively correlated with the oral cancer stage (correlation coefficient R=0.5969, P=0.0005; Fig. 1B). No significant difference was found between healthy and oral cancer patients regarding expression level of RNU-6 (Fig. 1C). Furthermore, the association between the expression level of miR-216a-3p and clinicopathological factors, including sex, age and smoking status, was examined using Fisher's exact tests (Table II). It was found that the expression level of miR-216a-3p was significantly associated with sex (P=0.0494) and oral cancer stage (P=0.0028) and two complications, including difficulty swallowing (P=0.0494) and speech problems (P=0.0608). However, the expression level of miR-216a-3p had no significant correlation with the smoking status and free bleeding in the mouth.

Inhibition of miR-216a-3p potently suppresses cell viability and induces apoptosis of oral cancer cells. To further investigate the effects of miR-216a-3p on oral cancer, inhibitors of miR-216a-3p were used. It was found that two miR-216a-3p inhibitors (cat. no. miR2150818102526-1-5; Guangzhou RiboBio Co., Ltd.) could significantly inhibit miR-216a-3p level on HSC-6 cells (P<0.01; Fig. 2A). Moreover, it was found that two miR-216a-3p inhibitors could significantly increase expression levels of apoptosis marker caspase3 in the HSC-6 cells (P<0.05; Fig. 2B). It was also found that the two miR-216a-3p inhibitors could significantly increase the protein level of apoptosis marker cleaved caspase3 in HSC-6 cells and decrease the protein level of pro-caspase 3 in HSC-6 cells (Fig. 2C). The two miR-216a-3p inhibitors could significantly

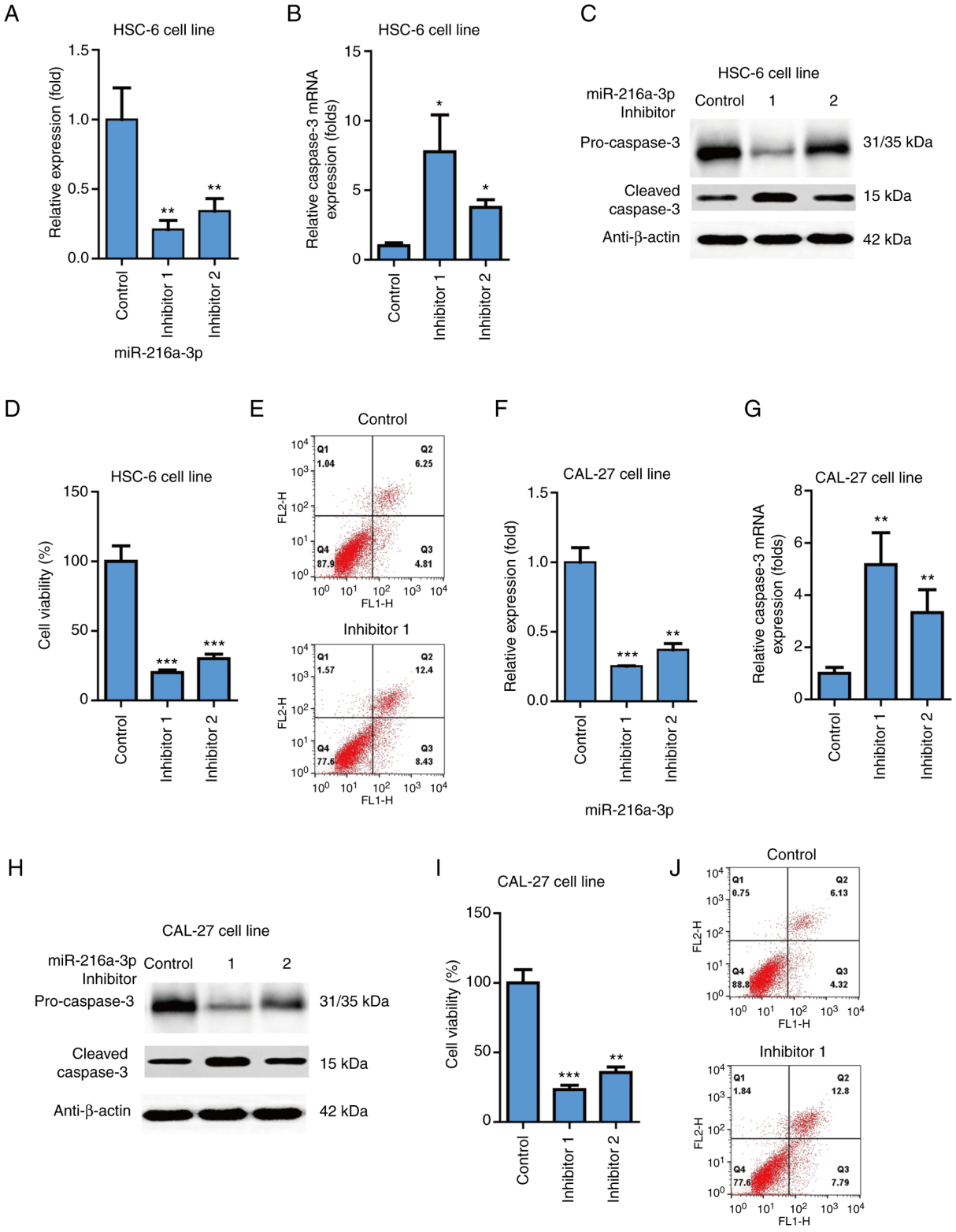

Figure 2. Inhibition of miR-216a-3p potently suppresses cell viability and induces apoptosis of oral cancer cells. (A) Two miR-216a-3p inhibitors significantly inhibited miR-216a-3p level in HSC-6 cells. (B) Two miR-216a-3p inhibitors significantly increased expression level of apoptosis marker caspase3 in the HSC-6 cell line. (C) miR-216a-3p inhibitors significantly increased the protein level of apoptosis marker cleaved caspase 3 in HSC-6 cells and decreased the protein level of pro-caspase 3 in HSC-6 cells. (D) miR-216a-3p inhibitors significantly decreased cell viability of cells of the HSC-6 cell line. (E) Following incubation with miR-216a-3p inhibitor 1, HSC-6 cells were stained with Annexin V-FITC and propidium iodide before fluorescence analysis by flow cytometry. The percentage of cells in the four different quadrants was calculated and the results present in different histograms where viable cells are Annexin V-/PI-, apoptotic cells Annexin V+/PI- and necrotic cells are PI+. miR-216a-3p inhibitor 1 significantly increased apoptosis in HSC-6 cells. (F) miR-216a-3p inhibitors significantly inhibited miR-216a-3p levels in CAL-27 cells. (G) miR-216a-3p inhibitors could significantly increase the expression level of apoptosis marker caspase 3 in the CAL-27 cell line. (H) miR-216a-3p inhibitors could significantly increase the protein level of apoptosis marker caspase 3 in the CAL-27 cell line. (I) miR-216a-3p inhibitors could significantly decrease the viability of cells of the CAL-27 cell line. (J) Following incubation with miR-216a-3p inhibitor 1, CAL-27 cells were stained with Annexin V-FITC and PI before fluorescence analysis by flow cytometry. The percentage of cells in the four different quadrants was calculated and the results present in different histograms where viable cells are Annexin V^-/PI^-, apoptotic cells Annexin V^+/PI^- and necrotic cells are PI^+. miR-216a-3p inhibitor 1 significantly increased apoptosis in CAL-27 cells. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs. control. miR, microRNA; PI, propidium iodide.

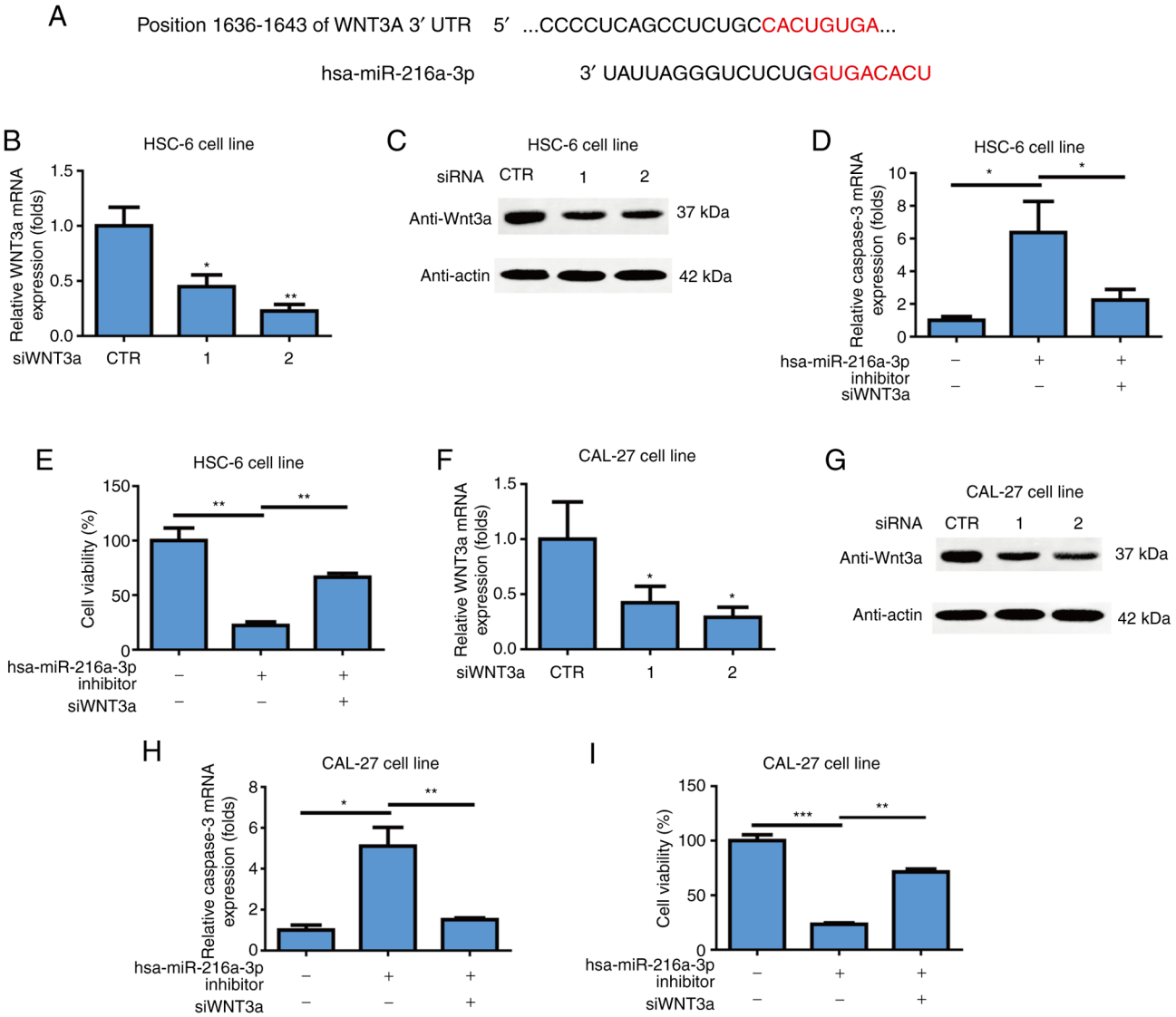

Figure 3. Effects of miR-216a-3p on oral cancer via Wnt3a. (A) Wnt3a was a target gene of miR-216a-3p as verified with the *in silico* method of TargetScan (https://www.targetscan.org/vert_80/). (B) siRNAs could successfully inhibit mRNA level of Wnt3a in the HSC-6 cell line. (C) siRNAs could successfully inhibit protein levels of Wnt3a in the HSC-6 cell line; (D) Wnt3a knockdown attenuated induction of miR-216a-3p inhibitors on apoptosis of oral cancer cells in the HSC-6 cell line. (E) Wnt3a knockdown attenuated inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the HSC-6 cell line. (F) Wnt3a siRNAs successfully inhibited mRNA level of Wnt3a in CAL-27 cell line. (G) siRNAs could successfully inhibit protein level of Wnt3a in the CAL-27 cell line. (H) Wnt3a knockdown attenuated induction of miR-216a-3p inhibitors on apoptosis of oral cancer cells in the CAL-27 cell line. (I) Wnt3a knockdown attenuated inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the CAL-27 cell line *P<0.05; **P<0.01; ***P<0.001 vs. CTR. miR, microRNA; si, small interfering; CTR, control.

decrease cell viability of HSC-6 cells (P<0.001; Fig. 2D). Apoptosis was also evaluated using flow cytometry, which indicated that miR-216a-3p inhibitor increased apoptosis in HSC-6 cells (Fig. 2E). Similarly, it was found that the two miR-216a-3p inhibitors significantly inhibited miR-216a-3p level on CAL-27 cells (P<0.01; Fig. 2F). It was found that the two miR-216a-3p inhibitors could significantly increase expression level of apoptosis marker caspase3 in CAL-27 cells (P<0.05; Fig. 2G); the two miR-216a-3p inhibitors could significantly increase the protein level of apoptosis marker cleaved caspase3 and decrease the protein level of pro-caspase 3 in CAL-27 cells (Fig. 2H); the two miR-216a-3p inhibitors could significantly decrease the cell viability of CAL-27 cells (P<0.001; Fig. 2I). Apoptosis was also evaluated using flow cytometry and indicated that miR-216a-3p inhibitor increased apoptosis in CAL-27 cells (Fig. 2J). Thus, inhibition of miR-216a-3p potently suppressed cell viability and induced apoptosis of oral cancer cells.

Effects of miR-216a-3p on oral cancer through Wnt3a. To evaluate the mechanism of action of miR-216a-3p on oral cancer, an *in silico* method of TargetScan (https://www.targetscan.org/vert_80/) was used to analyze the target gene of miR-216a-3p, which showed that Wnt3a was a target gene of miR-216a-3p (Fig. 3A). To investigate effects of Wnt3a on oral cancer, two siRNAs against Wnt3a were used to knockdown the gene and it was found that siRNAs could successfully inhibit mRNA level of Wnt3a in HSC-6 cell line (P<0.05; P<0.01; Fig. 3B). siRNAs could successfully inhibit the protein level of Wnt3a in HSC-6 cell line (Fig. 3C). Wnt3a

knockdown attenuated induction of miR-216a-3p inhibitors on apoptosis of oral cancer cells in the HSC-6 cell line (P<0.05; P<0.01; Fig. 3D). It was also found that Wnt3a knockdown attenuated the inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in HSC-6 cells (P<0.01; Fig. 3E). The two Wnt3a siRNAs successfully inhibited the mRNA level of Wnt3a in the CAL-27 cell line (P<0.05; Fig. 3F). siRNAs could successfully inhibit the protein level of Wnt3a in CAL-27 cells (Fig. 3G). Wnt3a knockdown attenuated induction of miR-216a-3p inhibitors on apoptosis of oral cancer cells in CAL-27 cells (P<0.05; P<0.01; Fig. 3H). Further, Wnt3a knockdown attenuated the inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in CAL-27 cells (P<0.01; Fig. 3I). While miR126 does not affect mRNA expression of WNT3a in both HSC-6 (Fig. S1A) and CAL-27 cells (Fig. S1B), knockdown of WNT3a significantly inhibits cell viability of HSC-6 cells (Fig. S1C) and CAL-27 cells (Fig. S1D), thus, the effects of miR-216a-3p on oral cancer was through Wnt3a.

Expression level of β-catenin is higher in oral cancer patients and positively correlated with tumor stage. The present study measured the expression level of miR-216a-3p in oral cancer patients and healthy controls using RT-qPCR. The protein level of β-catenin in oral cancer patients was much higher than in healthy controls, as measured using IHC method (Fig. 4A). The mRNA expression level of β-catenin in oral cancer patients was much higher than in healthy controls (P<0.0001; Fig. 4B). Of note, it was found that the expression level of β-catenin positively correlated with oral cancer stage (correlation coefficient R=0.3552, P=0.045; Fig. 4C).

Effects of miR-216a-3p on oral cancer through β-catenin. To further investigate the effects of β-catenin on oral cancer, siRNAs against β-catenin were used and it was found that siRNAs could successfully inhibit mRNA level of β-catenin in the HSC-6 cell line (P<0.05; P<0.01; Fig. 5A). siRNAs could successfully inhibit the protein level of β-catenin in HSC-6 cells (Fig. 5B). β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on mRNA level of caspase3 in HSC-6 cells (*P<0.05; Fig. 5C). β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on the protein level of cleaved caspase3 in HSC-6 cells and decrease the protein level of pro-caspase 3 in HSC-6 cells (Fig. 5D). Moreover, β-catenin knockdown attenuated the inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the HSC-6 cell line (P<0.01; Fig. 5E). The two β-catenin siRNAs successfully inhibited the mRNA level of β-catenin in the CAL-27 cell line (P<0.01; Fig. 5F). Two β-catenin siRNAs successfully inhibited the protein level of β-catenin in CAL-27 cells (Fig. 5G). β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on mRNA level of caspase3 in CAL-27 cells (P<0.01; Fig. 5H). β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on protein level of cleaved caspase3 and decrease the protein level of pro-caspase 3 in CAL-27 cells (Fig. 5I). Furthermore, β-catenin knockdown attenuated the inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the CAL-27 cell line (P<0.05; P<0.01; Fig. 5J), while miR-126 does not affect mRNA expression of β-catenin in HSC-6 cells (Fig. S1E) and CAL-27 cells (Fig. S1F).

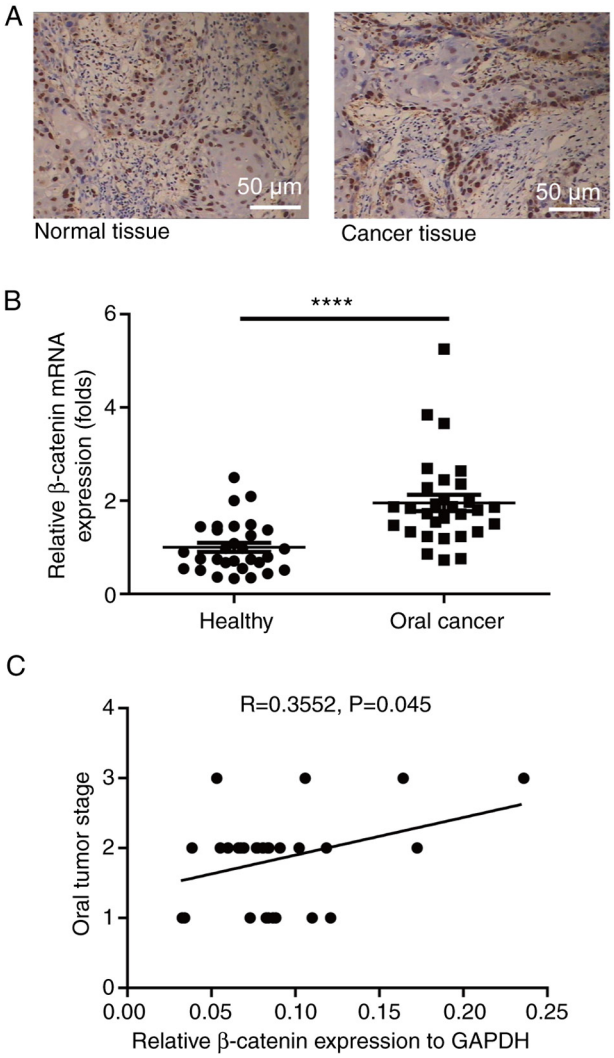

Figure 4. Expression of β-catenin is higher in patients with oral cancer and positively correlated with the tumor stages. (A) Protein level of β-catenin in oral cancer patients was much higher than in healthy controls measured using immunohistochemistry. Scale bar, 50 μm. (B) mRNA expression level of β-catenin in oral cancer patients was higher than in healthy controls (****P<0.0001). (C) Expression level of β-catenin was positively correlated to oral cancer stages (correlation coefficient R=0.3552, P=0.045).

Discussion

Oral cancer is a severe public health concern and is widespread in a number of countries, especially developing countries (17). More efforts are needed to uncover the underlying pathogenesis and develop novel drugs for treatment. miRNAs are considered important targets for developing anti-cancer drugs. The Wnt signaling pathway serves an important role in activities of cancer stem cells (18). In the present study, two oral cancer lines, iHSC-6 and CAL-27, were used. Effects of miR-216a-3p on oral cancer and the corresponding mechanism were investigated.

As small single-stranded non-coding RNA molecules, miRNAs have been confirmed to exert various functions such as cell growth, apoptosis, development, differentiation and inflammation, via RNA silencing and post-transcriptional regulation of gene expression (19). An increasing number of studies have confirmed that miRNAs are essential to regulate

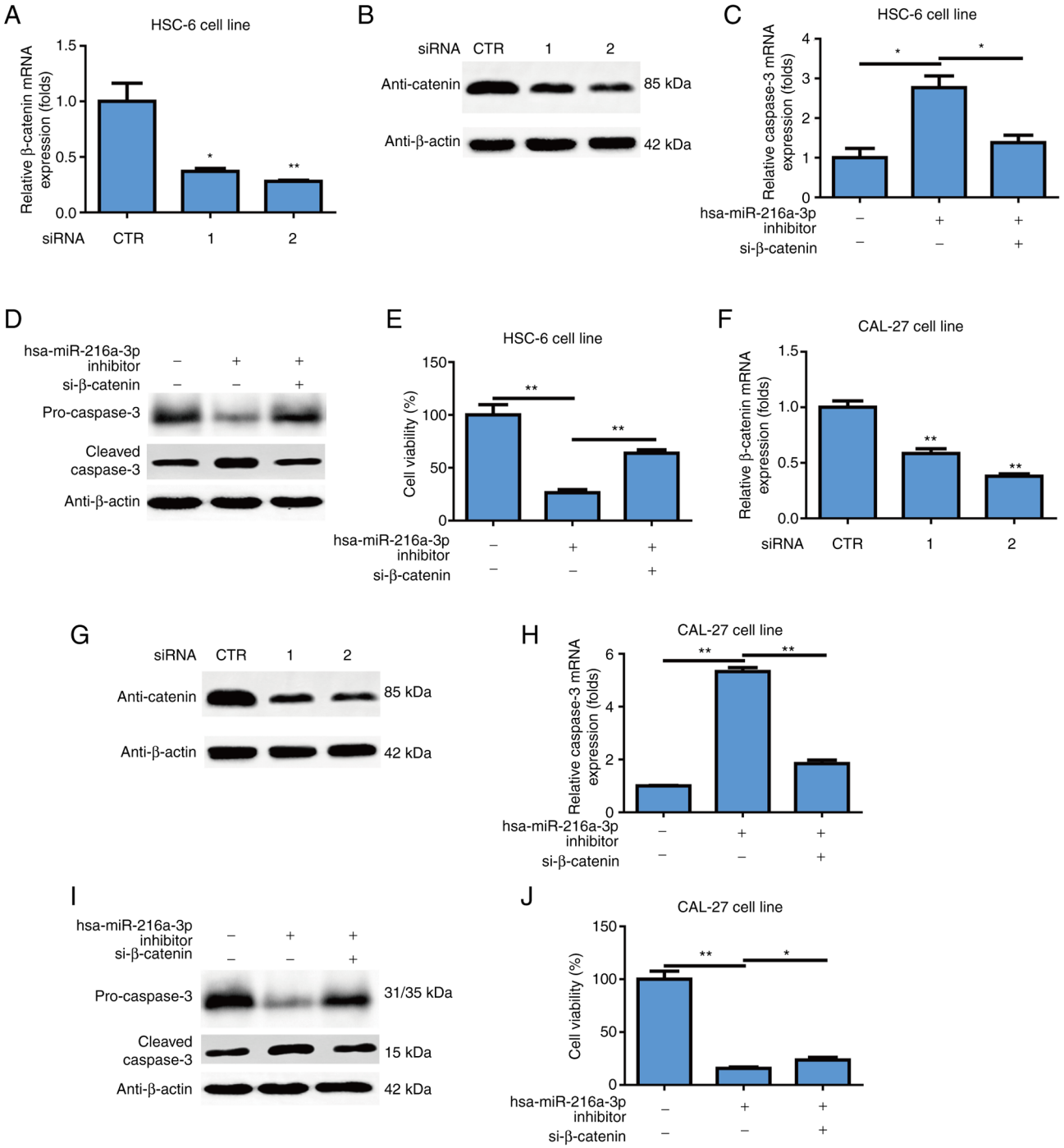

Figure 5. Effects of miR-216a-3p on oral cancer through β-catenin. (A) siRNAs successfully reduced the mRNA levels of β-catenin in the HSC-6 cell line. (B) siRNAs successfully reduced the protein levels of β-catenin in the HSC-6 cell line. (C) β-catenin knockdown attenuated the inhibitory effects of miR-216a-3p inhibitors on the increase of caspase 3 mRNA levels in the HSC-6 cell line. (D) β-catenin knockdown attenuated inhibitory effects of miR-216a-3p inhibitors on the increase of caspase3 protein levels in the HSC-6 cell line. (E) β-catenin knockdown attenuated inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the HSC-6 cell line. (F) β-catenin siRNAs successfully reduced the mRNA levels of β-catenin in the CAL-27 cell line. (G) β-catenin siRNAs successfully reduced the protein levels of β-catenin in CAL-27 cell line. (H) β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on increase of caspase3 mRNA level in CAL-27 cell line. (I) β-catenin knockdown attenuated induction of miR-216a-3p inhibitors on increase of caspase3 protein level in the CAL-27 cell line. (J) β-catenin knockdown attenuated inhibitory effects of miR-216a-3p inhibitors on cell viability of oral cancer cells in the CAL-27 cell line. *P<0.05; **P<0.01 vs. CTR. miR, microRNA; si, small interfering; CTR, control.

the activities of a variety of tumors. Terkelsen *et al* (20) found that miR-146a and miR-494 are richly expressed in the tumor interstitial fluid of breast cancer patients and several miRNAs are associated with tumor grade. In another study, authors found that 8 of the 10 miRNAs (miR-139-5p, miR-10b-5p, miR-486-5p, miR-455-3p, miR-107, miR-146b-5p, miR-324-5p and miR-20a-5p) are highly correlated with prognosis of breast cancer (21). Grzelczyk *et al* (22) found that the levels of serum expression of miR-31, miR-141, miR-149a, miR-182, LET-7a, miR-4853p, miR-122 and miR-33 are upregulated, which might be used to diagnose laryngeal squamous cell carcinoma with high sensitivity and specificity. For oral

Table II. Association of the expression level of miR-216a-3p and clinicopathological factors including sex, age and smoking status using Fisher's exact test.

Variable	n=30	Expression level of miR-216a-3p (fold)	P-value
Sex			0.0494
Male	20	0.022323192	
Female	10	0.027622647	
Oral cancer stage			0.0028
I	10	0.021805101	
II	17	0.020385862	
III	3	0.058574635	
Age, years			0.1429
<50	2	0.01580415	
50-60	15	0.027476579	
>60	13	0.025532913	
Smoking status			0.5100
Yes	13	0.021847226	
No	17	0.031098617	
Complications			
Difficulty swallowing (Dysphagia)			0.0002
Yes	8	0.042074396	
No	22	0.019958622	
Speech problems			0.0608
Yes	10	0.028519873	
No	20	0.024314014	
Free bleeding in the mouth			0.3981
Yes	15	0.030593512	
No	15	0.021118813	

miR, microRNA.

cancer, Li *et al* (23) found that miR-34a-5p binds to its direct downstream target AXL to suppress oral squamous cell carcinoma cell proliferation and metastasis. Cai *et al* (24) found that exosome-enclosed miR-29a-3p promotes tumor growth in nude mice with xenograft of oral squamous cell carcinoma. Similarly, the present study found that the expression level of miR-216a-3p was much higher in oral cancer patients than healthy controls and positively correlated with tumor grades. Thus, it appears that miR-216a-3p is a potential biomarker for oral cancer. Notably, it was also found that inhibition of miR-216a-3p potently inhibited cell viability and induced apoptosis in oral cancer cell lines. The results showed the important role of miR-216a-3p in oral cancer.

The Wnt signaling pathway is one of important pathways controlling various biological activities (17). In cancer studies, accumulating evidence has indicated that Wnt signaling serves an important role in regulating cancer activities. In colorectal cancer (CRC) with mutations of activated KRAS and SLC25A22, which indicates increased DNA methylation, activation of Wnt signaling to β-catenin increased expression of LGR5, proliferation, stem cell features and resistance to 5-fluorouracil (25). In hepatocellular carcinoma, it was found that activation of autophagy can induce MCT1 expression by activating Wnt/β-catenin signaling to promote metastasis and glycolysis (26). Regarding breast carcinomas, it was found that increased relative mRNA expression levels of Wnt3 were found in 54% cases (27). Deng *et al* (28) found that activation of Wnt signaling can facilitate pancreatic cancer progression. The present study found that Wnt was the target gene of miR-216a-3p. More importantly, it was found that the effects of miR-216a-3p on oral cancer were via Wnt3a. Li *et al* (29) found that serum β-catenin levels in the CRP and CRC patients are significantly higher compared with those in the healthy control group. Xu *et al* (30) found that β-catenin expression is higher in hepatocellular carcinoma patients compared with that in healthy controls and increased β-catenin expression is closely correlated with tumor differentiation, tumor size, serum α-fetoprotein level and transarterial chemoembolization treatment frequency. The present study found that the expression level of β-catenin was much higher in oral cancer patients compared with in healthy controls and positively correlated with tumor stages. The effect of miR-216a-3p on oral cancer was via β-catenin. Therefore, the Wnt-β-catenin signaling pathway probably serves an important role in oral cancer.

In conclusion, the present study demonstrated that the expression level of miR-216a-3p was higher in oral cancer patients compared with healthy controls and positively correlated with tumor stage. Inhibition of miR-216a-3p could potently inhibit the growth of oral cancer cells and this effect was through the Wnt-β-catenin signaling pathway. β-Catenin expression level was higher in oral cancer patients compared with healthy controls and positively correlated with tumor stage. Therefore, miR-216a-3p and Wnt-β-catenin signaling pathway might be appealing candidates for the development of effective therapies for oral cancers. It is considered that the findings of the present study will provide useful insights for developing novel therapies of oral cancer.

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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

YW, SL, YD, YQ, FL, WZ and WW performed the experiments. WZ and WW designed the research. YW and SL

wrote the manuscript and YD and YQ supervised the project. YW and YQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was obtained from the ethical board of The Fourth Hospital of Hebei Medical University (approval no. 2022KY392). Informed consent was obtained from all volunteers.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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