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RESEARCH ARTICLE

Deregulation of miR-21 and miR-29a in Cervical Cancer Related to HPV Infection

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Abstract: ***Background*:** Early diagnosis is an important factor to improve the survival of Invasive Cervical Cancer (ICC) patients. Molecular biomarkers such as micro RNA (miRNA) can be used in the early detection of ICC. The expression of miR-21 and miR-29a are deregulated in many types of human cancers.

***Objective*:** The aim of this study was to investigate the differences in miR-21 and miR-29a expression patterns in the Human Papilloma Virus (HPV) infection and various grades of cervical cancer among Iranian women.

***Methods*:** Small RNAs were extracted from positive for HPV, cervical cancer and healthy samples from 43, 50 and 46 individuals, respectively. Expression levels of miR-21 and miR-29a were analyzed by SYBR Green real-time RT-PCR using specific primers, and 5s rRNA as the internal reference gene.

***Results*:** Results have shown a significant increase in miR-21 and decrease in miR-29 in cancerous samples in comparison with the control groups ($P < 0.0001$).

***Conclusion*:** This study illustrated that miR-21 and miR-29a could be operated as an oncogene and tumor-suppressor in cervical cancer progression. More studies are needed to demonstrate the role of miR-21 and miR-29a as potential biomarkers for the diagnosis of cervical cancer in future investigations.

Keywords: Biomarker, cervical cancer, HPV, infection, microRNA, miR-21, miR-29a.

1. INTRODUCTION

Cervical Cancer (CC) is the third most common cancer in women in the world [1]. This cancer is developed through pre-malignant lesions known as Cervical Intraepithelial Neoplasia (CIN) from grades I to III [1-4]. More than 85% of the CC take place in developing countries. High-risk regions are Africa, Southern Africa, South-Central Asia, South America and Middle Africa. The clinical, epidemiological and laboratory data have demonstrated that persistent Human Papilloma Virus (HPV) infection is the main etiological agent in CC progression. However, other factors such as cellular, immunological, genetic, epigenetic, and environmental factors can affect the ultimate final outcome of dysplastic changes [1, 5-9].

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MicroRNAs (miRNAs), as endogenous non-coding RNAs with 18-25 nucleotides in length, are derived from coding or noncoding genes and have regulatory roles in cells [10, 11]. Based on their expression patterns in cancers, miRNAs categorize as either oncogenes or tumor suppressor genes [10, 12].

Recent studies have shown that various miRNAs have different expression in High Risk-HPV positive (HR-HPV) CC cells as compared with HPV-Negative CC cells or normal cervical tissues [10]. It has been demonstrated that HR-HPVs have roles on down-regulation of tumor suppressor or up-regulation of oncogenic miRNAs. In this way, differential miRNAs expression can be identified during CC progression. miRNAs have been revealed to involve in cellular events, including cell proliferation, apoptosis, angiogenesis, immune responses and tumor invasion and metastasis [10]. HPV infection leads to the expression of viral proteins that

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Table 1. Demographic clinical data of subjects.

Data Set	Women with CC	Women with HPV Positive and Free of Cancer	Women Free of HPV and Cancer (Healthy Controls)
Age/years			
Mean	44.34	33.77	33.87
SD	±10.56	±8.775	±8.013
Age (range)	23-70	20-58	20-55
HPV Genotypes			
HPV 16	35	6	
HPV 18	31	0	-
HPV 16 & 18 positive	21	0	
Other HPV Genotypes*	56	68	
Pathological Stage of Neoplasia			
CIN I	9(18%)	-	-
CIN II	6(12%)		
CIN III & ICC	35(70%)		
Total	50	43	46

*Other HPV types including: High Risk HPV: 52, 31, 56, 58, 59, 33, 66, 45, 68, 35, 51, 39, 53, 82, 26.Low Risk HPV: 6, 11, 54, 61, 70 and 89.

Abbreviations: CC, Cervical Cancer; HPV, Human Papilloma Virus; SD, Standard Division; CIN, Cervical Intra Neoplasia; ICC, Invasive Cervical Cancer

alters normal cell functions such as proliferation and differentiation [12, 13]. The aberrant expression of these proteins in CC has been suggested to be used as biomarkers for CC detection in the early stage [10, 11]. Additionally, it has been found that encoded proteins by HR-HPV can affect miRNA expression inside the host cell [2]. Among the cancers, associated-miRNAs, miR-21 and miR-29a have been suggested to be used as potential diagnostic and prognostic biomarkers in CC.

MiR-21 genomic locus is located in the fragile site FRA17B within the 17q 23.2 chromosomal region, which is one of the HPV16 integration loci [10, 14]. Therefore, over-expression of miR-21 in CC may be associated with HPV16 integration [10, 14, 15]. One of the targets for miR-21 is the tumor suppressor PTEN that its down-regulation leads to over-expression of matrix metalloproteinases MMP2 and MMP9 which promote cellular migration and invasion. Over-expression of these MMPs is recognized in CC and causes invasive cancer. Two other proteins, which have been implicated in the suppression of tumor invasion and metastases are programmed cell death 4 (PDCD4) and maspin. It seems that miR-21 acts directly on the PDCD4 miRNA. Some of the studies showed that PDCD4 was down-regulated in CC, indicating that miR-21 could play an oncogenic role in CC invasion and metastasis [10].

MiR-29a is mapped to chromosome 7 and is down-regulated in CC. MiR-29a decreases expression of Heat-shock protein 47 (HSP47), which is a super-family of serine protease inhibitors. The over-expression of HSP47 has an important role in cancer development and metastasis processes. On the other hand, miR-29a inhibits G1/S transition in cervical cell lines. The expression of this miRNA is down-regulated during CC development and loading to apoptosis insensibility and uncontrolled cell cycles with increasing Ying Yang 1 (YY1) motif and CDK6 protein expression. YY1 is an important transcription factor that inhibits apoptosis and CDK6, is a kinase that phosphorylates pRb releasing the transcriptional factor E2F [12, 16]. It is known that miR-29a targets the HPV-related genes. However, the molecular mechanisms through which HPV infection can down-regulate miR-29a have not been well understood yet [17].

In the present study, expression levels of miR-21 and miR-29a in HPV infected individuals and CC patients in comparison with the healthy control groups in Iranian women investigated.

2. MATERIALS AND METHODS

2.1. Study Population

In this study, 50 Liquid Based Cytology Samples (LBCs) samples were collected in 2012 at Mohebe-Yas Hospital, Tehran, Iran, from women diagnosed with Cervical Intraepithelial Neoplasia (CIN) and Invasive Cervical Cancer (ICC). In addition, 46 archived LBC samples from patients with neither CC nor HPV infection, were collected to serve as a negative control. Also, 43 LBC samples that were positive for HPV were collected. Samples were selected from archived samples of several private pathobiology laboratories. The study was approved by the Shahid Beheshti University Ethic Committee. The informed consent form was obtained from cancer patients. Demographic and clinical data of patients were shown in Table **1**.

LBC samples were transferred to the Molecular Biology Department of Reference Health Laboratory, Iran Ministry of Health and Medical Education, Iran and stored at -20°C freezer for further use.

2.2. miRNA Extraction and Evaluation

The high pure miRNA Isolation Kit (Roche, Germany) was applied for purification of small RNAs (<100 nucleotides) from the total RNA. The integrity of the miRNAs was checked using agarose gel electrophoresis stained with loading dye. The extracted RNAs were used for cDNA synthesis immediately.

2.3. cDNA Synthesis from Small RNA

cDNA was synthesized using the miRNA amplification kit (Pars Genome Co, Iran). PCR primers for miR-21, miR-29-a and 5s-rRNA were obtained from Pars Genome Company, Iran. The 5s rRNA was selected as the internal reference gene. According to the manufacturer's instructions, in the first step for poly-A tail synthesis: 10µl of small RNA (final conc. / amount: 1.5µg), was incubated with 2µl 10x reaction buffer, 1 µl ATP (final conc. / amount: 10Mm), 0.5 µl poly-A enzyme, and 6.5µl DEPC treated water and was incubated at 370C for 10 min. In the second step, for specific cDNA synthesis, 5µl RNA poly A tail (final conc. / amount: 1.5 µg) was mixed with 2µl 5x buffer, 0.5 µl reverse transcriptase enzyme, 0.5 µl of each miRs cDNA synthesis specific primer (final conc. / amount: 15pmol), and 1µl dNTPs (final conc. / amount: 10mM). The mixture was incubated in a thermal cycler at 44 OC for 60 min, the process was followed by one-minute inactivation of reverse transcriptase in 85°C. Finally, obtained cDNAs were stored at -20°C until later use.

2.4. Real-time RT-PCR

Real-time RT-PCR was performed using the Rotor-gene (Corbett) instrument. Reactions were carried out in a total volume of 20µl, including 10µl SYBR green master mix (Takara, Japan), 0.5µl forward and reverse mix primer (final conc./ amount: 10pmol), 8.5 µl nuclease-free water, and 1µl undiluted cDNA. All samples were performed in duplicate using PCR cycling Rotor-Gene Q (software version 2.3) with the initial denaturation at 95°C for 5 minutes, followed by 40 cycles of amplification at 95°C for 5 seconds, 61°C for 20 seconds, and 72°C for 30 seconds. ΔCT method was used for analysis of real-time RT-PCR results. Mean of Cycle Threshold (CT) values were calculated for the reference gene and each of miRNAs. Threshold (CT value) was determined as the number of PCR cycle in which the fluorescent signal crosses the threshold. ΔCT is the difference of CT values of each miRNA and the reference gene. $2^{-\Delta CT}$ which is considered as an expression of two studied miRNAs between cancerous and free of cancer group was used.

2.5. Statistical Analysis

Differences in the mean between miRNAs (miR-21 and miR-29a) expression and ΔCT in the subjects of cervical cancer group, HPV-Positive and healthy individuals were analyzed using unpaired-t-test (Mann-Whitney test). In addition, differences in mean between miRs expression in CIN I & CIN II group and CIN III & ICC group were analyzed. Statistical analysis was performed with Graph Pad Prism version 7. P-value < 0.05 was considered as significant level.

3. RESULTS

3.1. miR-21 Expression in Unpaired Case and Control Samples

The miR-21 was significantly up-regulated in CC samples compared with HPV-Negative control group (P<0.0001). Level of miR-21 expression in these groups is shown in Fig. (**1A**). The mean of ΔCT value for miR-21 in CC sample was 5.02 ± 0.7299 in comparison with 8.184 ±5.419 in HPV-Negative control samples (P< 0.0001).

The miR-21 was up-regulated in HPV-Positive samples compared with controls, but it was not significant (P=0.2075). Level of miR-21 expression in population study is shown in Fig. (**1B**). The mean of ΔCT value for miR-21 in HPV-Positive samples was 10.27 ± 5.879 as compared with 7.780±4.824 in controls. (P=0.0590).

The relative expression of miR-21 was compared in CC progression. The number of patients with CIN I & CIN II and CIN III &ICC was 15 and 35, respectively. Our study showed that miR-21 was up-regulated in CIN III & ICC group compared to CIN I & CINII, however, it was not significant. (P = 0.5879) (Fig. **1C**). The mean of ΔCT value for miR-21 in CINIII & ICC was 19.43 ± 6.094 as compared with 18.92±3.344 in CIN I & CIN II (P = 0.8853).

3.2. miR-29a Expression in Unpaired Cases and Control Samples

The miR-29a was significantly down-regulated in CC samples compared with controls (P < 0.0001). The expression level of miR-29a in controls and CC samples is shown in Fig. (**2A**). The mean of ΔCT value for miR-29a in CC samples was 20.08±6.137 in comparison with 5.221±0.7698 in HPV-Negative control samples (P < 0.0001). The miR-29a was down-regulated in HPV-Positive samples compared to healthy controls, however, it was not significant (P= 0.3624). The expression level of miR-29a in control and HPV-Positive from 46 and 43 individuals are shown in Fig. (**2B**). The mean of ΔCT value for miR-29a in HPV positive samples was 11.56 ± 5.385 in comparison with 10.38±5.241 in free of cancer samples (P = 0.3200).

Furthermore, the relative expression of miR-29a was compared in CC progression. Women suffered from CIN I & CINII and CINIII & ICC were 15 and 35, respectively. The miR-29a was down-regulated in CINIII &ICC patients compared with CIN I & CINII, however, it was not significant (P= 0.3818) (Fig. **2C**). The mean of ΔCT value in CINIII& ICC individuals was 22.02 ± 4.717 in comparison with 19.88 ± 4.309 in CIN I& CINII individuals (P = 0.2697).

4. DISCUSSION

Cervical Cancer (CC) as a genetic and pathologic complex disorder and de-regulation in the miRNA expression has a significant biological role in the malignancy [5, 10]. Several studies have shown that de-regulation of miRNA was detected in CC patients; it would appear these molecules could

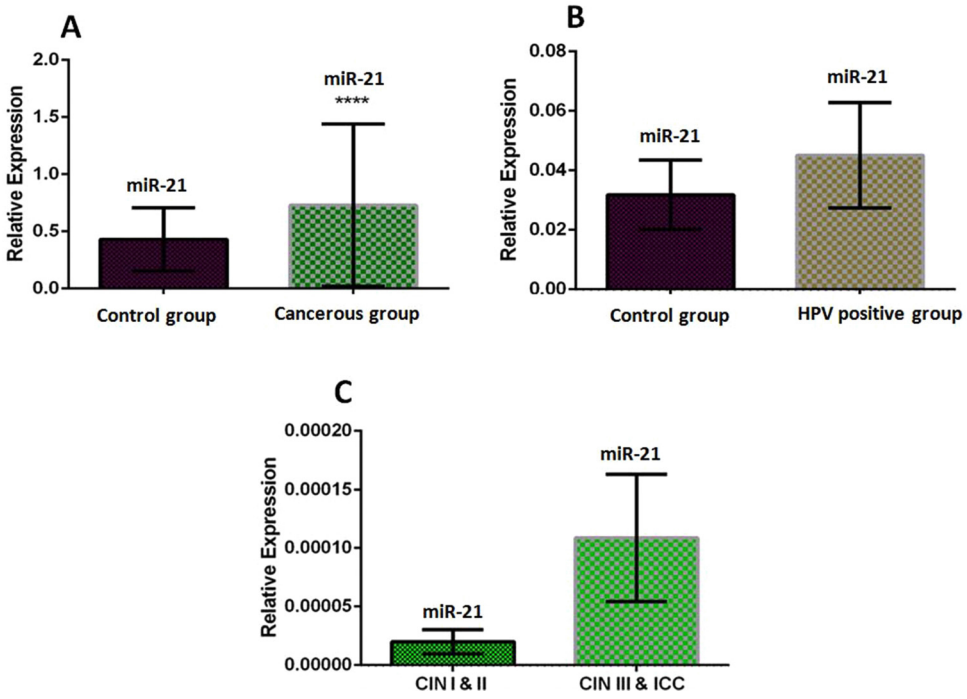

Fig. (1). (**A**): Relative expression levels of miR-21 in cancerous and control group (P < 0.0001). (**B**): Relative expression levels of miR-21 in HPV-Positive and control group (P = 0.2075). (**C**): Relative expression levels of the miR-21 in CIN I, II, III& ICC group (P= 0.5879).

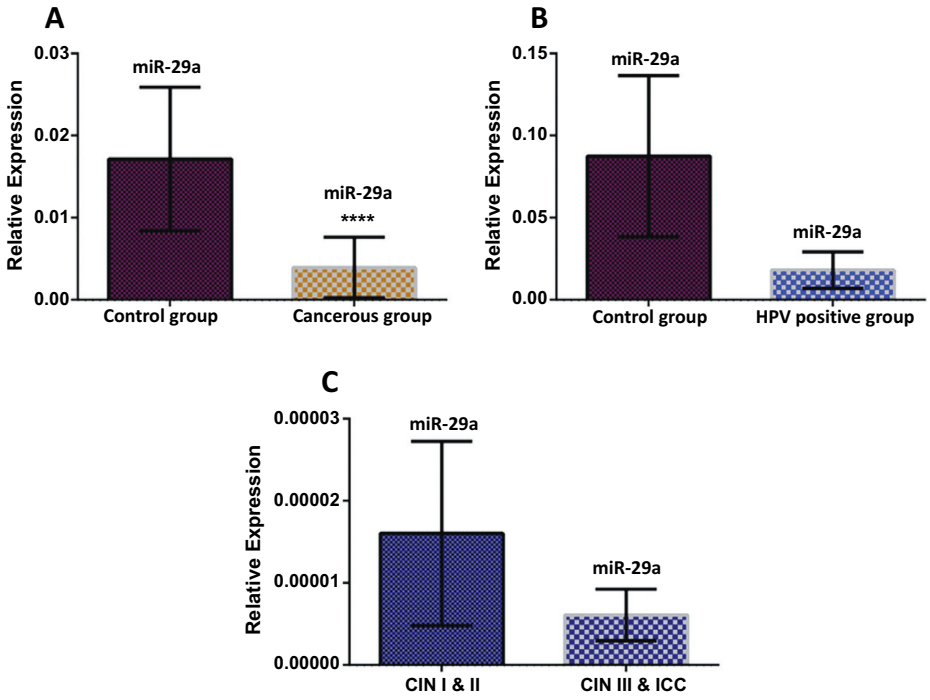

Fig. (2). (**A**): Relative expression levels of miR-29a in cancerous control group (P < 0.0001). (**B**): Relative expression levels of the miR-29a in control and HPV positive group (P= 0.3624). (**C**): Relative expression levels of the miR-29a in CIN I, II, III & ICC group (P= 0.3818).

be used as potential biomarkers. Other studies indicated some interactions between viral oncoproteins and miRNAs like miR-21 and miR-29a. It would appear that deregulated miRNAs have crucial roles in cervical tumor biology [17-19]. In order to explore the expression profile of miR-21 and miR-29a, a real-time quantitative RT-PCR has been carried out in HPV-Positive, HPV-Negative and CC samples.

MiR-21 up-regulation can be played a major role in CC progression by affecting cell proliferation, tumor invasion, metastasis, angiogenesis, immune responses and apoptosis inhibition [10].

Our findings suggested that the miR-21 expression is significantly increased in CC cells in comparison with normal cells which is in agreement with the finding of Ying Han

et al, and Chamsai Pientong *et al.* studies [18, 19]. Furthermore, we assessed the expression of miR-21 in HPV infected cells with no evidence of carcinogenic changes. Our results demonstrated that miR-21 expression was increased in the HPV-Positive group compared to the normal group. Even though, it was not significant. It would seem a comparison of miR-21 expression in different grades of dysplasia showed an up-regulation of miR-21 in CIN III& ICC in comparison of CINI & CINII, although it was not significant as well.

A number of studies showed the biological role of miR-29a in cellular processes such as angiogenesis, immune responses and pro-apoptosis in CC progression. Furthermore, it was demonstrated that miR-29a is the most highly down-regulated miRNA in the clinical samples, indicating miR-29a as a putative tumor suppressive miRNA in human cancers [3].

At the present study, miR-29a expression was significantly decreased in CC group comparing to the normal group which is consistent with the finding of Naohiko Seki *et al.* [9]. miR-29a expression was down-regulated in the HPV positive groups compared with the normal group, but it was not significant. Comparing expression levels of miR-29a in the CIN I& CIN II group and the CIN III& ICC group showed a reduced miR-29a expression in CIN I & CIN II, but it was not significant.

CONCLUSION

In conclusion, expression analysis of miR-21 and miR-29a in HPV-Positive and CC samples in Iranian women is a novel approach in this study. There were no significant de-regulation of these miRNAs among HPV-Positive free cancer and normal groups. The results demonstrated considerable de-regulation in miR-21 and miR-29a in the cancerous group which was statistically significant. It seems that these miRNAs can be used as biomarkers for early detection of CC. In order to provide necessary information on the critical role of miRNAs as a prognostic marker in CC prevention programs, more studies are required to determine their specific function, transcriptional targets, and mechanisms of regulatory actions on cellular processes and HPV related changes in CC in comparison to normal cervical cells as well.

LIST OF ABBREVIATIONS

CC	=	Cervical Cancer
CIN	=	Cervical Intraepithelial Neoplasia
CT	=	Cycle Threshold
HPV	=	Human Papilloma Virus
HR	=	High Risk
HSP47	=	Heat-shock protein 47
LBC	=	Liquid Based Cytology
LR	=	Low Risk
miRNA	=	MicroRNA
PDCD4	=	Programed Cell Death 4
SD	=	Standard Division
YY1	=	Ying Yang 1

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Shahid Beheshti University Ethic Committee.

HUMAN AND ANIMAL RIGHTS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

CONSENT FOR PUBLICATION

Informed consent was obtained from all individual participants included in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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