Determination of Expression of VEGF-mRNA in Cervical Cancer Tissues by Real-time Fluorescent Quantitative RT-PCR

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Abstract Objective To investigate the expression of vascular endothelial growth factor (VEGF) mRNA in cervical cancer tissues, and the relationships between VEGF-mRNAs expression and the tumor metastasis, prognosis were analyzed. **Methods** The levels of VEGF-mRNA expression in 48 cases of invasive carcinoma of cervix, 30 cases of carcinoma in situ and 36 cases of normal controls were detected by TaqMan real-time fluorescent quantitative RT-PCR. **Results** The level of VEGF-mRNA expression in invasive carcinoma of cervix was higher than that in all matched. And there was no significant relationship between the expression levels of VEGF-mRNA with the pathological types in invasive carcinoma of cervix. But the levels of VEGF-mRNA expression was significant associated with the clinical stage ,the grade of tumor pathology, lymph node metastasis, tumor size and the invasion of deep muscular layer ($P<0.05$). **Conclusion** VEGF may play an important role in genesis and development of invasive carcinoma of cervix, and may be used as a potential molecular target for the treatment of malignant tumors.

Key words Real-time fluorescent quantitative RT-PCR; Vascular endothelial growth factor; Cervical neoplasms

Cervical cancer is the second most familiar malignant tumor in the global women, which was only next to mammary cancer. Its incidence presents to go up and trends towards lower age, the half of the patients didn´t yet be menostasia[1]. The pathogenesis of the cervical cancer haven´t yet clarified completely yet, and there is still many problems to resolve in diagnosis, the same time, the treatments, besides the surgical operation, make progress slowly. The research about biotherapy to the tumor taking new vessel as the target has become a new hot spot in the last few years. The vascular endothelial growth factor (VEGF) is the most important angiogenesis factor which could promote the differentiation, accrementition, immigration and infiltration of vscular endothelial cells. It is related with the growth, infiltration and metastasis of the tumor[2,3]. Domestic and international scholars have researched plenty about the expression and meaning of VEGF in the cervical cancer. The qualitation or semiquantitative methods, such as the reverse transcription-polymerase chain reaction (RT-PCR), immunity and histochemistry, and hybridization in situ, which have been adopted in our clinic work, were discovered not enough ideal in the sensibility and specificity because they can´t reflect each characteristics of patients tumor and prognosis completely. In this article, we adopt the quantitative method, the real time fluorescence quantitative PCR (FQ-PCR) technique to investigate the VEGFmRNA expression and meaning in the cervical cancer and the normal cervical tissues.

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METERIALS AND METHODS

Clinic data

Fourty-eight samples of invasive carcinoma of cervix, 30 samples of carcinoma in situ and 30 samples of normal controls taken from the patients who were

operated in our hospital gynecology from March 2004 to December 2005. All patients hadn't received radiotherapy or chemotherapy preoperative and they have integrity clinical data. Of them, 15 samples were in Ⅰb stage, 20 samples in Ⅱa stage, 13 samples in Ⅱb stage according to FIGO criterion; 12 cases were adenomous cancer, 32 cases of SqCa, 4 cases of adenosquamous carcinoma according to histological type in WHO; and according to pathological differentiation degree, 6 cases were well-differentiated, 22 cases were moderately differentiated, and 20 cases poorly differentiated. Postoperation to confirm 29 cases with lymph nodes metastasis, and 19 cases without. 28 cases tumor diameter were less than 4cm, 20 cases tumor diameter were more than 4cm. 27 cases carcinoma was limited in light muscular layer, 21 cases have deep muscular layer infiltration.

Sample collection

After the excision, the samples were taken in germ free condition around the carcinoma tissues which grow active and put in liquid nitrogen after cutting them into piecees, then transferred to -70℃ freezer. We sampled the normal cervical tissues in the same way, which were all taken from patients suffered hysterectomy for hysteromyoma.

Reagents

Roche Cycle from Germeny, Trizol reagent from Gibco, TAKARA Exscript TM RT-PCR Kit(Perfect Real Time)from dalian baosheng company.

Methods

Detection of the total RNA According to the instruction, the RNA absorbency was measured under 260nm and 280nm using UV4501S, then calculate the ratio OD260/OD280. All the ratios should be in the range of 1.75~1.95. Less than the lower limit means containing protein impurity and a further detection need to be done. The RNA sample should be preserved in -70℃ freezer.

Detection of fluorescent quantitation RT-PCR reaction The application of sample was progressed on the ice, centrifugate the capillaries with reaction liquid for 15s at 3000r, progress the Real Time RT-PCR reaction in Light Cycler. Fluorescent quantitation RT-PCR reaction system: Premix Ex Taq™(2×)10μl, PCR Forward Primer 0.7μl, PCR Reverse Primer 0.7μl, fluorescent probe solution 0.8μl, DNA template (cDNA solution) 2.0μl, dH_2O 5.8μl, total 20.0μl, all the sample additions were done under glacial environment, 95℃, 5 seconds; 60℃, 20 seconds; total 40 cycles.

GAPDH endocounsel gene amplified with VEGF gene the same time and analysis the result together after the reaction, ABI PRISM 7500 fluorescent quantitation RT-PCR Amplifier is used to do the PCR reaction. The quantity of PCR product was tested once a amplification circle. All the reaction product were collected by the ABI PRISM 7500 Amplifier and stored in the corresponding analysis software. Calculate the relative quantitative expression of VEGFmRNA according to the forma as follows: △CT(destination gene)=destination gene CT-endocounsel gene CT, △△CT=△CT (destination gene)-△CT (certified value), destination gene relative total amount is $2^{-\triangle\triangle CT}$. CT value means the cycle at which the fluorescence signal reach the threshold, and it has an negative correlation with quantitative expression of VEGF mRNA: the higher the CT value, the lower the quantitative expression of VEGF mRNA.

Statistical analysis

Statistical analysis was performed with SPSS14.0 software, $P<0.05$ was considered as significance in statistics.

RESULTS

Expression of VEGF mRNA in the tissues of tumor, carcinoma in situ and the normal controls

The expression of VEGF mRNA in the tissues of tumor, carcinoma in situ and the normal controls were detected by means of quantitative method $2^{-\triangle\triangle CT}$ formula, the amount of expression of normal cervical tissues VEGF mRNA was used as standard. In this foundation the expressions of the VEGF mRNA in the tissues of tumor, carcinoma in situ were compared (see table2, figure1, 2). Statistics analysis showed that the VEGF mRNA expression in three kinds of tissues have

Table 1 Primer and detecting head sequence

Variable		Sequence	Tm value	Amfilication part(bp)
VEGF	primer	5′-AAGATCCGCAGACGTGTAAATGTT-3′	60	100
		5′-CGGCTTGTCACATGCAAGTA-3′	60	
	probe	5′-GCAAGGCGAGGCAGCTTGAGT-3′	60	100
GAPDH	primer	5′-CTTAGCACCCCTGGCCAAG-3′	60	150
		5′-GATGTTCTGGAGAGCCCCG-3′	60	
	probe	5′-CATGCCATCACTGCCACCCAGAAGA-3′	60	150

Table 2 Expression of VEGF mRNA in different tissues

Varials	Cases	△Ct value	△△Ct	$2^{-\triangle\triangle Ct}$	*P* value
normal cervical	36	12.72±1.58	0.00±1.58	1.00(0.33-2.99)	
carcinoma in situ	30	11.40±1.00	-1.32±1.00	2.50(1.25-4.99)	
cervical cancer	48	9.90±1.27	-2.82±1.27	7.06 (2.93-17.03)	*P*<0.05

Table 3 expression of VEGF mRNA in the cervical cancer with the clinical pathology factors

Varials	Cases	△Ct value	△△Ct	$2^{-\triangle\triangle Ct}$	*P* value
Clinical stage					
Ⅰb	15	11.15±0.83	-1.57±0.83	2.97(1.67~5.28)	*P*<0.05
Ⅱa	20	9.91±0.80	-2.81±0.80	7.01(4.03~12.21	
Ⅱb	13	8.43±0.45	-3.29±0.45	9.78(7.16~13.36)	
Histology typing					
adenocarcinoma	12	9.71±1.41	-3.01±1.41	8.06(3.03~21.41)	*P*>0.05
SqCa	32	9.96±1.30	-2.76±1.30	6.77(2.75~16.68)	
denosquamous	4	9.98±0.67	-2.74±0.67	6.68(4.20~10.63)	
Pathology grade					
G1	6	11.81±0.61	-0.91±0.61	1.88(1.23~2.87)	P<0.05
G2	22	10.44±0.77	-2.28±0.77	4.86(2.85~8.28)	
G3	20	8.73±0.56	-3.99±0.56	15.89(10.78~23.43)	
Lymph nodes					
metastasis	29	9.07±0.75	-3.65±0.75	12.55(7.46~21.11)	P<0.05
no metastasis	19	11.17±0.72	-1.55±0.72	2.93(1.78~4.82)	
Tumor diameter					
⩾4cm	20	8.84±0.62	-3.88±0.62	14.72(9.58~22.63)	P<0.05
<4cm	28	10.66±1.06	-2.06±1.06	4.17(2.00~8.69)	
Invasion					
deep muscular layer	21	8.86±0.59	-3.86±0.59	14.52(9.65~21.86)	P<0.05
in superficial layer	27	10.71±1.05	-2.01±1.05	4.03(1.95~8.34)	

significant difference (P<0.05). As shown, the VEGF mRNA expression is obviously high in carcinoma in situ than that in the normal control, in the other hand, expression in tumor is high than that in another two (P<0.05).

Relation between the VEGF mRNA expression and clinical pathology

The 48 cases of the tumor were divided into different teams as the standard of histology type, clinical

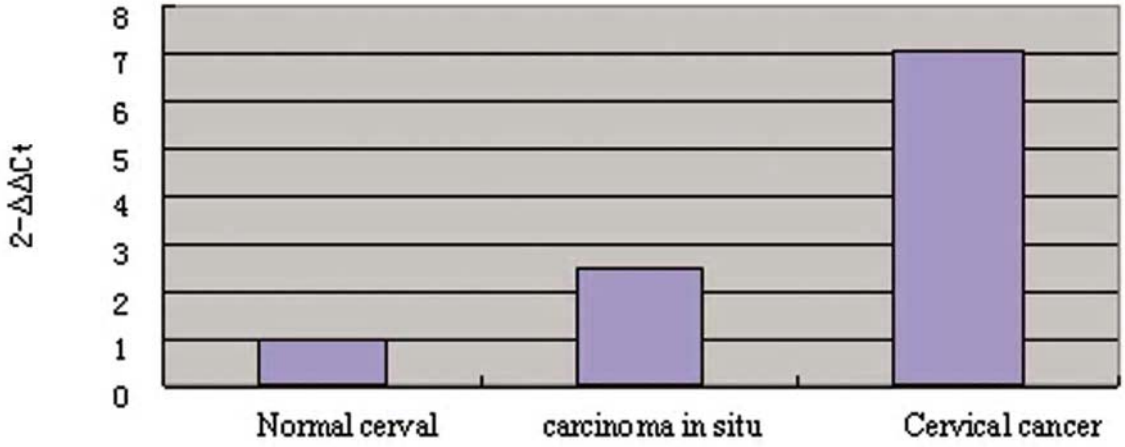

Fig. 1 Expression of VEGF mRNA in different tissues

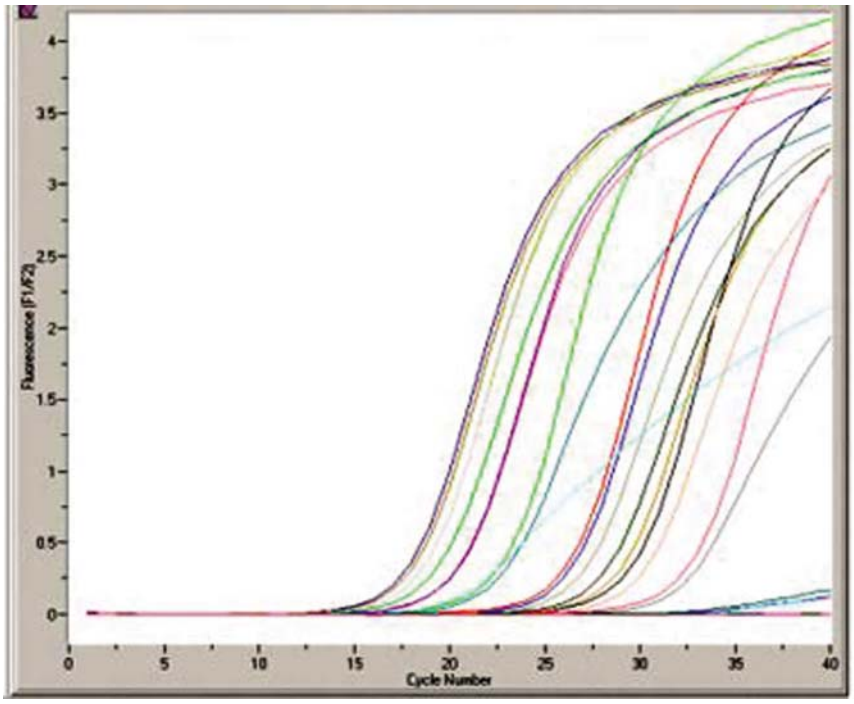

Fig. 2 Fluorescent quantitation RT-PCR amplification curve chart of VEGF mRNA in different tissues

stage, pathology differentiation, lymph nodes metastasis, tumor diameter and deep muscular layer invasion, taking the expression of the VEGF mRNA in normal control as a reference standard, the influence of the factors, such as the histology type, clinical stage, pathology differentiation, etc were detected. The results is shown in table 3, fig. 3 and 4, which showed that the expression of the VEGF mRNA has no significant different with the histology type of the tumor ($P>0.05$), but it closely related with the clinical stage, pathology differentiation, lymph nodes metastasis, tumor diameter and deep muscular layer invasion($P<0.05$). The high expression of the VEGF mRNA is related with the late clinical stage and the low pathological differentiation degree ($P<0.05$), as well as lymph metastasis, tumor diameter is more than 4 cm and deep muscular layer infiltration($P<0.05$).

DISCUSSION

The real time fluorescence quantitative PCR (FQ-PCR) is a new technique developed in recent years [5]. it is extensively applied on the clinic currently as a kind

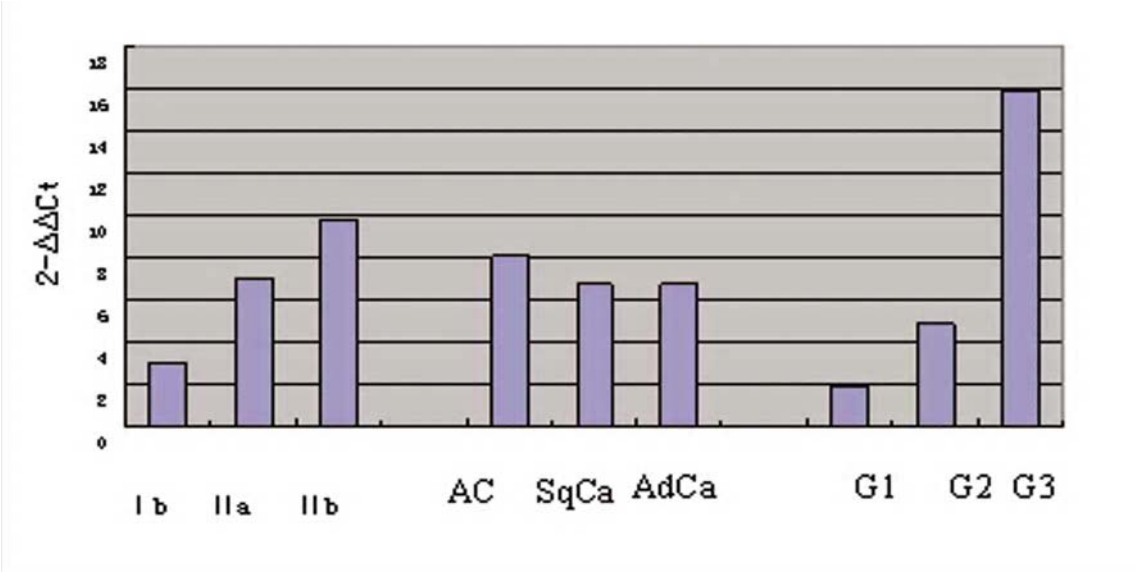

Fig. 3 clinical stage, histology type, differentiation of cervical carcinoma with expression of VEGF mRNA.

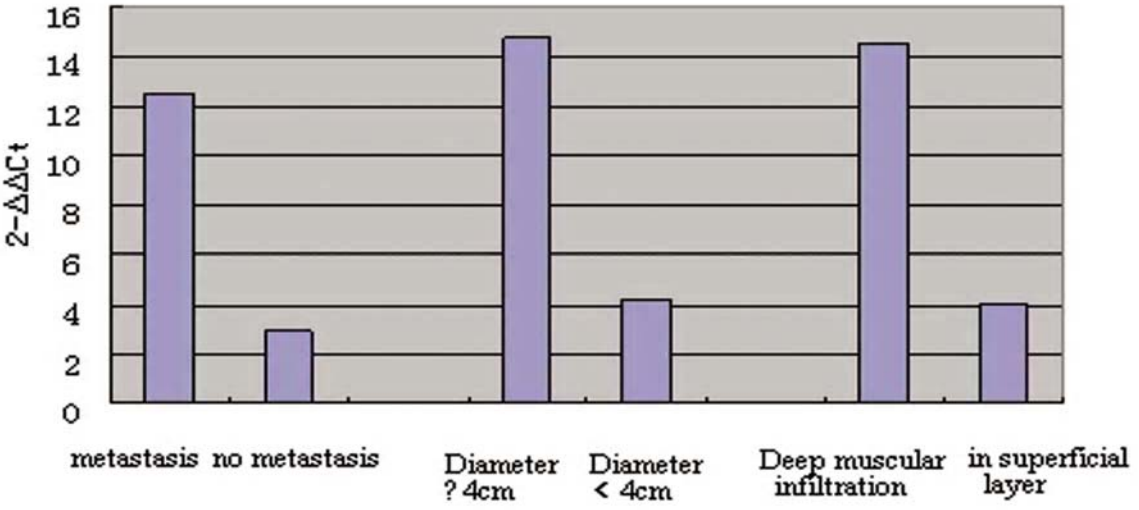

Fig. 4 the temple neck cancer the lymph node transfer, the tumor diameter,deep musclar layer expresses the relation of the quantity with VEGFmRNA gradually

of gene quantitation technique, it is not only to examine gene of mutation effectively, and can be accurately to examine the amount of expression of gene with related tumor. Thereby the earlier diagnosis and the types of tumor may be made, and the metastasis may be discovered earlier with FQ−PCR, furthermore to judge the prognosis in early days of the tumor. Moreover, applying FQ−PCR to examine the quantity of tumor marking in blood and quantity of gene mutation resulted from rotooncogene translocation may provide a more dependable basis in early days for the clinic in judging curative effect and establishment of the treatment project.

The VEGF is a kind of high particularity and selectivity mitogen of the vascular endothelial cell, it can induce the endothelial cell hyperplasia and immigration, and repress apoptosis in the meantime in vitro, and in vivo it is a important factor in angiogenesis, which can particularity stimulate multiplication, promote angiogenesis, increase blood vessel permeability, and correlate with relapse, metastasis and prognosis of various tumors. Similar to other entity tumors, the growth, transfer and

prognosis of the cervical carcinoma were closely related with angiogenesis. Research finished by Lee etc. [6] showed the VEGF have a function of promote the cervical cancer blood vessel formation. Fujimoto *et al.* [7] found the expression of VEGF121 and VEGF165 in cervix and the cervical cancer, and both expression level go up obviously in the advanced stage of the cervical cancer, which suggested the VEGF121 and the VEGF165 play important roles in the angiogenesis in the later period of cervical cancer. From damaged cervix endepidermis to cell canceration, the VEGF synthesis and secretion increase, and with the increasing of VEGF expression, its ability enhance. VEGF may participate in the positive regulation during the cervical cancer angiogenesis, and may be an important regulate factor in angiogenesis. There is another research [8] discovered overexpression of VEGF mRNA in the cervical cancer in early days, and besides the Ⅳb stage, each stage had something to do with the VEGFmRNA level, so VEGF was thought to play an important role in the cervical cancer earlier period.

This research applied the FQ-PCR technique to detect the VEGF mRNA level in 48 cervical cancer cases, 30 carcinoma in situ and 36 normal controls, the result manifested VEGF mRNA expression level in the cancer is obviously higher than that in the other two groups. Dai etc.[9] discovered the expression of VEGF goes up with the deterioration of infiltrating cervical cancer. This research shows that the VEGF mRNA expression level increases with the development of cervical cancer, which agrees with Dai, VEGF play an important role in the cervical cancer development.

The cervical cancer mainly metastasize through a lymphatic path, in the clinic the evaluation of prognosis and guiding the treatment commonly depend on whether lymphatic metastasis developed. So, predicting and detecting lymphatic metastasis in early days has an important and clinical meanings. Cheng etc.[10] reported, expression of VEGF in the cervical cancer with lymph nodes metastasis was higher than that in cervical cancer without lymph nodes metastasis, the excessive expression of VEGF leads to pelvic lymph nodes transfer, which agrees with our research. As for this relativity is whether the VEGF promotes directly a lymphatic vessels neogenesis is still unclear. Moreover, this research demonstrated VEGF expression was related with the cervical cancer cell grades, and the expression of VEGF in poorly differentiated group was significant higher than that in well-differentiated group, which indicated that expression of VEGF was related with the malignant degree of the tumor. Some research demonstrated the overexpression of VEGF was correlated with the tumor size, deep mesenchymal infiltration, the lymph vessels cancerous embolism, the latero-palace infiltration and pelvic lymph nodes metastasis in the cervical cancer, the results suggested the VEGF may be a worthy cancer prognostic indicator. Further analysis shown the relation beteen the varies clinical pathologic sign and cervical cancer VEGF mRNA level. The results of this experiment displayed VEGF mRNA level in tumor with deep muscle layer infiltration and in the tumor which diameter is >4cm went up obviously, the VEGF expression level possibility still reflecting to propagate activity of the tumor cell, which hinted in some time, the level of VEGF mRNA was related with tumor local infiltration and transfering potential, and prognosis. As a result, the author suggested that, for the patients with high VEGF mRNA level preoperative, attention should be given to the possibility of lymphatic metastasis and malignant cell infiltration, and a thorough scavenge of lymphaden is necessary. But the research of the relation about the VEGF expression and the cervical cancer occurrence, development, infiltration, transfer and prognosis etc, is still in a beginning stage, and need more extensively study.

In conclusionin, VEGF may play an important role in genesis and development of invasive carcinoma of cervix, and may be used as a potential molecular target for the treatment of malignant tumors.

REFERENCE

1 Fields A, Jones JG, Thomas GM, *et al.* Gynecologic Cancer. In: Lenhard RE Jr, Osteen RT, Gansler T, eds. Clinical Oncology. Atlanta, Ga: American Cancer Society, 2001: 455-496.

2 Harada Y, Ogata Y, Shirouzu K. Expression of vascular endothelial growth factor and its receptor KDR/Flk-1 as prognostic factors in human colorectal cancer. Int J Clin

Oncol, 2001, 6:221-228.

3 Zhang H, Wu J, Meng L, *et al.* Expression of vascular endothelial growth factor and its receptor KDR and Flk-1 in gastric cancer cells. World J Gastroenterol, 2002, 8: 994-998.

4 Burger H, Foekens JA, Look MP, *et al.* RNA expression of breast cancer resistance protein, lung resistance -related protein, multi-drug resistance-associated protein 1 and 2, and multi -drug resistance gene 1 in breast cancer: Correlation with chemotherapeutic response. Clin Cancer Res, 2003, 9: 827-836.

5 Ahrendt SA, Yang SC, Wu L, *et al.* Molecular assessment of lymph nodes in patients with resected stage I non-small cell lung cancer: preliminary results of a prospective study. J Thoracic Cardiovasc Surg, 2002, 123: 466-473.

6 Lee IJ, Park KR, Lee KK, *et al.* Prognostic value of vascular endothelial growth factor in Stage IB carcinoma of the uterine cervix. Int J Radiat Oncol Biol Phys, 2002, 54: 768-799.

7 Fujiwaki R, Hata K, Iida K, *et al.* Vascular endothelial growth factor expression in progression of cervical cancer: correlation with thymidine phosphorylase expression, angiogenesis, tumor cell proliferation, and apoptosis. Anticancer Res, 2000, 20: 1317-1322.

8 Fujimoto J, Sakaguchi H, Hirose R, *et al.* Expression of vascular endothelial growth factor (VEGF) and its mRNA in uterine cervical cancers. Br J Cancer, 1999, 80: 827-833.

9 Dai Y, Zhang X, Peng Y, *et al.* The expression of cyclooxygenase -2, VEGF and PGs in CIN and cervical carcinoma. Gynecol Oncol, 2005, 97: 96-103.

10 Cheng WF, Chen CA, Lee CN, *et al.* Vascular endothelial growth factor and prognosis of cervical carcinoma.Obstet Gynecol, 2000, 96: 721-726.