

Relationship between expression of vascular endothelial growth factor and intratumoral hemorrhage in human pituitary adenomas

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ABSTRACT

Aims and background. Although pituitary adenoma is a primary brain tumor that occasionally accompanies intratumoral hemorrhage, there are little reports about the molecular mechanism of intratumoral bleeding in pituitary adenoma. Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis and vascular permeability of various brain tumors. The authors studied the relationship between intratumoral hemorrhage and the expression of VEGF in human pituitary adenomas.

Methods. VEGF expression was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) in 71 pituitary adenomas. Clinical factors to investigate were age, gender, hormonal functioning, and radiological findings of pituitary adenomas. Radiological findings which were investigated by magnetic resonance (MR) images were intratumoral hemorrhage, cystic change, tumor size, and cavernous sinus invasion. The relationship between these factors and VEGF expression was statistically analyzed.

Results. VEGF was expressed in 25 cases (35.2%). Functioning tumors, hemorrhage, cystic change, and cavernous sinus invasion were 32 (45.1%), 18 (25.4%), 12 (16.9%), and 21 (29.6%) respectively. The expression of VEGF showed a significant relationship with the intratumoral hemorrhage of the adenomas ($P < 0.001$). However, age, gender, tumor size, hormonal functioning, cyst formation, and cavernous sinus invasion had no relationship with VEGF expression ($P > 0.05$).

Conclusions. This study suggests that VEGF expression may be responsible for intratumoral hemorrhage of pituitary adenomas. Therefore, VEGF can be a novel target to prevent a catastrophic apoplexy in pituitary adenomas and to establish roles in angiogenesis-based therapeutics of pituitary adenomas.

Introduction

Angiogenesis is an essential step for tumor growth and invasion, and angiogenic factors are played roles in the pituitary adenoma progression. Several angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are involved in neovascularization of pituitary adenomas¹⁻³. VEGF had been characterized as a heparin binding angiogenic factor which is 32 to 46 kD dimeric disulfide-bound glycoprotein displaying high specificity for endothelial cells⁴⁻⁷. Five VEGF isoforms are produced as a result of alternative splicing from a single VEGF pre-mRNA, although different in molecular size and biological property^{6,7}. Besides the angiogenic ability, VEGF is the most potent mediator among the known mediators of vascular permeability^{8,9}. Recent studies demonstrated that VEGF increased venular and capillary permeability by inducing and maintaining formation of the vascular endothelial fenestrations^{8,10-13}.

Key words: angiogenesis, intratumoral hemorrhage, pituitary adenoma, vascular endothelial growth factor.

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Conflict of interest notification: none

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Tumor-associated hemorrhage was reported in a series of glioblastoma with VEGF overexpression⁵. VEGF expression may be intimately related to pituitary tumor growth and vascularization. But, little is known about the role of VEGF in pituitary adenomas, even though pituitary adenomas have the high incidence of spontaneous intratumoral hemorrhage^{5,14-16}. The purpose of this study was to investigate the relationship between VEGF expression and clinical factors including intratumoral hemorrhage in pituitary adenomas.

Materials and methods

Clinical materials

We retrospectively obtained clinical data of the 88 patients with pituitary adenomas who had been operated at the single institute. Seventeen of the 88 cases were excluded because of poor condition of the tumor specimens. Age, gender, hormonal functioning were reviewed and magnetic resonance (MR) images were used to evaluate tumor size, cavernous sinus invasion, intratumoral hemorrhage, and cystic lesion in 71 cases of pituitary adenomas. Preoperative MR imaging were performed in all patients. Intratumoral hemorrhage was defined as a lesion with hyperintensity on the T₁-weighted image (T₁WI) and hypointensity on the T₂-weighted image (T₂WI), while a cystic component was defined as a lesion with hypointensity on the T₁WI and hyperintensity on the T₂WI (Figure 1). Intratumoral hemorrhage and cyst formation were confirmed intraoperatively. The Knosp's radiological classification was used to evaluate tumor invasion into the cavernous sinuses¹⁷. The cavernous sinus invasion was defined as extending lateral to the lateral tangent of the intra- and supracavernous internal carotid artery (ICA) or beyond that (Grade 3 or 4). The greatest diameter of tumor obtained on the gadolinium (Gd)-enhanced T₁WI was measured as tumor size (Table 1).

Collection of tumor specimens

Freshly excised pituitary adenoma tissues were collected at the time of surgery after the patient's informed consent had been obtained under a protocol reviewed and approved by the institutional review board of Hanyang University Medical Center, Korea. The tissues were stored quickly in a deep freezer at -70 °C until processing. Histopathologic confirmations of the brain tumor tissue were undertaken by a neuropathologist.

Reverse transcription polymerase chain reaction (RT-PCR)

Anonymized samples of frozen pituitary adenoma were obtained from the deep freezer. Their RNA was extracted by using the SV total RNA isolation system (Promega, Madison, WI, USA). RNA isolation from tumor tissue was processed according to manufacturer's

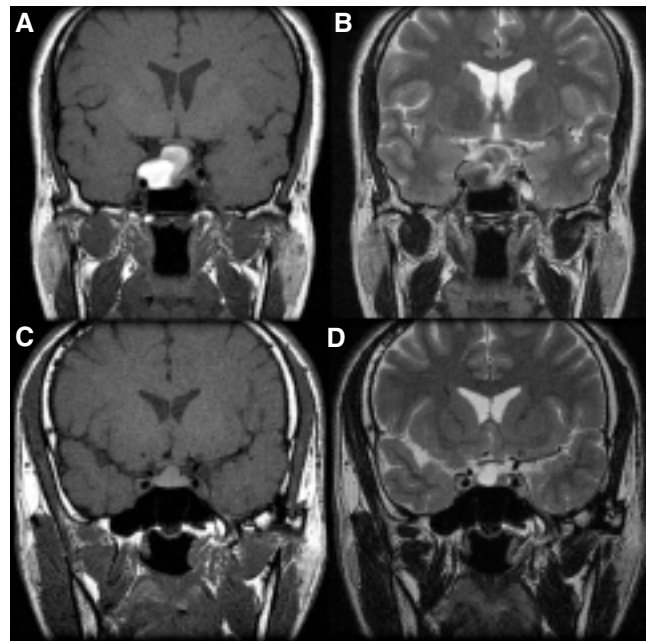
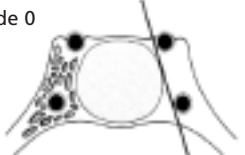

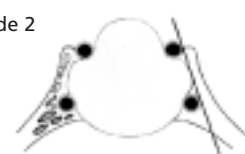




Figure 1 - Preoperative magnetic resonance (MR) images demonstrating the large intratumoral hemorrhage in pituitary adenoma; Hyperintensity on the coronal T₁-weighted image (A) and hypointensity on the T₂-weighted image (B). Preoperative MR images showing cyst in pituitary adenoma; Hypointensity on the coronal T₁-weighted image (C) and hyperintensity on the T₂-weighted image (D).

protocol. In briefly, the frozen tissues were rinsed three times with phosphate-buffered saline (PBS) and then, it was ground into a fine powder using a mortar and pestle, under liquid nitrogen, after which 1 ml of Trizol containing 250 µg of glycogen was added. The grounded tumor tissues were lysed and homogenized in 175 µl RNA lysis buffer including β-mercaptoethanol (BME) at the ratio of 1:50 until no visible fragments remain. Homogenates were prepared by adding 350 µl RNA dilution buffer and mixed by inverting 3-4 times. The homogenates were centrifuged for 10 min at 12000 x g at 4 °C and then the supernatants were transferred to fresh tubes. The cleared lysate was added by 200 µl 95% ethanol and mixed well by 3-4 timed pipetting. The mixture was transferred to Spin Basket Assembly and centrifuged at 12000 x g for 1 min, and then the elate was discarded. 600 µl RNA wash solution containing 95% ethanol was added and centrifuged at 12000 x g for 1 min, and then the elates was also discarded. Their supernatants were applied 50 µl of DNase incubation mixture that contained 40 µl yellow core buffer, 0.09 M Mn-Cl₂ 5 µl, and DNase I 5 µl, and incubated at room temperature for 15 min. 200 µl DNase stop solution that contained 95% ethanol 20 ml and DNase stop solution 5.3 ml was added and centrifuged at 12000 x g for 1 min. After adding 600 µl SV RNA wash solution, it was centrifuged for 1 minute and then, 250 µl SV RNA wash solution added. Following centrifugation for 2 minutes,

Table 1 - The Grade of the pituitary adenoma invaded into the cavernous sinus by Knosp's classification¹⁷

Grade 0		Normal findings within the cavernous sinus space, with enhancement of all venous compartments. Thus, the tangent of the medial aspects of the intra- and supracavernous ICA is not passed.
Grade 1		In grade 1, medial tangent is passed, but the extension does not go beyond a line drawn between the cross sectional centers of the intra- and supracavernous ICA.
Grade 2		Grade 2 is characterized by tumor extending beyond the intercrotid line, but not past the tangent on the lateral aspects of the intra- and supracavernous ICA.
Grade 3		Grade 3 is characterized by tumor extending lateral to the lateral tangent of the intra- and supracavernous ICA. Depending on the direction of tumor growth, the medial, superior, and/or inferior compartments of the cavernous sinus plexus will not be enhanced.
Grade 4		Grade 4 is characterized by total encasement of the intracavernous carotid artery. There is no enhancement of either of the compartments of the venous plexus. Usually, the superior and lateral walls of the cavernous sinus space are stretched and convex in contour.

ICA, internal carotid artery.

Spin basket was transferred to elution tube. After adding 100 μ l Nuclease-Free water to membrane to elution tube, it was centrifuged for 1 minute to elude the RNA and store at -70 $^{\circ}$ C.

For each 50 μ l reverse transcription reaction, combine 25 μ l AccessQuick Master mix 2x (Promega, Madison, WI, USA), 1 μ l upstream primer (5'-GCG AAT TCC TCC TGC CCG GCT CAC-3'), 1 μ l downstream primer (5'-AAG CCA TCC TGT GTG CCC CTG ATG -3'), 5 μ g RNA template, and nuclease free water to a final volume of 50 μ l. 1 μ l AMV reverse transcriptase was added to above mixture as the final component and mixed by gentle vortexing. Reaction tubes were incubated at 45 $^{\circ}$ C for 45 minutes.

The PCR reaction was carried out using 3 μ l of the reverse transcription reaction product. Thirty-five cycles of touchdown PCR were performed by manufacturer's protocol consisting of 95 $^{\circ}$ C for 3 minutes, a one-degree decline in annealing temperature 55 $^{\circ}$ C for 1 minute, extension reaction at 72 $^{\circ}$ C for 6 minute, and hold the reaction at 4 $^{\circ}$ C overnight. And then, PCR products were stored at -20 $^{\circ}$ C. A single reaction void of template was performed with each experiment as a negative-control.

After mixing 8 μ l of PCR products and 2 μ l loading buffer, mixtures were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination (Figure 2). VEGF mRNA was measured semi-quantitatively by spectrometry. The average quantity of VEGF mRNA for each tumor was compared to that of β -actin. Briefly, when if optical density of β -actin measured by spectrometry was supposed to 1, we calculated the relative amount of VEGF mRNA in respective pituitary adenomas.

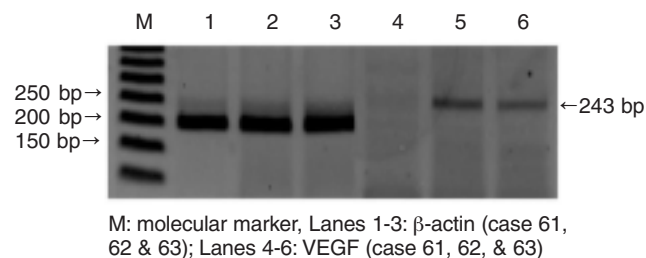


Figure 2 - Detection of vascular endothelial growth factor (VEGF) by reverse transcription polymerase chain reaction (RT-PCR) in pituitary adenomas. VEGF is expressed in lanes 5 and 6, but not in lane 4.

Statistical analysis

Data were analyzed using SPSS software (version 11; SPSS Inc., Chicago, IL, USA). Student's *t*-test and the Chi-square test were employed to compare the differences in gender, function and volume of tumor, cyst formation and cavernous sinus invasion between the VEGF positive and negative groups. All data was expressed as mean \pm standard deviation (SD). *P* value of less than 0.05 was regarded as statistically significant.

Results

Demographic characteristics

There were 41 (57.8%) males and 30 (42.2%) females with a mean age of 46.5 years old (ranged from 20 to 76 years old). Of the 71 patients, 32 patients were functioning tumors including 18 prolactinomas, 12 growth hormone (GH) secreting adenomas, and 2 thyroid stimulating hormone (TSH) secreting adenomas. Thirty-nine patients had non-functioning pituitary adenomas. Twenty five (35.2%) cases were positive in VEGF expression by RT-PCR and 46 cases (64.8%) were negative (Table 2).

Radiological features

The mean maximum diameter of the tumors was 22.6 \pm 13.5 mm (range, 4.0-60.0 mm). Sixty seven cases (94.4%) were macroadenomas (\geq 10 mm in size) and four cases (5.6%) were microadenomas (<10 mm in size). Cystic component was noted in 12 cases (16.9%)

Table 2 - Clinical summary of 71 patients with pituitary adenomas

Characteristics	Value
Total No. of patients	71
Age (years)	
Mean \pm SD	46.5 \pm 16.4
Range	20-76
Gender (male to female)	41:30 (57.8%:42.2%)
Hormonal function (cases)	
Nonfunctioning adenoma	39 (54.9%)
Functioning adenoma	32 (45.1%)
PRL	18
GH	12
TSH	2
Follow-up periods (months)	
Mean \pm SD	37.7 \pm 13.2
Range	6-64
VEGF expression by RT-PCR	
Positive	25 (35.2%)
Negative	46 (64.8%)

GH, growth hormone; PRL, prolactin; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TSH, thyroid stimulating hormone; VEGF, vascular endothelial growth factor.

and intratumoral hemorrhage was noted in 18 cases (25.4%). Tumor invasion into the cavernous sinus according to the Knosp's grading system was noted as follows: Grade 0 in 9 cases, Grade 1 in 16 cases, Grade 2 in 25 cases, Grade 3 in 14 cases, and Grade 4 in 7 cases (Table 3).

Table 3 - Radiological findings of 71 patients with pituitary adenomas

Characteristics	Value
Diameter of tumors (mm)	
Mean \pm SD	22.6 \pm 13.5
Range	4.0-60
Macroadenoma (\geq 10mm)	67 (94.4%)
Microadenoma (<10mm)	4 (5.6%)
Cystic component	
Present	12 (16.9%)
None	59 (83.1%)
Intratumoral hemorrhage	
Present	18 (25.4%)
None	53 (74.6%)
Invasiveness to CS	
Grade 0 (cases)	9 (12.7%)
Grade 1	16 (22.5%)
Grade 2	25 (35.2%)
Grade 3	14 (19.7%)
Grade 4	7 (9.9%)

CS, cavernous sinus; SD, standard deviation.

Relationship between VEGF expression and demographic data

Twenty-five (35.2%) patients showed the positive reaction in VEGF expression by RT-PCR. Forty-six patients (64.8%) did not express VEGF. The mean age of the positive VEGF patients was 52.8 years and that of the negative patients was 43.2 years. There was no correlation between VEGF expression and age ($P = 0.071$, Student t -test) (Figure 3A).

Fifty-seven percent of the positive reaction was expressed in male patients and 43% in female cases. Fifty-nine percent of the negative VEGF was shown in male and 41% in female. The expression of VEGF had no significant relationship with the gender ($P = 0.348$, Chi-square test) (Figure 3B).

Relationship between VEGF expression and tumor size

The mean tumor size of the cases with positive reaction in VEGF expression was 24.8 \pm 17.8 mm and that of the cases with negative reaction was 21.5 \pm 10.5 mm. There was no correlation between VEGF expression and mean tumor size ($P = 0.095$, Student t -test) (Figure 4).

Relationship between VEGF expression and hormonal functioning

Thirty-two patients (45.1%) had functioning adenomas, and 39 (54.9%) patients had non-functioning pituitary adenomas. Forty percent of the positive reaction was expressed in hormonal functioning adenomas and 60% in non-functioning cases. Thirty percent of the negative VEGF was showed in functioning adenomas and 70% in non-functioning cases. The expression of VEGF did not show relationship with the hormonal function of the adenomas ($P = 0.053$, Chi-square test) (Figure 5A).

Relationship between VEGF expression and intratumoral hemorrhage

Eighteen cases (25.4%) had an intratumoral hemorrhage. Six cases had a focal hemorrhagic formation and 12 cases had the lesion with hyperintensity on the T₁WIs and hypointensity on the T₂WIs. Fifty-seven percent of the positive reaction was expressed in intratumoral hemorrhagic cases and 43% in non-hemorrhagic cases. The negative expression of VEGF was mostly observed in non-hemorrhagic cases (89%). The expression of VEGF showed a significant relationship with the intratumoral hemorrhage of the adenomas ($P < 0.001$, Chi-square test) (Figure 5B).

Relationship between VEGF expression and cyst formation

Twelve cases (16.9%) had intratumoral cysts. Twenty-eight percent of the positive reaction for VEGF was expressed in cystic cases and 72% in non-cystic cases.

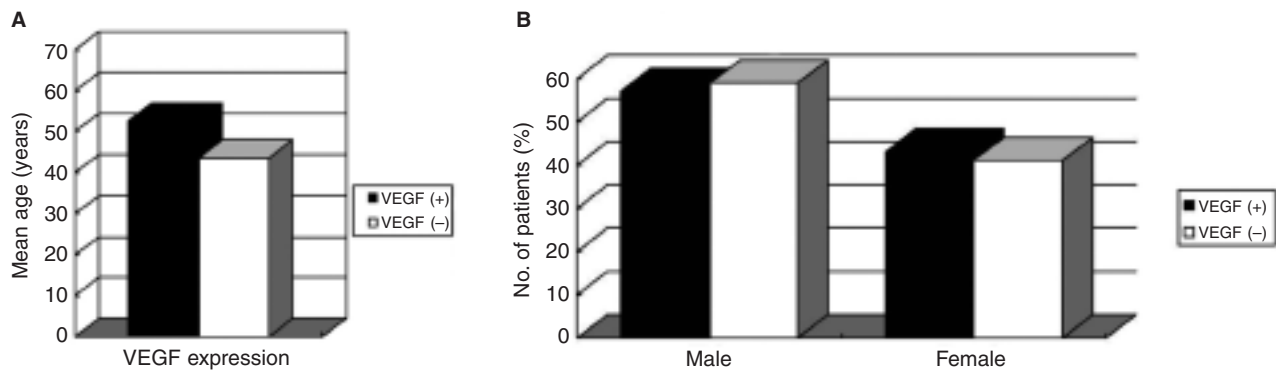


Figure 3 - A) Relationship between vascular endothelial growth factor (VEGF) expression and age. The mean age of the patients is 52.8 years in the positive group and 43.2 years in negative group, respectively ($P = 0.071$ by Student *t*-test). B) Relationship between vascular endothelial growth factor (VEGF) expression and gender. There is no significant difference of VEGF expression between male and female ($P = 0.348$ by Chi-square test).

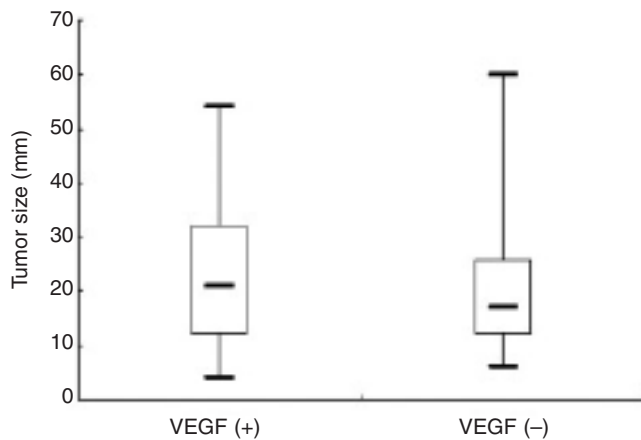


Figure 4 - Relationship between vascular endothelial growth factor (VEGF) expression and mean tumor size. Mean tumor size (mm) of the positive VEGF expression vs negative expression is 24.8 ± 17.8 vs 17.8 ± 10.5 , respectively ($P = 0.096$ by Student *t*-test).

Twelve percent of the negative reaction of VEGF was showed in patients with cystic formation and 88% in non-cystic cases. The expression of VEGF had no significant relationship with the cyst formation of the adenomas ($P = 0.192$, Chi-square test) (Figure 5C).

Relationship between VEGF expression and cavernous sinus invasion

The Knosp's grading system in 71 patients was noted as follows: Grade 0 in 9 cases, Grade 1 in 16 cases, Grade 2 in 25 cases, Grade 3 in 14 cases, and Grade 4 in 7 cases (Table 3). Twenty one cases (29.6%) of 71 patients were considered to have a cavernous sinus invasion. Fifty-seven percent of the positive reaction was expressed in cases with cavernous sinus invasion and 43% in cases with non-invasion. Twenty-one percent of the

negative reaction for VEGF was shown in patients with cavernous sinus invasion and 79% in cases with non-invasion. The expression of VEGF had no significant relationship with the cavernous invasion of the adenomas ($P = 0.906$, Chi-square test) (Figure 5D).

Discussion

VEGF is known to induce angiogenesis as well as permeability of the microvascular endothelium^{4,6,8,9,11,18,19}. The VEGF is a constellation of angiogenic and lymphangiogenic growth factors, consisting of 6 glycoproteins known as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factors 1 and 2^{3,20,21}. VEGF-A, usually referred to as VEGF, is a polypeptide synthesized and produced by a variety of normal and tumor cells^{3,21}. In normal pituitary gland, VEGF is expressed in ACTH, GH and pituitary-derived folliculo-satellite cells in conditioned medium^{4,22-25}. Both interleukin (IL)-1 and IL-6 have been shown to regulate VEGF levels in a variety of tissues²⁶. VEGF binds with high affinity to tyrosine kinase receptors and its binding leads to receptor dimerization, autophosphorylation of the receptor, and signal transduction²⁷.

In several kinds of brain tumor, overexpression of VEGF is known to induce tumor-associated cyst, peritumoral edema, and intratumoral hemorrhage^{5,16,28-30}. Vaquero *et al.*³⁰ suggested that the predominance of a cystic or solid macroscopic appearance of craniopharyngiomas may be influenced by the degree of vascular endothelial growth/permeability factor expression within the tumor cells. It is reported that the single overexpression of the VEGF by human glioblastoma cells causes reproducible and predictable intracerebral hemorrhage⁵. Overexpression of VEGF and matrix metalloproteinase (MMP) may also play a role in the metastatic brain tumor accompanying hemorrhage¹⁶.

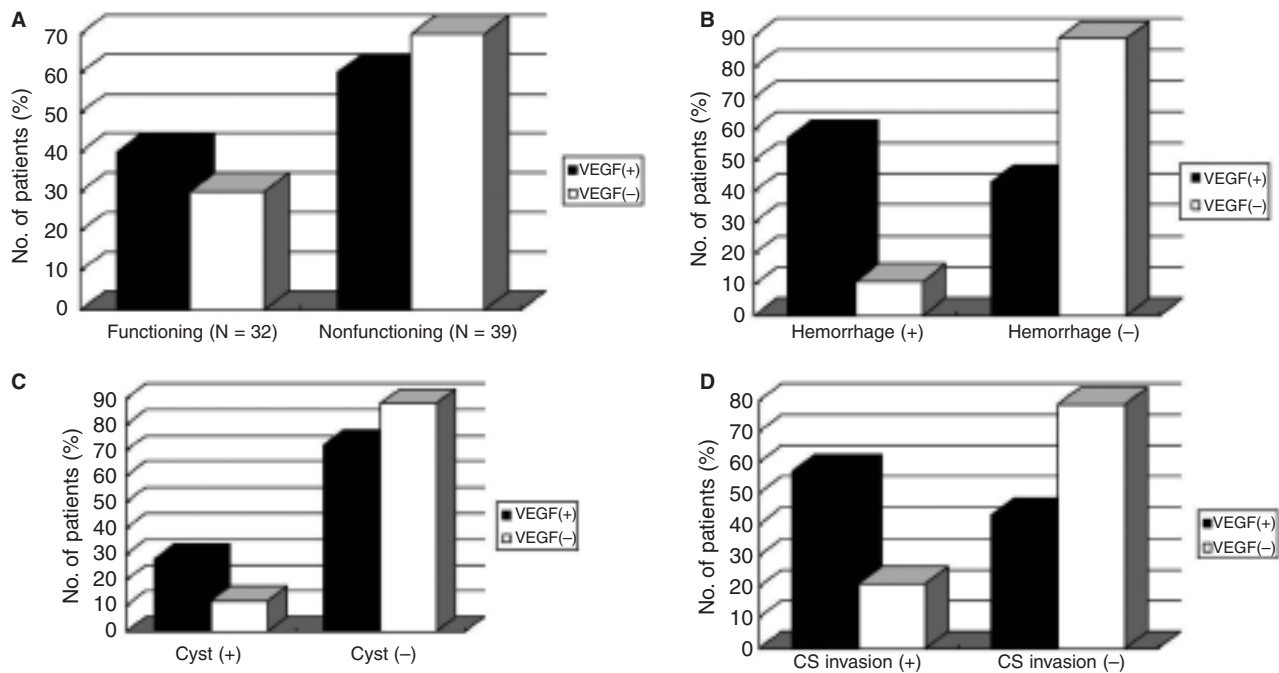


Figure 5 - A) Relationship between vascular endothelial growth factor (VEGF) expression and hormonal functioning. There is no difference in VEGF expression between the functioning group and the non-functioning group ($P = 0.053$ by Chi-square test). B) Relationship between VEGF expression and hemorrhage. There is a significant difference in VEGF expression between hemorrhagic cases and non-hemorrhagic cases ($P = 0.001$ by Chi-square test). C) There is no relationship between VEGF expression and cystic formation ($P = 0.192$ by Chi-square test). D) VEGF expression is also not related to the cavernous sinus invasion ($P = 0.906$ by Chi-square test). CS, cavernous sinus.

In this study, intratumoral hemorrhage was noted in 18 cases (25.4%) on the MR imaging and it is consistent with the previous incidence of which an intratumoral hemorrhage of pituitary adenomas has been reported as 9.6-46.0%^{2,31-33}. Although pituitary adenomas hold the high incidence of intratumoral hemorrhage, however, the potential roles of VEGF in pituitary adenomas remain poorly understood. The previous study has reported that VEGF expression is associated with intratumoral hemorrhage⁷. In contrast to this study, there are several reports that have been noted no correlation between VEGF expression and intratumoral hemorrhage in the pituitary adenomas^{9,19}. This study shows contradictory results: positive relationship between VEGF expression and intratumoral hemorrhage in the human pituitary adenomas was observed in this study. These differences are likely to be responsible for detection method of VEGF. Most studies have investigated VEGF protein expression using immunohistochemical staining, while this study used RT-PCR to detect VEGF expression at the transcriptional level. Advantage of the RT-PCR as a detection method is that VEGF mRNA for each tumor could be verified and quantified.

The VEGF expression showed no correlation with age, gender, cystic formation, and cavernous sinus invasion in this study. Contrary to the present result, Fukui *et al.*¹⁹ supposed that there was a close relationship between VEGF expression and cyst formation in pituitary adeno-

mas, and suggested that VEGF played a potential role in the pathogenesis of cystic formation in pituitary adenomas. Compared to the method used in the present study, they used semiquantitative assessment of the VEGF expression on the basis of immunohistochemical staining. The immunoreactivity of VEGF in the tumor section was assigned as either one of three grades (weak, moderate, and strong) based on the intensity of the visualized brown dots. In this regard, some major weak points can be revealed in immunohistochemistry: It is related to the technical ineffectiveness, such as, potential contamination in handling during lengthy successive staining procedure, and possible interobserver discrepancy between counters and grades. On the contrary to this, the RT-PCR method used in my study is capable of minimizing possible contamination by carrying out entire procedure as one step. Furthermore, by using RT-PCR, tiny amount of mRNA can be sufficiently detected and quantified by amplification. Although a combined immunohistochemical (IHC) and RT-PCR approach for the determination of expression of the VEGF mRNA in pituitary adenomas may be an more effective and efficient strategy, we could not perform all two methods. But, in several comparative studies of RT-PCR and IHC, RT-PCR has a better sensitivity than IHC study^{34,35}. Also, RT-PCR assays and IHC methods are created equal, and marked variations of results may be seen².

There was only anecdotal report on the relationship between VEGF expression and cavernous sinus invasion, but without clinical significance. Iuchi *et al.*³⁶ reported that VEGF expression was not correlated with serum hormone level and tumor volume, instead, it was correlated with tumor proliferative potential. Additionally, they demonstrated that proliferative potential and tumor volume were two independent factors related to the cavernous sinus invasion. Although VEGF expression was not a direct factor related to the cavernous sinus invasion, it may play an indirect role in activation of tumor aggressiveness that is required in cavernous sinus invasion. Despite their suggestion, their study was restricted only to the growth hormone producing pituitary adenoma, and the data from small surgical samples could not represent general characteristics of the whole adenomas.

We think that Knosp's radiological classification does not exclude the possibility of microinvasion into the cavernous sinus. To define the radiological invasion of pituitary adenoma into cavernous sinus, Knosp's radiological classification has been cited in numerous literatures since it was published in *Neurosurgery*, 1993³⁷⁻⁴⁰. But, it was few reported that the invasion into the cavernous sinus is accurately described as the pathologicoradiological criteria in pituitary adenomas. Up to date, Knosp's classification is a reliable radiological tool to evaluate the preoperative cavernous invasion of pituitary adenomas.

There are limitations in this study. One is the relatively small numbers of positive VEGF patients. The other is that we have not investigated whether the preoperative MR images were compatible with the microscopic findings of the surgical specimens.

Conclusions

This study revealed the VEGF expression at a transcriptional level, not a protein in pituitary adenomas. The results suggest that VEGF expression may be responsible for intratumoral hemorrhage of pituitary adenomas. VEGF may be a novel target to prevent a catastrophic apoplexy in pituitary adenomas and it would enlighten to play a role in angiogenesis-based therapeutics of pituitary adenomas. Further studies should be followed to define a molecular pathogenesis of intratumoral hemorrhage in pituitary adenomas by VEGF and to examine the pathophysiological basis for the hemorrhage in pituitary adenomas.

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