SPANDIDOS PUBLICATIONS

EXPERIMENTAL AND THERAPEUTIC MEDICINE 1: 921-926, 2010

Markers of vascular differentiation, proliferation and tissue remodeling in juvenile nasopharyngeal angiofibromas

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Received June 18, 2010; Accepted July 21, 2010

DOI: 10.3892/etm.2010.141

Abstract. Juvenile nasopharingeal angiofibroma (JNA) is a histologically benign locally aggressive tumor characterized by irregular vessels embedded in a fibrous stroma. Excessive vascularity results in bleeding complications, and the inhibition of angiogenesis is a promising strategy for managing extensive JNA tumors. To better characterize the endothelial components of JNA, we aimed to evaluate markers of vascular differentiation and proliferation, such as friend leukemia integration-1 (FLI-1) and endoglin, lymphatic markers, including podoplanin and vascular endothelial growth factor receptor 3 (VEGFR3) and its cognate ligand VEGFC, GLUT-1, a diagnostic marker that discriminates between hemangiomas and vascular malformations, and two markers of tissue remodeling, stromelysin 3 (ST3) and secreted acid protein rich in cysteine (SPARC). Antigens were assessed immunohistochemically in vessels and stromal cells of JNA archival cases (n=22). JNA endothelial cells were positive for endoglin, VEGFC and FLI-1, whereas podoplanin and VEGFR3 were negative in all cases. Both endothelial cells and fibroblasts stained for ST3 and SPARC. GLUT-1 was investigated in JNA cases, in infantile hemangiomas (n=123) and in vascular malformations (n=135) as controls. JNAs and vascular malformations were GLUT-1-negative, while hemangiomas showed positive staining. The presence of markers of endothelial differentiation and proliferation highlighted the hyper-proliferative state of JNA vessels. The absence of podoplanin and VEGFR3 underscores their blood endothelial cell characteristic. The absence of GLUT-1 discriminates JNAs from hemangiomas. ST3 and SPARC up-regulation in endothelial cells and fibroblasts may contribute to a compensatory signaling for controlling angiogenesis. Some of these markers may eventually serve as therapeutic targets. Our results may aid in the understanding of JNA pathophysiology.

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Key words: juvenile nasopharingeal angiofibroma, endoglin, friend leukemia integration-1, podoplanin, vascular endothelial growth factor receptor 3, GLUT-1, stromelysin 3, secreted acid protein rich in cysteine

Introduction

Juvenile nasopharyngeal angiofibroma (JNA) is a rare benign neoplasm characterized by proliferating irregular vascular channels within a fibrous stroma composed of spindle cells in a dense collagen matrix (1,2). Despite being classified as benign tumors, JNAs often exhibit an aggressive growth pattern with possible intracranial spreading. These features have stimulated numerous theories on tumor origin, focusing on the vascular or the stromal component (3). Nevertheless, the implicated neoplastic cell responsible for sustained growth remains a matter of debate (1,3,4).

Recent studies based on vascular irregularities (thickness of the vessel walls, discontinuous vascular basal laminae and focal lack of pericytes) have led to the hypothesis that JNA is a vasoproliferative malformation (2,3). This suggestion of a vascular tumor origin is supported by an embryological explanation, based on the involvement of plexus remnants deriving from incomplete regression of the first branchial arch artery, which were incorporated into the vascular tumor component (3,4).

The presence of proteins such as merosin (laminin α2) and collagens type VI and 1A2, which were recently described as being highly expressed in JNA vessels, is suggestive of vessels in an early developmental vascular differentiation state (5-7). Notably, merosin and type VI collagen were also elevated in several types of cancer and in proliferative hemangiomas. The latter are vascular lesions characterized by abnormal excessive endothelial cell proliferation (5-9).

A series of immunohistochemical studies have been performed to analyze angiogenic activators and vessel growth promoting factors in JNA samples. Vascular markers, such as CD31, CD34 and von Willebrand factor (vWF), were previously

documented in JNAs. However, such pan endothelium biomarkers did not discriminate between vascular and lymphatic cells, and some are not restricted to blood vessels (10). TGFβ1, FGF, vascular endothelial growth factor (VEGF A) and its receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR), were also reported to be expressed in JNA endothelial cells (11-19). Of note, several of these growth factors were also elevated in both hemangiomas and in tumor-associated blood vessels (8,20).

Thus, is would appear that JNA vessels exhibit malformative and endothelial proliferative features, as well as neoplastic markers, although they maintain benign characteristics. Similar aspects of angiogenesis in vascular anomalies and tumors were recently discussed (4).

Given that the most conspicuous feature of JNAs is the endothelial proliferative state, inhibition of angiogenesis is thought to be a promising strategy for their treatment. Our research has focused on identifying other markers that may provide additional information regarding the abnormal vascular component of JNAs. The first step in the present study was to evaluate the expression of endothelial markers involved in cell differentiation and cell growth, such as friend leukemia integration-1 (Fli-1) and endoglin. Fli-1 is a nuclear transcription factor that is considered to be a very reliable marker of endothelial differentiation. Fli-1 was reported to be involved in cellular proliferation and tumorigenesis, as well as in the suppression of the expression of genes such as Rb and Bcl-2 protein (21,22). Endoglin (CD105) is a transmembranic glycoprotein that is a marker of activated endothelium; its vascular expression is limited to proliferating endothelial cells, with no expression in normal vessels (23). To investigate the vascular differentiation state of JNA endothelial cells, specific markers of lymphatic vessels, such as podoplanin and VEGFR3 and its ligand VEGFC (24,25), were also analyzed. The erythrocyte transporter (GLUT-1) was described in fetal endothelial cells (26) in solid tumors with enhanced glycolitic demand (27), and is a universal trait of hemangiomas, discriminating these tumors from vascular malformations (28). Thus, we evaluated the immunohistochemical expression of GLUT-1 in JNAs and compared its expression to that displayed by hemangiomas and vascular malformations used as the control.

Studies focused on differences in gene expression in endothelial cells derived from tumor tissues have revealed the enrichment of genes involved in tissue remodeling, a hallmark of post developmental angiogenesis, such as stromelysin 3 (ST3) and secreted acid protein rich in cysteine (SPARC; or osteonectin). These are up-regulated in a number of pathological processes (29-31). ST3 (MMP11) is a member of the metalloprotease (MMP) family and is an established connective tissue-derived factor associated with embryogenesis, wound healing and tissue involution (31). SPARC is a matricellular glycoprotein that has counter adhesive and antiproliferative functions and is associated with developing vessels and tumor blood vessels (32). Its importance in the angiogenic events in JNAs has been previously hypothesized (33). Thus, the expression of ST3 and SPARC was investigated in the present study.

Materials and methods

Case selection. Twenty-two patients with JNA who underwent surgery between 2000 and 2006 at the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo were selected for this study. All patients were male and between 12 and 25 years of age at the time of surgery (median 16 years). Since the study was retrospective, informed patient consent was waived. However, any form of patient identification was avoided. The study was approved by the Ethical Committee for Research Projects of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

The tumors were staged using the Fisch system and ranged from stage II to IIIa. Recurrence was observed in 20% of the cases. Frozen tumor samples were obtained from 18 of the patients with JNAs; samples of paired turbinate tissues were obtained from 15 of the 18 patients and used for ST3 mRNA determination by Northern blot analysis.

In addition, 124 cases of proliferative hemangioma and 135 cases of vascular malformation excised at the Hospital do Câncer A.C. Camargo (São Paulo, Brasil) were examined. The age of the patients at surgery ranged from 1 to 208 months (median 42), and there was a predominance of female patients (69.5%).

The study was approved by the Hospital A.C. Camargo Institutional Ethics Committee.

Immunohistochemical analysis. A representative paraffin block was selected from each case, and 3 μm-thick sections were prepared for immunohistochemical staining.

Sections were deparaffinized and rehydrated through a graded series of ethanol. Antigen retrieval was performed in a pressure cooker using citrate buffer at a pH of 6.0. Endogen peroxidase activity was blocked by 3% perhidrol. Slides were rinsed with PBS (pH 7.4) and incubated at 4˚C for 18 h with the primary antibodies (Table I). This was followed by incubation with the respective appropriate secondary antibodies against podoplanin, GLUT-1, ST3, FLI-1, SPARC, endoglin, VEGFA and VEGFC, as well as their receptors (VEGFR1 and VEGFR3) and CD34, CD31 and vWF (factor VIII-related antigen).

StreptABComplex – HRP Duet mouse/rabbit (K0492; Dako) diluted 1:200 in PBS was used as the detection system. Slides were developed in diaminobenzidine solution and counterstained with Harris hematoxylin.

Immunoreactivity was assessed in both vascular endothelial cells and in the stroma of JNA cases by staff pathologists (H.T., H.C. and F.A.S.). The sections were scored according to the degree of positivity observed in the cells. According to our grading scale, cases presenting <10% of stained cells were considered negative. Sections incubated without the primary antibody served as negative controls.

RNA isolation and Northern blot analysis. The frozen tissues were pulverized and the total RNA was isolated by the guanidinium isothyocyanate method. Northern blot analysis was performed as previously described (34). An ST3 probe was radiolabeled using a rediprime DNA labeling system with [α-32p]dCTP (Amersham-Pharmacia). The ST3/MMP11 cDNA probe was donated by Dr P. Chambon of the Institute of Genetics and Molecular and Cellular Biology (Strasbourg, France). The autoradiograms were individually quantified by densitometry, and the ratio of each mRNA to 18S mRNA was compared to the average ratio of the controls. The mean value of the expression of the genes in normal turbinates was defined

Table I. Antibodies used for immunohistochemical analysis.

Antibodies	Clones	Sources	Dilutions
CD34	QBEnd 10	M7165; Dako, Glostrup, Denmark	1:100
CD31	JC/70A	M0823; Dako	1:50
Factor VIII	Polyclonal	A0082; Dako	1:300
VEGF (A-20)	Polyclonal	sc152; Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:600
GLUT-1	Polyclonal	A3536; Dako	1:500
Podoplanin	18H5	DM3500; Acris antibodies, Hiddenhausen, Germany	1:200
VEGFC	Goat polyclonal	sc1881; Santa Cruz Biotechnology	1:100
ST3/MMP11	SL3.05	MS1035P; NeoMarkers, Fremont, CA, USA	1:100
VEGFR3 (FLT4)	Rabbit polyclonal	RB1527; Thermo Scientific, Fremont, CA, USA	1:400
VEGFR1 (Flt-1)	Rabbit polyclonal	RB9255; Thermo Scientific	1:100
FLI-1	Mouse monoclonal	LLC CM274A,B; Biocare Medical, Concord, CA, USA	1:30
SPARC	Rabbit polyclonal	Ab1858; Chemicon, Temecura, CA, USA	1:2,000
Endoglin	SN6H	MS1290; NeoMarkers	1:100

as the basal or normal expression. An expression of >2-fold above that found in normal turbinate tissues was defined as overexpression. Immunohistology was performed to visualize ST3 protein expression in JNAs.

Results

A total of 22 cases of JNA were included in this study. Results regarding the frequency of positivity of the antibodies utilized are shown in Table II. Podoplanin was evaluated in the JNAs and, although it was evident in the endothelial lining of small lymphatic vessels, was consistently not detected in JNA vessels (Fig. 1B). VEGFC expression was noted in both stromal and endothelial cells in all cases (Fig. 1C). All cases were uniformly negative for VEGFR3 (Fig. 1D). The pan endothelial marker FLI-1 and the marker of activated endothelium, endoglin (CD105), were positive in the vessels in 100% of the cases. Endoglin was absent in the stromal cells. Single cells also stained positively for FLI-1, but it was not clear whether these cells were endothelial or stromal cells (Fig. 1E and F). SPARC expression was found primarily in the JNA endothelial cells, but was also observed in stellated shaped cells of the stroma (Fig. 1G). Of the 15 cases analyzed by immunohistochemistry, 66.7 and 33.3% showed ST3 positivity, respectively, in vessels and in numerous single stromal cells (Fig. 1H). Samples of JNA were analyzed for GLUT-1, and the rate of positivity was compared to that found in hemangiomas and in vascular malformation cases. GLUT-1 immunoreactivity was present in the majority of hemangiomas tested (Table III, Fig. 1A), but was absent in vascular malformations and nasofibromas. This difference was highly significant ($p<0.01$).

We also evaluated CD31, CD34 and FVIII, which highlighted the vascular endothelium in all cases. CD31 and FVIII were absent in stromal cells, but CD34 showed a weak positivity in 80% of the stromal cells. VEGFA and VEGFR1 were positive in the endothelial cells in all cases, whereas stromal cells showed a weak expression of both markers in 79% of the cases (data not shown).

Table II. Frequency of endothelial cell markers in juvenile-nasoangiofibromas vessels.

Markers	Frequency (%)
FLI-1	22/22 (100)
Endoglin (CD105)	22/22 (100)
Podoplanin	0/22 (0)
VEGFR3	0/22 (0)
VEGFC	22/22 (100)
ST3 (MMP11)	10/15 (66.7)
SPARC	22/22 (100)
VEGFA	21/21 (100)
CD31	22/22 (100)
CD34	22/22 (100)
FVIII	22/22 (100)

Northern blotting was performed to analyze the mRNA for ST3/MMP11, which was expressed in all tumor samples, showing increased levels as compared to normal turbinates (0.68 ± 0.36 vs. 0.01 ± 0.006) and resulting in significant distribution of these mRNA values ($p<0.001$). Representative autoradiographs from Northern blot analysis of ST3 are shown in Fig. 2.

Discussion

To better characterize the endothelial component of JNAs, our objective in the present study was to determine endothelial proliferation and differentiation markers that have not been previously analyzed in this rare tumor. As it has been suggested that the irregular vascular basement membrane described in JNAs and tumors may possibly result from tissue remodeling (35), we also analyzed the biomarkers of remodeling.

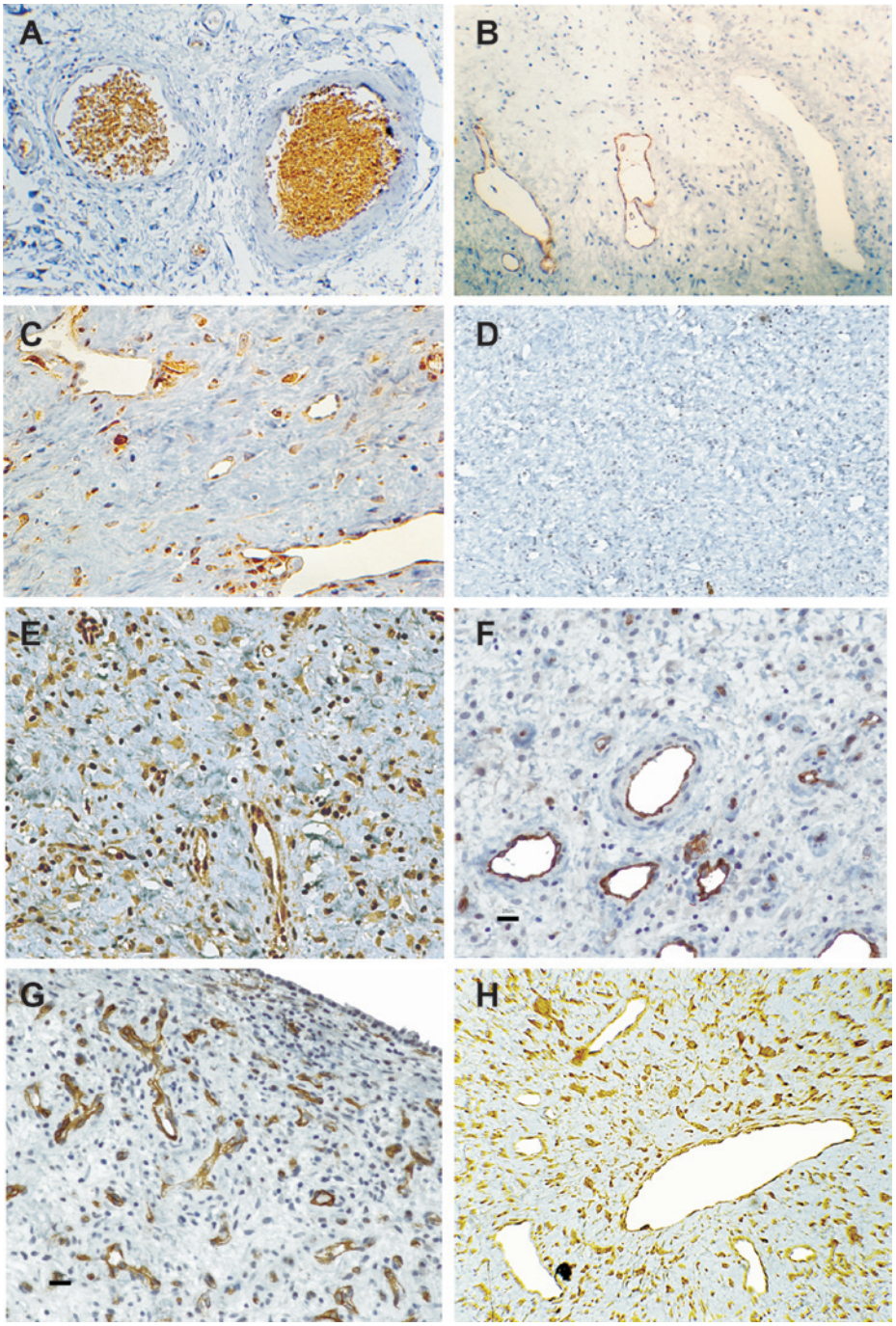

Figure 1. (A) GLUT-1 expressed in the endothelium of infantile proliferating hemangiomas (x200). (B, C, D, E, F, G and H) Immunohistochemical staining of juvenile nasopharyngeal samples for the antibodies used. Podoplanin was evident in the endothelial lining of lymphatic vessels and absent in blood vessels (x200) (B); VEGFC decorated the blood vessels and stromal cells (x200) (C); absence of VEGFR3 staining in JNA vessels (x100) (D); Endothelial cells showing a positive reaction for FLI-1 (x200) (E); endoglin-positive staining in JNA vessels (x200) (F); SPARC expressed by endothelial cells and by a few stromal cells (x200) (G); ST3 positivity in endothelial and stromal cells (x200) (H).

Table III. GLUT-1 immunoreactivity in vascular lesions.

	GLUT-1 (%)
Vascular malformations	0/135 (0)
Hemangiomas	101/124 (81.5)[a]
Angiofibromas	0/22 (0)

[a]GLUT-1-negative hemangiomas were in an involution phase. Positive cases showed immunoreactivity in more than 10% of the cells.

Our findings of positive endoglin and Fli-1 staining in the vessels of the cases analyzed here highlight the endothelial differentiation as well as the hyper-proliferative state of angiogenesis in JNAs. Although VEGFC acts primarily as a lymphangiogenic factor, it may also promote the formation of blood vessels (36).

The finding that JNA vessels were devoid of the expression of lymphatic endothelial cell specific markers (podoplanin and VEGFR3) underscores their blood endothelial cell characteristic, without expressing lymphatic competence. Lack of

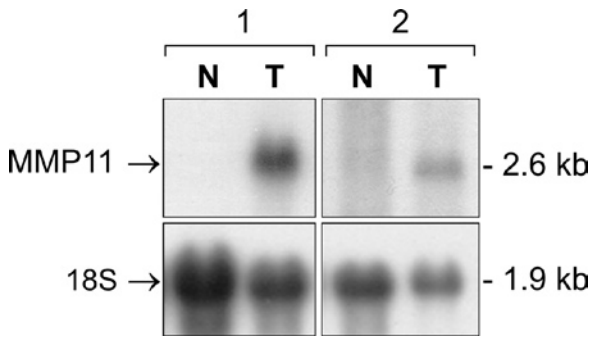

Figure 2. Northern blot analysis of ST3/MMP11 gene expression in JNAs. N, RNA from normal turbinates; T, RNA from JNAs.

GLUT-1 discriminated JNAs from hemangiomas, which is interesting in the context of the suggestion that JNAs might represent vascular malformations (2,3).

Higher levels of ST3 mRNA were found in JNA vessels as compared to inferior nasal turbinate specimens. Although several MMPs have been described in JNAs (37), there have been no reports of ST3 expression in this particular type of tumor. ST3 differs from other MMPs, since its mature form is incapable of cleaving type I and III collagens, the major components of the stromal and vascular ECM in JNAs (7). On the contrary, ST3 exhibits a collagenolytic function against the native α3 chain of collagen VI (38). Prominent collagen-type VI expression has been previously described in JNA vessels, possibly exerting growth stimulatory effects on endothelial cells (6,7).

Several studies reviewed by Clark and Sage (30) indicated that a stressed microenvironment increases the expression of SPARC, reflecting the loss of normal tissue homeostasis. The abundance of SPARC in the late stages of tumor progression and invasion may represent a failed attempt to restore tissue homeostasis within the tumor microenvironment.

According to Chlenski *et al* (39), SPARC has antiangiogenic properties and is capable of suppressing the activity of VEGF by directly binding to this ligand through a negative regulatory feedback mechanism, as VEGF-induced SPARC (40). Alternatively, SPARC enhances tumor stroma formation, stimulating the production and secretion of several extracellular matrix proteins, including collagen type I, thus favoring the increase of the fibrous component observed during JNA maturation (41). Our results suggest a role for ST3 and SPARC in altering extracellular matrix properties during angiogenesis in JNAs.

Of note, ST3 was reported to be induced by TGFβ (42), and a reciprocal regulatory loop has been demonstrated for SPARC and TGFβ, since SPARC was shown to induce TGFβ and vice versa. Endoglin is a type III/TGFβ1 receptor also up-regulated by TGFβ stimulation (23), and TGFβ type II receptor gene transcription is activated by Fli-1 (21). TGFβ was previously reported in both the stromal and endothelial cells of JNAs (12,15,16). Taken together, our data emphasize the potential role of TGFβ1 in the pathogenesis of JNA.

Acknowledgements

This study was supported by CNPq and FAPESP. We thank Dr Waldir Carreirão Neto for help with the collection of samples and the clinical characteristics of the patients and Dr J.P. Plese from the Neurology Department (FM, USP) for the careful review of the manuscript.

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