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Expressions of VEGF and miR-21 in tumor tissues of cervical cancer patients with HPV infection and their relationships with prognosis

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Abstract. – OBJECTIVE: To investigate the expressions of vascular endothelial growth factor (VEGF) and micro-ribonucleic acid-21 (miR-21) in cervical cancer patients with human papillomavirus (HPV) infection and determine the potential relationships with prognosis.

PATIENTS AND METHODS: Expressions of VEGF in cervical cancer tissues and cancer-adjacent tissues were detected by immunohistochemistry, and the expressions of miR-21 and VEGF in both tissues were quantitatively analyzed using reverse transcription polymerase chain reaction (RT-PCR). Patients with cervical cancer were followed up after operation, and the survival rates of patients with different expression levels of miR-21 and VEGF were compared.

RESULTS: VEGF was expressed in both cervical cancer tissues and cancer-adjacent tissues. The positive expression rate of VEGF in cervical cancer tissues (75.69%) was significantly higher than that in cancer-adjacent tissues (10.45%). RT-PCR results showed that the expression levels of miR-21 and VEGF in cervical cancer tissues were significantly higher than those in cancer-adjacent tissues ($p<0.05$). Correlation analyses revealed that miR-21 expression was significantly positively correlated with VEGF expression in cervical cancer tissues ($r^2=0.4174$, $p<0.0001$). Prognostic analyses showed that the 5-year survival rate of patients was relatively high when miR-21 and VEGF were lowly expressed.

CONCLUSIONS: VEGF and miR-21 are highly expressed in tumor tissues of cervical cancer patients with HPV infection. VEGF expression is significantly positively correlated with miR-21 expression, and the high levels of VEGF and miR-21 predict unfavorable prognosis of cervical cancer. Data provide a theoretical support for clinical treatment of cervical cancer patients with HPV infection.

Key Words:
HPV, Cervical cancer, VEGF, miR-21, Prognosis.

Introduction

Cervical cancer is one of the most common malignant tumors in women and ranks the second in the world among women tumors[1]. According to the latest report of the World Health Organization (WHO), there are over 500,000 new cases of cervical cancer each year, in which more than 80% of new cases occur in developing countries[2]. With changes in the living environment, the incidence rate of cervical cancer in China begins to be gradually increased, and it tends to occur in young people. Cervical cancer has become one of the important diseases that threaten women's health. Molecular epidemiological studies have shown that human papillomavirus (HPV) infection is an important factor leading to the occurrence of cervical cancer. Infection with high-risk HPV is a necessary but not sufficient condition for the development of cervical cancer in almost all patients[3]. Vascular endothelial growth factor (VEGF) has a strong growth-inducing effect on the growth of blood vessels, and it is the strongest pro-angiogenic factor known at present[4]. It has been confirmed that VEGF is directly involved in the formation of tumor blood vessels, and its overexpression in tumor tissues is closely related to the occurrence, development and other processes of tumors in the body[5]. Micro-ribonucleic acids (miRNAs) are a class of endogenous, non-coding and single-stranded RNA molecules that regulate gene expression

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by complementing and binding to target messenger RNA (mRNA)[6]. MiRNA can function as an oncogene (such as miR-221 and miR-373) or a tumor suppressor gene (such as miR127 and miR-152)[7,8]. The aim of this study was to investigate the expressions of VEGF and miR-21 in cervical cancer patients with HPV infection and analyze their relationships concerning prognosis.

Patients and Methods

Seventy-five patients with cervical cancer in our hospital from April 2010 to April 2011 were enrolled in this study. Cervical cancer tissues as well as cancer-adjacent tissues were collected. All the tissues were paraffin-embedded and serially cut into about 4 μm-thick sections. The study was reviewed and approved by Ethics Committee of our hospital and all patients participated in the study signed the informed consent.

Instruments and Reagents

Rabbit anti-human VEGF monoclonal antibodies were bought from Beijing Dingguo Changsheng Biotechnology Co., Ltd., (Beijing, China). 3,3-diaminobenzidine (DAB) reagents and citrate buffer powder were provided by Shanghai X-Y Biotechnology Co., Ltd. (Shanghai, China). Real-time quantitative fluorescence polymerase chain reaction (PCR) kits were collected from Guangzhou Vipotion Biotechnology Co., Ltd. (Guangzhou, Guangdong, China). TRIGene reagents were offered by Beijing GenStar Co., Ltd. (Beijing, China). Reverse transcription (RT) kits were obtained from Shanghai Yuduo Biotechnology Co., Ltd., (Shanghai, China). Tissue total protein extraction kits were acquired from Jiangsu Keygen Biotech Co., Ltd (Changzhou, Jiangsu, China).

Immunohistochemical Staining

Paraffin sections were dewaxed, hydrated and rinsed with phosphate-buffered saline (PBS). Blocking was conducted for 15 min, and sections were sealed using 10% serum at room temperature under the non-specific background for 15 min. The sections were added with primary antibodies (hMTERF3 and C-met monoclonal antibody, 1:200 dilution), and placed at a 4°C overnight. After the sections were taken out, they were rinsed with PBS and added with biotinylated secondary antibodies (1:1000 dilution) for incubation at room temperature for 30 min, followed by rinsing with PBS. Streptomyces antibiotic protein-peroxidase solution was added for incubation at room temperature for 30 min, followed by rinsing with PBS and 3,3'-diaminobenzidine (DAB) for color development. Next, the sections were washed with tap water, re-stained with hematoxylin, and sealed with neutral gels.

Detection of the Expressions of VEGF and miR-21 by RT-PCR

According to the instructions of TRIGene kits, the total RNAs were extracted from cervical cancer tissues and cancer-adjacent tissues, respectively. The concentrations and purities of the two kinds of total RNAs were determined using a spectrophotometer with A260/A280 value of 1.8-2.0. According to the instructions of the RT kit, primer sequences were synthesized by Shanghai Jiran Biotechnology Co., Ltd., (Shanghai, China) VEGF primer sequence: forward: 5'-TCGGGCCTCCGAAACCATGA-3', and reverse: 5'-CCTGGTGAGAGATCTGGTTC-3'. MiR-21 primer sequence: forward: 5'-GCTTCGCCTAGCTTATCAGACT-3', and reverse: 5'-CAGTGCTGGGTCCGAGTGA-3'. Reaction system with a total volume of 20 μL was reversely transcribed into cDNA on a RT-PCR machine. According to the instructions of Real-time fluorescence quantitative PCR kit (2×RealStar Green Power Mixture, GenStar, A311), 25 μL reaction system was prepared. Reaction conditions: 95°C for 10 min, 95°C for 30 s, 59.4°C for 30 s, 40 cycles, and 95°C for 15 s followed by cooling to 65°C. 5 μL products were taken for electrophoresis analysis with DL2000 DNA maker as a molecular weight control. An ultraviolet spectrophotometer was used for observation, and software was used to scan product gray value. The relative expression levels of miR-21 and VEGF were calculated with U6 small nuclear RNA and β-actin as internal references, respectively.

Evaluation of Experimental Results

A total of 100 cells counted in the fields of view to be observed were randomly selected, and the average number of cells in the fields of view was calculated as the positive cell number of the expressed protein within tissues. Scores of color depths: 0-2 points represented no coloring, weak coloring and strong coloring, respectively. Scores of stained cell positive rate: 1-4 points represented the percentage of positive cells, namely, [1,25], [26,50], [51,75] and [76,100], respectively. Products of the above two components: ≤ 2 points

Table I. VEGF expression in tissues.

	n	Expression degree				Positive rate (%)	χ^2	p
		-	+	++	+++			
Cancer-adjacent tissues	74	67	4	3	0	10.45	29.015	0.000
Cervical cancer tissues	74	18	20	24	12	75.68		

for negative, 3-4 points for weakly positive (+), 5-8 points for moderately positive (++), and ≥ 9 points for strongly positive (+++).

Follow-Up

Cervical cancer patients with HPV infection were followed up for 5 years after operation, and the survival status of all patients was recorded. The median expression levels of miR-21 and VEGF were taken. The expression higher than the median was recognized as high expression, while that lower than the median was recorded as low expression. Next, the correlation analysis was conducted.

Statistical Analysis

The professional statistical software Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 20.0 was used in this experiment for data analyses. Measurement data were expressed as mean ± standard deviation. *t*-test was used for comparisons of independent factors between two groups. Pearson's correlation analysis was used to assess the relations of VEGF and miR-21 expression. Count data were compared using χ^2-test. $p<0.05$ represented that the difference was statistically significant.

Results

Detection of the Expression of VEGF in HPV-Infected Cervical Cancer Tissues By Immunohistochemistry

Immunohistochemistry showed that VEGF was mainly expressed in the nucleus and cytoplasm. The positive expression rate of VEGF was 10.45% (7/67) in cancer-adjacent tissues and 75.69% (56/74) in cervical cancer tissues. The positive expression rate of VEGF in cervical cancer tissues was significantly higher than that in cancer-adjacent tissues ($\chi^2=29.015$, $p<0.05$) (Figure 1 and Table I).

MiR-21 Expression in HPV-Infected Cervical Cancer Tissues

By using U6 small nuclear RNA as an internal reference, the expression level of miR-21 in different samples was calibrated. It was found that the expression level of miR-21 in 74 cases of cervical cancer tissues was (2.25±0.13), while that in cancer-adjacent tissues was (0.93±0.08). The statistical scatter diagram analysis revealed that the expression level of miR-21 in cervical cancer tissues was significantly higher than that in cancer-adjacent tissues ($p<0.05$) (Figure 2).

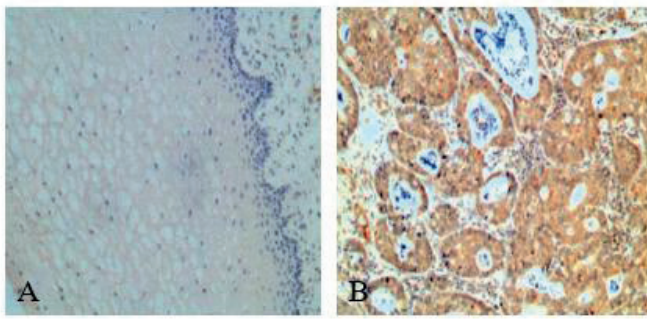

Figure 1. Detection of the expression of VEGF in tissues by immunohistochemistry (×100). ***A***, Cancer-adjacent tissues; ***B***, Cervical cancer tissues.

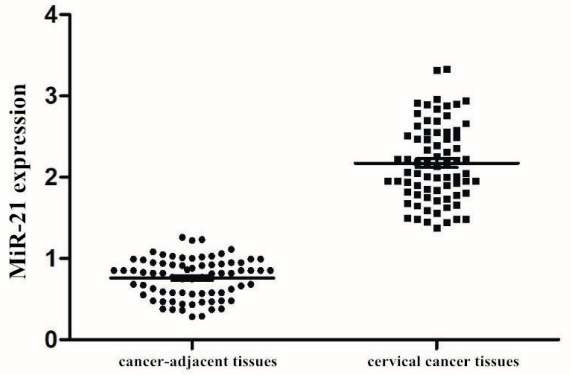

Figure 2. MiR-21 expression in cervical cancer tissues and cancer-adjacent tissues.

VEGF Expression in HPV-Infected Cervical Cancer Tissues

With β-actin as an internal reference, the expression level of VEGF in different samples was detected and showed that the level of 74 cases of cervical cancer tissues was (3.88±0.25), while that in cancer-adjacent tissues was (1.94±0.14), the difference of which was statistically significant (p<0.05) (Figure 3).

Correlation Between miR-21 Expression and VEGF Expression in Cervical Cancer Tissues

Statistical analyses were conducted and showed that there was a significant positive correlation between miR-21 expression and VEGF expression in cervical cancer tissues (r^2=0.4174 and p<0.05) (Figure 4).

Prognostic Analysis

Analysis showed that the 5-year survival rate of patients with lowly expressed miR-21 was higher than that of patients with highly expressed miR-21, and the difference was statistically significant (r^2=6.004 and p=0.0143). Similarly, the 5-year survival rate of patients with low level of VEGF was higher than that of patients with highly expressed VEGF, and the difference was statistically significant (r^2=7.575 and p=0.0059) (Figure 5A, 5B).

Discussion

Researches confirmed that the main cause of cervical cancer is the continuous infection with high-risk HPV. Although HPV vaccines are available on the market, the incidence rate of cervical cancer has not been decreased, especially in developing countries. Additionally, cervical cancer has emerged as one of the most serious diseases that threaten women's health. Therefore, it is particularly important to study the mechanism of cervical cancer's occurrence and development.

VEGF is encoded by a single gene and contains 8 exons and 7 introns. Investigations have shown that VEGF is mainly synthesized by tumor cells, vascular endothelial cells and macrophages. Through the binding with receptors on vascular endothelial cells by various methods, it promotes the process of endothelial cell growth, proliferation and vascular reconstruction[9]. At present, consensus has been reached on the fact that the further growth of solid tumors depends on the formation of tumor neovascularization. However, the formation of neovascularization is rather complicated and summarized in three steps: 1) The extracellular matrix (ECM) is largely hydrolyzed, 2) endothelial cells metastasize and continue to proliferate, and 3) differentiation

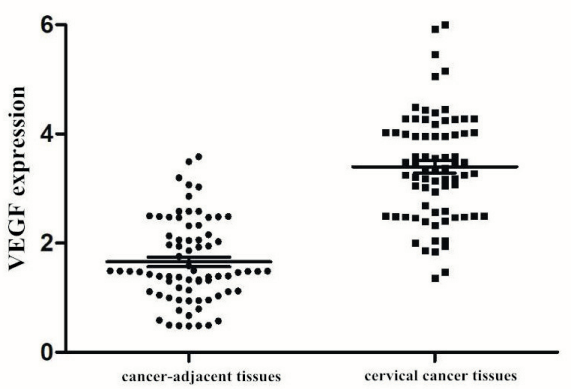

Figure 3. VEGF expression in cervical cancer tissues and cancer-adjacent tissues.

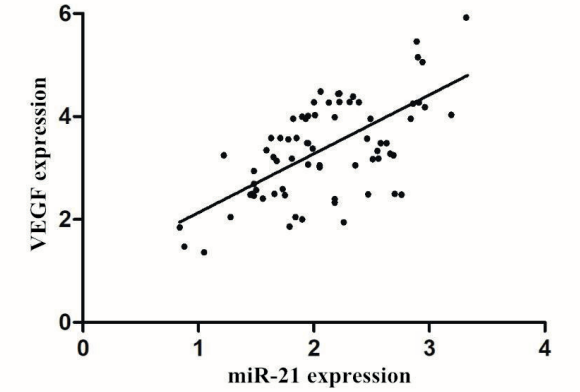

Figure 4. Correlation between miR-21 expression and VEGF expression in cervical cancer tissues.

and anastomosis occur in the blood capillaries[10-12]. VEGF is involved in all three stages. Liu et al[13] found that VEGF is relatively highly expressed in cervical cancer tissues but the level in normal cervical tissues is extremely low, indicating that the expression level is closely related to the clinical stage of cervical cancer and the possibility of cervical cancer metastasis occurrence. Peralta-Zaragoza et al[14] found that the degree of VEGF expression has a certain guiding role in the development of cervical cancer. However, Park et al[15] reported that the positive rate of VEGF expression in cervical cancer tissues increased with lymph node metastasis, suggesting that VEGF may be involved in the process of lymph node metastasis in cervical cancer patients. The results of immunohistochemistry in this study revealed that VEGF was mainly expressed in the nucleus and cytoplasm, while the positive expression rate of VEGF in cancer-adjacent tissues was significantly lower than that in cervical cancer tissues, consistently with previous research results. It has been found[16] that the abnormal expression of miRNA is associated with the occurrence of a variety of tumors, such as, cervical cancer. Mandic et al[17] showed that miR-21 was overexpressed in cervical cancer tissues, and the expression of miR-21 in HPV-infected cancer tissues was higher than that in cancer tissues without HPV infection, suggesting that miR-21 expression may be associated with the occurrence of HPV-infected cervical cancer to a certain degree. Shishodia et al[18] used pcDNA-HPV16 E7 to transfect cancer cells, which showed that miR-21 was abnormally expressed in cells and significantly higher than that in untransfected group, and cancer cell proliferation and invasion were significantly increased. In another study[18], pcDNA-HPV16 E6 and E7 genes were transfected into cancer cells, miR-21 expression began to rise, suggesting that miR-21 may be involved in the occurrence process of cervical cancer with HPV16 infection. In this study, it was found that the expression of miR-21 in tumor tissues of cervical cancer patients with HPV infection was significantly increased, which is consistent with the existing conclusion. Based on the analysis of the expressions of miR-21 and VEGF in cervical cancer tissues, it was found that there was a significant positive correlation between their expressions. Zhang et al[19] found that miR-21 can promote the proliferation and metastasis processes of cervical cancer cells by inhibiting the expression of phosphatase and tensin homolog (PTEN) gene. However, Yue et al[20] presented that the inhibition of PTEN expression in canine mammary tumors can significantly up-regulate the expression of VEGF and result in tumor formation. Therefore, it is speculated that this process may exist in tumor tissues of cervical cancer patients with HPV infection, but the specific mechanism remains to be further studied. Patients with HPV-infected cervical cancer were followed up, and the results revealed that when miR-21 and VEGF were lowly expressed, the survival rate of patients was significantly higher, which provides a new suggestion for clinical treatment of patients with HPV-infected cervical cancer.

Conclusions

We demonstrated that both VEGF and miR-21 are highly expressed in tumor tissues of cervical cancer patients with HPV infection, the levels between which are positively correlated. The combination of VEGF expression and miR-21 expression may offer novel insights for the prognosis of cervical cancer patients with HPV infection.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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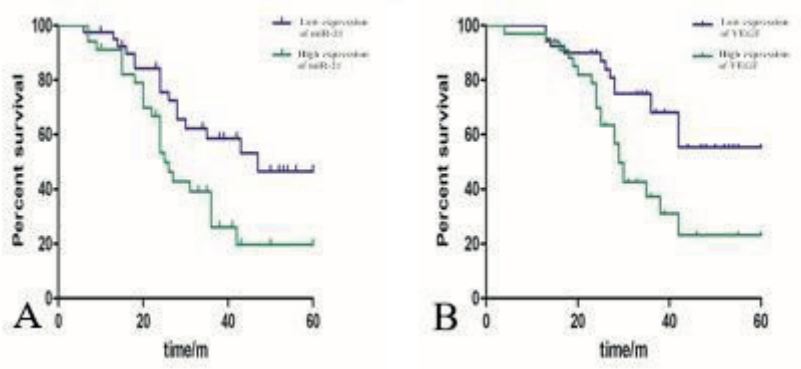

Figure 5. Prognostic analysis of patients with cervical cancer. Note: ***A,*** Prognosis of miR-21 expression; ***B,*** Prognosis of VEGF expression.

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