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MicroRNA-32 functions as a tumor suppressor and directly targets EZH2 in uveal melanoma

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ABSTRACT. MicroRNA-32 (miR-32) has been shown to be dysregulated in some human malignancies and this has been found to be correlated with tumor progression. However, its role in uveal melanoma formation and progression remains largely unknown. Thus, the aim of this study was to explore the expression and function of miR-32 in human uveal melanoma. Using quantitative reverse transcription-polymerase chain reaction, we detected miR-32 expression in uveal melanoma tumor tissues and cell lines. The effects of miR-32 on the biological behavior of uveal melanoma cells were also investigated. Finally, the potential regulatory function of miR-32 on EZH2 expression was confirmed. miR-32 expression levels were significantly downregulated in uveal melanoma samples and cell lines (both $P < 0.01$). Ectopic expression of miR-32 could inhibit uveal melanoma cell proliferation, migration, and invasion, and promote cell apoptosis *in vitro*. Further, EZH2 was confirmed as a direct target of miR-32 by using the luciferase reporter assay. These findings indicate that miR-32

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may function as a novel tumor suppressor in uveal melanoma and could be a potential therapeutic target for this disease.

Key words: Uveal melanoma; MiR-32; Proliferation; Invasion; Apoptosis

INTRODUCTION

Uveal melanoma is the most common primary malignancy of the eye and the second most common form of melanoma (Hurst et al., 2003). Uveal melanoma eventually spreads to the liver in up to 50% of patients and most patients have subclinical metastasis at the time of diagnosis (Bakalian et al., 2008). Despite the advances in surgery, chemotherapy and radiotherapy, the 5-year relative survival rate did not improved from 1973 to 2008 (Buder et al., 2013). Currently, the etiology and pathogenic mechanism of uveal melanoma have not been elucidated. Therefore, it is important to identify reliable biomarkers of uveal melanoma for its early diagnosis and effective therapy.

MicroRNAs (miRs) are a class of short (about 22 nucleotides in length), endogenous, single-stranded, and non-protein coding RNAs that play key roles in the regulation of gene expression (Bartel, 2009). By base-pairing with the complementary sites in the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs), miRs regulate target genes by increasing mRNA decay or repressing translation (Xu et al., 2008). Growing evidence indicates that miRs are involved in many biological processes, such as cell proliferation and apoptosis, development, differentiation, glucose and lipid metabolism, and signal transduction (Bartel, 2004). Recent research also suggests that miRs participate in human tumorigenesis and progression, which could provide a new but promising way to diagnose and treat human malignancies (Heneghan et al., 2010). Abnormal miR expression has been frequently reported in various human tumors including uveal melanoma (Ragusa et al., 2015; Sun et al., 2015a). Functional miRs may be applied for use in tumor diagnosis and prognosis, and also act as potential novel therapeutic targets.

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One of the tumor-related miRs is miR-32. It is observed to be downregulated in oral squamous cell carcinoma (Zhang et al., 2014a), non-small cell lung cancer (Zhu et al., 2015), gastric cancer (Zhang et al., 2014b), and osteosarcoma (Xu et al., 2014), where it acts as a potential tumor suppressor. However, miR-32 was also reported to be upregulated in breast cancer (Xia et al., 2015), hepatocellular carcinoma (Yan et al., 2015), colorectal cancer (Wu et al., 2013a), prostate cancer (Ambs et al., 2008), and kidney cancer (Petillo et al., 2009), indicating that the role of miR-32 may be different depending on the etiology of the tumor and type of cancer. The role of miR-32 and its downstream targets in uveal melanoma remain unknown.

In the present study, we focused on the expression and biological function of miR-32 in uveal melanoma. We demonstrated that miR-32 was downregulated in uveal melanoma cells and tissues. Ectopic expression of miR-32 could inhibit uveal melanoma cell proliferation and invasion and promote cell apoptosis *in vitro*. Furthermore, we identified enhancer of zeste homolog 2 (EZH2) as a direct target of miR-32. Our findings suggest that miR-32 may function as a novel tumor suppressor in uveal melanoma and could be a potential therapeutic target for this disease.

MATERIAL AND METHODS

Ethics statement

This study was approved by the Medical Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University, and all patients provided written informed consent.

Tissue specimens and cell lines

Tumor samples and their adjacent healthy uveal tissue counterparts were obtained from 22 uveal melanoma patients and were immediately frozen in liquid nitrogen. The uveal melanoma cell lines (MUM-2B, C918, MUM-2C and OCM-1A) and the human melanocyte cell line (D78) were obtained from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). The OCM-1A, MUM-2C, and D78 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MUM-2B and C918 were cultured in RPMI 1640 supplemented with 10% FBS.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

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Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. Reverse transcription reaction was carried out starting from 100 ng total RNA using the looped primers. Real-time PCR was performed using the standard Taqman MicroRNA assay protocol on ABI [illegible] real-time PCR detection system with cycling conditions of 95°C for 10 min, followed by [illegible] cycles of [illegible]°C for 15 s, 60°C for 30 s, and 74°C for 5 s. U6 small nuclear RNA was used as an internal control. The threshold cycle (Ct) was deðned as the fractional cycle number at which the ñuorescence passed the ðxed threshold. Each sample was measured in triplicate and the relative amount of miR-32 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta Ct = (Ct^{miR\text{-}32} - Ct^{U6})$.

Oligonucleotide transfection

MiR-32 mimics and negative control (miR-NC) were designed and synthesized by GenePharma Co. (Shanghai, China). Tumor cells were plated onto a six-well plate at a density of 3 x 10^5 cells/well for about 24 h prior to transfection. Transient transfections of miR-32 mimics or miR-NC (20 nM) were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. Twenty-four hours later, the cells were collected for further analysis.

MTT assay

Cell proliferation was analyzed using the colorimetric MTT assay. Briefly, approximately 1 x 10^3 cells were seeded onto a 96-well plate and incubated for 1, 2, 3, or 4 days. At the indicated time point, twenty microliters of MTT (5 mg/mL) (Sigma, USA) was added into each well and incubated for another 4 h. Then the supernatants were removed and 150 µL DMSO (Sigma) was added to terminate the reaction. The absorbance value (OD) was measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

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Detection of apoptosis by flow cytometry

Apoptosis was detected by ñow cytometric analysis. Brieñy, the cells were harvested, washed and resuspended in ice-cold PBS after miR transfection. Then, the cells were stained with propidium iodide (10 µg/mL; Sigma) and Annexin V-FITC (50 µg/mL, BD Biosciences) in the dark for 15 min at room temperature, and examined by flow cytometry (FACScan; BD Biosciences).

Cell invasion and migration assays

Six-well transwell chambers (8-µm pore size, Corning, NY, USA) were used to investigate cell invasion and migration. For the migration assay, about 1 x 10^5 tumor cells in serum free media were seeded into the upper chambers, and DMEM containing 10% FBS was added in the lower chamber as a chemoattractant. Following a 48 h-incubation, the cells located on the lower surface of the chamber were stained and counted using a microscope (Olympus Corp., Tokyo, Japan). For the invasion assay, the upper chambers were first coated with 5 mg/mL Matrigel and the same steps as described for migration were followed.

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Luciferase reporter assays

The pGL3-report luciferase vector was used for the construction of the pGL3-EZH2 and pGL3-EZH2-mut vectors. The pGL3-EZH2-mut vector was designed with EZH2 that had undergone site-directed mutagenesis of the miR-32 target site using the Stratagene Quik-Change site-directed mutagenesis kit (Stratagene, Germany). For the luciferase reporter assay, cells were cultured in 24-well plates, transfected with the plasmids and miR-32 mimics using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) 24 h after transfection.

Western blot analysis

Protein lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. After blocking, the membranes were incubated with rabbit anti-EZH2 antisera at 4°C overnight. The next day, the membranes were washed with PBS and then incubated with peroxidase-conjugated goat anti-rabbit IgG. Intensity of the bands was measured using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) and exposed on an X-ray film. β-actin was used as an internal reference for relative quantification.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). Data are reported as means ± standard deviation (SD). Differences between groups were analyzed using the Student *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Decreased miR-32 expression in uveal melanoma tissues and cell lines

Quantitative RT-PCR analysis showed that the expression of miR-32 was decreased in uveal melanoma specimens (means ± SD: 7.24 ± 1.92) compared with normal uveal tissues (means ± SD: 21.23 ± 4.39; P < 0.01; Figure 1A). In addition, miR-32 was also decreased in uveal melanoma cell lines (MUM-2B, C918, MUM-2C and OCM-1A) compared with the human melanocyte cell line D78 (Figure 1B). The MUM-2C cell line, which exhibited the lowest miR-32 expression among all tested uveal melanoma cell lines, was selected for miR-32 mimics transfection and further studies.

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Figure 1. Expression of mi[illegible]32 in [illegible]veal [illegible] t[illegible]sues [illegible]d cell lines. **A.** MiR-32 expression was significantly lower in uveal melan[illegible] spe[illegible]mens [illegible]an in [illegible]e corres[illegible]ding normal uveal tissues. MiR-32 expression levels were calculated by the [illegible] m[illegible]od and nor[illegible]alized to [illegible] small nuclear RNA. **B.** MiR-32 expression was downregulated in uveal m[illegible]lom[illegible]ll [illegible] MUM-2B, C[illegible], MUM-2C and OCM-1A compared to the human melanocyte cell line D78 (**P [illegible] 0.01).

Effects of mi[illegible]-32 [illegible] the biological behaviors of MUM-2C cells

As shown in Figure 2A, the expression level of miR-32 in miR-32 mimics-transfected cells was significantly higher compared to miR-NC transfected cells (P < 0.01). The MTT assay showed that cell proliferation was significantly impaired after transfection with miR-32 mimics (Figure 2B). We also observed increased cell apoptosis in miR-32 mimics-transfected cells (Figure 2C). Furthermore, transwell invasion/migration assays confirmed the inhibitory effect of miR-32 on MUM-2C cell invasion and migration (Figure 2D and E).

EZH2 is a target gene of miR-32

We used TargetScan (http://www.targetscan.org) to identify that the 3'-UTR of EZH2 contained the highly conserved miR-32 binding sites (Figure 3A). Transfection with miR-32 mimic significantly reduced EZH2 protein levels in MUM-2C cells (Figure 3B). Luciferase reporter gene assays demonstrated that miR-32 reduced luciferase activity of the pGL3-EZH2 plasmid but not the pGL3-EZH2-mut plasmid, suggesting that miR-32 directly targets the EZH2 3'-UTR (Figure 3C). These data indicate that EZH2 is a direct target of miR-32 in uveal melanoma.

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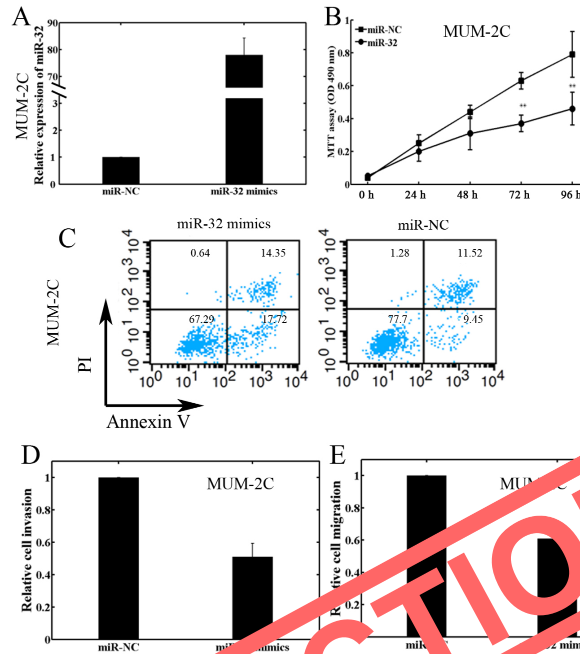

Figure 2. Effects of miR-32 on the biological behavior of MUM-2C cells. **A.** The expression level of miR-32 in miR-32 mimics-transfected cells was significantly higher compared to negative control (miR-NC) transfected cells (P < 0.001). **B.** Cell proliferation was measured by MTT assay in MUM-2C cells transfected with miR-32 mimics or negative control (**P < 0.01). **C.** Apoptosis of MUM-2C cells was detected by flow cytometric analysis after transfection with miR-32 mimics or miR-NC. **D. E.** MiR-32 suppressed MUM-2C cell invasion and migration *in vitro*. The transwell invasion and migration assays showed that the number of invading or migrating cells was significantly lower in the miR-32-transfected group than in the miR-NC-transfected group.

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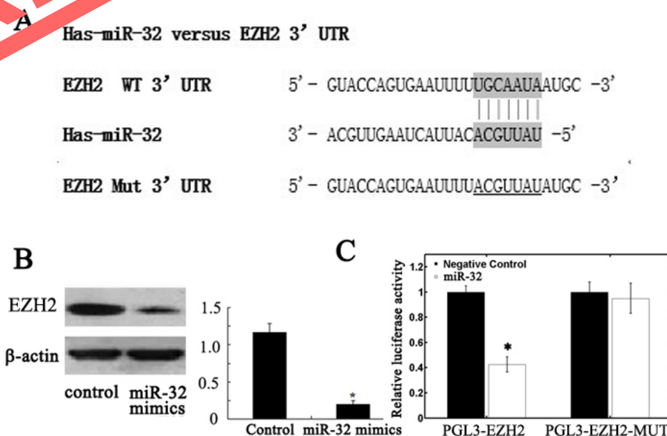

Figure 3. EZH2 is a direct target of miR-32. **A.** MiR-32-binding sites in EZH2 3'-UTR region. EZH2-mut indicates the EZH2 3'-UTR with a mutated miR-32 binding site. **B.** Western blot showing that ectopic expression of miR-32 decreased EZH2 protein expression. **C.** Luciferase reporter analysis demonstrated that overexpression of miR-32 reduced luciferase activity in the EZH2 wild-type reporter gene but not the mutant reporter in MUM-2C cells (*P < 0.05).

DISCUSSION

Dysregulation of miRs has been demonstrated to be involved in tumorigenesis and progression in various types of tumors; however, elucidation of their potential roles in uveal melanoma remains in the early stages of development. In the present study, we found that miR-32 was downregulated in uveal melanoma cell lines and primary tumor samples. Ectopic expression of miR-32 could inhibit uveal melanoma cell proliferation and invasion and promote apoptosis *in vitro*. Finally, EZH2 was identified as a direct target of miR-32. To our knowledge, this is the first study to analyze the expression and biological function of miR-32 in uveal melanoma.

There are increasing reports on the tumor suppressor functions of miR-32 in several human malignancies. *In vitro* studies have demonstrated that upregulation of miR-32 could suppress tumor proliferation and invasion and induce apoptosis in gastric cancer (Zhang et al., 2014b), non-small cell lung cancer (Zhu et al., 2015), oral squamous cell carcinoma (Zhang et al., 2014a), and osteosarcoma (Xu et al., 2014). Clinical research revealed that decreased miR-32 expression was significantly correlated with aggressive clinicopathological features including lymph node metastasis, advanced tumor stage, and poor overall survival in non-small cell lung cancer and oral squamous cell carcinoma (Zhang et al., 2014a; Zhu et al., 2015). However, the expression of miR-32 was reported to be upregulated in colorectal cancer tissues and cell lines, functioning as a novel oncogenic miR contributing to colon tumorigenesis by targeting the tumor suppressor gene PTEN (Wu et al., 2013a; Wu et al., 2013b). Therefore, miR-32 plays dual functions in cancer pathogenesis and progression, and the role of miR-32 is tumor specific and possibly dependent on its targets in different cancer types.

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MiRs execute their function by inhibiting the expression of target genes (Liu et al., 2014); therefore, elucidation of their target gene is crucial. EZH2, as a tumor-promoting gene, has a major influence on biological processes including cellular proliferation, apoptosis, migration, and invasion (Liu et al., 2015; Zhou et al., 2015; Zingg et al., 2015). Alterations in EZH2 may play a role in tumorigenesis of many cancers such as breast cancer (Wang et al., 2015), non-small cell lung cancer (Geng et al., 2015), clear cell renal cell carcinoma (Xu et al., 2015), and colorectal cancer (Song-Bing et al., 2015). Overexpression of EZH2 is considered an independent predictor of poor prognosis for patients with oral squamous cell carcinoma (Kidani et al., 2009), glioblastoma (Zhang et al., 2015), and prostate cancer (Melling et al., 2015). Moreover, EZH2 is overexpressed in uveal melanoma and associated with tumor aggressiveness (Chen et al., 2013). Knockdown of EZH2 resulted in inhibition of uveal melanoma cell migration and invasion. In our study, EZH2 was identified as a direct target of miR-32. This may provide a possible molecular mechanism of the anti-tumor effect of miR-32 in uveal melanoma cells. However, miRs may function in accordance with the combinatorial circuit model, in which a single miR can target multiple mRNAs and several coexpressed miRs may target a single mRNA. EZH2 is not the only miR-32 target dysregulated in melanoma; other functional targets of miR-32, such as PTEN and SOX9, also modulate melanoma pathogenesis (Cheng et al., 2015; Sun et al., 2015b). Therefore, the potential regulatory circuitry afforded by miR-32 is enormous, and the accurate mechanisms on how miR-32 influences uveal melanoma progression need further clarification.

In conclusion, the present study demonstrated that miR-32 is downregulated in uveal melanoma tissues and cell lines. Ectopic expression of miR-32 inhibits uveal melanoma cell proliferation and invasion. Further investigation revealed that EZH2 is a direct target of miR-

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32. These findings suggest that miR-32 might serve as a potential therapeutic target in patients with uveal melanoma.

Conflicts of interest

The authors declare no conflict of interest.

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