

## RESEARCH ARTICLE

# Roles of MiR-101 and its Target Gene Cox-2 in Early Diagnosis of Cervical Cancer in Uygur Women

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### Abstract

**Aims:** Early diagnosis is important for cervical cancer treatment. This study aimed to characterize the microRNA profile and target gene protein levels of cervical cancers in Uygur women for application in early diagnosis. **Methods:** The profiles of miRNA in cervical cancer and chronic cervicitis were analyzed with miRNAmicroarray V4.0. The expression of miR-101 was detected by real-time PCR and locked nucleotide acid *in situ* hybridization (LNA-ISH). Cox-2 protein levels were assessed by immunohistochemistry. **Results:** The microarray identified a set of 12 miRNAs significantly decreased in cervical cancer in comparison to the control group. Quantitative RT-PCR analysis showed miR-101 to be significantly downregulated in cancer tissues ( $p < 0.05$ ) while LNA-ISH showed miR-101 positive rates of 80% (20/25) and 8% (5/25) ( $p < 0.05$ ) in the control and cervical cancer groups. Cox-2 positive rates of cervical cancer and control groups were 84% (21/25) and 8% (2/25) ( $p < 0.05$ ). **Conclusions:** Use of down-regulation of miR-101 and up-regulation of Cox-2 as markers may play a role in early diagnosis of cervical cancer in Uygur women.

**Keywords:** MicroRNA - cervical cancer - Uygur ethnicity - miR-101 - Cox-2

*Asian Pac J Cancer Prev*, 15 (1), 45-48

### Introduction

Cervical cancer is a malignancy with second highest morbidity and mortality in women worldwide. Its incidence in developing countries is higher than developed countries (Arbyn et al., 2011). There are 131, 500 new cases each year in China alone. In Xinjiang, especially in the southern border region with poor economic conditions, cervical cancer is one of the leading causes of death in Uygur women with a prevalence rate of 527/100 000 and an early occurrence with the average age of 45.04 years (50.85 years for Han). About 80% cases have progressed to advanced stage when patients come to hospital for treatment (Lalaisuzuke et al., 2006). Prevention, early diagnosis and treatment of cervical cancer increasingly attract attention.

The microRNAs (miRNA) play a critical role in the gene regulatory networks. MiRNA is a well conserved class of endogenous non-coding small RNA molecules with approximate 22 nt. They are the final products processed from precursors (pre-microRNAs) that are 70-90 nt single stranded RNAs with a hairpin structure by the enzyme Dicer (Bumgarner et al., 2009). The mature microRNA can be selectively integrated into the RNA-induced silencing complex body (RISC) in combination with the target mRNA 3'UTR (untranslated region) with complete or incomplete complementary pairing, causing

suppression of protein translation or degradation of target mRNA (Bartel, 2009). microRNAs have been shown to be involved in inflammation, tumorigenesis, and other physiological and pathological processes.

In this study, we were to characterize miRNAs expression profiles in cervical cancer of Uygur women to identify the biomarkers for early diagnosis.

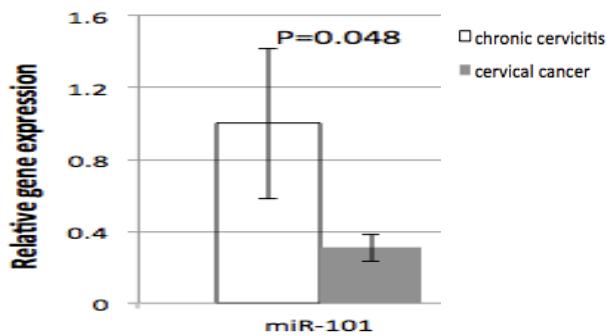
### Materials and Methods

#### Patients

**Experimental group:** Fresh tissue specimens collected from 15 Uygur women with cervical cancer from September 2009 to April 2010 in the Xinjiang Tumor Hospital gynecological hospitalization. All procedures were approved by the Institutional Review Board of Xinjiang Tumor Hospital. A written informed consent was obtained from each participant. Three cases were randomly selected for microarray experiments, 15 cases for the real-time PCR experiments.

**Control group:** at the same period, five cases of cervical tissue were collected from Uygur patients underwent hysterectomy due to non-cancerous diseases and identified as chronic cervicitis tissues by pathological diagnosis. Three cases were randomly selected for microarray experiments and five cases for real-time PCR experiments.

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**Figure 1. The RNA Levels of miR-101 Were Analyzed by Real-time PCR**

#### Reagents

**miRNA microarray chip:** mammalian miRNAs Microarray V4.0 (Beijing Boao Biotech Co., Ltd.); Trizol Reagent (Invitrogen life technologies); LightCycler FastStart DNA Master SYBR Green I (Roche Company); M-MLV reverse transcriptase enzyme (Invitrogen); of Recombinant RNasin RNase Inhibitor (Promega); miR-101 probe (Danish the Exiqon companies); Universal situ hybridization detection kit III MK1031 (Wuhan Boster Biological Engineering Co., Ltd.); Cox-2 rabbit anti-human polyclonal antibody, immunohistochemistry SP hypersensitivity kit and DAB reagent (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.).

#### MiRNA microarray screening

The total RNA was extracted with Trizol reagent according to manufactures protocol. Its concentration was measured by UV spectrophotometer and quality was checked by formaldehyde denaturing gel electrophoresis. miRNA was isolated from 20µg of total RNA using PEG solution precipitation method and then treated with alkaline phosphatase CIP (NEB, USA) to remove the 5' phosphate group and labeled with CU-CY3 (Dharmacon, USA) with T4 RNA ligase. The labeled miRNA was precipitated with absolute ethanol and resuspended in 20 µl of the hybridization solution (15% formamide; 0.2% SDS; 3 × SSC; 5 × Denhardt's) which was hybridized to the chip at 42°C overnight. After hybridization, the chip was first washed 4 min at 42°C in washing solution containing 0.2% SDS, 2 × SSC and then at room temperature for 4 min in 0.2 × SSC. The chips were scanned with a LuxScan 10K/A single channel laser scanner (CapitalBio, China). Data were extracted from the TIFF images using LuxScan™ 3.0 software (CapitalBio Corp.). The microarray intensity data were analyzed by using Significance Analysis of Microarrays (SAM) software. Two class unpaired t-tests were performed to identify miRNA expressed differences among the three groups. Genes possessing a q-value equal to 0 and a fold-change >2 were considered significantly different.

#### Real-time PCR

The expression of specific miRNA was analyzed with TaqMan MicroRNA SYBR Green detection kit on a Roche LightCycler 1.2 using total RNA template. The PCR primers were synthesized by Invitrogen Corporation. The oligonucleotide sequences were TACAGTACTGTGATA

ACTGAA (forward) and GTCGTATCCAGTGCAGGG TCCGAGGTATTTCGCACTGGATACGACTTCAGT (reverse) for has-miR-101. The U6 small RNA was used as an internal control. The experiment was repeated three times.

#### Locked nucleic acid in situ hybridization (LNA-ISH)

Using semi-quantitative integration method, the staining intensity was divided into three classes: no staining, light purple, and dark purple. It was given 1 point if the proportion of positive tumor cells was ≤ 25%, 2 points for 26% to 75%, and 3 points for ≥ 76%. The final score for each slice was determined by multiplying the scores twice and ≥ 3 was taken as positive and <3 as negative.

#### Immunohistochemistry

The Cox-2 antibody was used with 1:100 dilutions from Cox-2 rabbit anti-human polyclonal antibody immunohistochemistry SP hypersensitivity kit according to the manufacturer's instruction. PBS incubation served as a negative control. Using semi-quantitative integration method, the staining intensity was divided into three classes: 1 point for no staining, 2 for light purple, and 3 for dark purple. It was given 1 point if the proportion of positive tumor cells was ≤ 25%, 2 points for 26% to 75%, and 3 points for ≥ 76%. The final score for each slide was calculated by multiplying the point values from the two sets of scores and ≥ 3 was taken as positive and <3 as negative.

#### Statistical Analysis

Statistical analysis was performed with SPSS16.0 statistical package and the results were expressed as mean ± standard deviation. The means were compared using the two tails t-test and  $p < 0.05$  was designated as statistically significant.

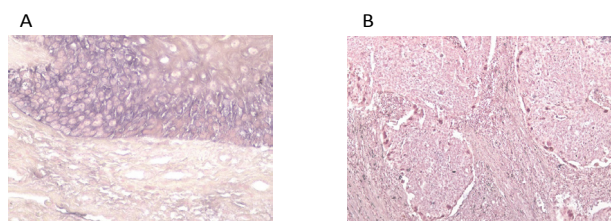
## Results

#### The different miRNA profile between cervical cancer

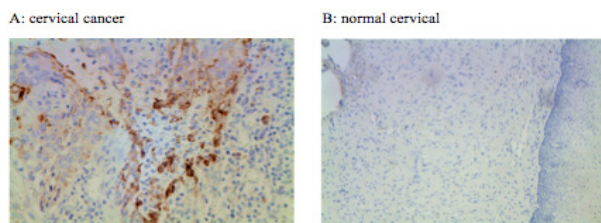
The expression of miRNA clearly showed different patterns between normal cervical tissue and cervical cancer. When compared with normal cervical tissue, there were 12 miRNAs significantly downregulated in cervical carcinoma (fold change ≤ 0.5). These 12 cervical cancer downregulated miRNAs were: hsa-miR-101, hsa-miR-572, hsa-miR-424, hsa-miR-409-3p, hsa-miR-365, hsa-miR-675, hsa-miR-187\*, hsa-miR-1247, hsa-miR-634, hsa-miR-1224-3p, hsa-miR-1238, and hsa-miR-125a-5p.

#### The differential expression and clinicopathological significance of miR-101 in cervical cancer tissue

The expression level of miR-101 was further analyzed in 5 chronic cervicitis and 15 cervical cancer tissues of Uyghur women. The expression levels of miR-101 were  $1.4 \pm 0.8$  and  $3.19 \pm 0.9$  in cervical cancer and normal cervical tissue ( $p=0.001$ ) (Table 1). The miR-101 expression pattern was further investigated with locked nucleic acid in situ hybridization (LNA-ISH). In control group, miRNA was



**Figure 2. The miR-101 Level Was Analyzed by LNA-ISH.** In the control group miR-101 was moderately positive (A) but was weakly expressed in cervical cancer (B)



**Figure 3. Cox-2 Protein Was Strongly Detected in Cervical Cancer Tissue (A) by Immunohistochemistry but Weak to None in Control Cervical Tissue (B)**

expressed in the majority of cervical squamous cells and it showed pale purple to deep purple color in cytoplasmic lumps (Figure 2A) but it had substantially weaker showing in cervical carcinoma (Figure 2B). The miR-101 positive rate control group was 80% (20/25) and 8% (5/25) for cervical cancer group ( $p = 0.018$ ). The differences of miR-101 expression rate among different ages, tumor grades and clinical stages were not statistically significant ( $P > 0.05$ , Table 1).

#### The expression of Cox-2 protein in cervical cancer

In cervical cancer tissues, Cox-2 was mostly stained in the cytoplasm of tumor cells as light yellow, brown or tan particles (Figure 3A). The Cox-2 positive rates in the control group and cervical cancer group were 8% and 84%, respectively ( $P = 0.001$ , Table 2).

## Discussion

Human cancers often exhibit altered expression profiles of miRNAs (Garzon et al., 2009). Recent studies have revealed that cancer-associated miRNAs play important roles in tumorigenesis and may serve as diagnostic and prognostic biomarkers in various cancers including cervical cancer (Chen et al., 2013). Our study by array analysis identified 12 differentially expressed miRNA in cervical cancer in Uygur women, indicating aberrant miRNA expression plays a critical role in pathogenesis of cervical cancer. Rao et al. (2012) identified 18 significantly upregulated miRNAs (1.9%) and 19 significantly downregulated miRNAs (2.1%) in cervical cancer tissues. The differences might be resulted from A) racial or ethnic difference; B) different control tissues; and C) differences in sample source. Our founding supply the microRNA data base associated with cervical cancer.

Of all the different expression microRNA, the significant downregulation of miR-101 in cervical cancers of Uygur women is consistent with the reported decrease

**Table 1. miR-101 Expression in the Cervical Tissues of Uygur Women by LNA-ISH**

Group	+	-	n	positive rate (%)
Control	20	5	25	80.0
Cervical cancer	5	23	25	8.0
Total	25	28	50	44.0

**Table 2. Cox-2 Expression in the Uygur Cervical Cancer Patients**

Group	+	-	n	positive rate (%)
Control	2	23	25	8.0
Cervical cancer	21	4	25	84.0
Total	23	27	50	46.0

of miR-101 in prostate cancer (Pang et al., 2010; Hao et al., 2011), colon (Chandramouli et al., 2012; Strillacci et al., 2013), liver cancer (Zhang et al., 2012), non small cell lung cancer (Zhang et al., 2011), breast cancer (Wang et al., 2012), and gastric cancer (Wang et al., 2010). Our data showed that miR-101 expression was significantly reduced in cervical cancer, which may play a critical role in the occurrence and development of cervical cancer. The downregulation of miR-101 was not varied among the clinical stage of cervical cancer, suggesting its potential as a biomarker for early detection of cervical cancer.

The target genes of microRNA often to be a group of genes with similar functions. The depressed expression of miR-101 would result in elevated expression of its target genes and the promotion of tumorigenesis. Su et al. (2009) found that the level of miR-101 was significantly reduced in hepatocellular carcinoma. Ectopic expression of miR-101 inhibited cancer cell proliferation in vitro and tumor growth in vivo. In luciferase assay, miR-101 significantly inhibited the expression of luciferase gene carrying mcl-1 3' untranslated region. It also reduced Mcl-1 endogenous protein level; on the contrary, miR-101 inhibitor significantly up-regulated the expression of Mcl-1 and inhibited apoptosis.

As an aspect of the relationship with the microRNA-101, the prediction based on PicTar and TargetScan DNA analysis software suggest that Cox-2 could be one direct target for miR-101 because miR-101 has a seed region, which is able to bind to the Cox-2 mRNA 3-UTR. Cyclooxygenase-2 (Cox-2) was a target gene of miR-101. Cox-2 gene is located on chromosome NO.1 1q25.2-q25.3, length 8.3 kb, consists of 10 exons and 9 introns, encoding 604 amino acid residues from the polypeptide consisting of base. Regulation carcinogenic mechanism of Cox-2, is now more accepted view is that Cox-2 can promote cell proliferation, inhibition of apoptosis, angiogenesis, suppression of the immune function involved in tumor development and progression. Such as colon cancer (Strillacci et al 2009) and prostate cancer (Hao et al., 2011). Our data showed that the expression of Cox-2 protein level was significantly increased in cervical cancer. The levels of miR-101 and Cox-2 were negatively correlated in cervical cancer of Uygur women, which may serve as biomarkers of diagnosis markers for cervical cancer in Uygur women.

## Acknowledgements

This research is supported by Xinjiang Uygur Autonomous Region Natural Science Foundation (No. 2011211B24) and PHD Scientific Research Foundation of Xinjiang Medical University (No. 2012-9).

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