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Original Article

miR-320 inhibited ovarian cancer oncogenicity via targeting TWIST1 expression

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Abstract: Ovarian cancer is the most lethal gynecological cancer in most countries. Increasing studies have demonstrated that dysregulation of microRNAs (miRNAs) can contribute to cancer progression. In this study, we showed that miR-320 was underexpressed in ovarian cancer samples compared to their non-tumor tissues. The expression of Twist homolog 1 (TWIST1) in ovarian cancer tissues was upregulated compared with that in the non-tumorous tissues. We found that the expression of TWIST1 was inversely correlated with that of miR-320 in the ovarian cancer. Overexpression of miR-320 suppressed cell proliferation, cell cycle and invasion in ovarian cancer. We identified TWIST1 as a direct target gene of miR-320 in the ovarian cancer cell. Overexpression of TWIST1 promoted the ovarian cancer cell proliferation, cell cycle and invasion. Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion. These findings revealed that miR-320 was a tumor suppressive gene that supressed cell prloferation, cycle and invasion through targeting TWIST1 in ovarian cancer.

Keywords: Ovarian cancer, microRNAs, miR-320, TWIST1

Introduction

Ovarian cancer is the most lethal gynecological cancer in women [1-3]. Due to lack of early diagnosis markers, the early diagnosis rate for ovarian cancer is still low [4-7]. Despite the significant achievements in surgery, chemotherapy and radiotherapy, the 5-year overall survival of ovarian cancer is still dissatisfied [8-11]. Hence, it is essential for elucidating the molecular mechanisms for ovarian cancer and finding the target for its treatment.

MicroRNAs (miRNA), a type of nonprotein-coding and small RNAs, regulate gene expression through affecting both tranlation and stability of target mRNAs [12-15]. Emerging evidences have demonstrated that miRNAs play an important role in diverse biological processes such as development, immunity and metabolism [12, 16-19]. Recent studies have found deregulation of miRNAs in various cancers including hepatocellular carcinoma, renal cell carcinoma, lung cancer, breast cancer and also ovarian cancer [20-24]. Moreover, deregulated miRNAs are correlated with cancer initiation, progression and promotion by regulating a lot of tumor suppressor genes or oncogenes [25-27].

In our study, we showed that miR-320 was underexpressed in ovarian cancer samples and cells. Overexpression of miR-320 suppressed the ovarian cancer cell proliferation, cell cycle and invasion. We identified Twist homolog 1 (TWIST1) as a direct target gene of miR-320 in the ovarian cancer cell.

Materials and methods

Tissue collection, cell culture and transfection

Human ovarian cancer and its non-tumorous tissue was abtained at the time of surgery from The Second Affiliated Hospital of Wenzhou Medical University. Written informed consent was collected from each patient and this study was also approved the Institutional Ethics Review Board of the Second Affiliated Hospital of Wenzhou Medical University. Four ovarian

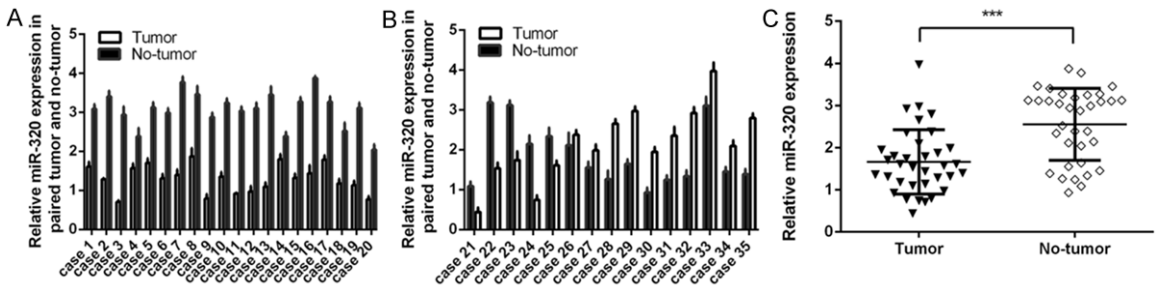

Figure 1. miR-320 was downregulated in tumor tissues of ovarian cancer. A. The expression of miR-320 in 20 ovarian cancers and non-tumorous tissues was shown. B. The expression of miR-320 in additonal 15 ovarian cancers and non-tumorous tissues was shown. C. The level of miR-320 expression in ovarian cancer tissues was reduced compared with that in the non-tumor tissues. ***P<0.001.

cancer cell lines (HO8910PM, HO8910, ES2 and SKOV-3) and a normal human fallopian tube epithelial cell line (FTE187) were bought from Cell Resource Cente of the Chinese Academy of Sciences (Shanghai, China). The cell lines were kept in the RPMI-1640 medium supplemented with 10% FBS (Invitrogen, CA, USA). miR-320 mimic and sramble oligonucleotide was synthesized from RiboBio (Guangzhou, China) and transfected to cell line using Lipofectamine 2000 (Invitrogen) following to the manufacturer's protocol.

Western blot analysis

Total protein was separated on the 12% SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinyl fluoride) membranes (Amersham, UK). The membrane was blocked with 5% milk for 1 hour and incubated with primary antibody (TWIST1 and GAPDH, Abcam) at 1:2,000 dilution overnight. The membrane was then incubated with second antibody (zsgb-bio, Beijing) and measured using ECL (enhanced chemiluminescence-plus reagent) according to the manufacturer's protocol.

Luciferase reporter assays

Cells were cultured on the 96-well plate. A mixture of miR-320 mimic or scramble and pGL3-TWIST1-3'UTR-wt or pGL3-TWIST1-3'UTR-mut and Renilla luciferase plasmids was transfected to cells using Lipofectamine 2000 according to manufacturer's instruction. After 48 hours, renilla and firefly luciferase activities were detected using a Dual-Luciferase Reporter System (Promega). The Renilla luciferase activity was used as the internal control.

Cell proliferation and migration assay

Cell proliferation was assessed using the MMT (3-(4,5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide) assay according to the manufacturer's protocol. The cells were cultured on the 96-well culture plate and cell proliferation was determined 24, 48 and 72 hours after transfection. The OD (optical density) was measured at 490 nm on the multiwell spectrophotometer (BioTek, VT, USA). For cell migration.

Quantitative real-time PCR

Total RNA was isolated from tissue or cell with Trizol reagent (Invitrogen, CA, USA). Expression level of miR-320 and TWIST1 were measured by using SYBR Green reagent (Invitrogen, USA) according to the manufacturer's protocol. The sequences primers were used as following: TWIST1-forward: 5'-GGAGTCCGCAGTCTTACG-AG-3'; RECK-reverse: 5'-TCTGGAGGACCTGGT-AGAGG-3'; GAPDH-forward: 5'-AAGGTCGGAG-TCAACGGATTTG-3'; RECK-reverse: 5'-CCATGG-GTGGAATCATATTGGAA-3'.

Statistical analysis

Data was expressed as mean ± SD (standard deviation). Statistical significance between two groups was measured by Student's t-test and one-way ANOVA was used for more than two groups. P<0.05 was deemed to be statistically significant.

Results

miR-320 was downregulated in tumor tissues of ovarian cancer

Our study firstly measured the expression of miR-320 in 35 ovarian cancer tissues. The

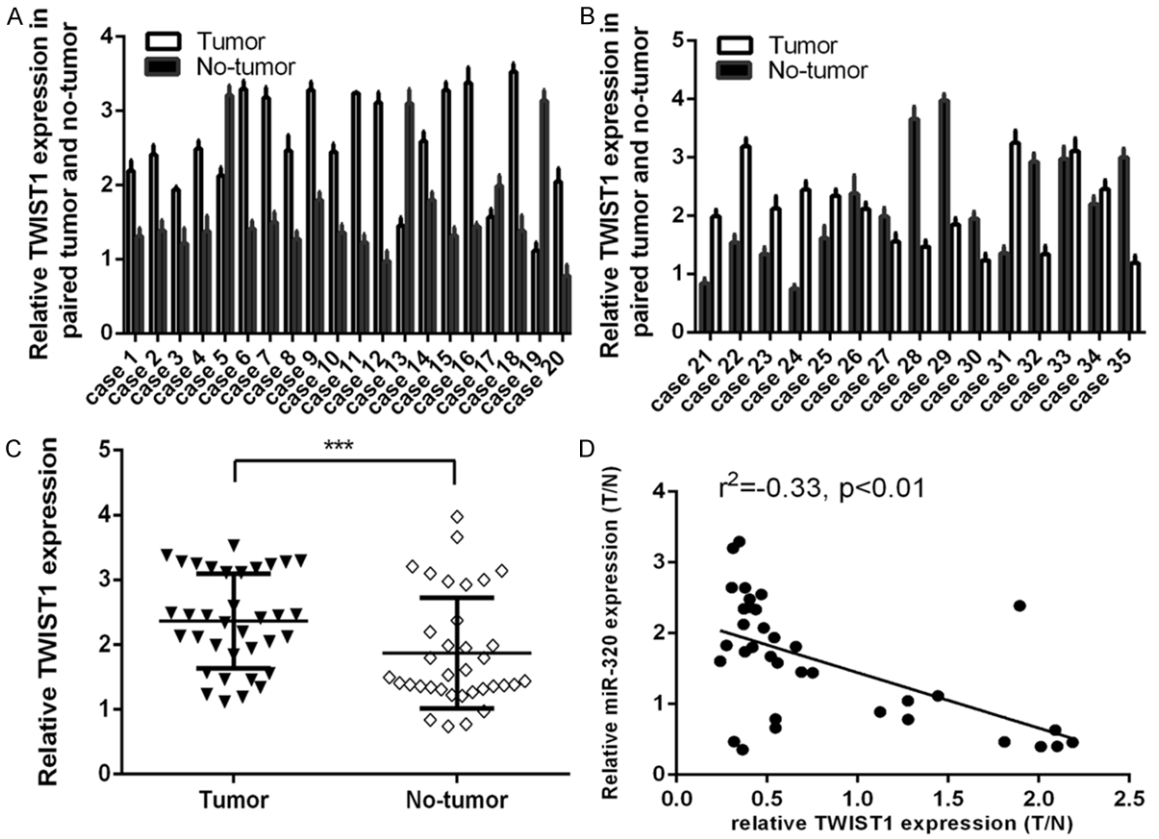

Figure 2. TWIST1 was upregualted in ovarian cancer tissues. A. The expression of TWIST1 in 20 ovarian cancers and non-tumorous tissues was shown. B. The expression of TWIST1 in 15 ovarian cancers and non-tumorous tissues was shown. C. The expression level of TWIST1 in ovarian cancer tissues was upregulated compared with that in the non-tumortissues. D. We found that the expression of TWIST1 was inversely correlated with that of miR-320 in the ovarian cancer. ***P<0.001.

expression of miR-320 in 35 ovarian cancers and non-tumorous tissues was shown in the **Figure 1A** and **1B**. The level of miR-320 expression in ovarian cancer tissues was reduced compared with that in the non-tumor tissues (**Figure 1C**).

TWIST1 was upregualted in ovarian cancer tissues

We next determined the expression of TWIST1 in 35 ovarian cancer tissues. The expression of TWIST1 in 35 ovarian cancers and non-tumorous tissues was shown in the **Figure 2A** and **2B**. The expression level of TWIST1 in ovarian cancer tissues was upregulated compared with that in the non-tumor tissues (**Figure 2C**). Interesting, we found that the expression of TWIST1 was inversely correlated with that of miR-320 in the ovarian cancer (**Figure 2D**).

Overexpression of miR-320 suppressed ovarian cancer cell proliferation, cell cycle and invasion

The expression of miR-320 was downregulated in four ovarian cancer cell lines (HO8910PM, HO8910, ES2 and SKOV-3) compared to a normal human fallopian tube epithelial cell line (FTE187) (**Figure 3A**). The miR-320 expression was upregulated in the HO8910PM cell after treated with miR-320 mimic (**Figure 3B**). Ecoptic expression of miR-320 suppressed the HO8910PM cell proliferation (**Figure 3C**). Moreover, overexpression of miR-320 inhibited the HO8910PM cell cycle (**Figure 3D**). miR-320 overexpression suppressed the HO8910PM cell invasion (**Figure 3E**).

TWIST1 was a direct target gene of miR-320 in ovarian cancer TargetScan was used to determine the target of miR-320. As shown in the

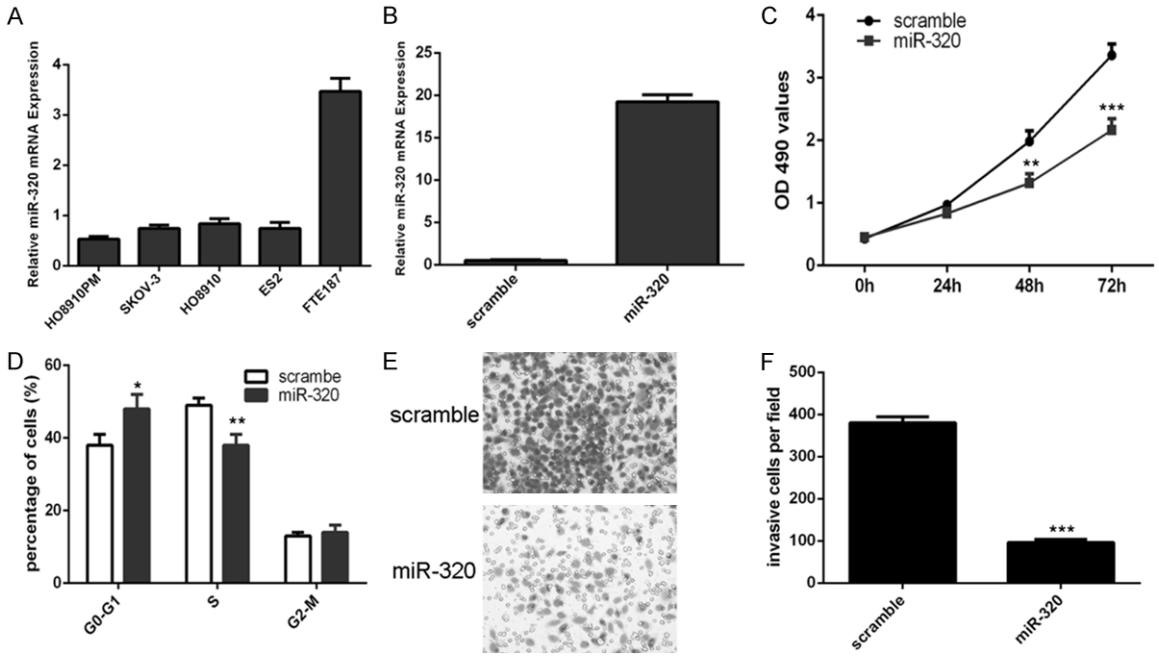

Figure 3. Overexpression of miR-320 suppressed ovarian cancer cell proliferation, cell cycle and invasion. A. The expression of miR-320 in four ovarian cancer cell lines (HO8910PM, HO8910, ES2 and SKOV-3) and a normal human fallopian tube epithelial cell line (FTE187) was measured by qRT-PCR. B. The miR-320 expression was upregulated in the HO8910PM cell after treated with miR-320 mimic. C. Ecoptic expression of miR-320 inhibited the HO8910PM cell proliferation. D. Overexpression of miR-320 suppressed the HO8910PM cell cycle. E. Ecoptic expression of miR-320 suppressed the HO8910PM cell invasion. F. The relative invasive cells were shown. *P<0.05, **P<0.01, and ***P<0.001.

Figure 4A, there was a putative miR-320 binding seed site in the 3'UTR of TWIST1. The luciferase activity was decreased in the wild 3'UTR of TWIST1, and the luciferase activity of the mutation putative miR-320 target site was no change (**Figure 4B**). Overexpression of miR-320 suppressed the expression of TWIST1 (**Figure 4C** and **4D**). The expression of TWIST1 was upregulated in four ovarian cancer cell lines (HO8910PM, HO8910, ES2 and SKOV-3) compared to a normal human fallopian tube epithelial cell line (FTE187) (**Figure 4E**).

Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion

The protein expression of TWIST1 was upregulated in the HO8910PM cell after treated with TWIST1 vector (**Figure 5A**). The mRNA expression of TWIST1 was also increased in the HO8910PM cell after treated with TWIST1 vector (**Figure 5B**). Ecoptic expression of TWIST1 promoted the HO8910PM cell proliferation (**Figure 5C**). Moreover, overexpression of TWIST1 increased the HO8910PM cell cycle (**Figure 5D**). TWIST1 overexpression promoted the HO8910PM cell invasion (**Figure 5E** and **5F**). Ectopic expression of TWIST1 rescued the miR-320-overexpressing HO8910PM cell proliferation (**Figure 5G**), cell cycle (**Figure 5H**) and invasion (**Figure 5I** and **5J**).

Discussion

In our study, we demonstrated that miR-320 was underexpressed in ovarian cancer samples compared to their non-tumor tissues. The expression of TWIST1 in ovarian cancer tissues was upregulated compared with that in the non-tumorous tissues. We found that the expression of TWIST1 was inversely correlated with that of miR-320 inovarian cancer. Overexpression of miR-320 suppressed the ovarian cancer cell proliferation, cell cycle and invasion. We identified TWIST1 as a direct target gene of miR-320 in the ovarian cancer cell. Overexpression of TWIST1 promoted the ovarian cancer cell proliferation, cell cycle and invasion. Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion. These findings revealed

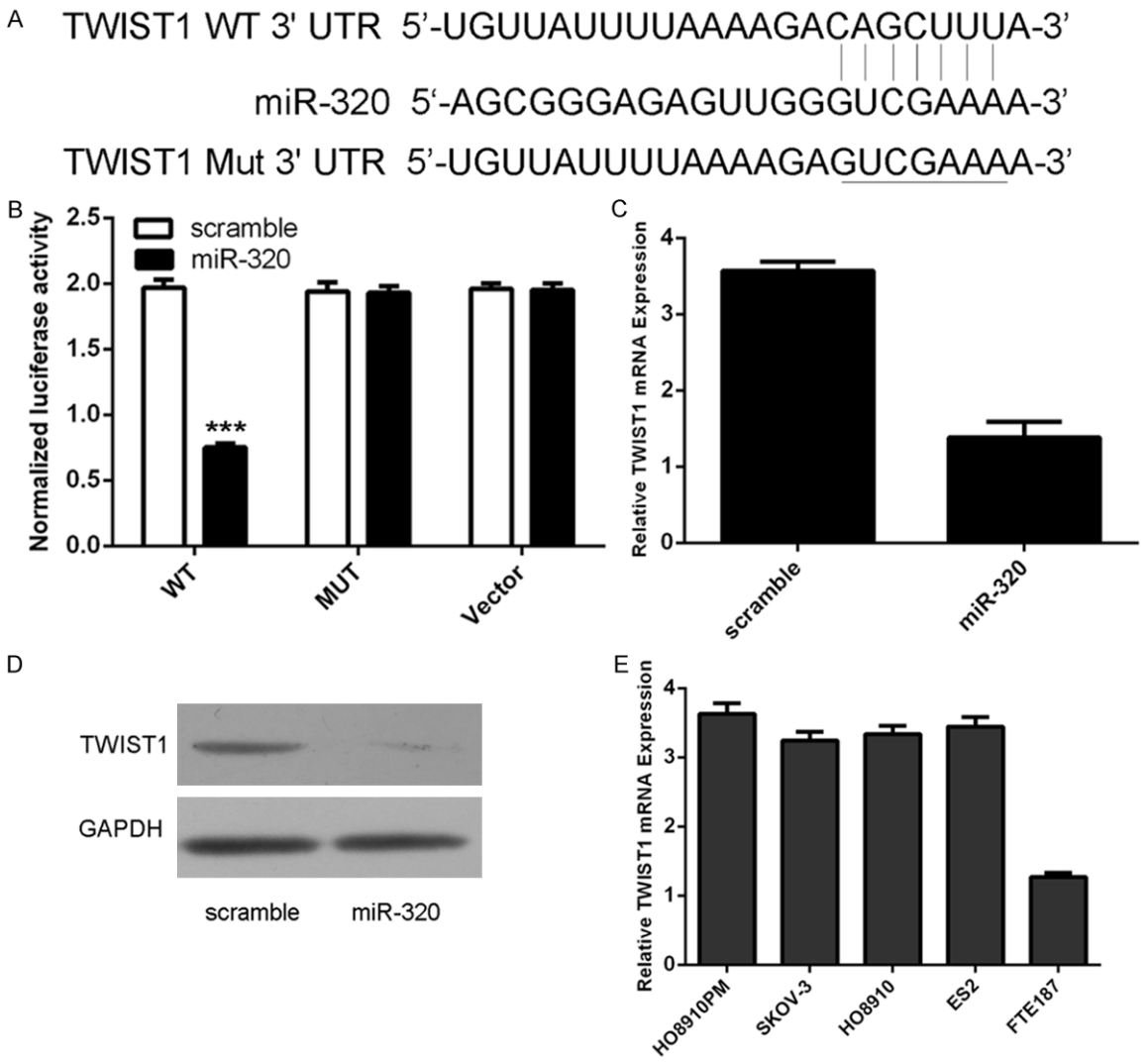

Figure 4. TWIST1 was a direct target gene of miR-320 in ovarian cancer. A. There was a putative miR-320 binding seed site in the 3'UTR of TWIST1. B. The luciferase activity was decreased in the wild 3'UTR of TWIST1, and the luciferase activity of the mutation putative miR-320 target site was no change. C. Overexpression of miR-320 suppressed the mRNA expression of TWIST1. D. Overexpression of miR-320 suppressed the protein expression of TWIST1. E. The expression of TWIST1 was upregulated in four ovarian cancer cell lines (HO8910PM, HO8910, ES2 and SKOV-3) compared to a normal human fallopian tube epithelial cell line (FTE187). ***P<0.001.

that miR-320 was a tumor suppressive gene that supressed cell prloferation, cell cycle and invasion in ovarian cancer.

miR-320 had been previously studied in a lot of tumors and played a critical role in these cancers [28-33]. For example, Lei et al [34]. Showed that miR-320 was decreased in non-small cell lung tumor and miR-320 overexpression suppressed the non-small cell lung cancer cell migration, proliferation and invasion through regulating fatty acid synthase expression. Vishnubalaji et al [35]. Demonstrated that miR-320 was downregulated in the primary colorectal cancer and miR-320 overexpression suppressed colorectal cancer growth and migration in vitro, sensitized colorectal cancer cells to 5-Fluorouracil, and suppressed cancer formation in the mice through targeting SOX4, FOXM1, and FOXQ1. Zhang et al [36]. Determined that miR-320 expression was decreased in the cervical cancer tissues and etopic expression of miR-320 suppressed cervical cancer cell invasion, migration, proliferation and tumorigenesis by inhibiting Mcl-1 expression. Until now, there is no report about the role of miR-320 in the ovarian cancer development. In our study, we firstly detected the expression of miR-320 in

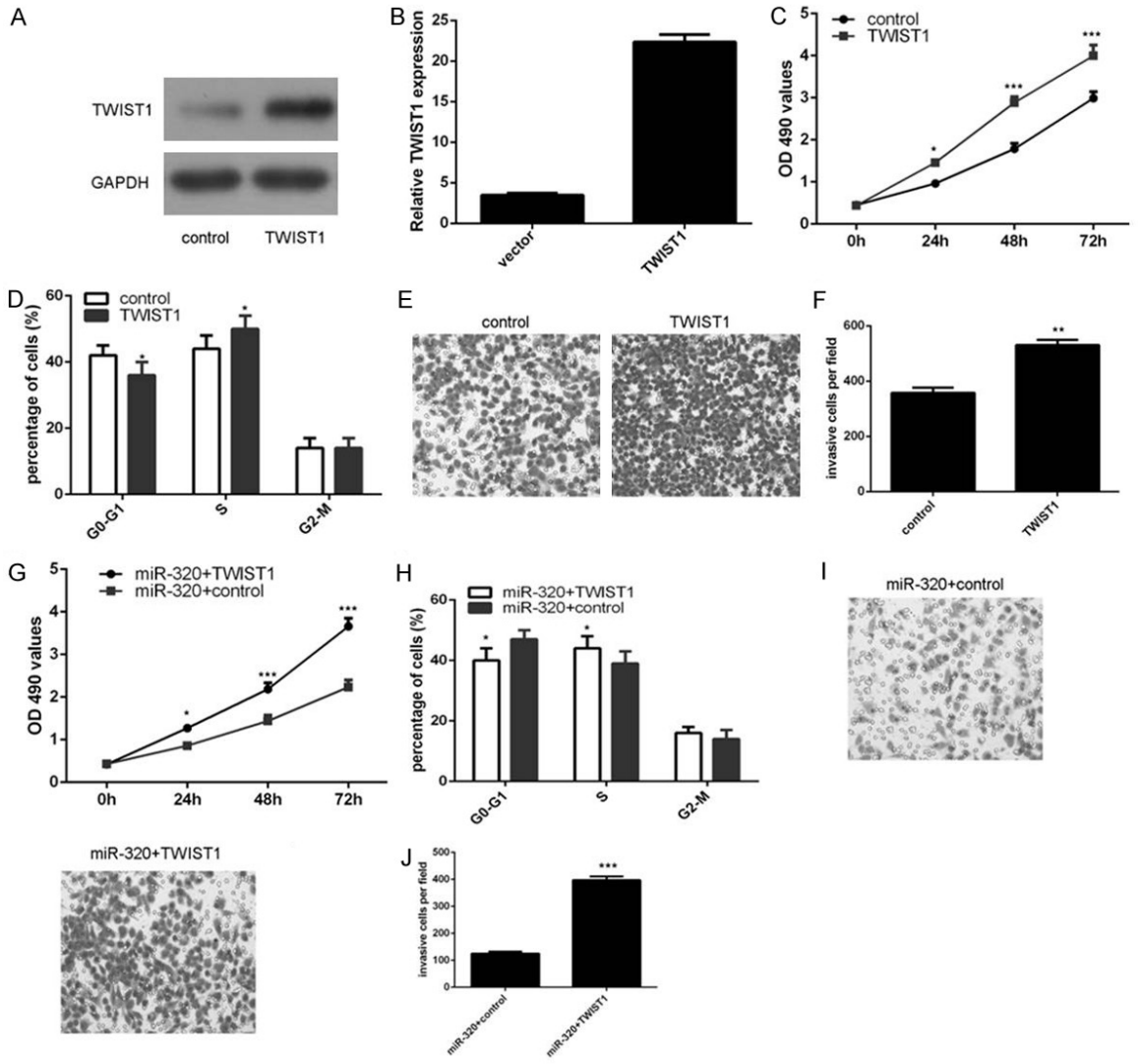

Figure 5. Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion. A. The protein expression of TWIST1 was upregulated in the HO8910PM cell after treated with TWIST1 vector. B. The mRNA expression of TWIST1 was also increased in the HO8910PM cell after treated with TWIST1 vector. C. Ecoptic expression of TWIST1 promoted the HO8910PM cell proliferation. D. Overexpression of TWIST1 increased the HO8910PM cell cycle. E. TWIST1 overexpression promoted the HO8910PM cell invasion. F. The relative invasive cell was shown. G. Ectopic expression of TWIST1 rescued the miR-320-overexpressing HO8910PM cell proliferation. H. Ectopic expression of TWIST1 rescued the miR-320-overexpressing HO8910PM cell cycle. I. Ectopic expression of TWIST1 rescued the miR-320-overexpressing HO8910PM cell invasion. J. The relative invasive cell was shown. *P<0.05, ** P<0.01, and ***P<0.001.

the ovarian cancer tissues and cell lines. We found that the level of miR-320 expression in ovarian cancer tissues was reduced compared with that in the non-tumor tissues. Moreover, Overexpression of miR-320 suppressed the ovarian cancer cell proliferation, cell cycle and invasion. These data suggested that miR-320 play an important role in the development of ovarian cancer.

Another crucial finding was that we identify TWIST1 as a new direct target gene of miR-320 in the ovarian cancer cell. TWIST1 is one member of the basic helix-loop-helix transcription factor twist family that plays important roles in the mesenchymal phenotypes and serves as one powerful oncogene [37-39]. TWIST1 was found to be overexpressed in various cancers such as gastric cancer, breast cancer, hepato-

cellular cancer, colon cancer and bladder cancer [40-45]. Overexpression of TWIST1 promotedtumor cell migration, proliferation and invasion [42, 46, 47]. Recently, several studies have showed that miRNA plays important roles in the regulation of TWIST1 in ovarian cancer [48-50]. For example, Sun et al [50]. Demonstrated that miR-548c was downregulated in the ovarian cancer tissues and suppressed ovarian cancer cell proliferation, invasion and migration through targeting TWIST1 expression. Zhu et al [49]. Demonstrated that miR-186 inhibited the ovarian cancer cell esenchymal-to-epithelial transition, G1 cell-cycle arrest and promoted cell apoptosis by regulating TWIST1. In our study, we identified TWIST1 was a direct target gene for miR-320. Western blot and Luciferase assay were determined to study the effect of miR-320 on the translation of TWIST1. Overexpression of miR-320 inhibited the luciferase activity of the wild-type TWIST1 vector reporter gene but not with the mutant TWIST1 3'UTR vector. Moreover, overexpression of miR-320 suppressed the protein expression of TWIST1 in the HO8910PM cell. Furthermore, we showed that overexpression of TWIST1 promoted the HO8910PM cell proliferation, cell cycle and invasion. Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion.

In conclusion, we showed that miR-320 was underexpressed in ovarian cancer tissues and cells. Overexpression of miR-320 suppressed the ovarian cancer cell proliferation, cell cycle and invasion. We identified TWIST1 as a direct target gene of miR-320 in the ovarian cancer cell. Our data showed that the expression of TWIST1 was inversely correlated with that of miR-320 in the ovarian cancer. Ectopic expression of TWIST1 restored the effects of miR-320 on cell proliferation, cell cycle and invasion. These findings revealed that miR-320 was a tumor suppressive gene that supressed cell prloferation, cell cycle and invasion through targeting TWIST1 in ovarian cancer.

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Disclosure of conflict of interest

None.

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