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MiR-613 promotes cell proliferation and invasion in cervical cancer via targeting PTPN9

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Abstract. – OBJECTIVE: To investigate the potential effects of miR-613 on the development of cervical cancer (CC) and the relevant mechanism.

PATIENTS AND METHODS: The expression level of miR-613 was detected in CC tissues and cells (siHa) by comparing with corresponding adjacent normal tissues and normal human embryonic kidney cells (293T). Luciferase assay was performed to evaluate the interaction between miR-613 and PTPN9. The effects of the miR-613 on siHa cells were determined by subsequent experiments including cell proliferation, invasion and migration.

RESULTS: In our study, miR-613 was found up-regulated in CC tissues and the same result was found at cellular level. The potential target of miR-613 was analyzed by three public databases. We found that tyrosine-protein phosphatase non-receptor type 9 (PTPN9) was a direct target of miR-613, and Luciferase assays confirmed our hypothesis. The subsequent experiments showed that decreased expression of PTPN9 resulting from up-regulation of miR-613 could promote the cell proliferation, invasion and migration of CC cells.

CONCLUSIONS: We showed the promotion function of miR-613 on CC by targeting PTPN9 and revealed that miR-613/PTPN9 axis might be a potential therapeutic target for the treatment of CC.

Key Words:
MiR-613, Cervical cancer (CC), Tyrosine-protein phosphatase non-receptor type 9.

Introduction

Cervical cancer (CC) is one of the most common female malignant tumors worldwide triggered by canceration of female cervical epithelial cells. Its incidence rate ranks second following breast cancer in malignant tumors[1]. According to the statistics of the World Health Organization (WHO), there are about 500,000 new cases of CC globally each year, among which about 300,000 cases are from economically underdeveloped countries or regions. Each year, there are approximately 270,000 deaths from CC, and the number of new cases in the economically underdeveloped countries or regions accounts for about 80% of the total number worldwide, and the deaths account for about 85%[2]. In China, there are about 131,500 new cases of CC, accounting for around 1/4 to 1/3 of the total annual incidence of CC in the world[3]. Therefore, the incidence and mortality rates of CC are high in China.

Research on the pathogenesis of CC remains not very clear so far. At present, it is widely believed that human papillomavirus (HPV) infection is the main cause of CC[4-6], but studies[7,8] have revealed that high-risk HPV infection is a risk factor triggering CC but not inducing the transformation and canceration of cervical epithelial cells. The processes of occurrence and development as well as metastasis period of CC are relatively long, and there is a lack of effective tumor markers. Thus, cancer generally cannot be detected effectively and timely for early diagnosis, delaying the optimal treatment period. Currently, surgical treatment, chemotherapy and radiotherapy are the main treatment methods for CC. Clinical treatment effects of CC have been remarkably improved in the past decades. However, the 5-year survival rate of patients with advanced CC is only about 50% due to the great difficulty in surgical operation and easy recurrence after operation[9,10]. Therefore, conducting studies on basic molecular mechanisms such as the occurrence and development of CC is of important guidance value for early detection and individualized treatment.

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Micro ribonucleic acids (miRNAs) generally consist of non-coding sequences of single-stranded RNA molecules with the length of more than 20 nucleotides. They are featured with their tissue specificity, time sequence of expression, and high-degree conservation. Besides, miRNAs are considered as potential markers for auxiliary diagnosis, individualized treatments and prognostic judgments of the disease[11]. The post-transcriptional regulation of genes by miRNAs, as a breakthrough scientific discovery in this century, is considered very important. More than 1000 types of human miRNAs have been found to change the expression levels of around1/3 of human genes by regulating transcription up to now, thus controlling changes in cell function[12,13].

As a member of the miRNA family, miR-613 had shown its unique advantages in the diagnosis and treatment of a variety of cancers including non-small cell lung cancer[14], ovarian cancer[15], breast cancer[16], and bladder cancer[17]. However, there were few reports on the role of miR-613 in the occurrence and development of CC and its related molecular mechanism. In this study, the role of miR-613 in the occurrence and development of CC and its related molecular mechanism were clarified through analyzing the expression of miR-613 and the effects of miR-613 on biological behaviors of CC.

Patients and Methods

Cervical Cancer Cases and Cells

This study included 28 CC patients undergoing a surgical procedure in West China Second University Hospital. All patients underwent pathological diagnoses to be confirmed CC. Preoperative chemotherapy or radiotherapy treatment was forbidden. The liquid nitrogen was used to freeze the CC tissues and corresponding adjacent normal tissues, and then those tissues were kept in -80ºC refrigerator. The adjacent normal tissues had to be concerned by Biological biopsy to be sure that they do not include CC cells. Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of West China Second University Hospital. Signed written informed consents were obtained from all participants before the study.

The CC cell lines (siHa) together with normal human embryonic kidney cell lines (293T) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in RPMI- 1640 (Roswell Park Memorial Institute) 1640 medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 cell culture incubator.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that Tyrosine-protein phosphatase non-receptor type 9 (PTPN9) was a target gene of miR-613. The binding sequence of miR-613 at the 3'-end of PTPN9 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated PTPN9 (Mut-type) and non-mutant PTPN9 (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant PTPN9 was transfected into siHa cells after lentivirus intervention on the 24-well plate. The same treatment was performed on the pGL3-Basic vector connected with the non-mutant PTPN9 according to the steps in the Luciferase Reporter Gene Assay Kit. Then, the luciferase activity was detected in a multi-function microplate reader.

Transfection

MiR-613 mimics and si-PTPN9 were synthesized and transfected to CC cell line to analyze biological function of miR-613. Three groups were established to study the potential relevance between miR-613 and siHa cell: NC group (negative control), miR-613 mimics (siHa cell transfected by miR-613 mimics) and mimics + PTPN9 (siHa cell transfected by miR-613 mimics and si-PTPN9). All the stuff was purchased from RiboBio (Guangzhou, China), and were transfected by using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

SiHa cells were detected *via* real-time fluorescence quantitative polymerase chain reaction (qPCR). Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of PTPN9, expression and endogenous controlled

by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) was used to measure the level of miR-488 expression normalized to miRNA U6. The primer sequences were as the follows: PTPN9 (F: 5'-GGCCCCGTCGTTGTAATAAAGCCTCCAG-3', R: 5'-GACAAATCGTTTTCATTTCAATCGTAG-3'), microRNA-488 (F: 5'-GCGGCGCCCAGAUAAUG-3', R: 5'-GTGCAGGGTCCGAGGT-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'), U6 (F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3').

Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) lysate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was employed to extract the total protein in the cells. The proteins were first separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Sigma-Aldrich, St. Louis, MO, USA) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After that, the membranes were blocked with 5% milk and incubated with antibodies, PTPN9 and GAPDH [diluted at 1:1000, Cell Signaling Technology (CST) Inc. Danvers, MA, USA] (at 4°C overnight). The next day, the corresponding secondary antibodies (CST, Inc. Danvers, MA, USA) were used for incubation at room temperature, followed by development *via* enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA), exposure in gel imaging system, fixation and observation of results. With β-actin as an internal reference, the relative changes in protein expression were detected.

Cell Proliferation

When cells grew to the logarithmic growth phase, they were collected, diluted into 1×10^6 cell suspension, and added into a 96-well cell culture plate (5×10^3/100 μL per well). The wells only added with medium were used as blank controls. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. 15 μL MTT reagents (500 μg/mL) was added into each well for the culture for another 2 h, after which the absorbance was measured using an enzyme-labeled spectrophotometer, followed by zero setting using blank wells.

Cell Invasion and Migration Assays

After 48 h of transfection, cell migration and invasion abilities were measured using a Transwell chamber (Corning Incorporated, Corning, NY, USA) with a pore size of 8 μm, in which Matrigel (BD, Franklin Lakes, NJ, USA) at a concentration of 1:9 was paved in the upper chamber for detection of invasion ability. Subsequently, the upper chamber was added with 250 μL serum-free medium while the lower chamber was added with 700 μL medium containing 10% fetal bovine serum. Cells (5×10^4/well) were then added to the upper chamber and placed in an incubator for incubation. After 24 h, the chamber was removed, and the remaining cells in the upper chamber were gently wiped off with a cotton swab. The lower chamber cells were then fixed with paraformaldehyde and stained with crystal violet. Finally, five fields of view were randomly selected under an inverted microscope (×100) for counting, and the average was calculated.

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. All *p*-values were two-sided and $p < 0.05$ were considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

miR-613 Expression Found Reduced Both in Tissues and Cells of CC

To investigate the expression level of miR-613 in CC, its expression in 28 cases of CC and cancer-adjacent tissues was detected using quantitative polymerase chain reaction (qPCR) first. The results manifested that the relative expression level of miR-613 in CC tissues notably increased compared with that in cancer-adjacent tissues (Figure 1A). Then the expression level of miR-613 in the CC cell line siHa and normal human embryonic kidney cell line 293T were detected. The results showed that the relative expression level of miR-613 in the CC cell line was significantly higher than that in 293Ts (Figure 1B), which was consistent with the expression difference in tissues.

PTPN9 is a Direct Target of miR-613 in CC Cell

To investigate potential target of miR-613, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA to elucidate

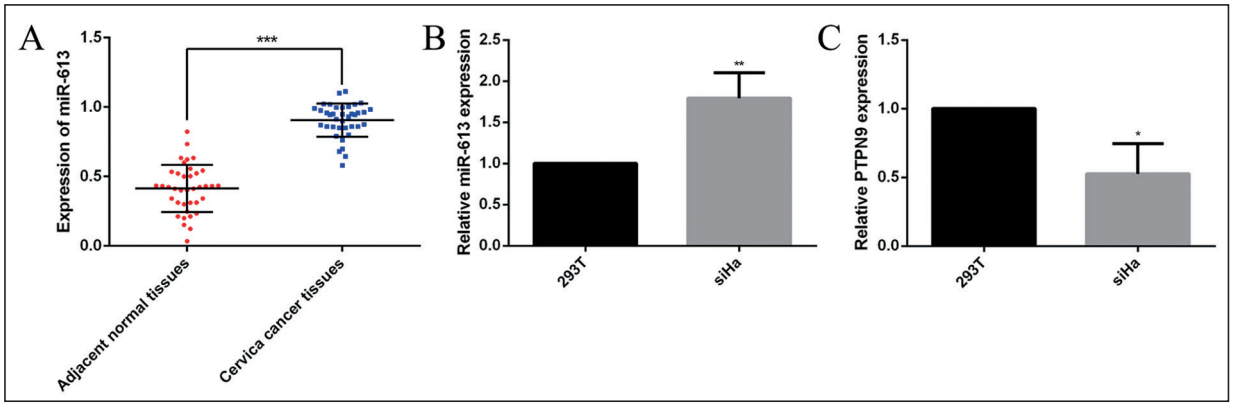

Figure 1. The expressions of miR-613 and PTPN9 in cervical cancer (CC) tissue samples and cells comparing with corresponding adjacent normal tissues and normal human embryonic kidney cells (293T). ***A,*** Difference in the expression of miR-613 between CC tissues and corresponding adjacent normal tissues (***$p < 0.001$ compared with adjacent normal tissue). ***B,*** The expression of miR-613 in CC cells (siHa) and normal human embryonic kidney cells (293T) (**$p < 0.01$ compared with 293T). ***C,*** The expression of PTPN9 in siHa cells and 293Ts (*$p < 0.05$ compared with 293T).

the putative and possible targets of miR-613. We found the PTPN9 has checked a supposed target of miR-613 (Figure 2A). Thus, PTPN9 had caught our attention and was implemented in our further studies. Firstly, we detected the level of PTPN9 expression in cell lines by qRT-PCR, and we found thatPTPN9 expression was down-regulated in CC cells by comparing with 239t cells (Figure 1C). Then, we established luciferase reporter vectors containing the wild or mutant-type miR-613 seed sequences of the PTPN9 30UTR. Increased the expression of miR-613 with mimics result in the decrease of the luciferase activity of the wide-type PTPN9 30UTR reporter gene, but it had no effect on mutant-type (Figure 2B), which suggested the expression of PTPN9 could be regulated by miRNA 613. In conclusion, these results suggested miR-613 and PTPN9 might have some correlation on effect during the progression of CC.

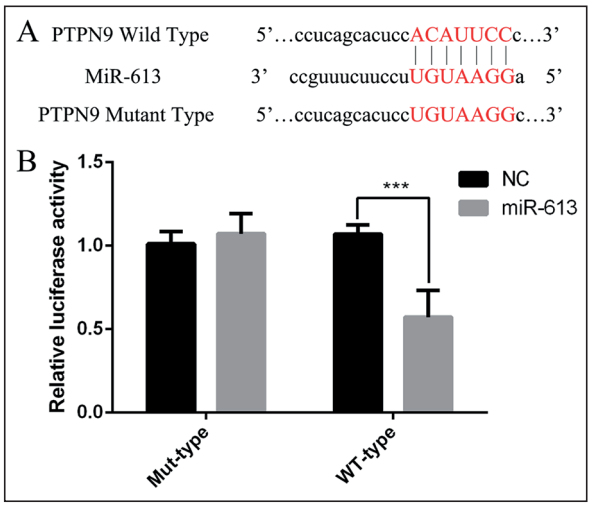

Figure 2. PTPN9 is a direct and functional target of miR-613. siHa cell transfection with miR-613 mimics and inhibitor. ***A,*** Diagram of putative miR-613 binding sites of PTPN9. ***B,*** Relative activities of luciferase reporters (***$p < 0.001$).

MiR-613 Decreased the Expression Level of PTPN9

Three groups were established to take similar experiments (miR-NC group, miR-613 mimics group and the mimics PTPN9 group) in siHa cell. The results showed that the expression level of PTPN9 was decreased by up-regulation of miR-613 in siHa cell in both PCR and Western blot analysis (Figure 3B-3D). The data further illustrated the regulating effect of miR-613 on the expression of PTPN9.

MiR-613 Suppressed Proliferation of CC Cell

We took MTT assay to detect the cell proliferation rates after the transfection. The MTT results suggested that the cell proliferation rates of siHa cell were accelerated after miR-613 mimics transfection. In contrast, the cell growth of CC cells was limited increased in mimics PTPN9 group (Figure 3A).

MiR-613 Inhibited Invasion and Migration of CC Cell

Migration and invasion are two most key factors in cancer cell metastasis. In the transwell

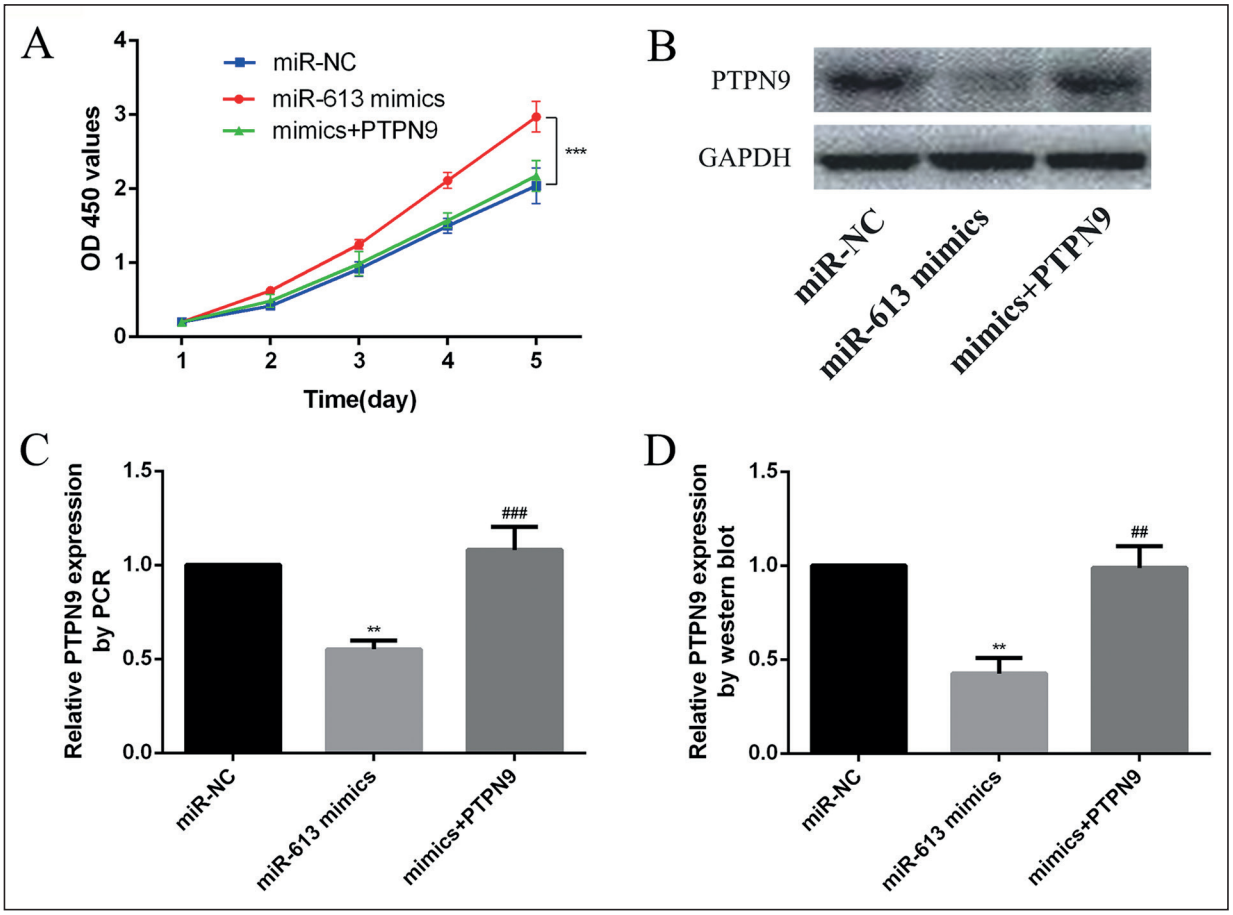

Figure 3. ***A,*** MiR-613 accelerates the proliferation of CC cell (**$p < 0.01$), ***B-D,*** MiR-613 decreases the expression level of PTPN9. Data were presented as means ± standard deviations (**$p < 0.01$ *vs.* NC group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* Mimics group).

experiments, the migration and invasion abilities of siHa cells were detected after up-regulation of miR-613 expression level. The results illustrated that compared with those in the control group, the migration and invasion abilities of SIHA cells were obviously increased after the expression level of miR-613 was up-regulated. However, after up-regulating the expression of PTPN9, the promoting effect of miR-421 was counteracted (Figure 4).

Discussion

Cervical cancer (CC) is one of the most common malignant tumors in the female reproductive system. Cancer data statistics in China in 2015 revealed that the incidence rate of CC exhibiting an annual increasing trend, and its fatality rate ranks as high as second among female tumors[18]. CC severely threatens women's health. Human papillomavirus HPV is the main risk factor for CC and is closely associated with the occurrence in more than 90% of patients with CC. However, it must be admitted that the abnormal expressions of oncogenes and tumor suppressor genes are essential for the occurrence of CC[19]. As oncogenes or tumor suppressor genes, miRNAs participate in the development of various tumors, and they are abnormally expressed in almost all tumor cells through detection. MiRNA-based targeted therapy has become a hot topic in cancer therapy research in recent years, and targeted therapy achieved by interfering with gene expression patterns is gradually becoming a new direction in CC research[20]. It has been currently confirmed that miR-613 exerts important effects in tumors and participates in the occurrence and development of non-small cell lung cancer, hepatocellular carcinoma, breast cancer and bladder cancer.

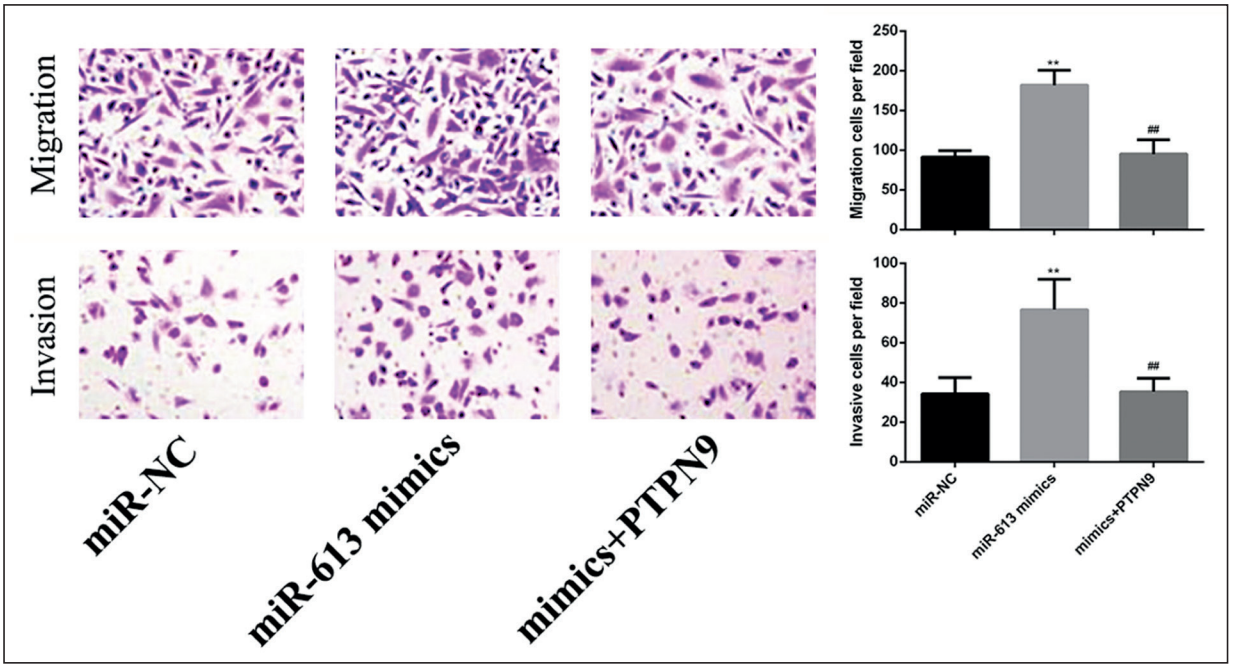

Figure 4. MiR-613/PTPN9 axis inhibits the invasion and migration of CC cell. PTPN9 overexpression attenuates the promotive effect of miR-613 on siHa cell. ***A,*** The invasion test by transwell assay. ***B,*** The migration test by transwell assay. (**$p < 0.01$ *vs.* NC group; ##$p < 0.01$ *vs.* Mimics group).

However, its role in CC has not yet been clarified. This study illustrated that the expression level of miR-613 in CC tissues of clinical patients was notably higher than that in cancer-adjacent tissues, and the same results were obtained in cell experiments *in vitro*.

MiRNAs complementarily bound to the three prime untranslated regions (3'UTR) of target genes, thus inactivating or degrading target genes and achieving post-transcriptional regulation of target genes[21]. To study the role of miR-613 in the pathogenesis of CC, three online websites were employed for screening of possible potential target genes of miR-613. Based on bioinformatics analysis, PTPN9 is a direct target gene of miR-613, and qPCR results manifested that PTPN9 markedly declined in CC cells. In addition, according to the dual luciferase report results, miR-613 mimics significantly impeded the luciferase activity of wild-type (WT) PTPN9-3'UTR but not the luciferase activity of mutant-type (Mut) PTPN9-3'UTR. To sum up, these results indicated that PTPN9 is the direct target gene of miR-613.

Tyrosine-protein phosphatase non-receptor type 9(PTPN9) is a member of the classical protein tyrosine phosphatases (PTPs) family, which was first found in the human megakaryoblastic leukemia cell line MEG-01 and confirmed to be a protein phosphatase widely distributed in organisms[22,23]. PTPN9, as an important molecule involved in the intercellular junction and signal transduction, regulates various functions such as cell proliferation, migration and differentiation, and plays an important biological role[24]. Current studies[25,26] have confirmed that PTPN9 not only promotes the dephosphorylation of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER-2) so as to indirectly suppress the activation of EGF-related signal transducer and activator of transcription 3 (STAT3), but also directly catalyzes the dephosphorylation of Try705 residues of STAT3. In breast cancer, PTPN9 has been proved to be a tumor suppressor gene, which can inhibit tumor cell proliferation, invasion and various other functions[27]. To further understand whether PTPN9 intimidated the proliferation, migration, and invasion of CC cells mediated by miR-613, MTT assay and detection of cell migration and invasion would be conducted for cells after transfection of SiHa cells with PTPN9 small interfering ribonucleic acid (siRNA) and/or miR-613 mimics. The results demonstrated that PTPN9 siRNA remarkably sup-

pressed the proliferation-promoting ability of miR-613 and reduced SiHa cell migration and invasion abilities after transfection with miR-613. The above results indicate that PTPN9 is a functional target of miR-613 in CC.

Conclusions

We showed that miR-613 expression was significantly up-regulated in CC tissues. The regulation of the expression of PTPN9 promoted the proliferation, invasion and metastasis of CC cells. As such, miR-613 acts as an oncogene in CC, which will provide experimental evidence for searching for new molecular therapeutic targets for CC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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