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Original Article

Expression of miR-138 and miR-720 in squamous carcinoma of cervix

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Abstract: This study aims to investigate the expression of miR-138 and miR-720 in squamous carcinoma of cervix (SCC). Cervical tissues from 103 cases of Uighur SCC patients and 82 cases of Han SCC patients were collected. And the normal cervical tissues as control were also collected from 40 Uighur individuals and 40 Han individuals. Quantitative RT-PCR was applied to detect the expression of miR-138 and miR-720 in cervical tissues. Western blot was used to detect the expressions of γH2AX and Ezh2 in different cervical tissues. It was found that expressions of miR-138 and miR-720 were significantly down-regulated in cancer tissues of SCC than in normal control tissues. MiR-720 expression was significantly decreased in cancer tissues from Uighur women than in Han women, while there was no significant difference for miR-138 between Uighur women and Han women. It was found that expressions of miR-138 and miR-720 were significantly increased after chemotherapy ($P<0.05$). H2AX protein was significantly decreased in cervical tissues after chemotherapy compared with before chemotherapy, while Ezh2 protein expression was not changed. In summary, expressions of miR-138 and miR-720 were decreased in SCC cancer tissues and were increased after chemotherapy. MiR-720 expression was significantly lower in cancer tissues from Uighur women.

Keywords: MiR-138, miR-720, γH2AX, Ezh2, squamous carcinoma of cervix

Introduction

Expressions of miRNAs are closely related to the development and progression of tumor, and abnormally expressed miRNAs play important roles in regulating the processes of tumorigenesis [1]. Cervical cancer is one of the most common malignancies among women, and several miRNAs have been reported to regulate biological behavior of tumor cells through regulating associated target genes. For example, miR-205, miR-1, miR-2, and miR-4 were significantly up-regulated in cervical cancer tissues [2, 3]. Actually, many miRNAs were found to be associated to the development and progression of cervical cancer [2, 3].

In our previous study, we applied miRNA microarray 4.0 to screen the differentially expressed miRNAs in cervical cancer tissue [4]. MiR-138 and miR-720 were identified to be differentially expressed between Uighur SCC patients and Han SCC patients. For further investigating the roles of miR-138 and miR-720, qRT-PCR was used to validate the differential expression in cervical cancer tissues of Uighur and Han SCC patients. The change of expression before and after therapy was also compared. Western blot was used to detect expressions of H2AX and Ezh2.

Materials and methods

Sample collection

This study enrolled 185 cases of SCC patients, including 103 Uighur women and 82 Han women. SCC cancer tissues were collected from these 185 patients. Among Uighur SCC patients, there were 18 cases in Ib stage, 33 cases in IIa stage, 29 cases in IIb stage, and 23 cases in III-IV stage. And in Han SCC patients, it

Table 1. The primer sequences for qRT-PCR

Gene	Primer Sequence 5'→3'
miRNA Uni-primer	GTGCAGGGTCCGAGGT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
miR-720	UCUCGCUGGGGCCUCCA
miR-720 (Reverse Transcription)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACtggagg
miR-720 (qRT-PCR)	GCTCTCGCTGGGGCCT
miR-138	AGCUGGUGUUGUGAAUCAGGCCG
miR-138 (Reverse Transcription)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcggcct
miR-138 (qRT-PCR)	GCGAGCTGGTGTTGTGAATCA

included 32 cases in Ib stage, 33 cases in IIa stage, 10 cases in IIb stage, and 7 cases in III-IV stage. Tissues from 36 patients among 185 were collected before and after new adjuvant chemotherapy respectively, among which 1 patient with well differentiation, 30 patients with intermediate differentiation, and 5 patients with poor differentiation were included. The chemotherapy regimens were paclitaxel plus platinum, and 28 cases were response while 8 cases were not response to the chemotherapy. We also collected normal cervical tissues from 40 Uighur women and 40 Han women with benign diseases including patients without cervical lesions (benign tumors in uterine, ovary, and oviduct) and patients with cervical lesions (Cervical intraepithelial neoplasia, cervical myoma, and the cervical papilloma), and hysterectomy was used for all patients. All the cervical cancer patients were confirmed to be cervical squamous cell carcinoma by biopsy pathology. The criteria of diagnosis and staging were based on FIGO, and the diagnosis was double checked by 2 physicians in gynecological examination. All patients had no other serious medical illness and cancer. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Xinjiang Medical University.

The cancer tissues were determined by eye, and 0.5 cm^2 tissue was cut and its peripheral part was used for pathological examination. After washing with sterile saline and drying, tissues were stored in liquid nitrogen, and then transferred to -80°C refrigerator. The remaining specimens were sent to department of pathology for routine paraffin test.

QRT-PCR

Total RNA was extracted by Trizol (Invitrogen, California, USA) method, and RNA quality was checked by gel electrophoresis and the ratio of 260/280 by Spectrophotometer. The cDNAs of mRNA were reversed transcribed from total RNA, while miRNA cDNA was reversely transcribed by TaKaRa Prime Script® RT reagent Kit (TaKaRa, Dalian, China). The reaction system was 10 μl system including: 2 μl of 5xPrimeScript® Buffer, 0.5 μl of PrimeScript® RT Enzyme Mix I, 0.5 μl of specific primer (2 μM), 1 μl of total RNA and 6 μl of RNase Free dH_2O. The relative expressions of miR-138 and miR-720 were detected by TaKaRa SYBR® Premix Ex TaqTM Perfect Real Time Kit (TaKaRa, Dalian, China). The U6 was used as internal reference. The primers were shown in **Table 1**. The reaction system was 20 ul, including: SYBR® Premix Ex Taq (2x) 10 μl , miRNA reverse primers (10 uM/ul) 0.4 μl uni-primer (10 uM/ul) 0.4 μl, Rox Reference Dye II (50x) 0.4 μl, cDNA template 2 μl, ddH_2O 6.8 μl. The cycle conditions were the following: 95°C for 5 min, and followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. The relative expression was calculated by the $2^{-\Delta\Delta T}$ method of miRNA/U6.

Western blot

Total proteins were extracted based on standard protocol by protein lysis, and the protein concentration was detected by BCA assay kit. After boiling with loading buffer for 5 min, 20 ug proteins was loaded into 10% SDS-PAGE and then transferred to PVDF membrane under ice bath (constant voltage 100 V for 2 h). After blocking by 5% slim milk, the primary antibody was added. The primary antibody was rabbit anti-human polyclone H2AX and Ezh2 (1:1000,

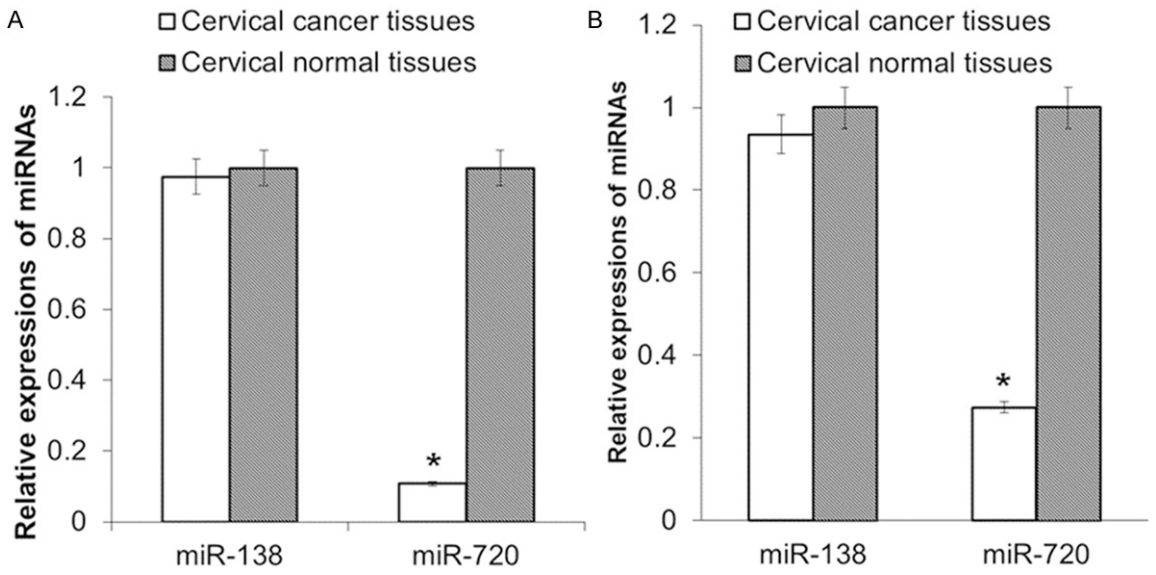

Figure 1. Expressions of miR-138 and miR-720 in SCC. A. Expressions of miR-138 and miR-720 in cancer tissues and normal tissues in Uighur patients. MiR-720 expression was significantly decreased in cervical cancer tissues in Uighur patients ($P<0.01$). B. Expressions of miR-138 and miR-720 in cancer tissues and normal tissues in Han patients. MiR-720 expression was significantly decreased in cervical cancer tissues in Han patients ($P<0.05$).

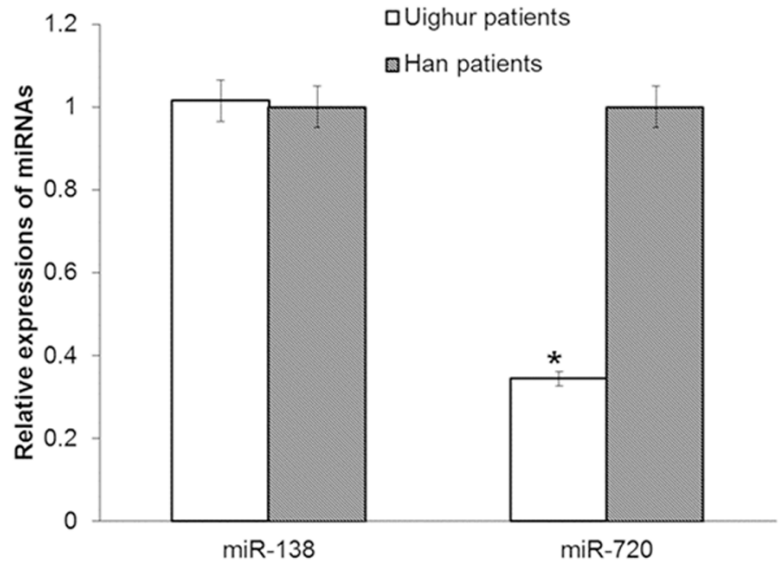

Figure 2. Expressions of miR-138 and miR-720 between Uighur and Han patients with SCC. MiR-720 expression was significantly decreased in cervical cancer tissues in Uighur patients than in Han patients ($P<0.05$).

Cell Signaling Company, Boston, USA) and rabbit anti-human β-actin antibody (1:1000, Cell Signaling Company, Boston, USA). The second antibody was HRP-conjugated goat anti-rabbit IgG (1:10000, ZSGB-Bio ORIGENE, Beijing, China). Finally, the membrane was developed by enhanced chemiluminescence reagent. The developed film was scanned and analyzed by image lab 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as an internal control to calculate the relative expression of H2AX and Ezh2.

Evaluation criteria of clinical efficacy

The Solid Tumor Evaluation Criteria (RECIST) revised in 1998 was used to estimate the response efficacy at 2 weeks after first chemotherapy. Complete remission (CR): disappearance of the tumor and no new lesions were occurred; Partial remission (PR): tumor volume reduced more than 50% and no new lesions were occurred; Stable Disease (SD): tumor volume decreased not more than 50%; Progression Disease (PD): no reduction of tumor volume or even new lesions occurred.

Statistical analysis

The SPSS 16.0 software was used to do statistical analysis. For SCC patients before and after

Table 2. Expressions of miR-138 and miR-720 in normal tissues between Uighur patients and Han patients

Target gene	ΔCt_{Uighur} (n=40)	ΔCt_{Han} (n=40)	*t* Value	*P* Value
miR-138	13.1795±2.3160	13.9070±2.0878	-1.476	0.144
miR-720	2.6137±2.3905	2.1513±1.9875	0.903	0.370

chemotherapy, paired t test was used, and two independent sample t-test was used to compare normal tissues and cancer tissues. One-way ANOVA was used to compare difference among multiple groups. All data were shown in mean ± SD, and followed normal distribution. P<0.05 was considered as statistically significant.

Results

Expressions of miR-138 and miR-720 in cervical cancer tissues

To detect the expression changes between SCC patients and benign patients, we applied qRT-PCR to detect the expressions of miR-138 and miR-720 in cervical cancer tissues and normal tissues. Compared with normal tissues, miR-138 expression was not significantly increased in Uighur patients with SCC (P>0.05, as shown in **Figure 1A**). And miR-720 expression was significantly different between Uighur SCC patients and Uighur normal women (P<0.05, as shown in **Figure 1A**). The results indicate that miR-720 may play regulatory roles in pathological processes of SCC.

We also compared the difference of miR-138 expression and miR-720 expression between SCC cancer tissues and normal tissues in Han women. As shown in **Figure 1B**, miR-720 expression was significantly different between cancer tissues and normal tissues (P<0.05), while miR-138 expression had no significant difference.

Comparison of miR-138 and miR-720 expression between Uighur patients and Han patients

To check the expressions of miR-138 and miR-720 between Uighur patients and Han patients, qRT-PCR was performed. Among SCC patients, Hysterectomy and pelvic lymph node dissection was used, and 58 cases were Uighur women and 55 cases were Han women. There was no significant difference in age and other clinical information between Uighur patients and Han patients (P>0.05). MiR-720 expression was significantly different between cancer tissues of Uighur patients and cancer tissues of Han patients (P<0.05), while miR-138 expression had no significant difference (as shown in **Figure 2**). In the normal cervical tissues, the expressions of miR-138 and miR-720 had no significant difference between Uighur patients and Han patients (as shown in **Table 2**). The results indicate that miR-720 might play different roles in the development of SCC between Uighur and Han patients.

Expressions of miR-138 and miR-720 before and after chemotherapy

To investigate the roles of miR-138 and miR-720 in response with chemotherapy in SCC patients, we detected the expressions of miR-138 and miR-720 in cervical cancer tissues before and after chemotherapy. According to the evaluation criteria for solid tumor by WHO, total 36 SCC patients were divided into response group and non-response after neoadjuvant chemotherapy. In 28 cases of response group, miR-138 expression was significantly increased after chemotherapy (P<0.01). For miR-720, compared with expression in tissues before chemotherapy, the expression was also significantly increased after chemotherapy (P<0.05). The details were shown in **Table 3**. In the 8 non-response group, the expressions of miR-138 and miR-720 were not significantly different after chemotherapy when compared with before chemotherapy.

Expressions of γH2AX and Ezh2 in cancer tissues with adjuvant chemotherapy

To determine whether γH2AX and Ezh2 that were targeted by miR-138 and miR-720, we detected the expressions in 31 paired patients from 36 cases of patients with adjuvant chemotherapy by Western blot. As shown in **Figure 3**, in response group, γH2AX expression was significantly down-regulated after chemotherapy compared with before chemotherapy (P<0.01), while Ezh2 had no significant difference before and after chemotherapy. In non-response group, either γH2AX expression or Ezh2 expression had no significant different before and after chemotherapy (as shown in **Figure 3**). The results indicate that γH2AX expression might play roles in response process of adjuvant chemotherapy.

Table 3. Expressions of miR-138 and miR-720 in cancer tissues before and after neo-adjuvant chemotherapy

	Before chemotherapy (n=28)	After chemotherapy (n=28)	$\Delta\Delta Ct_{(Before - After)}$	$2^{-\Delta\Delta Ct}{}_{(Average)}$	t Value	*P* value
mir-138-ΔCT	13.4330±2.0533	12.5529±1.5445	1.0383±1.2594	0.7306	3.378	0.002
mir-720-ΔCT	3.3323±1.3287	2.7312±1.2257	0.5814±1.1567	0.8638	2.768	0.01

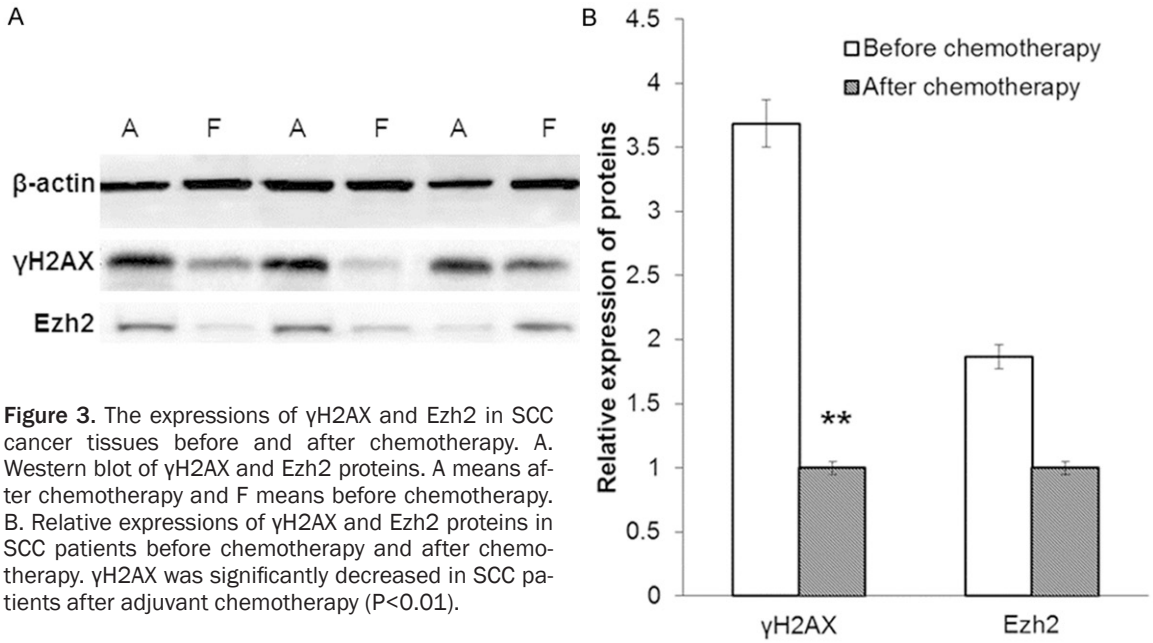

Figure 3. The expressions of γH2AX and Ezh2 in SCC cancer tissues before and after chemotherapy. A. Western blot of γH2AX and Ezh2 proteins. A means after chemotherapy and F means before chemotherapy. B. Relative expressions of γH2AX and Ezh2 proteins in SCC patients before chemotherapy and after chemotherapy. γH2AX was significantly decreased in SCC patients after adjuvant chemotherapy (P<0.01).

Correlation between expressions of miR-138 and miR-720 with expressions of γH2AX and Ezh2

To further investigate the regulatory relationship between miR-138 and miR-720 with γH2AX and Ezh2, linear regression analysis was performed. It was shown that miR-138 expression was not correlated to its target gene γH2AX and miR-720 was not correlated to γH2AX expression. For Ezh2, miR-138 and miR-720 can regulate its expression through directly target it. It was found that miR-138 expression was positively correlated to Ezh2 expression (r=0.3148, P=0.0127) and miR-720 expression was also positively correlated to Ezh2 expression (r=0.2822, P=0.0263). The results indicate that miR-138 and miR-720 might participate in the pathological processes of SCC through regulating Ezh2.

Discussion

MiRNA is a class of endogenous, highly conserved non-coding single-stranded small molecular RNAs, which is widely expressed in animals, plants, virus and other multicellular eukaryotes [5]. Many studies showed that miRNA was better as biomarkers in diagnosis, which had higher sensitivity and specificity than other types of biomarkers [6, 7]. Kurashige et al. found that miR-21 could be used as diagnosis biomarker in esophageal squamous carcinoma [8]. Li et al. reported that miR-21, miR-218, and miR-223 were expressed in early stage of gastric cancer [9]. Taylor et al. found that 8 miRNAs were highly expressed in serum of ovary cancer in different stages, and the abnormal expression was correlated with the staging [10]. Cervical cancer is one of the most common cancers in women. Many miRNAs were reported to be expressed in cervical cancer [2, 3]. In this study, we found that miR-720 expression was down-regulated in cervical cancer tissues compared with normal tissues, and miR-720 expression was decreased in Uighur patients than Han patients, which indicates the potential of miR-720 as a diagnosis biomarker in SCC.

In our previous study, we found that miR-138 and miR-720 were differentially expressed in Uighur SCC patients [11]. MiR-138 was down-regulated in several cancers, which was regarded as tumor suppressor gene to play roles in cancers [12-14]. It was found that miR-138 inhibited the epithelial-mesenchymal transition in nasopharyngeal squamous cell carcinoma, and down-regulated miR-138 could promote cell migration and invasion as a multifunctional regulator [15]. In glioma stem cells (GSC), highly expressed miR-138 was related to the recurrence and survival, and miR-138 inhibited tumorigenesis in vivo and inhibited the formation of nerve ball of GSCs [16]. And it was also found that induced pluripotent stem (IPS) cells by miR-138 and four factors (Oct4, Sox2, Klf4, and c-Myc) was similar to embryonic stem cells about the pluripotency [6]. In vestibular schwannomas, miR-720, miR-21, miR-221, and miR-431 were up-regulated, which were all located in chromosome 14q32 [7]. In esophagus cancer, it was found that SnoN/SKIL controlled the proliferation of cancer cells through down-regulating miR-720 expression. Although miR-720 was found to be increased in other cancer tissues, it was down-regulated in cancer tissues of Uighur SCC patients.

Chemotherapy is an indispensable method in the treatment of cervical cancer. In this study, we showed that miR-138 and miR-720 were significantly up-regulated after chemotherapy in response group, while there was no significant difference in non-response group, which indicated that miR-138 and miR-720 may become novel biomarkers to predict the chemotherapy efficacy in SCC.

MiRNAs regulate biological and pathological processes in tumorigenesis through directly binding to 3'UTR of target mRNAs. Through reviewing publication, bioinformatics prediction (TargetScan, miRanda, and PicTar), and dual luciferase assay, it was validated that H2AX was target gene of miR-138 and Ezh2 was target of miR-138 and miR-720. It was found that miR-138 induced the chromosome instability after DNA damage through down-regulating H2AX [17, 18]. The mechanisms for most chemotherapy drugs are to induce DNA damage, and H2AX prevents DNA repairing by inhibiting upstream kinases to phosphorylate H2AX, [19, 20]. Parikh et al. found that the drug sensitivity was significantly increased in head and neck cancer cells when H2AX gene was deleted [21]. In this study, miR-138 and miR-720 was significantly increased after chemotherapy, while γH2AX was significantly down-regulated after chemotherapy, which indicated that miR-138 and miR-720 may play roles through regulating γH2AX expression. EZH2 locates in chromosome 7q35 [22], which regulates cell proliferation through methylating lysine in target gene to inhibit transcription [23]. In gastric cancer, cervical cancer and other cancers, Ezh2 was highly expressed, which was an important regulator to influence prognosis [24-26]. It is reported that E7 protein induced by high-risk HPV infection can activate Ezh2 gene to inhibit tumor cell apoptosis, and to promote tumor proliferation [27]. After inhibiting Ezh2 expression, the malignancy degree of tumor was significantly inhibited [28]. In this study, Ezh2 protein expression was not correlated with miR-138 expression and miR-720 expression before and after chemotherapy. But it was positively correlated to the expressions of miRNAs, which indicated that Ezh2 might be the predictors for miRNA-138 and miR-720.

In summary, we found that miR-720 expression was significantly different in SCC cancer tissue between Uighur patients and Han patients, which might be used as diagnosis biomarker for SCC. γH2AX and Ezh2 may be used to predict the sensitivity of chemotherapy. Our findings may provide basis for designing personalized treatment strategy on cervical cancer.

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Disclosure of conflict of interest

None.

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