miRNA Expression Profiles of HPV-Infected Patients with Cervical Cancer in the Uyghur Population in China

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Abstract

The study aimed to investigate the state of human papillomavirus (HPV) infection in patients with cervical cancer in the Uyghur population in China and to identify miRNA as biomarker for cervical cancer and HPV infection. We also performed genotyping to determine the variation in the types of HPV. Using microRNA (miRNA) microarray technology, differential miRNA expression between HPV-infected cervical cancer and uninfected normal cervical tissues was determined; the microarray results were verified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using 20 samples of both the tissues. The infection rate of HPV in patients with cervical cancer was 96.7% (29 of 30), and the main subtype identified was HPV16 (29 of 29). HPV16 integration assay demonstrated that the majority of infectious cases were of the integrated form (26 of 29). Analysis of 140 miRNAs demonstrated greater than two-fold change in miRNA expression in HPV-infected cervical cancer tissue as compared to that in uninfected cervical tissue. The qRT-PCR analysis verified that the expression of miR-15a-5p, miR-17-5p, miR-20a-5p, miR-21-5p, miR-96, miR-106b-5p, and miR-3653 was higher, while the expression of miR-497-5p was lower in cancer tissues than in normal tissues. The results demonstrate significant changes in miRNA expression in cervical cancer tissues associated with HPV infection as compared to that in normal tissues. These molecular markers may be useful for an early diagnosis and prognosis of cervical cancer in specific human populations.

Introduction

Cervical cancer is prevalent worldwide and is often fatal [1]. Although the factors leading to its occurrence are not fully understood, human papillomavirus (HPV) infection, as a causative agent of cervical cancer, has been well documented [2–4]. There are several subtypes of HPV, and those that cause cancers are generally referred as high-risk HPV (hrHPV). There are more than 20 common subtypes of hrHPV, with HPV16 being the most prevalent [5]. A previous study has shown a high degree of association between the carcinogenicity of hrHPV and its
gene fragments E6 and E7 [6]. Protein translation from E6 and E7 is continuous throughout their integration into the host genome, which promotes cell proliferation and metastasis, leading to tumor progression [7, 8]. Disruption of E2 is an important event leading to the development of cervical lesions [9]. A previous study has indicated that smaller the ratio of E2/E6, higher the number of malignant cervical lesions [10].

The Uyghur population is one of the largest minority populations in China, particularly in the Xinjiang Province. The infection rate of hrHPV in the Uyghur population is below the average rate in China [11]; however, the incidence and mortality rates of cervical cancer in this population are very high [12]. Uyghur population maintains a traditional lifestyle—women tend to marry earlier, have more children, and have poor health conditions. These reasons may result in a difference in the occurrence of cervical cancer in the Uyghur population in China. Therefore, addressing how cervical cancer develops in this population is of significant importance. Conventional treatments for cervical cancer lead to a poor prognosis due to a gradual increase in resistance to chemotherapy [13]. In recent years, the discovery of molecular biomarkers and the development of targeted therapy has improved the prognosis and quality of life of patients with cervical cancer [14]. Therefore, identifying molecular targets of cervical cancer in the Uyghur population has become a primary task, necessary for improving early diagnosis and prognosis of cervical cancer probably for the development of targeted therapy.

MicroRNAs (miRNAs) are a class of RNA molecules that play important roles in maintaining normal cellular function by regulating mRNA transcription. They are, generally, 18–25 nucleotides long, and can be found in various eukaryotes [15]. miRNAs play vital regulatory roles in cell growth, apoptosis, cell migration, and metastasis [16]. Moreover, they are also important in the development of cervical cancer. A previous miRNA microarray study demonstrated aberrant changes in miRNA expression in the cervical cancer tissues as compared to those in the normal tissues [17]. However, no study has investigated the changes in miRNA expression in the Uyghur population with HPV-infected cervical cancer as compared to that in the uninfected normal cervical tissue. Using miRNA microarrays, in this study, we evaluated the differences in miRNA expression in patients with HPV-infected cervical cancer and with uninfected normal cervical tissues within the Uyghur population. In addition, we validated our results using clinical samples, which allowed identification of miRNA with aberrant expression specific to the Uyghur population.

Methods

Patient and tissue samples

Cervical cancer tissues and uninfected normal cervical tissues were obtained from the Obstetrics and Gynecology Department of Xinjiang Medical University Cancer Hospital. All tissues were obtained between June 2012 and June 2013 as fresh specimens from surgical resection, and stored at -80°C until use. There were 30 cervical cancer tissues and 26 normal cervical tissues. Thirty cervical tissues were used to detect HPV infection—six cervical cancer tissues and six normal cervical tissues were used in miRNA microarray, and twenty cervical cancer tissues and twenty normal cervical tissues were used for validation of qPCR. The number of cases of cancer tissues from stage I to III is 5, 21, and 4, respectively. All patients belonged to the Uyghur population of Xinjiang Province, China. All cervical specimens were confirmed as squamous cell carcinoma through pathological examination. Prior to tissue sampling, patients had not received any chemotherapy treatment. Samples from the control group were determined to be normal by ThinPrep cytologic tests. This study was approved by the Ethics Committee of Xinjiang Medical University Cancer Hospital (K-201343), and an informed consent was obtained from all the patients.
Detection of HPV infection

Total DNA was isolated from 30 cervical cancer tissues using the TIANamp Genomic DNA kit (Tiangen Biotech Co. Ltd, Beijing) according to the manufacturer’s instructions, and DNA concentrations were determined using NanoDrop 2000 spectrophotometer (Thermo Fisher scientific). PCR was performed on a gradient PCR instrument (Bio-Rad, USA), and the DNA polymerase used was Taq polymerase (Thermo Fisher Scientific). All PCR primers used are listed in Table 1. Detection of HPV infection was performed by applying MY09 and MY11 primers in the first round of PCR, and the PCR product was then diluted 1:100 before adding GP5+ and GP6 + primers to perform the second round of PCR [18]. For detection of HPV16 subtypes, GP-E6-3F and GP-E6-5R primers were used in the first round of PCR, and the PCR product was diluted 1:100 before adding HPV16Fz and HPV16Rz primers to perform the second round of PCR. The final product was electrophoresed on 1% agarose gel. For qPCR based on amplified fluorescence of multiple Taqman probes, the pCR-XL-TOPO-HPV16 plasmid (State Key Laboratory of Biological Resources and Genetic Engineering, School of Life Sciences and Technology, Xinjiang University) was used to construct a standard curve and was regarded as the positive control for HPV16 infection. Based on the amplification results of the standard HPV16 plasmid, HPV infection of each sample was determined to be either of an episomal or of an integrated form.

miRNA microarray analysis

Six cervical cancer tissues infected with HPV16 along with six normal cervical tissues were randomly selected. Total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the

Table 1. Details of all primers used in the study.

| Primer name   | Sequence (5’ to 3’)                     | Number of bases | Length of PCR product (bp) |
|---------------|----------------------------------------|-----------------|---------------------------|
| MY09          | CGTCCMARRGGAWACTGATC                   | 20              | 450                       |
| MY11          | GCCAGGGWXATAAYAATGG                   | 20              | 450                       |
| GP5+          | TTTGTACTGTTAGATACTAC                  | 23              | 150                       |
| GP6+          | GAAAAATAAAGCTGAAAATCATTTCC            | 25              | 150                       |
| GP-E6-3F      | GGGCGKAACCTGAAATCGGT                  | 19              | 609                       |
| GP-E6-5R      | CTAAGGTGTCATTAATGGTCTCA               | 23              | 609                       |
| HPV16Fz       | CACAGTTATGAGAGCATGC                   | 21              | 457                       |
| HPV16Rz       | CATATATTGATGCAATGTAGGTGTGA            | 25              | 457                       |
| HPV16 E2-F    | GAAACACAGACATATCCTA                   | 20              | 609                       |
| HPV16 E2-R    | TCCGTCCTTTGTTGGACGTGT                 | 21              | 609                       |
| HPV16 E2 TaqMan probe | HEX-CACAGATCAGACAGACACAC-Eclipse | 20          | 609                       |
| HPV16 E6-F    | GAAATGTGTGACTGCAAGCA                 | 20              | 609                       |
| HPV16 E6-R    | GTTGATATGGCTGGCTCTTAATGTGGA          | 24              | 609                       |
| HPV16 E6 TaqMan probe | FAM-CACATATGGATCCCATCTC-Eclipse   | 23              | 609                       |
| hsa-miR-106b-5p-F | UAAAGUGUCGACAGUCGAGAU               | 21              | 609                       |
| hsa-miR-106b-5p-R  | GCCAGAAAGTGCAGCAGCTA                | 22              | 609                       |
| hsa-miR-3188-F  | AGAGGCCUUUGCGGAUACGGG               | 23              | 609                       |
| hsa-miR-3188-R  | GCCAGAGACTTTGTTGCGGTTAC             | 24              | 609                       |
| hsa-miR-3653-F  | UAAAGAGUUGUAGAGAAG                  | 23              | 253                       |
| hsa-miR-3653-R  | GCCAGCCTAAGGACTGCTAGA               | 24              | 253                       |
| hsa-miR-497-5p-F  | CGACAGCAGCAGCAGCGG                 | 21              | 253                       |
| hsa-miR-497-5p-R  | CGACAGCAGCAGCAGCGTGGT              | 21              | 253                       |
| hsa-miR-590-5p-F  | GACGCUAUAUAUAAUGUUC                 | 22              | 253                       |
| hsa-miR-590-5p-R  | CCCGACCTTATTCATAAAAGTGCAG         | 25              | 253                       |

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manufacturer’s instructions and total RNA was treated with DNase. RNA concentrations were
determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA quality
and integrity were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA).
All RNA samples used for miRNA microarrays exhibited a RIN (RNA integrity number)
of ≥ 6.0, and ratio of 28S/18S ≥ 1.7. Subsequently, reverse transcription (RT) was performed
on these samples using 1.0 μg of total RNA to generate cDNA using the miScript II RT kit
(QIAGEN, Germany).

Labeling and hybridization experiments were conducted by Urumqi OE Biotech Company,
according to the protocol of Agilent human 8×60K miRNA microarray system. Microarrays
were scanned using the Agilent Scan Control software, and all analyses were performed using
the Agilent Feature Extraction software (version 10.7.1.1, Agilent Technologies). Raw data
were normalized using the Quantile algorithm and Gene Spring Software (Agilent Technolo-
gies, USA).

Quantitative real time polymerase chain reaction analysis (qRT-PCR)
Cervical cancer tissues infected with HPV16 and normal cervical tissues were randomly
selected (n = 20/group), and total RNA was isolated using the TRIzol reagent (Invitrogen,
USA), according to the manufacturer’s instructions. Total RNA was treated with DNase and
RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo
Fisher scientific). Total RNA (1 μg) was converted into cDNA using the miScript II RT kit
(QIAGEN, Germany). qPCR was performed using SYBR Select Master Mix reagent (Invitro-
gen) on an ABI Prism 7500 detection system (Invitrogen). The PCR conditions were as follows:
denaturation at 95°C for 15 sec, followed by annealing and extension at 60°C for 1 min. The
primers for miR-218-5p, miR-17-5p, miR-96, miR-15a-5p, miR-20a-5p, and miR-21-5p were
obtained from Tiangen Biotech Co. Ltd (Beijing, China). The other primers are listed in
Table 1. miRNA expression level was calculated using the 2^−ΔΔCt method, the internal control
used was U6.

Statistical analysis
Differential miRNA expression was based on the following three conditions: (1) miRNA was
expressed in at least 75% of the samples; (2) the difference in the miRNA expression between
the two groups was equal to or greater than two-fold change; and (3) the Student’s t-test
between the two groups yielded a p-value of < 0.05. All microarray assays were expressed as
log2 of microarray signals, and a multiple correction test was applied to microarray data.

The results of the qRT-PCR assay were analyzed using the SPSS 17.0 software (IBM, USA).
All experiments were repeated thrice. Data were expressed as mean ± standard deviation. Sta-
tistical differences between the two groups were determined by Student’s t-test. Differential
expression was considered statistical significant at a p-value < 0.05 between the two groups.

Results
Patient data
The samples used for miRNA microarrays were from six cervical cancer tissues infected with
HPV16 and six uninfected normal cervical tissues. The average age of the patients in the cervi-
cal cancer group was 58 years 7 months (range: 53–64 years), and the average age of those in
the control group was 40 years 7 months (range: 28–45 years). There was no significant differ-
ence in the age between these two groups (p > 0.05). For qRT-PCR experiments, 20 cervical
cancer tissues infected with HPV16 and 20 uninfected normal cervical tissues were analyzed.
Fig 1. (A) Identification of cervical cancer tissues infected with HPV; (B) Identification of HPV genotype. The target bands are located at 150 bp and 457 bp.

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Table 2. The status of HPV16 infection of 29 patients with cervical cancer.

| case | expression of E2 | expression of E6 | Ratio(E2/E6) | status     |
|------|-----------------|-----------------|--------------|------------|
| T1   | 196.5367652     | 640.0229518     | 0.307077683  | integrated |
| T2   | 254075.384      | 332267.4344     | 0.764671339  | integrated |
| T4   | 4143353.075     | 9174234.224     | 0.45162931   | integrated |
| T5   | 229864.7201     | 971148.8077     | 0.236693613  | integrated |
| T6   | 38685.87163     | 121544.2711     | 0.318286261  | integrated |
| T7   | 12768525.01     | 21444957.29     | 0.595409207  | integrated |
| T8   | 2592824.161     | 529007.961      | 0.490129484  | integrated |
| T9   | 27341.7162      | 580030.2212     | 0.047138434  | integrated |
| T10  | 1016.361072     | 2259.709895     | 0.449775024  | integrated |
| T11  | 325937.6378     | 1238904.809     | 0.263085296  | integrated |
| T12  | 270.5257598     | 22545.03815     | 0.011999348  | integrated |
| T13  | 464698.7518     | 328704.5304     | 1.413727858  | integrated |
| T14  | 2517.288164     | 388929.2755     | 0.006472355  | integrated |
| T15  | 60.9897222      | 34909.30275     | 0.001747093  | integrated |
| T16  | 2086187.558     | 1669490.81      | 0.124959551  | integrated |
| T17  | 875.2390537     | 112046.0591     | 0.007811422  | integrated |
| T18  | 1275.454153     | 5681.330491     | 0.2244992    | integrated |
| T19  | 9012165.704     | 14284546.84     | 0.630903157  | integrated |
| T20  | 668.2854947     | 2289.563661     | 0.291883343  | integrated |
| T21  | 28904.79162     | 145312.2892     | 0.198914984  | integrated |
| T22  | 97.48535055     | 7198.336394     | 0.013542761  | integrated |
| T23  | 1330101.242     | 3827263.998     | 0.347533184  | integrated |
| T24  | 6095.375778     | 37592284.47     | 0.000162144  | integrated |
| T25  | 57.54174018     | 2937.976581     | 0.0195855    | integrated |
| T26  | 136.464152      | 21049.88812     | 0.006482905  | integrated |
| T27  | 694.3840603     | 46472.76647     | 0.014941741  | integrated |
| T28  | 1890.88282      | 19456.45244     | 0.097185357  | integrated |
| T29  | 171146.3166     | 97057.2826      | 1.763353677  | dissociated |
| T30  | 87743.32766     | 46407.93468     | 1.890696672  | dissociated |

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The average age of the patients in the cervical cancer group was 47 years 7 months (range: 41–62 years), and the average age of those in the control group was 44 years 5 months (range: 25–69 years). There was no statistically significant difference in the age between these two groups (p > 0.05). All the samples were obtained from women belonging to the Uyghur population.
High detection rate of HPV16 in cervical cancer tissues from the Uyghur population

We initially examined the infection state of HPV in the cervical cancer tissues of 30 patients. The DNA electrophoresis results showed that 29 of the 30 patients had HPV infection, with an infection rate of 96.67% (Fig 1A). Subsequent HPV genotyping revealed that 29 cases were caused by the HPV16 subtype. These results demonstrate the high HPV infection rate in patients with cervical cancer within the Uyghur population in Xinjiang Province (Fig 1B). Among the 29 cases of HPV16 infection, the expression of $E6$ was higher than that of $E2$ in 26 cases (Table 2), which suggests that these infections were of the integrated form.

miRNA microarray analysis of HPV-infected cervical cancer tissues and uninfected normal cervical tissues

Of 2028 miRNAs, 140 demonstrated at least 2-fold change in expression in cancer tissues infected with HPV16 as compared to those in normal cervical tissues. Among these, the expression of 64 miRNAs was higher and 76 miRNAs was lower in cancer cells (Fig 2).

Validation of differentially expressed genes by qRT-PCR analysis

qRT-PCR analysis was performed on 11 randomly selected miRNAs that were differentially expressed based on the microarray evaluation of 20 HPV-infected cervical cancer and 20 uninfected normal cervical tissues. These miRNAs included miR-106b-5p, miR3653, miR-3188, miR-497-5p, miR-218-5p, miR-17-5p, miR-96, miR-15a-5p, miR-20a-5p, miR-21-5p, and miR-590-5p (Table 3). The qRT-PCR results demonstrated that miR-106b-5p ($p = 0.000$), miR-3653 ($p = 0.000$), miR-17-5p ($p = 0.000$), miR-96 ($p = 0.000$), miR-15a-5p ($p = 0.000$), miR-20a-5p ($p = 0.000$), and miR-21-5p ($p = 0.000$) were highly expressed in cancer tissues, while miR-497-5p ($p = 0.016$) was expressed at very low levels. However, the expression changes in miR-3188 ($p = 0.975$), miR-218-5p ($p = 0.213$), and miR-590-5p ($p = 0.193$) were not statistical significant between the two groups. Among the 11 miRNAs, the expression of 8 miRNAs matched the microarray results, thus yielding a consistency rate of 72.7% (Fig 3).

Discussion

Using miRNA microarray followed by qRT-PCR, we identified miRNAs related to the development of HPV-associated cervical cancer in women belonging to the Uyghur population.
Fig 3. Relative quantification of differentially expressed miRNAs in 20 cervical cancer and 20 normal cervical tissues. qPCR analysis was performed in three separate experiments (*p < 0.05; **p < 0.01).

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Through interaction with the 3′UTR region of mRNA, miRNA inhibits mRNA function at the transcriptional level. Here, we identified eight types of miRNAs, namely, the highly expressed miR-15a-5p, miR-17-5p, miR-20a-5p, miR-21-5p, miR-96, miR-106b-5p, and miR-3653 as well as the poorly expressed miR-497-5p. The aberrant expression of these miRNAs could have a role in the development of cervical cancer.

Some microarray studies have reported an abnormal expression of miRNA in cervical cancer. A total of 18 miRNAs were screened in the pre-invasive and invasive stages [19]. In another study, 9 miRNAs, including miR-218 and miR-21, were validated by qPCR between different stages of cervical neoplasm [20]. These studies indicated that miRNA played an important role in the progress of cervical cancer.

Among the identified miRNAs, the differential expression of miR-96 was the most pronounced (FC = 26.31), and its overexpression has been observed in several cancers [21, 22]. Furthermore, it has been reported that the expression of miR-96 is significantly increased during cervical cancer metastasis [23], suggesting its possible role in the metastatic process.

HPV DNA often integrates into the host genome after infection. A recent study demonstrated that persistent HPV infection and disease progression harbored the integrated form of HPV16 DNA rather than the episomal form [24]. E6/E7 are responsible for cancer development. The expression of miRNAs, including those regulated by miR-15a-5p, could be upregulated via the interaction of E6 with c-MYC [25, 26]. Moreover, E6 can degrade p53 [27, 28], thus upregulating the expression of miR-17-5p, miR-20a-5p, and miR-106b-5p [29]. E7 can mediate the degradation of pRB [30], which reduces the pRB-E2F1 complex and increases the expression of the transcription factor E2F1, resulting in an increase in miRNAs, including miR-106b-5p [31]. miR-21 expression can also be affected by E6/E7, as these viral proteins can upregulate the expression of the transcriptional activator of miR-21, p65 NF-κB [32, 33]. These findings imply that miR-15a-5p, miR-17-5p, miR-20a-5p, miR-21-5p, and miR-106b-5p are molecular targets of HPV in vivo.

In addition, miR-497 can inhibit the proliferation of cervical cancer cells by acting on cyclin E1 [34]. miR-497 is also closely associated with the prognosis of many types of cancers, such as kidney and pancreatic cancers [35, 36]. As it is a newly discovered miRNA, there have been very few reports on miR-3653, and further studies are needed to determine its role in the development of cervical cancer.

In this study, we were unable to examine miRNA expression in different stages and types of cervical cancer due to limited samples. Thus, further research will focus on overcoming this limitation through the involvement of multiple medical centers and large-scale studies.

In conclusion, we identified a series of miRNAs that can be used to detect HPV infection associated with cervical cancer in the Uyghur women of Xinjiang Province. These miRNAs provide new insight into the molecular basis of cervical cancers. They can therefore serve as biomarkers to improve early diagnosis, prognosis, and treatment of cervical cancers.

Supporting Information
S1 Protocol. Trial protocol. (DOCX)

Author Contributions
Conceptualization: DG XW.
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Funding acquisition: DG XW.
Investigation: MZ SL.
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Supervision: XW.
Validation: XW YZ.
Visualization: YZ MZ.
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Writing – review & editing: DG XW.

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