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The Correlation of the MicroRNA129-2 and SOX_4 Genes Expression in HPV-infected Cervical Cancer Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author ZNG designed the study, wrote the protocol and wrote the first draft of the manuscript. Author KO performed the statistical analysis and managed the analyses of the study. Author EM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Cervical cancer is one of the most common neoplasias in females worldwide. The miRNAs affect cell proliferation and apoptosis in cancers. Low levels of the miR129-2 expression have been linked to human cancer while the SOX_4 gene expression has been identified as a necessary factor in cell proliferation and survival. The purpose of the present study was to determine the correlation of MicroRNA129-2 and SOX_4 genes expression in cervical cancer patients infected with HPV and compare the expression level with not infected patients and control group.

Materials and Methods: The total of samples were including 30 HPV infected cervical cancer, 30 cervical cancer patients with no infection and 20 normal Pap smear were collected from Mirzakochak Khan Hospital, Tehran, Iran in 2015. RNA was extracted; MIR129-2 and SOX_4 genes expression was investigated. Cervical cancer was determined by Pap smear and tissue sampling test. HPV 16 and 18 infections assessed by RT High-Risk HPV assay. The RNA was extracted and the expression of the MIR129-2 and SOX_4 genes was determined using Real Time PCR. The data was analyzed using Graph pad prism.

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Results: According to the results, there was a decreased MIR129-2 expression in HPV positive cervical cancer compare to control and not infected patients (P=0.0001, P=0.0002 respectively). Although there was a decrease in MIR129-2 gene expression in not infected cervical cancer patients compare to control group but, statistically it was not significant (P=0.0728). Also, the SOX_4 gene expression was increased in HPV infected cervical cancer patients compare to control and not infected patients which statistically was significant (P=0.0001, P=0.0032 respectively). Comparison of SOX_4 gene expression among patients showed an increased significant association in not HPV infected patients compare to control group (P=0.0439). Also, no significant difference observed on MIR129-2 and SOX_4 genes expression on the base of age and stage of disease.
Conclusion: Decreased MIR129-2 expression might increase SOX_4 gene expression. So, determining of the MIR129-2 and SOX_4 expression levels might be a useful indicator for prognosis of the HPV-infected cervical cancer.

Keywords: MicroRNA 129-2; SOX_4 gene; HPV; cervical cancer.

1. INTRODUCTION

Cervical cancer is one of the most common neoplasias in females which, progress from the precursor stage, manifested by cervical intraepithelial neoplastic lesions, to invasive tumors (Lee et al., 2011). Cervical cancer is known second cancer in women society in the worldwide [1] and HPV is presented in 99.7% of cervical cancers [2]. Even though standard treatment as radiotherapy and chemotherapy exist for the cervical cancer, there is no molecular marker to predict the clinical diagnosis of the patients [1]. Infection with high-risk human papilloma viruses (HPVs) such as HPV type 18, 16, or 31 is responsible for most cases of human cervical tumors (Lee et al., 2011).

MicroRNAs (miRNAs) are small, non-coding RNAs that post transcriptionally control gene expression of their target messenger RNAs (mRNAs) and potentially regulating a host of cellular signaling pathways [3]. The miRNAs affect cell proliferation, apoptosis, cell differentiation and the epithelial-mesenchymal transition in various types of cancers [4]. Some microRNAs expression increased which target tumor suppressor protein known as oncomirs and those with decreased expression which generally target oncogenes, referred to as tumor suppressor microRNAs [3]. Several microRNAs with altered expression such as miR-21, miR -135b, miR -141 and miR-146 in cervical cancer have been identified [3].

The miR-129 is transcribed from of two genes including miR129-1 and miR129-2. Two mature miRNAs, miR-129-5p and miR129-3p, are processed from both 5′ and 3′ precursors, respectively [5]. The miR129-1 is identified in genomic regions near a common fragile site on human chromosome 7 while the miR129-2 is located on chromosome 11 [6]. Low levels of the miR129-2 expression have been linked to human cancers [5]. Recent studies suggested microRNAs are crucial in the occurrence and development of the HPV-associated cancers [7]. For instance, HPV-16 promotes cell growth of esophageal cancer via down regulation of microRNA [8]. The miR129-2 expression has been shown to be involved in the progression of the several types of cancers and patient survival (Zhang et al. 2015). It is reported, miR-129 has role in cervical cancer where interferon-β induced the miR129-5p down-regulates HPV-18 E6 and E7 viral gene expression in cervical cancer cells [9].

The SOX (sex-determining region Y (SRY)-related high mobility-group (HMB) box transcription factor) gene family is found in the animal kingdom and twenty members of this family have so far been identified in vertebrate [10]. The SOX is overexpressed in a variety of cancers, including prostate, lung, bladder, breast, gastric and endometrial cancers [11]. The SOX expression was shown to be elevated in a wide variety of tumors [12]. For instance, SOX_4 acts as a pro-oncogene and associated with increased cell proliferation, cell survival, epithelial-to-mesenchymal transition and metastasis in endometrium [13]. Repression of the miR129-2 by DNA hypermethylation correlated with overexpression of SOX_4 in endometrial and gastric cancers [11]. Also, demethylation of the miR129-2 resulted in partial downregulation of SOX_4 expression [11]. It is reported expression of the SOX_4 was inversely associated with the miR129-2-but not of miR129-1 in gastric cancer [5]. As miRNAs play an important role in HPV induced cervical

carcinogenesis and role of the miR129-2 and SOX_4 in cervical cancer, to date, never been reported, we hypothesize to determine the correlation of the miR129-2 and SOX_4 genes expression in HPV-infected cervical cancer patients.

2. MATERIALS AND METHODS

2.1 Patients and Samples

Formalin-fixed paraffin-embedded tissue specimens were obtained from 30 HPV-infected cervical cancers, 30 cervical cancer patients with no HPV infection and 20 normal cases referred to the Mirza Kuchak Khan Hospital, Tehran, Iran, 2015. The clinical staging and clinicopathological classifications were determined according to the International Federation of Obstetrics and Gynecology (FIGO) and were recorded by an experienced pathologist. In this case-control study, only patients with IC (invasive carcinoma) and CIN3 were selected. All tissue samples used in the present study were obtained with the consent of each patient and with institutional research ethics committee approval (Code No: 29830517941019). All patients aged between 21-46 years old with no previous use of anti-pregnancy medications, smoking and immune system disorders at least for past 5 years. The Pap smear and tissue sampling tests were done to determine the incidence of the cervical cancer. The Pap smear results compared with their histopathology report to determine invasive carcinoma.

2.2 Human Papillomavirus (HPV-16 and HPV-18) Infections

RT High-Risk HPV assay was performed using Technology-DNA PCR Amplification Kit (HPV 16 & 18 Iot: 8045) to determine the HPV 16 and 18 types infections. Briefly, After PCR, the samples allocated into flash PCR Gene detector (Technology-DNA) and results reported by Gene v4 software. The HPV test results compared with cervical cytology (Castle et al. 2005). The patients who were no invasive carcinoma and HPV 16 and 18 negative infections allocated into control group.

2.3 RNA Extraction and cDNA Synthesis

The tissue samples was deparaffinized by xylene (1000 µl) at 37ºC for 5 minutes. Then micro tubes centrifuged at 3800 rpm for 5 minute, supernatant removed and then 1000 µl of ethanol was added and inverted for 5 minutes. Finally, samples centrifuged at 13000 rpm at 6ºC for 5 minute and the ethanol and xylene of the micro tubes entirely removed. Total RNA was extracted using the RNX plus™ kit (Cinnagen, Tehran, Iran) based on the manufacturer recommendations. A 100 µl of the tissue sample homogenized with the 500 µl of the RNX-PLUS solution and incubated at room temperature for 5 minutes. Chloroform (200 µl) was added to the solution and centrifuged at 12000 rpm for 15 minutes. Supernatant transferred to another tube and equal volume of isopropanol was added. The mixture was centrifuged at 12000 rpm for 15 minutes and the resulting pellet was washed in ethanol (70%) and dissolved in DEPC-treated water. The purity and the integrity of the extracted RNA was evaluated by optical density measurements and visual observation of sample electrophoresis on 2% agarose gel [14] using NanoDrop spectrophotometer for DNA (260 nm) and RNA (280 nm) (Jiang et al., 2010). cDNA was synthesized from total RNA using the Taqman miRNA reverse transcription kit. The Random hexamer used as primer (1 µl) and the Oligo dt (1 µl) were added, vortex and spine. A 10 µl of RNA added and incubated at 65ºC for 5 minutes. Then nucleuse free water (4.5 µl), MMULV buffer (2 µl) and MMULV (0.5 µl) were added to final volume of 20 µl. the solution spin and RUN at 42ºC for 60 minutes.

2.4 Real Time PCR

Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system (Applied Biosystems, USA). The expression of miR129-2, SOX_4 and GAPDH genes were defined on the threshold cycle (Ct) and relative expression levels calculated after normalization with reference to expression of small nuclear RNA [15]. The steps of mRNA RT-PCR were performed based on the procedure described by Kaka et al. [14]. Briefly, 1 ng of total RNA obtained from samples, transcribed with oligo (dT) and then RT-PCR was performed. The primers used for miR129-2, SOX_4 and GAPDH were follows: miR129-2-forward: GTGAAGCCCTTACCCCAAA (51.09℃), miR129-2-reverse: GTGCAGGGTCCGAGGT (51.06℃); SOX_4-forward: GGCCTGTTTCGCTGTCGGGT, SOX_4-reverse: GCCTGCATGCAACAGACTGGC; GAPDH -forward: ATGGAGAAGGCTGGGGCT3´ (124 bp, 61℃); GAPDH-reverse: ATCTTGAGGCTGTTGTCATACTTCTC 3´ (124 bp, 61℃) [16]. The cycling conditions for

miR129-2 and GAPDH were initial denaturation at 95℃ for 10 seconds followed by 35 cycles of the 95℃ and a final extension 60℃ for 34 seconds. Each experiment was repeated at least 3 times in order to ensure reproducibility. The size of the digested products was checked on 2% agarose gel electrophoresis. The relative expression levels of miR129-2, SOX_4 and GAPDH were calculated using the comparative ΔΔCt method [17]. The fold changes in these genes were calculated using the ΔΔCt method. All experiments were performed at least in triplicate [17].

2.5 Statistical Analysis

The data of GAPDH, miR129-2 and SOX_4 genes expression between groups were subjected to the *t*-Student and Mann–Whitney tests. The results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed with SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (ver. 5.0). P< 0.05 was considered as significant.

3. RESULTS

The baseline information of the women included into the study is presented in Table 1. After the amplification reaction, the Ct of the samples converted to the relative quantification and determined using ΔΔCt method. The RQ of the obtained results of the normal, positive HPV-infected- and negative HPV-infected cervical cancer patients were compared. The result of the miR129-2 expression is shown in Fig. 1. A significant decrease in miR129-2 expression was detected among HPV infected cervical cancer patients compare to control group (P=0.0001). Although there was a decrease in miR129-2 gene expression in not infected cervical cancer patients compare to control but, statistically it was not significant (P=0.0728), (Fig. 2). We observed a positive association in miR129-2 gene expression between infected cervical cancer patients compare to not infected group and it statistically was significant (P=0.0002), (Fig. 3). The results of the miR129-2 gene expression in HPV infected and not infected cervical cancer patients based on their age and stage of the disease, showed no significant difference in miR129-2 gene expression in patients aged >35 compared to <35 years old (P=0.813, P=0.375 respectively).

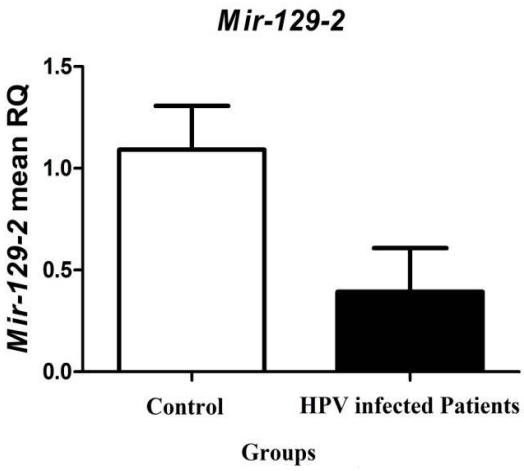

Fig. 1. The miR129-2 expression in normal and HPV infected patients (P=0.0001)

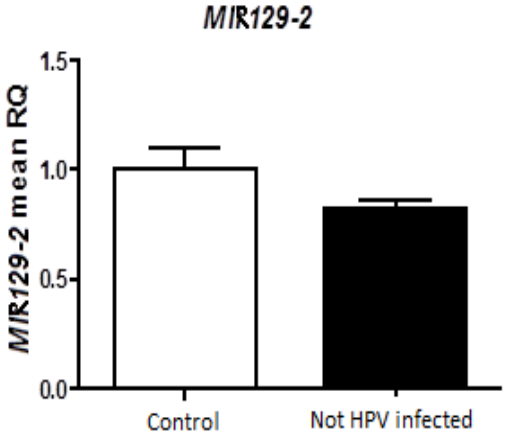

Fig. 2. The miR129-2 expression in normal compared to not infected patients

Regarding SOX_4 gene, we observed an increase in gene expression in patients infected with HPV compare to the control (P=0.0001), (Fig. 4). Also increase in gene expression was found among patients with no infection compared to healthy individuals P=0.0439), (Fig. 5). Comparison of SOX_4 gene expression among patients showed an increased significant association in HPV infected patients compare to not infected group (P=0.0030) (Fig. 6). SOX_4 gene expression in HPV positive and HPV negative infected cervical cancer patients based on the age and stage of the disease also found statistically not to be significant (P=0.0923, P=0.0868 respectively).

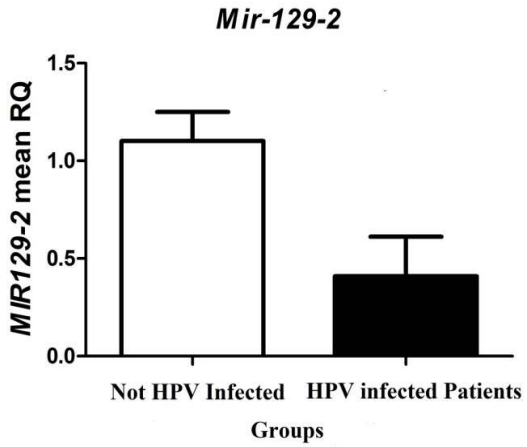

Fig. 3. The miR129-2 expression in HPV infected compared to not infected group (P=0.0002)

4. DISCUSSION

Cervical cancer is an important health problem in developing countries, where it ranks as the second leading cause of cancer-related death [1]. Indeed, several lines of evidence exist supporting the correlation between genital HPV infections and development of cancer of uterine cervix, vagina, vulva and adenocarcinoma in women [1]. HPV16 and 18 is the most prevalent types associated with cervical cancer [2]. Despite early detection programs, nearly 50% of these patients are diagnosed with locally advanced stages [18]. Standard treatment for these patients consists of radiotherapy in combination of chemotherapy. Unfortunately, nearly 50% of patients do not respond to standard treatment [18]. Despite Pap testing has acceptable results in cervical cancer detection there is growing interest for HPV DNA molecular testing in women with cervical lesions [19]. Because of the accuracy of the DNA detection based technique, in this study we used RT High-Risk HPV assay.

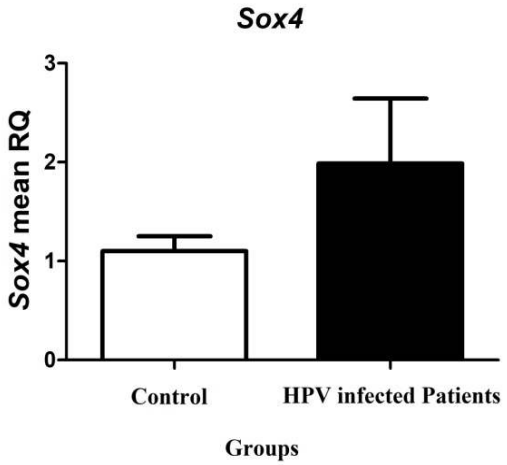

Fig. 4. The SOX_4 gene expression in normal and cervical cancer patients infected with HPV (P=0.0001)

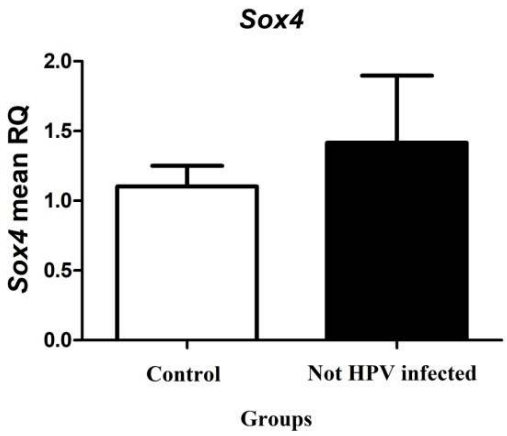

Fig. 5. The SOX_4 gene expression in normal compared to not infected patients (P=0.0439)

Table 1. The baseline information of the women included into the study

		Normal (%)	Patients (%)
Age	35<	10 (50)	23 (38.3)
	≤35	10 (50)	37 (61.7)
Marriage status	Married	18 (90)	43 (71.7)
	Single	2 (10)	17 (28.3)
Number of children	≤ 1	10 (50)	27 (45)
	≥2	10 (50)	33 (55)
Stage of disease	IC	-	21 (35)
	CIN3	-	39 (65)

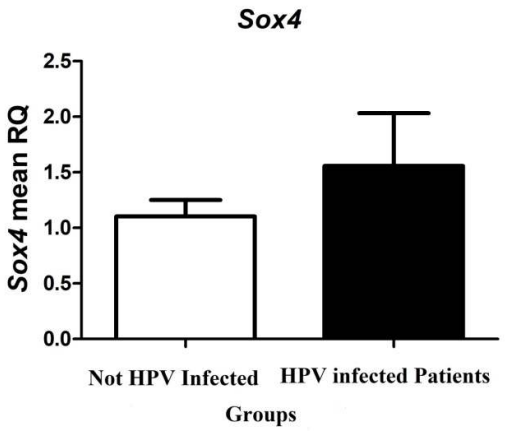

Fig. 6. The SOX4 gene expression in HPV infected compared to not infected cervical cancer (P=0.0030)

The miRNAs regulate mRNA expression by forming imperfect pairing at the 3′-end of untranslated regions (3′UTRs) of a target locus, which inhibits translation and may even promote degradation of the target mRNA [20]. Expression of the MicroRNAs has potential value as tumor biomarkers, which can use for diagnosis, prognosis and monitoring of the [17]. These days several researches were done to determine the cellular mechanisms on expression and biogenesis of the MicroRNAs in cancer [21]. According to the results, miR129-2 gene expression decreased in HPV positive compared to HPV negative infected cervical cancer patients. No significant difference observed on miR129-2 gene expression in IC compared to C_3 stages. The miR-129 is among the candidate miRNAs with potential tumor suppressor activity [22]. The miR129-2 precursor produces mature miR129-5p and miR129-2-3p [5]. Low levels of miR129-2 expression have been linked to human cancers [5]. It is reported miR129-1-3p, miR129-2-3p, and miR129-5p genes expression significantly lower in gastric cancer cells [5]. As observed, in the current study, the miR129-2 gene expression decreased in cancer which was similar to previous reports. Restoration of miR129-2 induced apoptosis [22]. Over-expression of miR129-5p reduces the proliferative activity of endometrial tumor cells and bladder cancer cells (Dyrskjot et al., 2009). It is reported over-expression of miR-129 results G_0/G_1 phase arrest, S phase cells decreased, cell proliferation was inhibited and leads to cell death [23]. miR129-2-3p and miR129-5p play key role in cell proliferation via down-regulation of CDK6 in cancer [5].

As observed, the SOX_4 gene expression increased in cervical cancer patients. SOX_4 acts as a pro-oncogene and is associated with increased cell proliferation, cell survival and metastasis with reduced apoptosis [13]. Based on the results suggested decreased miR129-2 expression might increase SOX_4 gene expression. There are several reports about the relationship between miR129-2 and SOX_4 [5]. Restoration of miR129-2 decreased SOX_4 expression and proliferation of endometrial cancer cells (Huang et al. 2009). On the other hand, methylation-mediated repression of miR129-2 increases oncogenic SOX_4 expression involved in hepatocellular carcinoma tumorigenesis (Chen et al. 2013). Also, Yu et al. [5] reported SOX_4 is targeted by miR129-2-3p and miR129-5p but not by miR129-1-3p in gastric cancer which they obtained result of the current study (correlation between miR129-2 and SOX_4 gene expression) was in agreement with previous reports. Regulation of SOX_4 expression by miR129-2, serves as an upstream regulator. An inverse association between the expression of miR129-2 and SOX_4 was found in cancer [24]. Silencing of miR129-2 by an epigenetic event, DNA hypermethylation, resulted in lost expression of this miRNA in endometrial cancer, while also resulting in overexpression of SOX_4 [13]. Shen et al. [22] reported the epigenetic repression of miR-129 led to the overexpression of SOX_4 in gastric cancer. Therefore, miR-129 may act as a tumor suppressor during the progression of gastric cancer [25]. Several researches were done to determine the cellular mechanisms on expression and biogenesis of the MicroRNAs in cancer [21]. The mechanism of for how miR-129 regulated the expression of the SOX_4 gen is still not clear, but it is probably miR129-5p interacts with the 3′UTR of MDR_1 mRNA (Zhang et al., 2015). SP_1 is a sequence-specific DNA binding protein and has a miR129-5p binding site in its 3′UTR. The upstream regulatory regions of HPV-18 genes contain the SP_1 binding site [9]. So, the SP_1 expression could be down-regulated by over-expressed miR129-5p [9]. Interferon-β dependent induction of miR129-5p cells reduced E6 and E7 expression via targeting of SP_1 in cervical cancer. Despite, there is no report on involvement of the SOX_4 gen in this pathway [24] but it is clear miR129-2 is involved in SOX_4 silencing and it is also regulated by promoter methylation. It seems, cellular researches needed to determine the direct mechanism(s) of action for observed results. In conclusion these results suggested decreased miR129-2

expression might increase SOX_4 gene expression. So, determining of the miR129-2 and SOX_4 levels might useful indicator for prognosis of the HPV-infected cervical cancer.

5. CONCLUSION

In conclusion our study has demonstrated that expression level of miR129-2 was down regulated and SOX_4 gene was up regulated in cervical cancer patients and it was associated with progression and high-risk HPV infection. These findings provide new insights into MicroRNAs and target genes involvement in HPV infected cervical cancer and indicate a potential role for these factors in the disease development. It also suggesting a potential role for prognosis and target for cervical cancer therapies, but further studies are needed to establish the potential role for MiceroRNAs and its target genes.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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