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Received, April 30, 2009.

Accepted, November 30, 2009.

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Expression of Angiogenic Factors in Craniopharyngiomas: Implications for Tumor Recurrence

BACKGROUND: The primary treatment for craniopharyngiomas is total excision, but recurrence is common. However, current knowledge on the mechanisms of recurrence is limited.

OBJECTIVE: We hypothesized that recurrence is linked to the angiogenesis of the tumor. Recurrent and nonrecurrent tumor samples were compared with regard to expression of angiogenesis-related factors and angiogenic capacity in a corneal angiogenesis model.

METHODS: Specimens of 4 recurrent and 6 nonrecurrent tumors were selected from 57 patients with adamantinomatous craniopharyngiomas. Sections were immunohistochemically stained with antibodies for vascular endothelial growth factor (VEGF), fibronectin, fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)-A, PDGF-B, platelet-derived growth factor receptor (PDGFR)- α , and PDGFR- β . Expression levels were graded using a 4-point scoring system and were compared. For corneal angiogenesis assay, tissue samples were inoculated in a micropocket created on the rat eye, and microvessels were counted on days 3, 5, 7, and 9 to evaluate angiogenic potential.

RESULTS: Expression of PDGFR- α and FGF-2 were significantly higher for recurrent tumors ($P = .02$ and $P = .01$). However, recurrent and nonrecurrent tumors did not differ in the expressions of other ligands and receptors (PDGF-A, PDGF-B, and PDGFR- β). Recurrent tumors displayed a higher angiogenic potential starting from the fifth day of corneal angiogenesis assay.

CONCLUSION: These findings suggest a relationship between recurrence of craniopharyngiomas and angiogenesis. New treatment modalities with selective PDGFR- α blockers may represent a novel and effective therapeutic option for the treatment of craniopharyngiomas.

KEY WORDS: Craniopharyngioma, Fibroblast growth factor 2, Platelet-derived growth factor, Platelet-derived growth factor receptors, Tumor angiogenic potential, Tumor recurrence

Neurosurgery 66:744-750, 2010

DOI: 10.1227/01.NEU.0000367553.65099.14

www.neurosurgery-online.com

Craniopharyngiomas are benign but biologically aggressive epithelial neoplasms, usually located at the sellar or suprasellar area.¹ They constitute 2.5% to 4% of all brain tumors and 6% to 9% of all pediatric brain tumors.² Craniopharyngiomas are thought to originate from the Rathke pouch, which, during embryogenesis, gives rise to the anterior pituitary, a part of the endocrine system.^{3,4} With a prevalence of

approximately 2 in 100 000, these tumors usually occur in children between 5 and 10 years of age, with another peak in the fourth decade of life.⁵ Histopathologically, these tumors consist of squamous epithelium surrounded by radially arranged cells, often with calcium deposition and papillary appearance. The ability of neuroimaging studies to detect the calcium deposition within the tumor tissue can aid in the diagnosis of the tumor.⁶ Although craniopharyngiomas are benign in nature, histopathologically they are in close contact with the normal tissues surrounding the tumor. Therefore, even after total excision of the tumor, radiation therapy, or both, a recurrence rate between 5% and 30% is observed.⁷⁻⁹

ABBREVIATIONS: **AVM**, arteriovenous malformation; **GBM**, glioblastoma multiforme; **FGF**, fibroblast growth factor; **PDGF**, platelet-derived growth factor; **PDGFR**, platelet-derived growth factor receptor; **VEGF**, vascular endothelial growth factor

Angiogenesis is a signal-dependent process in which new capillary vessels develop from already existing vessels.¹⁰⁻¹² In various tumor types, genes involved in angiogenesis are abnormally expressed, with several studies showing a correlation between tumor recurrence and angiogenesis.¹³⁻¹⁷ Angiogenesis promotes the growth and invasion of the tumors through provision of the nutrients and growth factors, which is consistent with the studies suggesting a direct relationship between tumor recurrence and angiogenesis.^{18,19} Vascular endothelial growth factor (VEGF) is an important growth factor involved in neovascularization, and some studies have focused on the role of VEGF expression in recurrent craniopharyngiomas.²⁰⁻²² However, to our knowledge, the role of other angiogenic factors in the recurrence of craniopharyngioma has not been studied previously.

Platelet-derived growth factor (PDGF) pathway is a key signaling pathway that connects extracellular signals to angiogenesis and proliferative response by a series of signal transduction events. Platelet-derived growth factor inhibitors have become an important target in tumor treatment owing to high level of expression of PDGF in many tumor types and its well-characterized role in tumor angiogenesis. Four isoforms of PDGF have been identified: PDGF-A, B, C, and D. These isoforms exert their effect on the cells through tyrosine kinase receptors α and β . Ligand receptor dimerization results in receptor autophosphorylation and signal transmission into the cell, which eventually causes the expression of genes associated with angiogenesis.²³ Fibroblast growth factor (FGF) and VEGF are growth factors with defined roles in angiogenesis response. Fibronectin is an important extracellular matrix protein. Extracellular matrix reorganization is one of the steps required for angiogenesis to take place.²⁴

Recurrent and nonrecurrent craniopharyngiomas were compared with regard to expression of angiogenesis-related factors—FGF-2, fibronectin, PDGF-A, PDGF-B, Platelet-derived growth factor [PDGFR]- α , and PDGFR- β —and angiogenic capacity as evaluated by corneal angiogenesis model, in an attempt to examine the role of angiogenic potential in tumor recurrence.

PATIENTS AND METHODS

Patients

Between 1988 and 2008, 75 patients underwent microsurgical craniopharyngioma resection at the Faculty of Medicine, Neurosurgery Department and Institute of Neurological Sciences of Marmara University, Istanbul, Turkey. For 68 patients undergoing surgery after 1998, fresh-frozen tumor samples were available (11 papillary and 57 adamantinomatous craniopharyngiomas). Among samples from patients with adamantinomatous craniopharyngioma in whom total excision was radiologically confirmed with postoperative 24-hour magnetic resonance imaging examination, 10 (4 recurrent and 6 nonrecurrent) were randomly selected for immunostaining and corneal angiogenesis model assessments. One of the recurrent tumors and 2 of the nonrecurrent tumors were cystic, whereas other tumors were solid. Clinical and demographic properties of these patients are shown in Table 1. Presence

of any radiologic findings suggestive of the tumor within 2 years after surgery was regarded as a tumor recurrence. All patients were followed up for at least 2 years.

Immunostaining

The analyses were performed at neurooncology and pathology laboratories of Marmara University Institute of Neurological Sciences. In each case, multiple serial sections were cut from the fresh-frozen tissue using a cryostat and were prepared for immunohistochemical analysis for structural proteins and angiogenic factors. Tissue sections were cut and rehydrated in an alcohol gradient before analysis with standard streptavidin-biotin technique. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ (0.6 mL of H₂O₂, 2.7 mL of methanol, and 2.7 mL of distilled H₂O). Slides were blocked with 5% normal goat serum and then incubated overnight with human monoclonal rabbit antifibronectin, VEGF, PDGF-A, PDGF-B, PDGFR- α , PDGFR- β , or FGF2 antibodies (all from Santa Cruz Biotechnology, Inc, Santa Cruz, California) at 4°C. Each was then incubated for 30 minutes at 25°C with secondary biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc, Burlingame, California). The final step was incubation with streptavidin-peroxidase complex, using the Vectastain ABC Elite kit (Vector Laboratories, Inc). Chromogenic reactions were completed with 3,3'-diaminobenzidine, yielding a positive brown stain (Vector Laboratories, Inc). Each slide was counterstained with hematoxylin and then mounted and examined under the light microscope.

Levels of expression were graded using a 4-point scoring system, as follows: grade 0, no expression; grade 1, moderate expression; grade 2, marked expression with focal distribution; and grade 3, marked expression with diffuse distribution. The slides were simultaneously evaluated by 1 neuropathologist (O.K.) and 2 neurosurgeons (T.K. and H.I.S.). For each case, an expression grade was assigned to histologic zones of the lesion by consensus.

Corneal Angiogenesis Model

Male Sprague-Dawley rats weighing 300 to 400 g were used for the corneal angiogenesis model. All experiments were approved by the Animal Care and Use Committee of the Marmara University Faculty of Medicine.

Four to 5 hours before the experimental procedures, each tissue sample in liquid nitrogen was brought to room temperature, washed with dimethyl sulfoxide, and cut into suitably sized pieces (approximately 2-3 mm in diameter) under a microscope.

Procedures for the corneal angiogenesis model have been previously described by our team.²⁵ Accordingly, each rat was anesthetized with an intraperitoneal injection of ketamine, and all manipulations were performed under the microscope and sterile conditions. Both corneas of each animal were anesthetized with topical 0.5% propacaine, and each globe was gently proptosed with jeweler's forceps. Using an operating microscope, a paracentral intrastromal linear keratotomy (approximately 4 mm long and at a right angle to the limbus) was performed with an arachnoid blade. Then, a microhook was used to form a micropocket within

TABLE 1. Demographic and Clinical Properties of the Patients Included in the Trial

Patient No.	Age, y /Sex	Localization of the Tumor	Surgical Approach	Pathology	No. of Operations
1	22/F	Suprasellar	Pterional	Adamantinomatous	1
2	34/M	Suprasellar	Pterional	Adamantinomatous	1
3	27/M	Suprasellar	Pterional	Adamantinomatous	1
4	26/F	Suprasellar	Pterional	Adamantinomatous	1
5	37/M	Suprasellar	Pterional	Adamantinomatous	1
6	21/F	Suprasellar	Pterional	Adamantinomatous	1
7	47/M	Suprasellar	Pterional + transcallosal	Adamantinomatous	2
8	52/F	Suprasellar	Transcallosal + pterional (2)	Adamantinomatous	3
9	29/M	Suprasellar	Pterional + transcallosal	Adamantinomatous	2
10	20/F	Suprasellar	Transcallosal + pterional	Adamantinomatous	2

the corneal tissue. A uniform amount of tissue was implanted into the micropocket between the 2 epithelial layers of the cornea.

Ten craniopharyngioma tissue specimens were implanted into the cornea of animals to compare the angiogenic potentials of recurrent and nonrecurrent tumor samples. Glioblastoma multiforme (GBM) and arteriovenous malformation (AVM) tissues were used as positive controls, and normal brain tissue served as a negative control.

Progression of angiogenesis was followed by microscopy and photographed for days 3, 5, 7, and 9 after tumor implantation. If signs of ocular infection (discharge, redness around the eye) appeared in either eye of a rat, it was replaced by another rat using the same procedure and tissue sample. For data collection, each cornea was photographed using a digital video system attached to a biomicroscope (Carl Zeiss Co., Oberkochen, Germany), and the degree of angiogenesis was assessed by counting the number of visible vessels by a person blinded to the study.

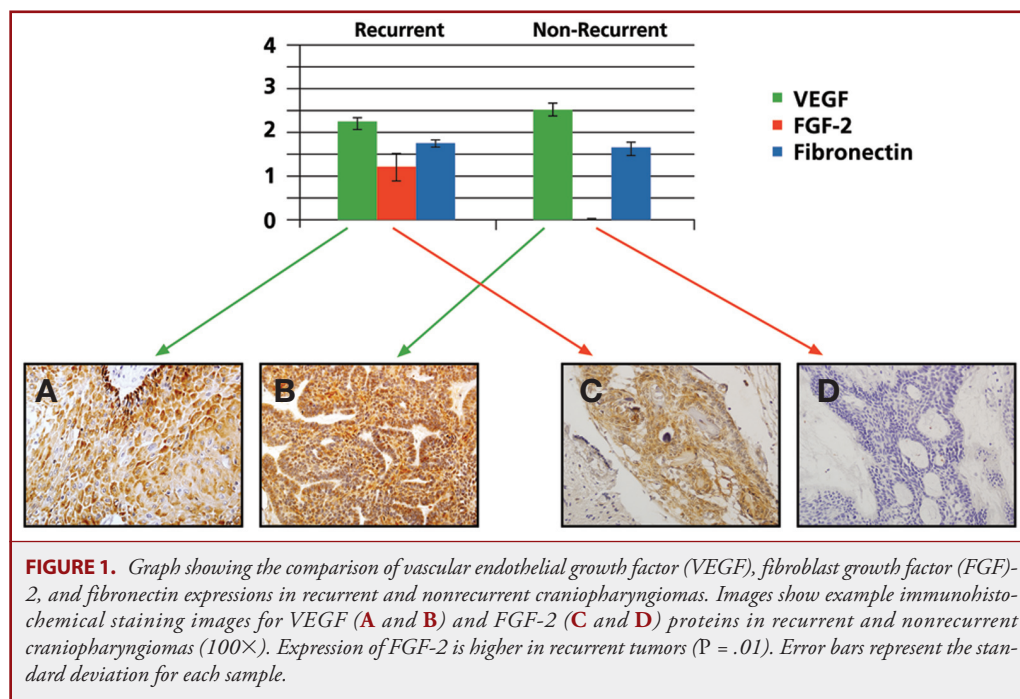
Statistical Analysis

The levels of fibronectin, VEGF, PDGF-A, PDGF-B, PDGF- α , PDGF- β , and FGF-2 expressions were compared using the Mann-Whitney *U* test. A *P* value < .05 was considered significant. The results for microvessel count were interpreted and compared as a function of time by the use of cell line charts with error bars indicating 1 standard error of the mean.

RESULTS

Fibroblast Growth Factor 2, Vascular Endothelial Growth Factor, and Fibronectin Expressions

Comparisons of FGF-2, VEGF, and fibronectin expressions are shown in Figure 1. Fibroblast growth factor 2 was expressed in recurrent craniopharyngiomas; however, there was no FGF-2 expression in nonrecurrent tumors (*P* = .01). On the other hand, although VEGF and fibronectin was expressed in both type of tumors, the levels of expression were similar for recurrent and nonrecurrent tumors (*P* = .61 and *P* > .99, respectively).



Platelet-Derived Growth Factor Receptor and Ligand Expressions

Expression levels of PDGF receptor and ligands are shown in Figure 2. Although both types of tumors expressed PDGF ligands (PDGF-A and PDGF-B), immunohistochemical staining intensities were similar ($P = .76$ and $P = .61$, respectively). On the other hand, PDGFR- α expression was significantly higher in recurrent craniopharyngiomas compared with nonrecurrent tumors ($P = .02$), whereas such an association could not be observed for PDGFR- β ($P = .11$).

Corneal Angiogenesis Assay

Corneal angiogenesis experiments showed significant differences between recurrent and nonrecurrent tumors in terms of angiogenic potential based on microvessel counts, starting from the fifth day of the experiment (Figure 3, A and B). As expected, positive controls (GBM and AVM samples) showed higher and negative controls showed lower angiogenic potential, compared with both tumor groups.

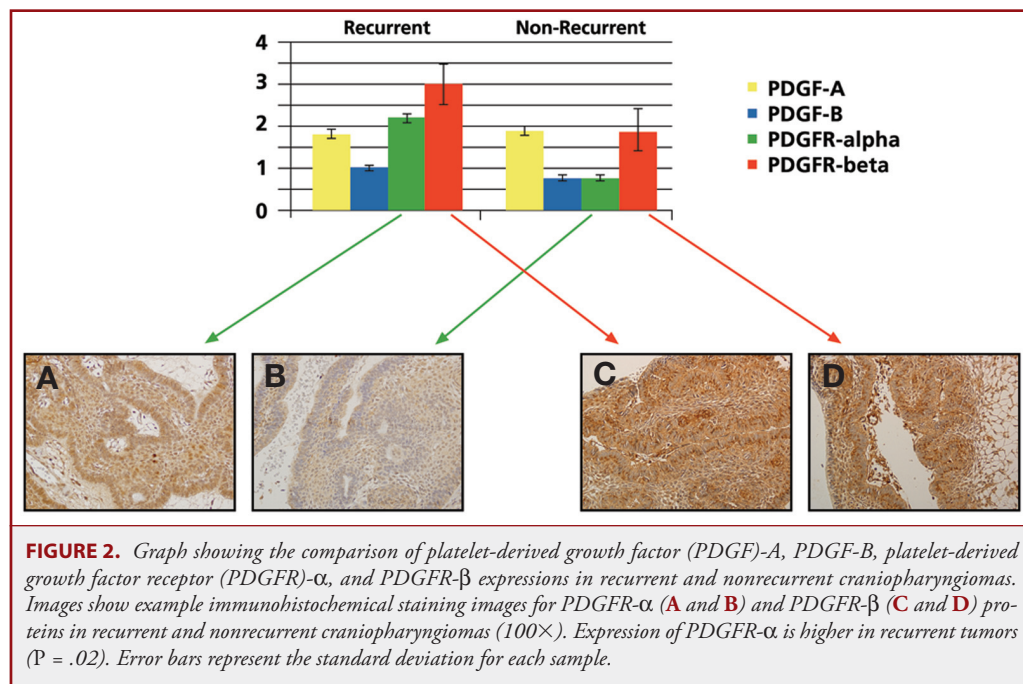
DISCUSSION

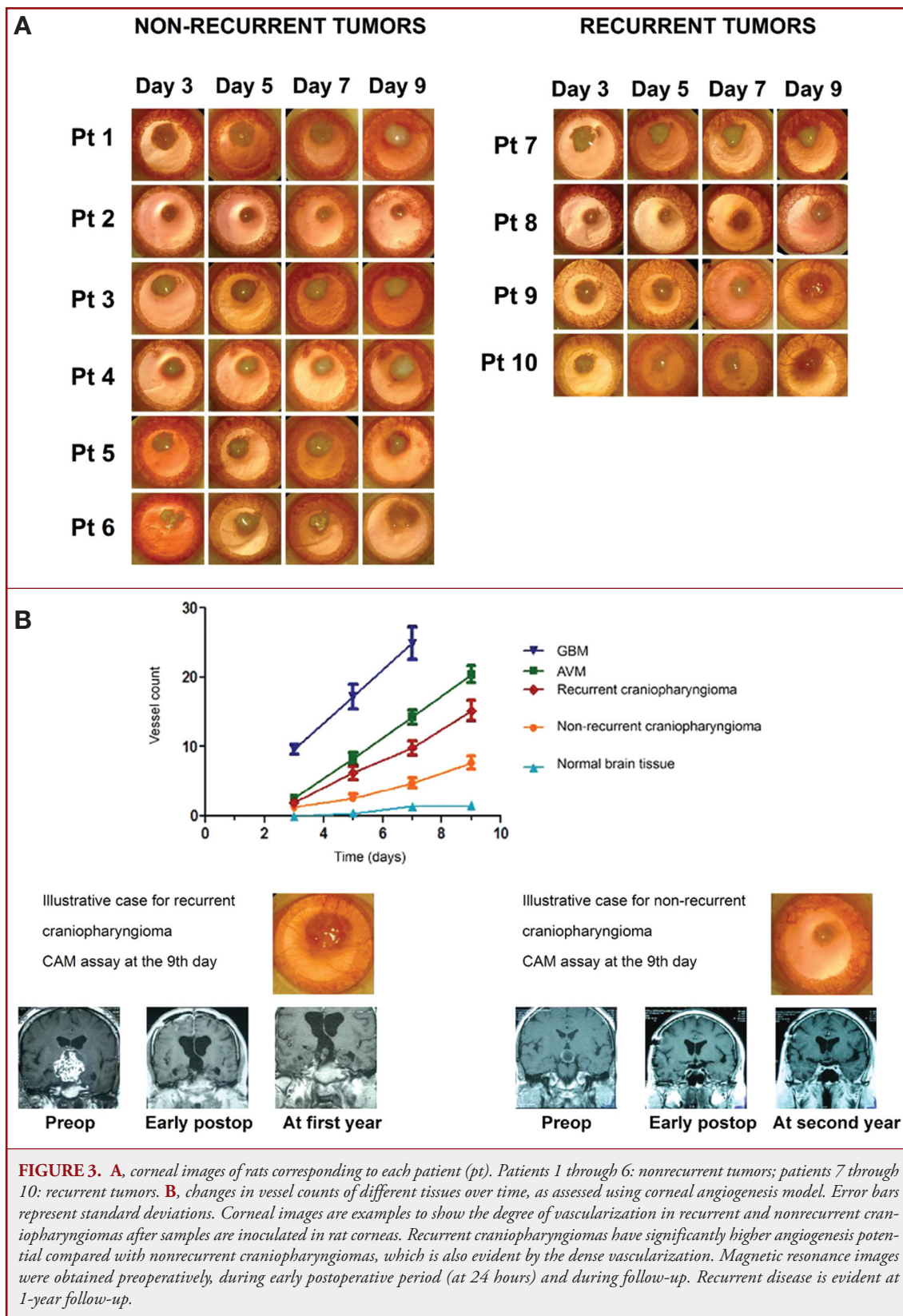
Craniopharyngiomas are benign tumors that develop in the sellar or suprasellar area and generally interact with normal brain tissue.²⁶ Consequently, total excision of the tumor is a challenge for neurosurgeons. Even after total excision, recurrences are relatively common in patients undergoing surgery for craniopharyngiomas,⁷ with little insight into the factors associated with the risk of recurrence. Although residual tumor tissue is implicated in many cases of recurrent craniopharyngioma, previous reports suggest no increase in the size of the residual tumor, and recurrence may develop even

after total excision.^{5,27-31} The potential role of angiogenesis in predicting the risk of recurrence has been assessed in certain tumor types.^{18,19} Choi et al¹⁸ found increased angiogenesis in recurrent colorectal cancer using an immunohistochemical method with antibodies against factor VIII-related antigen and by observing the vascularization in the tumor area. Similarly, Agozzino et al³² microscopically detected more vascularization in recurrent craniopharyngiomas. Accordingly, we compared the angiogenic potential in recurrent and nonrecurrent craniopharyngiomas. In our study, we also assessed the potential for neovascularization in recurrent vs nonrecurrent craniopharyngiomas by comparing angiogenic propensity as measured by the extent of neovascularization in rat cornea implanted microsurgically with the respective tumor cells. Glioblastoma multiforme, the most invasive type of brain tumor with a high degree of vascularization, was used as the positive control, as was AVM, which was shown to possess a significant potential for neovascularization by our team previously.²⁵ Normal brain tissue was used as the negative control. Our assessments showed significantly higher angiogenic potential for recurrent craniopharyngiomas with more corneal new vessel formation than nonrecurrent tumors. As with some other tumor types, these findings suggest a possible association with the recurrence and angiogenic potential in patients with craniopharyngiomas.

Angiogenesis is the process of formation of new capillary vessels from already existing vessels in a signal dependent manner, and it has been shown to play an important role in tumorigenesis as well as in some cerebrovascular malformations.^{16,33-35} Several growth factor pathways, such as FGF and PDGF, have also been shown to play an important role during this process. In an experimental setup removing inhibitory elements on PDGF-B messenger RNA, Shih et al³⁶ were able to eventually increase internal PDGF-B levels. They clearly observed that increased PDGF signaling resulted in higher tumor angiogenesis in glial tumors. Also, significant increase in tumor grade was observed after the increase in PDGF signaling. The role of FGF in tumor growth and angiogenesis is also well defined.³⁷ Mireux et al³⁸ have implanted glioma cells expressing a dominant negative form of FGF-2 receptor in null mice. Magnetic resonance imaging results showed a significant loss in tumor size compared with control. Also, there was a decrease in tumor angiogenesis and vessel stability when FGF pathway integrity is disrupted in implanted glioma cells.

Craniopharyngiomas are angiogenically active tumors that





express growth factors related to angiogenesis.^{21,22,39} Our results show a statistically significant difference in the expression of angiogenesis related proteins FGF-2 and PDGFR- α in recurrent vs nonrecurrent craniopharyngiomas as demonstrated by an immunohistochemical analysis ($P = .01$ and $P = .02$, respectively; Figures 1 and 2), suggesting a possible role for angiogenesis in craniopharyngioma recurrence. However, one should be cautious in extrapolating the results of descriptive studies and bear in mind the limitations related to our small sample size (Table 1) and difficulties inherent in the interpretation of immunohistochemical methods.

The PDGF pathway is inactive in normal brain tissue as a result of minimal neovascularization. Immunohistochemical staining of the brain tissue shows expression of PDGF-A and PDGFR- β , without the expression of their corresponding ligands and receptors. This prevents self-activation of the pathway by autocrine signaling and formation of a closed loop.⁴⁰⁻⁴⁴ Expression of both ligands and receptors in the PDGF pathway is a clear indication of formation of a closed loop in recurrent craniopharyngiomas, which supports the role of angiogenesis in recurrent craniopharyngiomas. As such, Fakhari et al⁴⁵ achieved a regression in tumor size in recurrent spinal ependymomas with the administration of imatinib (Gleevec), a selective PDGF blocker. This new therapeutic option also suggests a possible role for the inhibitors of angiogenesis that selectively block PDGF receptor autocrine loops in patients with craniopharyngiomas.⁴⁶⁻⁴⁸

CONCLUSION

In our corneal angiogenesis model, we have shown a higher angiogenic potential in recurrent craniopharyngiomas compared with nonrecurrent craniopharyngiomas. Also, immunohistochemical assays showed increased levels of proteins such as PDGFR- α and FGF-2 that play an important role in the angiogenesis, in recurrent craniopharyngiomas. These findings suggest a direct relationship between the pathologic behavior of the tumor and vascularization, which can be exploited to search for novel and effective therapeutic options for the treatment of craniopharyngiomas. New therapy options with selective PDGF blockers such as imatinib may be efficient for the prevention of recurrence.

Disclosure

Turker Kilic, MD, PhD, is a member of Turkish Academy of Sciences. This study was supported by The Scientific and Technological Research Council of Turkey and Turkish Academy of Sciences. The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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Acknowledgment

The authors thank A. Nur Sozudogru, MD, for his valuable contributions to the preparation of the manuscript.

COMMENT

This is an interesting article suggesting a relationship between craniopharyngiomas and angiogenesis. The authors have suggested that there is a correlation between platelet-derived growth factor receptor- α and fibroblast growth factor 2 and recurrence. Although the observation is interesting, certain questions cannot be answered. It would be important to know whether the original craniopharyngioma had the same angiogenic potential as the recurrence. In future studies, investigators ought to test primary resected specimens in a similar fashion so that recurrences can be correlated. It is not entirely clear to me whether the predominantly cystic tumors had as high an incidence of angiogenesis as did the mainly solid tumors. It would also be interesting to look at other tumor recurrences to see whether this is a pattern that might suggest that angiogenesis is greater in recurrent tumors because of the change in anatomical planes from the first intervention rather than from an intrinsic difference in the tumor.

The authors should be congratulated for presenting a provocative proposal.

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