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Original Article

MiR-320a inhibits cell proliferation and metastasis of esophageal squamous cell carcinoma cell lines by targeting CBX3

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Abstract: The aim of this study was to investigate whether miR-320a can suppresses esophageal squamous cell carcinoma cell proliferation and migration by targeting CBX3. We identified that miR-320a was significantly down-regulated in esophageal squamous cell carcinoma (ESCC) cell lines by using qRT-PCR. Moreover, qRT-PCR and western blot assay found that CBX3 mRNA and protein were up-regulated in ESCC cell lines. CBX3 was a downstream target of miR-320a by TargetScan online tool. CBX3 was down-regulated by miR-320a which was confirmed by luciferase activity assay. Knockdown of CBX3 by siRNA dramatically suppressed cell proliferation *in vitro* which was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-liumbromide (MTT) assay. Moreover, miR-320a was able to inhibit ESCC cell metastasis *in vivo*. In conclusion, miR-320a/CBX3 played a role in ESCC cell proliferation.

Keywords: CBX3, esophageal squamous cell carcinoma, invasion, miR-320a, migration

Introduction

Esophageal squamous cell carcinoma (ESCC) is the fourth death related cancer worldwide. There are 320,800 male and 157,200 female new cases in 2015 of china. The ESCC caused 253,800 male deaths and 121,300 female deaths in 2015 of china [1]. The risk rises with age and the average diagnosed age is 67 [2, 3]. About 90% of ESCCs are either adenocarcinomas or squamous-cell carcinomas [4]. The pathogenesis of ESCC remains obscure. It has urgent need to find new therapeutic methods for treatment of ESCC.

MicroRNA (miRNA) is non-coding RNA about 22 nucleotides long [5, 6]. MiRNAs play a pivotal role in various biological processes, such as cell proliferation, growth, migration, invasion and apoptosis. Mounting researches demonstrated that miRNAs function as tumor promotors or suppressors in tumorigenesis [7, 8]. MiR-320a is a member of miR-320 family and often functions as a tumor suppressor in many cancers. MiR-320a significantly inhibited lung cancer A549 and LTEP-a-2 cell proliferation and induced cell apoptosis by targeting signal transducer and activator of transcription 3 (STAT3) [9]. In human liver cancer, miR-320a highly inhibited cell proliferation and caused arresting of G_0/G_1 growth *in vitro*. Moreover, β-catenin was identified as a direct target of miR-320a [10]. The potential role of miR-320a in ESCC remains unclear.

CBX5, CBX1 and CBX3 are members of heterochromatin protein 1 family. CBX3 plays an important role in various biological processes, such as gene expression regulation, DNA repair and telomere function [11, 12]. Smallwood *et al.* reported that CBX3 bind to gene regions which are highly associated with gene activity in various cell types. Knockdown of CBX3 induces inhibition of a number of genes and results in fast accumulation of unprocessed transcripts [13]. In colon cancer, CBX3 promotes cell cycle and growth *in vivo* and *in vitro*. CBX3 demonstrates this function by regulating CDK6/P21 [14]. The function of CBX3 in ESCC has not been identified.

In our study, we identified that miR-320a was significantly down-regulated in ESCC cell lines compared with normal esophageal cells. CBX3 was a down-stream target of miR-320a. The novel miR-320a/CBX3 axis provides a new therapeutic target for treatment of ESCC and deepens insight of ESCC tumorigenesis.

Material and methods

In our study, we hypothesized that miR-320a inhibited ESCC cell proliferation and metastasis by targeting CBX3. Firstly, qRT-PCR and western blot were used to detect miR-320a expression level and CBX3 expression level in ESCC cell lines. The U6 snRNA and GAPDH were used as internal normalization standard for miR-320a and CBX3, respectively.

Secondly, CBX3 was hypothesized as a down-stream target of miR-320a. This hypothesis was confirmed by TargetScan online tool and luciferase reporter assay. The relative luciferase activity was detected to identify whether CBX3 was the target gene of miR-320a.

Finally, we investigate the effect of miR-320a on cell proliferation, migration and invasion. MTT assay and Transwell assay were used to perform these experiments.

Cell lines

ESCC cell lines TE10 and KYSE150 were used in our study. The cell lines were cultured in medium supplemented with fetal bovine serum. The human normal esophageal cell line Het-1 A was purchased from American Type Culture Collection (ATCC).

Western blotting

Total proteins were extracted from cells using the cell lysis buffer (Takara, Dalian, China) following the manufacture's instruction. The Bradford assay kit (Takara, Dalian, China) was used to quantify the concentration of proteins. The proteins were separated by the SDS-PAGE, and then were electrotransferred to PVDF membrane (Bio-Rad, California, USA). The membrane was blocked in Tween-20 containing 5% non-fat milk for 1 h and then incubated with primary antibodies. The antibodies used in our study were as follows: rabbit anti-human CBX3 (1:1000, Sigma-Aldrich) and rabbit anti-human GAPDH (1:1000, Sigma-Aldrich). The protein bands were visualized using the Bio-Rad Gel Doc XR instrument (Bio-Rad, California, USA). Every experiment was performed for three times in duplicate.

Plasmid construction and cell transfection

In our study, the miR-320a mimic and LNA anti-miR-320a were constructed to over-express and knockdown the miR-320a. The siRNA for CBX3 was synthesized and purchased from Thermo Fisher Scientific corporation for knockdown of CBX3 (Stealth RNAi™ siRNA, Thermo Fisher Scientific).

The transfection of cell lines was performed using the Lipofectamine 2000 instrument (Invitrogen, California, USA) and RNAiMAX (Invitrogen, California, USA) [15]. The transfection experiments were carried out following the instruction of Invitrogen Corporation.

Total RNA extraction and quantitative real-time PCR

The total RNA was extracted using the RNAprep pure Tissue kit (Tiangen, Beijing, China) according to the manufacture's instruction. The cDNA was synthesized using the FastKing RT Kit (with gDNase) (Tiangen, Beijing, China). Primers were designed and synthesized in our study as follows: (F: 5'-AAAAGCTGGGTTGAGAGGGCGA-3'; R: 5'-GCGAGCACAGAATTAATACGAC-3') and U6 snRNA (F: 5'-CTCGCTTCGGCAGCACA-3'; R: 5'-AACGCTTCACGAATTTGCGT-3'). GAPDH mRNA levels were used for normalization of CBX3. Primers were used for mRNA detection as follows: CBX3 (F: 5'-TGGCCTCCAACAAAACTACA-3'; R: 5'-TCCCATTCACTACACGTCGA-3') and GAPDH (F: 5'-TTGTTGCCATCAATGACCC-3'; R: 5'-CTTCCCGTTCTCAGCCTTG-3'). Real-time PCR was performed using the Roche LightCycler 480 instrument (Roche, Basel, Switzerland). The U6 snRNA levels were used for normalization of miR-320a. The qRT-PCR was carried out under following condition: denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 10 s, 60°C for 30 s. $2^{-\Delta\Delta CT}$ method was used to calculate gene expression ratio.

Luciferase reporter assay

The target gene of miR-320a was predicted on the TargetScan website (www. targetscan.org). In this website, we identified CBX3 was a poten-

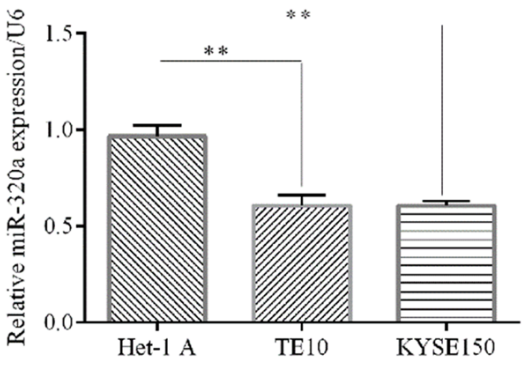

Figure 1. MiR-320a expression levels were detected in ESCC cell lines TE10, KYSE150 and normal cells. Values are means ± SE of three independent experiments. **P<0.01.

tial downstream target of miR-320a and the binding site of CBX3 for miR-320a was at the 3'-UTR. The 3'-UTR sequence of CBX3 was amplified by PCR and cloned into the PsicheckTM-2 vector (PsicheckTM-2-CBX3-WT). The binding site of CBX3 for miR-320a was mutated and cloned into the PsicheckTM-2 vector (PsicheckTM-2-CBX3-MT). The lipofectamine 2000 (Invitrogen) was used to carry out the luciferase activity assay. The luciferase activities were measured by using the Dual-luciferase Reporter Assay System (Promega, Fitchburg, WI, USA).

Migration and invasion assays

The migration of TE10 and KYSE150 cell lines was tested using chamber (8 μm pore, Corning, USA). Approximate $1*10^6$ cells were added to upper chamber and cell medium was add into the lower chamber. Cells were incubated for 24 h at 37°C. The crystal violet was used to stain the migrated cells and visualized using the inverted microscope. The upper chamber was filled with Matrigel (BD, San Jose, CA, USA) for the invasion test. The number of invading cells was calculated by counting three different fields of view using light microscopy.

Detection of metastasis activity of miR-320a in vivo

Mice were maintained in accordance with NIH Animal Care and Use Committee Guidelines. $1*10^6$ cells were injected into mice via tail vein. The health conditions and body weights of mice were monitored. 4 weeks after injection, mice were sacrificed. Livers were excised and tumor nodules were counted. Each experiment group contained 5 mice.

Cell proliferation assay

In this study, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-liumbromide (MTT) was employed to investigate cell proliferation. The cells were incubated for 4 h at 37°C and then add the MTT. After the supernatants were removed, the formazan crystals were dissolved by the DMSO (200 μl/well). The absorbance at 490 nm of each sample was determined using the Thermo Scientific Evolution 300 instrument (Thermo Fisher Scientific, Massachusetts, USA).

Statistical analysis

The SPSS 17.0 software was adopted for statistical analysis. Independent t-test was employed to carry out the comparison between means of two groups. Results were considered significant if *P* value was<0.5.

Results

MiR320a was down-regulated in ESCC cell lines compared with normal esophageal cells

The expression levels of miR-320a were determined by qRT-PCR. The results showed that miR320a expression were significantly decreased in ESCC cell lines TE10 and KYSE150 compared with the normal esophageal cells (P<0.05) (**Figure 1**). These results suggested that the expression level of miR-320a may function as a biomarker for diagnosis of ESCC.

Over-expression of miR-320a significantly inhibited cell migration and invasion in vitro

In order to better understand the mechanism of miR-320a, we over-expressed miR-320a using miR-320a mimic. TE10 cell line was transfected with miR-320a mimic or negative control. The successful over-expression of miR-320a by transfection of miR-320a mimic was confirmed by qRT-PCR (**Figure 2A**).

The transwell assay indicated that the cell migration and invasion were dramatically inhibited when TE10 cell line was transfected with miR-320a mimic compared with negative control (**Figure 2B**).

CBX3 was a downstream target of miR-320a and was down-regulated by miR-320a

In our study, we used the TargetScan to predict the potential role of miR-320a. CBX3 was iden-

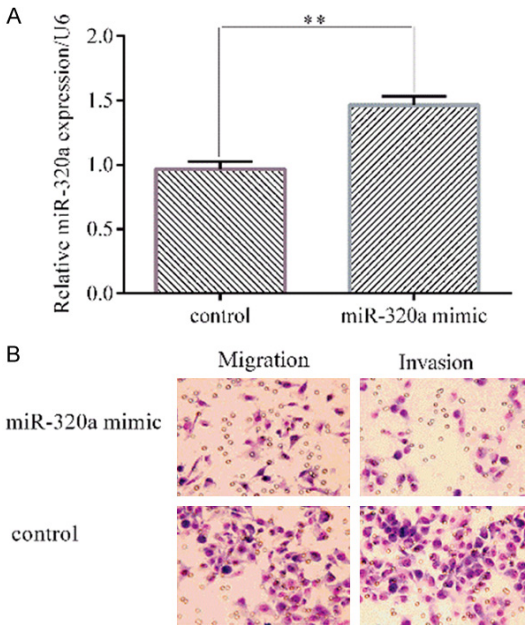

Figure 2. Over-expression of miR-320a highly inhibited cell migration and invasion *in vitro*. A. The miR-320a expression levels of TE10 cell line which was transfected with miR-320a mimic was detected by qRT-PCR. B. The effect of miR-320a on migration and invasion of ESCC cell line was detected by Transwell assay. Values are means ± SE of three independent experiments. **P<0.01.

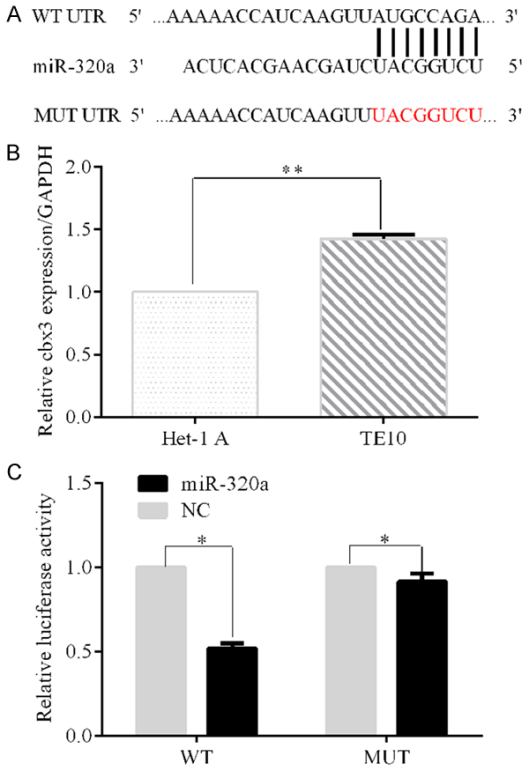

Figure 3. CBX3 was predicted as a potential target of miR-320a and down-regulated by miR-320a. A. The binding site of CBX3 for miR-320a was located at its 3'-UTR which was predicted by TargetScan online tool. The mutated sequences were presented in red. B. The CBX3 expression level was analyzed by qRT-PCR. C. Luciferase activity of TE10 cell line, which was co-transfected with PsicheckTM-2-CBX3-WT or PsicheckTM-2-CBX3-MT and miR-320a mimic was detected by luciferase reporter assay. Values are means ± SE of three independent experiments. *P<0.05; WT, wild-type; MT, mutated.

tified as a putative target of miR-320a. The binding site of CBX3 for miR-320a was at 1203-1209 bp (**Figure 3A**).

The expression level of CBX3 in ESCC TE10 cell line was detected by qRT-PCR (P<0.05) (**Figure 3B**). The results indicated that CBX3 was significantly up-regulated in ESCC cell line compared with normal esophageal Het-1 A cells.

The luciferase activity was dramatically inhibited when TE10 cell line was co-transfected with PsicheckTM-2-CBX3-WT and miR-320a mimics (**Figure 3C**). The decline extent of luciferase activity was attenuated when cell line was transfected with PsicheckTM-2-CBX3-MT and miR-320a mimics (**Figure 3C**). This demonstrated that CBX3 was a putative target of miR-320a and was down-regulated by miR-320a.

Knockdown of CBX3 inhibited ESCC cell proliferation in vitro

In order to investigate the effect of CBX3 on ESCC cell proliferation, siRNA for CBX3 was transfected into TE10 cell line to knock down CBX3. The successful knockdown of CBX3 was confirmed by western blot (**Figure 4A**). CBX3 protein level was highly declined by transfection of siRNA.

Proliferation of TE10 cell line was detected by MTT assay. The results indicated that knockdown of CBX3 significantly inhibited cell proliferation *in vitro* (**Figure 4B**).

MiR-320a dramatically suppressed cell metastasis in vivo

In order to investigate the metastatic activity of miR-320a on ESCC cells, TE10 cell line which stably expressed miR-320a or control vector was injected into mice. The numbers of liver nodules induced by transfection of TE10-miR-320a cells were dramatically reduced than those induced by TE10-control (**Figure 5**).

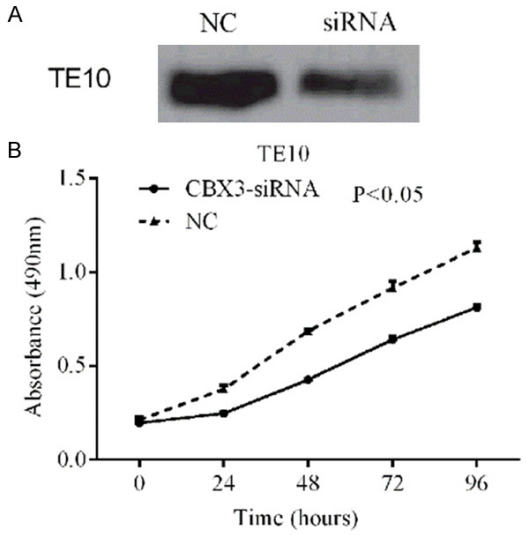

Figure 4. Knockdown of CBX3 inhibited ESCC cell proliferation. A. siRNA for CBX3 was transfected into TE10 cell line. The CBX3 protein level was determined by western blot. B. Proliferation of TE10 cell line was detected by MTT assay. Values are means ± SE of three independent experiments. *P<0.05.

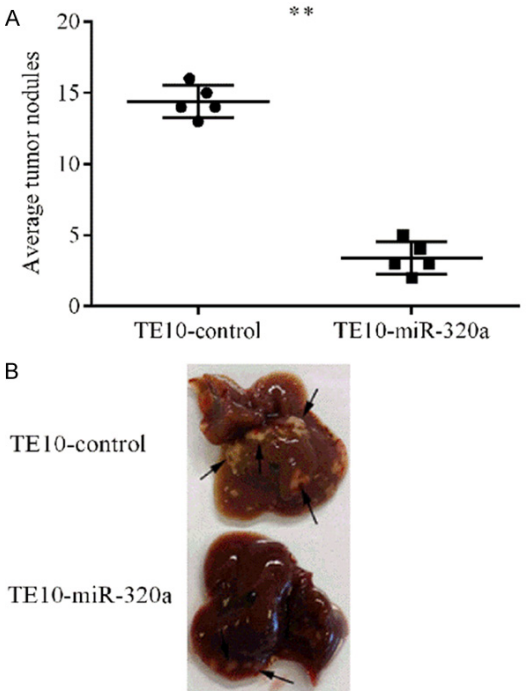

Figure 5. MiR-320a inhibited ESCC cell metastasis *in vivo* TE10 cell line which stably expressed miR-320a or control vector was injected into mice. The formation of nodules in liver surface was significantly suppressed by miR-320a. A. The average tumor nodules was counted. B. The livers were excised from mice. **P<0.01.

These observations demonstrated that miR-320a significantly inhibited ESCC cell metastasis *in vivo*.

Discussion

In our study, we identified that miR-320a was significantly down-regulated in TE10 and KYSE150 cell lines compared with normal cells. Over-expression of miR-320a highly inhibited cell migration and invasion.

In many previous studies, miR-320a has been identified as a tumor suppressor. MiR-320a expression was decreased in human glioma cells and cell lines. Over-expression of miR-320a inhibited cell proliferation, invasion and migration [16]. These results were consistent with ours. In human glioma, miR-320a and SND1 was prognostic biomarkers [16]. We did not analyze the potential role of miR-320a on ESCC prognosis. In our further study, we will carry out this research. Lv *et al.* also reported that miR-320a expression was significantly decreased in hepatocellular carcinoma (HCC) tissues and related with migration and metastasis [17]. Xie *et al.* also investigated the effect of miR-320a on HCC proliferation. They identified that miR-320 suppressed HCC cell proliferation by directedly targeting c-Myc [18]. In our study, we identified that miR-320a significantly inhibited ESCC cell metastasis.

In our study, we identified that CBX3 was a downstream target of miR-320a and was suppressed by miR-320a. CBX3 was highly up-regulated in ESCC cell line compared with normal esophageal cells. CBX3 binds at important DNA regions and regulate gene expression genome-widely [13]. Saini *et al.* reported that CBX3 was highly expressed in osteosarcoma tissues [19]. We suggested that CBX3 was often functioned as a tumor promoter in many cancers. CBX3 depletion also contributed anticancer ability of T cells [20]. In our study, we identified that knockdown of CBX3 highly inhibited ESCC cell proliferation. Consistent with our study, CBX3 could increase colon cancer cell proliferation *in vitro* [14]. This is the first report that CBX3 could function as a biomarker in ESCC.

There still exist some drawbacks in our study. For example, we did not analyze the patient specimens in our study. On the other hand, the novel identified miR-320a/CBX3 axis lacked

clinical experiments. In our further study, we will carry out associated experiments.

In summary, the newly identified miR-320a/CBX3 axis provided better understanding of ESCC mechanisms and functioned as a new biomarker for therapeutic treatment.

Disclosure of conflict of interest

None.

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