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Original Paper

Vascular Endothelial Growth Factor is Implicated in Early Invasion in Cervical Cancer

J. Kodama, N. Seki, K. Tokumo, A. Hongo, Y. Miyagi, M. Yoshinouchi, H. Okuda and T. Kudo

Department of Obstetrics and Gynecology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

The association between the expression of vascular endothelial growth factor (VEGF) and clinicopathological factors has scarcely been examined in cervical cancer. This study examines the level of VEGF messenger RNA (mRNA) expression in invasive cervical cancer and its association with clinicopathological features including microvessel density. The level of VEGF mRNA was assessed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) using β-actin as an internal control in 66 patients with stages Ia–IVb invasive cervical cancer. In 42 patients who underwent surgery, the microvessel count was also assessed by immunostaining for factor VIII-related antigen in the most neovascularised area of the specimen. The highest level of VEGF mRNA expression was observed in early invasive cervical cancers. Except for stage IVb, the stage of the disease inversely correlated with the level of VEGF mRNA ($P < 0.05$). There was no significant difference in the level of VEGF mRNA with respect to histological cell types. 38 patients with stages Ib–IIb cervical cancer underwent radical hysterectomy and pelvic lymphadenectomy. There was no significant difference in the level of VEGF mRNA with respect to lymph node metastasis, depth of stromal invasion, tumour size, parametrial involvement or vaginal involvement among these patients. A significant relationship was found between the microvessel density and the level of VEGF mRNA ($P < 0.01$). These findings provide evidence that the expression of VEGF is involved in the promotion of angiogenesis in cervical cancer and plays an important role in early invasion. © 1999 Elsevier Science Ltd. All rights reserved.

Key words: vascular endothelial growth factor, angiogenesis, cervical cancer, reverse transcription–polymerase chain reaction
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INTRODUCTION

ANGIOGENESIS, THE development of new blood vessels, is essential in tissue development, reproduction and wound healing [1]. Solid tumours require angiogenesis for progression and metastasis. In fact, tumour growth beyond 1–2 mm is strictly dependent on angiogenesis [2]. Angiogenesis also contributes to the metastatic process, carrying cancer cells into the circulation [3].

Tumour tissues secrete angiogenic factors that activate neovascularisation around tumours [1]. Vascular endothelial growth factor (VEGF) was originally detected in the conditioned medium of bovine pituitary folliculostellate cells. Vascular permeability factor (VPF) was identified in tumour ascites and was found to be identical to VEGF [4, 5]. VEGF is detectable in a number of tumour cell lines and tumour tissues and is thought to be a selective growth factor for endothelial cells [6]. Four molecular isoforms of VEGF are generated by alternative splicing, rendering proteins containing 206-, 189-, 165- and 121-amino acid residues [7]. The two shorter isoforms, VEGF165 and VEGF121, are secreted proteins which may act as diffusible agents, whereas the longer isoforms remain cell associated [8, 9].

Recently, it was reported that microvessel density correlates with immunoreactivity of VEGF in stages Ib—IIb cervical cancer [10]. There has been no study investigating the

Correspondence to M. Yoshinouchi, e-mail: m_yoshi@cc.okayama-u.ac.jp
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level of VEGF mRNA in invasive cervical cancer. The present study examines both the expression of VEGF mRNA in stage Ia–IVb invasive cervical cancer tissues by semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) and its association with clinicopathological features including microvessel density.

MATERIALS AND METHODS

Tissue samples

The patient population consisted of 66 patients with a diagnosis of invasive cervical cancer at the Department of Obstetrics and Gynecology of Okayama University Medical School, Okayama, Japan, who underwent treatment from 1995 to 1996. Biopsy specimens obtained by colposcopy were available at the time of admission and each specimen was bisected. One portion was snap frozen and stored at − 80°C until RNA was extracted. The other portion was fixed in 10% formaldehyde solution for histopathological examination. Specimens that did not contain significant cancer cells by histopathological diagnosis were excluded from the study.

The histological cell types of the tumours were assigned according to the World Health Organisation (WHO) classification: 35 were classified as squamous cell carcinoma, 20 as adenocarcinoma and 11 as adenosquamous carcinoma. Staging was reviewed based on the International Federation of Gynecology and Obstetrics (FIGO) staging system: 4 were stage Ia, 14 were stage Ib, 34 were stage II, 10 were stage III, 2 were stage IVa and 2 were stage IVb (lung metastasis). Simple hysterectomy was carried out in 4 patients with stage Ia disease. Radical hysterectomy and pelvic lymphadenectomy was carried out in 34 patients with stage Ib–IIb disease who were in good physical condition.

RNA preparation

Total RNA was extracted from each specimen using the RNeasy Total RNA kit (Qiagen, California, U.S.A.) according to the manufacturer's protocol. Tissue with RNA displaying high-quality 18S and 28S bands on ethidium bromide-stained gels was selected.

Semi-quantitative reverse transcription–polymerase chain reaction

RT-PCR was carried out according to the RNA PCR Kit (Takara, Kyoto, Japan) protocol for reverse transcription of 1 μl total RNA with subsequent amplification of cDNA. Transcribed products were subjected to PCR for VEGF (sense primer, 5′-CGAAGTGGTGAAGTTCATGGTG-3′; antisense primer, 5′-TTCTGTATCAGTCTTTCCTGGTGAG-3′) [8] and β-actin (sense primer, 5′-CTCACCATGGATGATGATAT-3′; antisense primer, 5′-TGGGTCATCTTCTCGCGGTT-3′) [11]. All oligodeoxynucleotides were synthesised on a Model 394 DNA synthesiser (PE Applied Biosystems, California, U.S.A.). Amplification for VEGF cDNA was started with a 3-min denaturation at 94°C followed by cycles of 30-sec denaturation at 94°C, 30-sec annealing at 65°C, 30-sec extension at 72°C and final extension at 72°C for 15 min. The PCR profile for β-actin consisted of a 3-min initial denaturation at 94°C followed by cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C and 1-min extension at 72°C. The PCR mixture was then kept at 72°C for 15 min for final extension. The composition of the PCR mixture has been described elsewhere [12]. Final PCR products were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. Ultraviolet (UV)-illuminated gels were photographed using Polaroid Type 667 films. Photographs were quantitated with an image scanner GT-9500 (Epson, Suwa, Japan) and analysed with Basic Quantifier software (Bio Image, Ann Arbor, Michigan, U.S.A.). The intensity of β-actin amplification was used as an internal standard.

To validate target mRNA quantitation, the number of amplification cycles required for linearity was tested. Band intensities from the final products were examined in representative cases. Linear range was obtained at 27 cycles for VEGF and at 23 cycles for β-actin. Then, the relative ratio of VEGF/β-actin PCR products (V/A ratio) was calculated with these numbers of PCR cycles.

Immunohistochemical staining for microvessels

Expression of factor VIII-related antigen was assessed in 42 formalin-fixed, paraffin-embedded sections obtained at the time of surgery with the abidin–biotin complex (ABC) procedure as described elsewhere [13]. Antifactor VIII-related monoclonal antibody (Dakopatts, Copenhagen, Denmark) was used as a primary antibody. The entire tumour was scanned under low-power magnification to select areas with the most intense vascularisation. The number of microvessels was recorded by counting any positively stained endothelial cell or endothelial cell cluster as a single, countable microvessel in a 100×microscopic field (0.618 mm^2) in 10 neovascularised areas. The mean value of the top three counts was used as the microvessel density for each case. The number of microvessels was determined by an investigator who had no knowledge of the level of VEGF mRNAs.

Statistical analysis

The relationship between levels of VEGF mRNA and clinicopathological features including microvessel density were evaluated by a Mann–Whitney *U*-test. Probability values lower than 0.05 were considered statistically significant.

RESULTS

VEGF mRNA expression and clinicopathological features

Although VEGF 206 transcripts were not amplified, VEGF 189, 165 and 121 were routinely detected in this series of cervical cancer. The VEGF/β-actin (V/A) ratios ranged from 0.6 to 9.7, with an average of 3.9 ± 2.6. Figure 1 shows a representative final RT-PCR product for VEGF and β-actin and the corresponding values of the V/A ratios. The highest expression of VEGF mRNA was observed in early invasive cervical cancer. The stage of disease correlated significantly with a decrease in the V/A ratio, except in stage IVb ($P < 0.05$) (Figure 2). V/A ratios of stage IVb were significantly higher than those of stage III and IVa ($P < 0.05$) (Figure 2). There was no significant difference in V/A ratios with respect to histological cell types (Figure 3). 38 patients with stages Ib–IIb cervical cancer underwent radical hysterectomy and pelvic lymphadenectomy. There were no significant differences in the V/A ratios with respect to tumour size, depth of stromal invasion, parametrial involvement, lymph node metastasis or vaginal involvement among these patients (Table 1). The observation period in this study was too short for survival analysis.

VEGF mRNA expression and microvessel density

Microvessel density was assessed by immunohistochemical staining for factor VIII-related antigen in 38 patients who

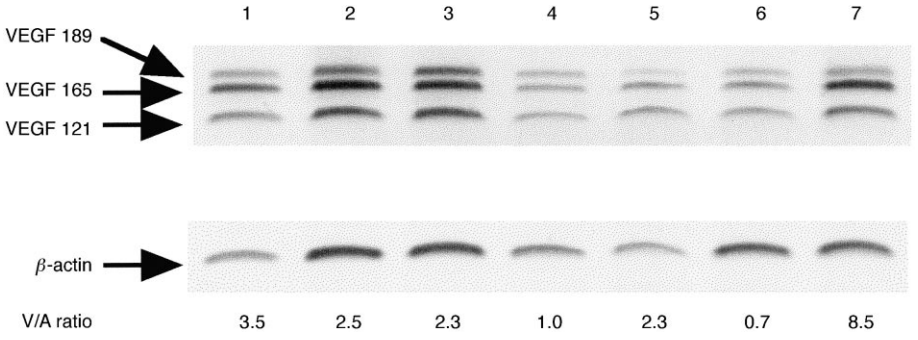

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of vascular endothelial growth factor (VEGF) mRNA levels. One microlitre of total RNA prepared from each biopsy specimen was subjected to RT-PCR for VEGF and β-actin. Three fragments representing VEGF 189, 165 and 121 were routinely amplified. The VEGF/β-actin ratios (V/A ratios) were calculated from total intensities of VEGF PCR products and those of β-actin as an internal standard.

underwent radical surgery. In most cases, the microvessel density was higher at the invasive edge of the tumour than within the tumour. Microvessel counts varied from 12.1 to 73.5 (100×microscopic field, 0.618 mm^2), and the average microvessel counts was 34.8 ± 14.0. The patients were stratified into two subgroups with high (≥ 3.3) and low (< 3.3) V/A ratios on the basis of the median value. The microvessel densities of patients with high V/A ratios were significantly higher than those of patients with low V/A ratios ($P < 0.01$) (Figure 4).

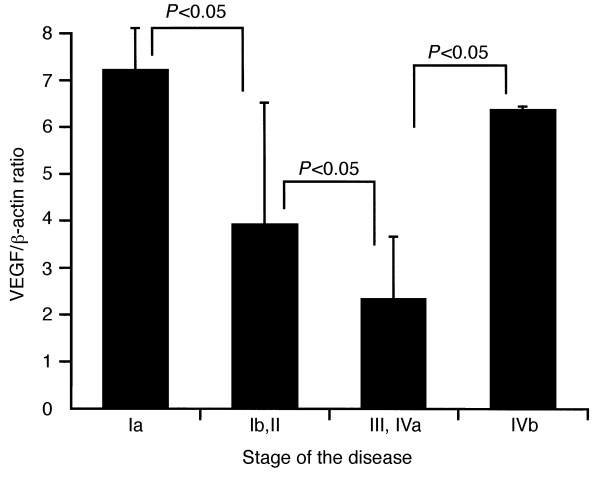

Figure 2. Relationship between the vascular endothelial growth factor (VEGF) β-actin ratio and stage of disease. The stage of disease was significantly correlated with a decrease in the VEGF/β-actin (V/A) ratio, except in stage IVb.

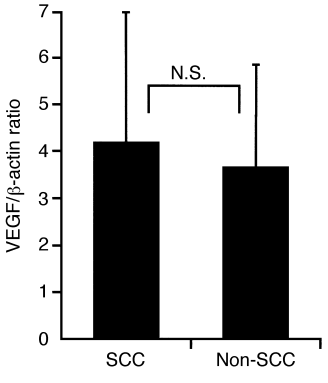

Figure 3. Relationship between vascular endothelial growth factor (VEGF) β-actin ratios and histological cell types. There was no significant difference in VEGF/β-actin ratios with respect to histological cell types. SCC, squamous cell carcinoma versus non-SCC.

DISCUSSION

Several angiogenic factors have been described, such as acidic and basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), transforming growth factor-β, VEGF, placenta growth factor (PlGF), interleukin-8, tumour necrosis factor-α and platelet-derived endothelial cell growth factor/thymidine phosphorylase [14–18]. In invasive cervical cancer, the association between the expression of the most important angiogenic factor, VEGF, and clinicopathological factors including microvessel density has scarcely been examined. If VEGF is the main mediator of angiogenesis in cervical cancer, it may provide novel opportunities for therapeutic intervention.

It has been demonstrated, using *in situ* hybridisation techniques, that VEGF mRNA expression is significantly

Table 1. *Vascular endothelial growth factor (VEGF)/β-actin ratio and pathological characteristics in* 38 *patients treated by radical hysterectomy and pelvic node dissection*

Variable	Number of Patients	VEGF/ β-actin ratio*	*P* value
Tumour size (cm)			
> 3	12	4.4 ± 3.2	NS
≤ 3	26	3.7 ± 2.4	
Cervical infiltration depth			
> 2/3	23	4.2 ± 3.1	NS
≤ 2/3	15	3.6 ± 1.8	
Parametrial involvement			
Positive	15	4.8 ± 3.4	NS
Negative	23	3.3 ± 1.9	
Vaginal involvement			
Positive	7	5.3 ± 3.3	NS
Negative	31	3.6 ± 2.4	
Pelvic lymph node metastasis			
Positive	10	3.6 ± 2.8	NS
Negative	28	4.1 ± 2.6	
Lymphovascular space involvement			
Positive	21	3.8 ± 2.7	NS
Negative	17	4.1 ± 2.6	

*Mean ± S.D. NS, not significant.

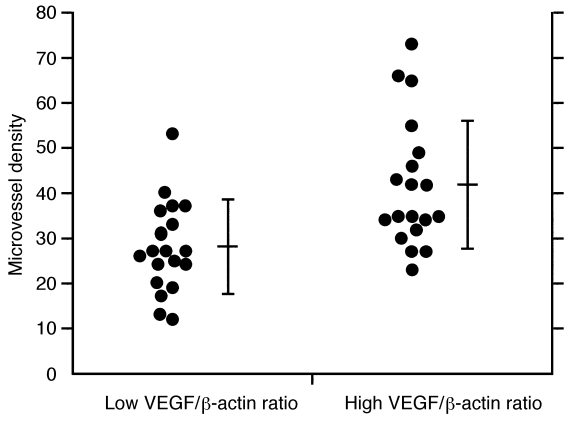

Figure 4. Association between microvessel density and vascular endothelial growth factor (VEGF)/β-actin ratios. The microvessel densities of tumours with high VEGF/β-actin ratios (≥3.3 median value) were significantly higher than those of tumours with low VEGF/β-actin ratios (<3.3 median value) (P<0.01, Mann–Whitney U-test).

increased in invasive carcinoma and in high-grade intraepithelial lesions compared with low-grade intraepithelial lesions and benign squamous epithelium [19]. No reports have focused on VEGF mRNA levels in invasive cervical cancer. The present study was designed to identify and quantify VEGF mRNA in invasive cervical cancer by RT-PCR. Analysis of mRNA levels in tumours by semi-quantitative RT-PCR requires cautious interpretation, because solid tumours are composed not only of cancerous cells but also of stromal cells. It is inevitable, therefore, that the level of VEGF mRNA in tissues with significant contamination with stromal cells would be underestimated. In this study, it was carefully verified that the available specimens were not significantly contaminated with stromal cells.

It was first shown that VEGF mRNA was extremely elevated in early invasive cervical cancer. VEGF seems to play an important role in early invasion in cervical cancer. Yoshiji and colleagues showed that VEGF was critical during the initial growth of human breast carcinoma cells in nude mice, but was not an absolute requirement for continued growth after tumours had reached a certain size. Other angiogenic factors, such as bFGF and TGF-α, can substitute for VEGF [20]. It is postulated, therefore, that other angiogenic factors may be upregulated following suppression of VEGF with further stromal invasion in cervical cancer. The most intriguing observation of this study was that VEGF mRNA levels were significantly lower in locally advanced cervical cancer, i.e. stages III and IVa. It appears that increased levels of VEGF mRNA are not required for local progression, and downregulation of VEGF mRNA may occur. In this study, diseases with distant metastases showed a relatively elevated level of VEGF mRNA. It is well known that the expression of VEGF promotes distant organ metastasis [21, 22]. Therefore, it is possible that high expression of VEGF mRNA might be associated with the haematogenous spread of cervical cancer. However, there were only 2 cases with stage IVb diseases in the current study and further investigation is needed to clarify this issue.

This study also examined whether the level of VEGF mRNA correlates with vascularisation in invasive cervical cancer. The neovascular hot spots appeared around the tumour margins in cervical cancer [10]. Since biopsy specimens were not applicable in this investigation, the cases without surgery were excluded. The expression of VEGF mRNA correlated closely with tumour vascularity. This agrees with the previous demonstration that the microvessel density correlated significantly with VEGF expression in stages Ib–IIb cervical cancer by immunohistochemical methods [10]. It has also been reported that high VEGF mRNA expression associated with increased microvessel density in squamous intraepithelial lesion and invasive squamous cell carcinoma of the cervix [19]. A significant correlation has been confirmed between VEGF expression and microvessel density in cervical intraepithelial neoplasia [23]. These findings indicate that VEGF exhibits a potent angiogenic effect in cervical intraepithelial neoplasia and invasive cancer.

Anti-VEGF monoclonal antibody inhibits both primary and metastatic tumour growth with minimal side-effects in nude mice [24]. Thus, VEGF could be an important target of antitumour agents in cervical cancer. It is well known that highly vascularised invasive squamous cell carcinoma of the cervix is sensitive to irradiation and chemotherapy [25, 26]. If tumour vascularity could be estimated before treatment, those patients with highly vascularised tumours may benefit from neoadjuvant chemotherapy or irradiation therapy.

Microvessel density is related to metastases and predicts patient prognosis in diverse types of malignancies arising from the breast, lung, prostate, head and neck [27–30]. There have been conflicting studies addressing whether microvessel density predicts prognosis in cervical cancer. Several studies demonstrated that high microvessel density is a useful indicator for the prognosis of cervical cancer [31–36]. In contrast, another study reported no correlation between vascularity and prognosis in squamous cell carcinoma of the cervix [37] and patients with low microvessel density were found to have had poor recurrence-free intervals [38]. In the present study, it was shown that the VEGF mRNA level is dependent on stages. Therefore, the question of whether microvessel density is an important prognostic predictor in cervical cancer should be analysed in uniform stages.

In conclusion, the present findings provide evidence that the expression of VEGF is involved in the promotion of angiogenesis in cervical cancer. The results also suggest that VEGF plays an important role in early invasion.

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