The downregulation of microRNA-375 in human cervical squamous cell carcinoma promotes invasion and migration by targeting JAK2

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Summary

Micro RNAs (miRNAs) contribute to the progression and expansion of many cancers, including cervical cancer. Reports reveal the down-regulation of MicroRNA-375 (miR-375) within cervical cancer tissues. Nevertheless, the mechanism for regulation of cervical cancer progression by miR-375 is still unknown. The current work deals with the molecular mechanisms orienting the regulatory impacts of miR-375 on invasion and migration in human cervical carcinoma. miRNA expression within cervical cancer tissues was assessed through Stem-loop RT-PCR. The invasion and migration of miR-375 mimic-transfected SiHa cells were observed by the Transwell test and wound healing test, respectively. Bioinformatics analysis was performed for predicting the target gene of miR-375, which was validated by the dual-luciferase test. Real-time RT-PCR and Western blot analysis were utilized for detecting protein expression and mRNA transcription. In cervical cancer, miR-375 expression was 4.2 ± 0.15-fold lower compared to the adjacent noncancerous tissues. The number of invasive and migrated SiHa cells (32 ± 3.6; 21.86 ± 6.4) transfected with miR-375 mimics were considerably lower in comparison with the NC group (101 ± 7.5; 41 ± 5.6) after 24 hours. Real-time RT-PCR and Western blot analysis confirmed JAK2 as a downstream target gene of miR-375. The protein expression and mRNA level of JAK2 in miR-375 mimic-transfected SiHa cells were decreased. The present study indicated that the invasion and migration of SiHa cells are regulated by miR-375 through down regulating JAK2 expression. This study provides a theoretical and experimental basis for reversing the metastasis of cervical cancer and developing new candidate drugs.

Key words: miR-375; Cervical squamous cell cancer; Invasion; Migration; JAK2.

Introduction

Cervical cancer is one of the most widespread malignant neoplasms in females worldwide, with 567,200 novel cases and 265,700 deaths reported in 2012, of which 80% occurred in developing countries [1, 2]. Each year, 75,000 new cervical cancer cases are reported in China [3] accounting for one-seventh of the total new cases worldwide. Although improvements in surgical techniques and cisplatin-based concurrent have chemoradiotherapy enhanced the overall survival and recurrence rates [4-6], the 5-year survival rates for stage-3 and 4 patients were maintained at 53.0% and 23.7%, respectively. These low survival rates are attributed to recurrence and metastasis [8]. Therefore, it is important to elucidate the molecular mechanism of the incidence and progress of cervical cancer and find new therapeutic targets.

Numerous clinical investigations proved that the main cause of cervical cancer is chronic human papillomavirus (HPV) infection [9, 10]. HPV includes high and low-risk types, among which the high-risk HPV has been detected in 99% of cervical cancer tissue samples [11]. Although the exact carcinogenic mechanism of HPV has not been fully elucidated, several investigations indicated that the abnormal miRNA expression in cervical cancer is associated with HPV infection [12-15].

miRNAs are endogenous non-coding RNAs of approximately 17-25 nucleotides (nt) in length that inhibit the translation of target mRNAs or participate in their post-transcriptional degradation via incomplete or complete pairing with the 3'-untranslated area (3'-UTR). miRNAs regulate various biological procedures such as cell growth, energy metabolism, differentiation, proliferation, metastasis, and apoptosis [16-18].

miR-375 is located on chromosome 2q35 and the mutation of miR-375 is related to the occurrence of several tumors. The miR-375 expression is out of balance in various cancers [19-22]. Wang et al. indicated that the miR-375 expression was downregulated in cervical cancer tissues compared to normal tissues. The miR-375 expression is associated with clinicopathological parameters like cervical cancer lymph node metastasis [1]. Nonetheless, the mechanism and contribution of miR-375 in the occurrence and expansion of cervical cancer have been confirmed.

In the current work, migration and invasiveness of cervical cancer cells were investigated following treatment with miR-375 mimics. JAK2 was recognized as the miR-375...
The downregulation of microRNA-375 in human cervical squamous cell carcinoma... target gene by bioinformatics analysis and verified by the double luciferase method. The up-regulation of JAK2 expression was found at protein and mRNA levels. The results of this study provide a theoretical and experimental basis for the reversal of cervical cancer metastasis and the discovery of new therapeutic drugs.

Materials and Methods

Twenty paraffin-rooted sections of cervical cancer tissues and nearby tissues were found from the patients in Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. No radiotherapy or chemotherapy was performed for the patients in this work prior to surgery. The patients were within the mean age of 49 ± 4.3 years (range of 32-65 years). Data on clinico-pathological parameters were obtained from the patients’ clinical records and pathology reports.

HEK 293 cell lines and human cervical cancer SiHa were prepared from the Shanghai Cell Bank (Shanghai, China). SiHa and HEK 293 cells were preserved in Dulbecco’s DMEM (modified Eagle medium) (GIBCO, USA) supplemented with ten percent fetal bovine serum (hyclone, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin (GIBCO, USA). Culturing the cells was performed at 37 °C in humidified air comprising 5% CO₂. miRNA mimics/controls were purchased separately and transfected into the cells at an ultimate concentration of 100 nM/L. miRNA transfection was performed using lipid reagents transfected with INTERFERin.

Finally, matrixx gel (BD Biosciences, Bedford, MA) was inserted to the upper surface of the chamber filter (8 mm). The cells (1 x 10⁵ cells/well) were inoculated in the upper chamber (millipore, Ma, USA) with a medium containing a low concentration of serum (1%). The lower chambers were occupied with a medium containing a high concentration of serum (20%) as a chemoattractant. The culture was incubated for 48 hours. Then, formaldehyde and crystal violet were used respectively to fix and stain the membranes. The invading cells were counted using an optical microscope, and five fields of view were selected for image acquisition.

SiHa cells were inoculated into 24 well plates (1 x 10⁵/well). After 16 hours, an incision was made in the central area of the fused cells, and an artificial wound was made with the tip of a rapid pipette. Then, the cells were rinsed with serum-free medium and negative control (NC) or mir-375 mimics were added to the culture dish. Non-specific miRNA mimics were used as a negative control. Cell cultures were cultured in a serum-free medium in five percent CO₂ humidified air at 37 °C. Wounds were photographed at 0, 24, and 48 hours (Olympus, Japan).

To extract the total microRNA from cells, the masy Mini Kit (Qiagen, Germany) was utilized based on the recommendations of the manufacturer. Then, mRNA was transcribed reversely utilizing the taqmantm microRNA Reverse Transcription Kit (Applied Biosystems, USA). A real-time premix ex TaqMan kit was used to verify the mRNA expression by RT-PCR. Cycle conditions include primary holding for 2 min at 50 and then holding at 95 °C for 5 min. A two-stage PCR procedure was then conducted containing 40 cycles of 60 °C for 40 s and 95 °C for 12 s. U6 microRNA was utilized as an internal control, and the 2^(-ΔΔCT) technique was utilized for calculating the relative expression.

Every specimen was examined in triplicate, and all the tests were re-performed three times. The primer sequences were as follows:

miR-375-FP, ACAGAGATTTGTTGTTGTCG U6-FP, TCTGGTTGTCGACGACA
U6-RP, AACGCTTCACGAATTGCGT

Targeted scanning technique (http://www.targetscan.org/) database was employed for predicting the target genes as well as 3-utr binding sites of mir-375. According to the database, transfer related JAK2 was identified as a direct target of miR-375.

A 200 bp JAK2 3’UTR fragment was synthesized, which contained the preceding binding sites of hsa-mir-375 and flanking sequences of each side. A second 240 bp fragment comprising the mutation-binding site sequences was also amplified and synthesized. Cloning the amplified product was performed into the pGL3 vector of the firefly luciferase reporter gene, Renilla luciferase reference gene downstream. The two recombinant reporter vectors were named MT (JAK2 mutant) and WT (JAK2 wild type) and confirmed by sequencing. Each vector and 500 ng of pGL3 and 60 nm/L mir-375 mock or mock control were transfected into 293T cells utilizing the transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) based on the instructions of the manufacturer. After 36 hours of incubation, cells were harvested, moreover, a dual-luciferase reporter system (Promega, Madison, Wisconsin) was used to measure luciferase activity in fire-
flies and kidneys. The RNeasy Mini Kit (Qiagen, Germany) was utilized for isolating Total RNA from the cells based on the manufacturer’s protocol. Then, mRNA was reverse transcribed through the Random Hexamine™ first-strand cDNA synthesis kit (genecopoeia, USA). RT-PCR analysis of mRNA expression was conducted utilizing the Real-Time Premixed ex TaqMan Kit (Takara, China). The CT value was standardized to the β-actin value, and the relative expression was determined through the $2^{-\Delta\Delta CT}$ technique. Every specimen was examined three times and all the tests were re-performed three times. Thermocycling conditions included a primary holding stage for two min at 50 °C and
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then at 95 °C for five minutes. Then, a two-stage PCR program was performed including a 95 °C stage for 12 seconds and a 60 °C stage for 40 seconds, for 40 cycles.

The primer and probe sequences included:

JAK2,
forward 5′-GCCTTCTTTCAAGCACCACAT-3′
reverse 5′-GTGTTAGATCCCGGTCTTCAA-3′
MMP-2,
forward 5′-AATCCATGAGGAGAGCAAG-3′
reverse 5′-GAGTGCTCCTTACCGTCAAAG-3′
β-Actin,
forward 5′-TCTGGCAACCGGTGAAGGTGAC-3′
reverse 5′-CACCTCCTCTGTGGACTT-3′.

The cells were taken and lysed with cell lysate 72 hours after transfection. Then, the BCA protein analysis kit (ThermoScientific, USA) was used to measure the protein concentration. The lysate was exposed to SDS-page, conveyed to the PVDF membrane, sealed in 5% skimmed milk (biorad, USA), and incubated with primary and secondary antibodies at 4 °C for one hour at room temperature (RT). The visualization was performed utilizing ECL detection reagents (GE Healthcare, UK). A chemical Doppler imager (Bio-Radar, USA) was used to obtain the exposures. The main utilized antibodies included anti JAK2 (CST, rabbit, 1 : 500), MMP-2 (BioWorld, 1 : 500) and HRP bound anti-β-actin (sigma, 1 : 10000). The secondary used antibodies included HRP anti-mouse (1 : 2000) and HRP anti-rabbit (1 : 2000).

The results

The miR-375 expression in clinical squamous cell carcinoma and adjacent tissues was detected by stem-loop PCR. miR-375 expression was 3.6 ± 0.46-fold lower in cervical cancer tissues than the nearby non-cancerous tissues (p-value of less than 0.01). miR-375 expression in cervical cancer tissues was 3.6 ± 0.46-fold lower than adjacent non-cancerous tissues (p-value of less than 0.01, Figure 1). miR-375 is included in the cervical cancer metastasis [1, 23, 24]. The impacts of miR-375 on SiHa cells invasion and migration from cervical cancer transfected with miR-375 mimics/NC were evaluated using Transwell and wound healing assays, respectively. The Transwell assays revealed that the number of invading SiHa cells was considerably reduced in miR-375 transfected (28 ± 5.4) compared to NC transfectants (93 ± 6.4, p < 0.01) after 24 hours (Figure 2). The wound healing assays revealed that the migration of SiHa cells was considerably inhibited in miR-375 transfectants (18.02 ± 3.8%) compared with NC transfectants (30 ± 3.6%, p < 0.01) after 24 hours (Figure 3). The target scan database was utilized for predicting the target genes of miR-375. A total of 3196 transcripts were deposited, including 328 conserved sites and 3732 low conserved sites. These genes were classified by the KEGG pathway and annotated by GO. The analysis revealed that JAK2 was associated with invasion and metastasis. The authors found that JAK2 has a putative miR-375 binding site (Figure 4).

To confirm the direct targeting and binding between miR-375 and the JAK2 3′-UTR, a double Luciferase report was performed. A pair of vectors was used encoding a partial sequence of the 3′-UTR comprising the putative miR-375.

JAK2 was confirmed as a miR-375 direct target gene by dual-luciferase reporter examine. The authors further conducted qRT-PCR and Western blotting experiments to assess whether miR-375 overexpression in SiHa cells reduced JAK2 expression. The protein expression and mRNA levels of JAK2 were decreased significantly in miR-375-transfected cells in comparison to Mir controls (Figures 6A and 6B).

Discussion

Tumour metastasis contributes to cancer-related death. It consists of different sequential phases, including cancer cell spreading, cancer cell dissemination, and the creation
of secondary tumors in other tissues and organs [26]. Although many studies have attempted to elucidate the molecular mechanisms of metastasis, these mechanisms are imprecise. Reports exist on the aberrant expression of miRNAs associated with cell migration and invasion in several human cancers such as cervical cancer [27-30]. Former investigations revealed the down-regulation of miR-375 expression in cervical cancer, which is related to the advanced lesions and lymph node metastasis of cervical cancer. The current work deals with the contribution of miR-375 in regulating cell invasion and migration in cervical cancer, and the core mechanisms. First, the authors confirmed that miR-375 is downregulated in cervical cancer, moreover, overexpression of miR-375 inhibits invasion, and migration of cervical cancer cells. Furthermore, JAK2 was predicted as a target gene of miR-375 and validated by a dual luciferase reporter assessment. Ultimately, the authors indicated that by overexpressing miR-375 in cervical cancer cells, the expression of JAK2, and its downstream gene, MMP2 was decreased. In total, these data suggest that miR-375 regulates the invasion and migration of cervical cancer cells by targeting JAK2.

It has been reported that the downregulation of miR-375 happens in different kinds of cancers including cervical cancers [30-33]. Wang et al. indicated that miR-375 expression was considerably decreased in cervical tissues relative to normal cervical tissues [1]. Moreover, they found a considerable negative association between miR-375 expression and clinical tumor stage and lymph node metastasis [1]. In line with such reports, the current results proved the miR-375 downregulation in cervical cancer tissues relative to the nearby noncancerous tissues.

The miR-375 expression is aberrant in several cancers related to the clinical tumor stage and lymph node metastasis [1, 23, 32-34]. Li et al. revealed the down-regulation of miR-375 expression in 89% (85/96) of NSCLC specimens compared to matched non-cancerous tissue samples. There was a significant association between miR-375 reduction and advanced disease and lymph node metastasis [30]. Xu et al. indicated that the miR-375 expression in human colorectal cancer tissues was considerably lower compared to the corresponding non-cancerous tissues (NCT). miR-375 up-regulation could inhibit the migration and invasion of colorectal cancer cells in vitro and reduce tumor metastasis. Thus, the present work showed that miR-375 could block the migration as well as invasion of cervical cancer cells.

To elucidate the mechanism of miR-375 regulating invasion and migration of cervical cancer cells, we predicted
JAK2 as the target gene of miR-375. Moreover, JAK2 as the third member of the protein tyrosine kinase family was dysregulated in many cancers [35-37]. Several reports showed that JAK2 was included in the migration and invasion of many cancers via JAK2/STAT3/MMP signaling pathways [38-40]. Aberrant JAK2 expression in gastric, breast, and lung cancer, as well as in colorectal carcinoma, activates the downstream target genes including MMP2, and MMP9 of JAK2/STAT3/MMP signaling pathway to promote carcinogenesis [41-43]. Xu et al. indicated that miR-375 may be included in the invasion and migration of gastric cancer cells by targeting JAK2 [44]. Consistent with these reports, the current work revealed that overexpression of miR-375 in SiHa cells leads to the downregulation of JAK2. Hence, it is indicated that miR-375 prevents the invasion and migration of SiHa cells from cervical cancer by targeting JAK2. In conclusion, these data provide a theoretical and experimental basis for reversing metastasis and developing novel drug candidates for treating cervical cancer.

Ethics approval and consent to participate

Ethical Committee approval was obtained from the Drug Clinical Trial Ethical Committee of Huazhong University of Science and Technology and all subjects signed an informed consent form.

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Conflict of Interest

We declare that we do not have any commercial or associational interest that represents a conflict of interest in connection with the work submitted.

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