The Role of Angiogenesis in the Transformation of Plexiform Neurofibroma into Malignant Peripheral Nerve Sheath Tumors in Children With Neurofibromatosis Type 1

Benjamin Gesundheit, MD, PhD,† Patricia Parkin, MD,‡ Mark Greenberg, MD,† Sylvain Baruchel, MD,† Christof Senger, MD,§ Josef Kapelushnik, MD,‖ Charles Smith, MD,§ and Giannoula Lakka Klement, MD¶*

Purpose: The role of angiogenesis in the transformation of peripheral neurofibroma (PNF) to malignant peripheral nerve sheath tumor (MPNST) in neurofibromatosis type 1 (NF1) remains elusive and forms the objective of this study.

Experimental Design: Archival tissue from 5 children with NF1 and PNF, who developed MPNST between the ages of 8 and 15 years were analyzed for differences in microvasculature. The role of proangiogenic growth factors such as Vascular Endothelial Growth Factor (VEGF), and its receptors Flk-1 and Flt-1, and vessel maturity, defined as von Willebrand factor (vWf), α-smooth muscle actin$^+$ (SMA$^+$), were evaluated by immuno-histochemistry.

Results: A qualitative evaluation of the vasculature showed predominantly α-SMA+/vWf+ more stable vessels in PNF, and an irregular meshwork of α-SMA−/vWf+ endothelial cells structures in MPNST. In NF and PNF tumor cells were VEGF$^-$, in contrast to VEGF$^+$ tumor cells in MPNST. If present, the VEGF stain was confined mainly to the perivascular spaces in PNF, unlike the mainly stromal VEGF stain in MPNST. VEGF receptors also manifested a tumor stage-specific pattern. Flk-1 and Flt-1 were restricted to the mature, well-formed vasculature in PNF, but exhibited a diffuse pattern in MPNST.

Conclusion: Our study provides a rare opportunity to document consistent and histologically detectable differences in the vascular organization of PNF and MPNST. It permits a pair-wise evaluation of the malignant conversion of benign PNF into its malignant counterpart, in the same patients. The phenotypic variations and characteristics of the vessels in these tumors are consistent with the idea that a strong proangiogenic drive contributes to the progressive growth in MPNST.

Key Words: neurofibromatosis, angiogenesis, vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-1 (VEGFR-1), vascular endothelial growth factor receptor-2 (VEGFR-2), α-smooth muscle actin (α-SMA), peripheral neurofibroma (PNF), malignant peripheral nerve sheet tumor (MPNST)

Received for publication May 19, 2009; accepted May 14, 2010.

From the *International Center for Cell Therapy and Cancer Immunotherapy, Tel Aviv; ‖Department of Pediatric Hematology and Oncology, Beer Sheva, Israel; †Division of Hematology and Oncology; Departments of ‡Pediatrics; §Department of Laboratory Medicine-Hospital for Sick Children, Toronto, Ontario, Canada; and ¶Floating Hospital for Children at Tufts Medical Center, Boston MA.

Reprints: Giannoula Lakka Klement, MD, Department of Pediatric Oncology, Floating Hospital for Children at Tufts Medical Center, 800 Washington Street, Boston, MA 02111 (e-mail: giannoula.klement@tufts.edu).

(*J Pediatr Hematol Oncol* 2010;32:548–553)

Neurofibromatosis type 1 (NF1), or von Recklinghausen Disease, named after its first describer Friedrich von Recklinghausen (1833 to 1910) in 1882,[1] is a progressive multisystem disorder with a high propensity for tumor development in humans.[1–4] Two distinct clinical phenotypes are known as a result of mutations of 2 separate NF genes: *NF1*, coding for the Neurofibromin, is the more commonly mutated gene and the associated syndrome occurs in about 1 of 4000 live births. *NF2*, also know as bilateral acoustic NF (BAN), is much rarer and occurs in about 1 of 40,000 live births[5,6]; NF1 is autosomal-dominant disease and in 50% of the patients results from a germline *de novo* mutation of the *NF1* gene. It is characterized by diverse, progressive cutaneous, neurologic, skeletal, and neoplastic manifestations, and its diagnosis is based on criteria summarized by the National Institute of Health (NIH) Consensus Conference.[3,6,7] It includes the development of skin stigmata and of typical tumors such as neurofibromas, neurofibrosarcomas, neurogenic sarcoma, astrocytoma, juvenile chronic myelogenous leukemia, pheochromocytoma, rhabdomyosarcoma, and, more rarely, Triton tumors.[8] There may be additional clinical hallmarks such as hypertension, osseous abnormalities such as scoliosis, various dermatologic lesions, and vasculopathies,[9] and focal and developmental neurologic deficits with learning disabilities.[10–12] Most relevant to this study is the increased risk of cancer in patients with NF1.[4] They have an increased risk of developing tumors of the central and peripheral nervous system (5%),[13] childhood leukemia,[14] plexiform neurofibromas (27%),[15] optic gliomas (15% to 20%),[15] pheochromocytomas (1%),[15,16] malignant peripheral nerve sheath tumors (5%),[15] and neurofibrosarcomas (6%).[4,17]

The most common extracranial neoplasias, peripheral neurofibromas (PNFs), are found in ±15% of NF1 patients.[18] They can remain indolent, grow, and cause clinical symptoms owing to compression of adjacent structures, or in 3% to 5% of cases undergo malignant transformation to neurogenic sarcoma (NS) also called neurofibrosarcoma or peripheral malignant nerve sheath tumors (PMNST).[19,20] These later tumors are highly aggressive, readily metastasize, and no treatments, except perhaps for radical surgical resection, are presently available. Regrettably, even after radical local surgery only 30% of the patients will remain disease free after 5 years.[21] As

such, even without the potential for malignant transformation, progressive growth of a benign PNF can be a life-threatening problem, especially in younger children below the age of 10 years.[22]

Current therapeutic modalities for PNF; that is surgical intervention, radiation and chemotherapy offer limited success. The infiltrative nature of these tumors, the number of metachronous lesions, and the high rate of tumor regrowth limit the efficacy of surgery. Both radiation and chemotherapy may also potentially induce secondary malignant neoplasms in this cancer predisposed background.[4,13] Novel biologic therapies such as cis-retinoid-acids and interferon-α-2a have shown only limited efficacy so far.[23,24] More than a century has passed since the original description of neurofibromatosis, and much remains unknown concerning the physiologic and angiogenic processes leading to malignant transformation from PNF to NS.

The recent finding that malignant transformation and tumor progression of several solid tumors are associated, and dependent upon, the induction of angiogenesis, or "angiogenic switch,"[25–27] may provide new insights to the pathology of NF1-associated tumors as well. The "angiogenic switch" can be turned on by either a reduction of naturally occurring angiogenesis inhibitors and/or by increased expression and activation of angiogenesis inducers.[28] An activation of angiogenesis inducers has been documented to occur in response of oncogenic mutations such as *ras*.[29,30] Among the natural inhibitors, angiostatin, tumstatin, and endostatin have already been shown to suppress the growth of a variety of human tumor xenograft models. Among the inducers, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) are believed to play pivotal roles in the process of tumor angiogenesis with the latter being specific for angiogenesis owing to the expression of VEGF receptors predominantly, but not exclusively, on endothelial cells. The ligands, bFGF, and VEGF, are less restricted to the endothelium, and are commonly produced by tumor cells[31] and stroma.[32] They act in a paracrine fashion to stimulate the proliferation of tumor-associated endothelium. Both the ligands and the receptors have now been successfully targeted for therapy and promise a valuable therapeutic option in neurofibromatosis as well.

Detection of active tumor angiogenesis by measuring elevated serum and urine levels of bFGF[33] and serum levels of VEGF have been shown to be associated with the extent and malignant potential of tumors, but are not known to decline reliably with treatment. The involution of hemangiomas of infancy in which urinary bFGF levels might be 20 to 50 fold those of healthy controls at the peak, a measurable change in serum, and urinary bFGF may be appreciated after involution of the lesion.[34,35] In comparison, many of the clinical trials that used VEGF and bFGF as a marker of disease response found it much less of a useful angiogenesis marker. Interestingly, the inhibitory effects of IFN α-2a on both the steady-state gene expression and protein production of bFGF were dose, dependent[36] suggesting a finely tuned control between the positive and negative regulators of angiogenesis.

There are some early antiangiogenic therapies in clinical and preclinical studies providing new possibilities for patients with NF1.[37–40] These include therapeutic trials with small molecule inhibitors of VEGF receptors in adults with neurogenic sarcomas,[37] and an open-label Phase I trial of thalidomide for the treatment of plexiform neurofibroma for patients with NF1, in which a 25% reduction in tumor size was observed in 4/20 patients treated.[41] Our goal here was to explore whether angiogenesis plays a role in the transformation of PNF to PMNST and whether identifying differential expression patterns of major proangiogenic growth factors may lead to refinement of these therapies.

MATERIALS AND METHODS

Tissue Specimens

Paraffin-embedded archival tissues from 5 patients with NF1 in whom both PNF and PMNST tissue samples were available were processed for histology, and in all cases the PNF and PMNST tissue was analyzed before any radio/chemotherapeutic treatment. The patients were seen in the special NF clinic at the Hospital for Sick Children, Toronto, Canada from 1986 to 2000, in which, approximately 200 children with NF and PNF were followed for a comprehensive multidisciplinary care. Characteristics and clinical course of the patients are summarized in Table 1. All 5 patients (3 boys + 2 girls) were diagnosed with NF1 at the age of 2.5 ± 0.5 years. Two had PNF at the time of their presentation to the clinic; the others were diagnosed with PNF within 1 year. PMNST developed in different locations over the next 7.5 ± 2.5 years. Despite debulking surgeries, radiation, and chemotherapy after the histologic diagnosis of PMNST, 3 patients died and 2 remain living with a follow-up of 3.5 ± 0.5 years.

TABLE 1. Patient Clinical Characteristics

Patient No. and sex	Age (in Years) at Diagnosis			Extension of Tumor, Site of Biopsy, and age at Biopsy	Size of Tumor by CT (in cm)	Treatment	Out-come
	NF	PNF	PMNST				
1 (M)	3	4	13	Retroperitoneal mass (13 y)	$5 \times 5 \times 4$	Multiple surgeries	Died (at age of 14 y)
2 (M)	3	3	8	Neck, intraspinal, mediastinum (7 y)	$4.5 \times 4 \times 3$	Multiple surgeries and radiation	Died (at age of 8 y)
3 (F)	2	2	8	Neck, face, mediastinum, right popliteal fossa (8 y)	$6 \times 5 \times 4$	Surgeries	Alive (3 y follow-up)
4 (M)	2	4	13	Right neck (13 y)	$8 \times 5 \times 4$	Radiation, surgery	Alive (4 y follow-up)
5 (F)	3	7	13	Left lumbosacral (13 y)	$7.5 \times 5 \times 4$	Radiation, surgery, palliation	Died (at age of 14 y)

CT indicates computed tomography; F, female; M, male; NF, neurofibromatosis; PNF, peripheral neurofibroma; PMNST, peripheral malignant nerve sheath tumors.

TABLE 2. Primary Antibodies and Pretreatment of Histologic Sections

Antibody Target	Source	Clone	Dilution	Pretreatment
H & E	Dako Carpinteria, CA (cat # Z0334)	Polyclonal	1:1000	None
vWf	Dako Carpinteria (cat #A008)	Polyclonal	1:500	Protease I*
α-SMA	Dako Carpinteria (cat #M0851)	1A4	1:40	None
VEGF	Santa Cruz, CA (cat #sc-152)	Polyclonal	1:500	Protease I*

*Proteolytic enzyme provided by Ventana Medical Systems, Tucson AZ (cat # 250-2018).
α-SMA indicates α-smooth muscle actin; H&E, hematoxylin; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor.

Immuno-histochemistry

All tissues were fixed in 10% formalin, embedded in paraffin, cut at 5 μm, and mounted on positively charged microscope slides before staining. Tissue sections were baked overnight at 60°C, dewaxed in xylene, and rehydrated with distilled water through decreasing concentrations of alcohol. Immunohistochemical procedures for von Willebrand factor (vWf), α- smooth muscle actin (SMA), and VEGF were carried out on the Ventana Gen II autoimmuno/in-situ stainer (Ventana Medical Systems, Tuscon, AZ), with a closed ABC system using the DAB (3–3′-Diaminobenzidine) Ventana Detection System (Cat #250-001). All tissue sections were pretreated for endogenous peroxidase. The counterstain of preference was hematoxylin, for clear nuclear detail. The stains for Immuno-histochemistry with their primary antibodies and pretreatments are summarized in Table 2. Commercially available inhibitory peptides were used as negative controls.

We used 6 complementary staining techniques to show the angiogenic features (Fig. 1). The standard staining H&E gives an impression of the tissue architecture and shows some of the typical features of PNF and malignant peripheral nerve sheet tumor (MPNST). VWf and α-SMA were used to underscore morphologic differences in blood vessels and to determine the degree of maturity and stability. The von Willebrand factor (vWf) is stored in the cytoplasm of the endothelial cells and released during coagulation, thus staining endothelial cells with a high degree of specificity and sensitivity. It does not, however, distinguish between established, stable, mature vessels, and immature newly formed ones. To show the degree of vessel stabilization as a surrogate of maturation, we correlated the α-SMA staining of pericytes, which stains actin within the smooth muscle cells and pericytes surrounding the blood vessel. This vessel phenotype has been associated with higher endothelial tube stability and suggests a higher level of maturation of the blood vessels.[42] For the differential expression of major known proangiogenic growth factors between PNF and PMNST, we stained for VEGF and its respective receptors, VEGFR1/Flt-1, and VEGFR2/Flk-1. This endothelial growth factor, produced and secreted from the tumor cells and the supporting stroma, is pivotal for the induction, maintenance, and propagation of angiogenesis.[32]

RESULTS

PNF showed large vessels with open lumina, and a clearly defined and pericyte-supported vascular wall (Fig. 1A). On hematoxylin/eosin stain of PMNST, a marked inflammatory component is evidenced by leucocyte margination and chronic micro-hemorrhages visible as iron-staining and hemosiderrin laden macrophages (Fig. 1B.). In contrast to the $vWf^+/\alpha\text{-}SMA^+$; well defined, mature vasculature in PNF, PMNST showed a pattern of numerous small $vWf^+/\alpha\text{-}SMA^-$ vessels with a mesh-like network of multidirectional, irregular vascular structures with indiscernible lumens. Many of these large vascular formations are consistent with glomeruloid structures reported by Pettersson et al.[43] Most of the PNF vessels are SMA^+ (Fig. 1E), which is in stark contrast to the SMA^- vessels and predominantly stromal smooth mucle stain of MPNST (Fig. 1F).

A very similar, yet more distinct pattern is evidenced with VEGF staining; tumor cells are predominantly negative with only the abluminal surface of the vessels staining for it in PNF (Fig. 1G), whereas in PMNST, a uniform cytoplasmic staining within the tumor cells and stroma (Fig. 1H). The receptors for VEGF are restricted to the tumor-associated vessels in PNF, with no staining in the vessels of surrounding normal tissues or in the tumor cells. In the MPNST, in addition to the vessel wall staining, the tumor cells and stromal fibroblasts seem to be strongly positive for both receptors, but this expression is limited to the perivascular cuffs of tumor and is not shared by the intervening connective tissue.

DISCUSSION

Our study provides a rare opportunity to compare benign and malignant counterparts within the tissues of a single patient. Although the association of VEGF and angiogenesis signaling with malignant progression has been documented earlier, these earlier studies were done by comparing groups of patients with colonic adenomas versus those with adenocarcinoma. The pathogenesis of malignant progression in neurofibromatosis has not been rigorously studied, and it is not widely appreciated that the pathognomic sign of cancer progression in neurofibromatosis may be angiogenesis. Unlike studies that compare a malignant tissue of 1 patient with premalignant lesion of another, the differences in protein expression in these cases are much more likely to reflect factors involved in malignant transformation when a single host is studied. This manuscript is the first to suggest an equivalent of an angiogenic switch induced by the oncogenic transformation during the development of MPNST, and offers a unique view into the specific subtypes of cells involved in the generation of a malignant, proangiogenic phenotype.

There is inflammatory cell margination in PNF (Fig. 1A), and whereas this can often be seen in tissue response to stress and may be owing to the surgery itself, it is equally likely, that it is reflective of the degree of tissue turnover and remodeling in response to tumor growth. In PNF, most vessels are vWf^+ and have open patent lumina (Fig. 1C), indicating a mature and stable vascular architecture that is underscored by presence of SMA^+ perivascular smooth muscle cells (Fig. 1E). We have used the coexpression of SMA and vWf as an index of maturity.[42] It is a relative

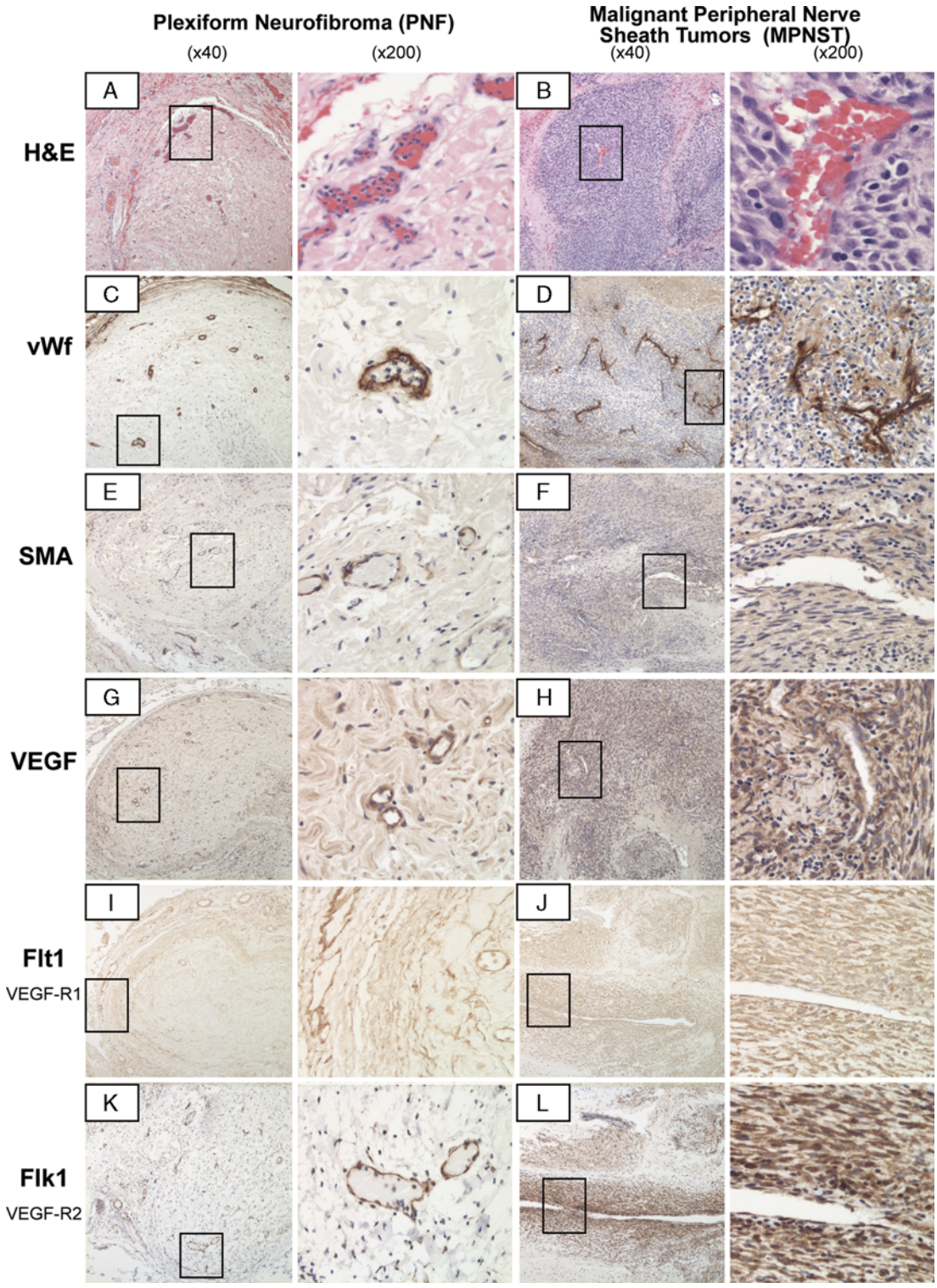

FIGURE 1. Immunohistochemical staining of angiogenesis-related markers of benign PNF and malignant peripheral nerve sheet tumor (MPNST). Archived tissue biopsies obtained from patients (n=5) presenting with peripheral neurofibroma (PNF), followed later by diagnosis of MPNST (Table 1) were sectioned (5 μM), prepared, and stained according to Materials and Methods using the indicated antibodies (Table 2); H&E (hematoxylin and eosin), vWf (von Willebrand factor), SMA (smooth muscle actin), VEGF (vascular endothelial growth factor), Flt-1 (VEGF-R1) vascular endothelial growth factor receptor-1, and Flk-1 (VEGF-R2) vascular endothelial growth factor receptor-2.

maturity index because the association of pericytes and smooth muscle cells provides a stabilizing (''maturing'') influence on endothelial cell tubes. The VEGF-independent, quiescent, nonproliferative vasculature is less prone to destruction in the absence of VEGF. A very different pattern of vWf- and SMA-staining was found in PMNST, in which mainly small, stag horn-type, lumenless, elongated vessels are evident. These PMNST-associated microvascular structures are immature with poorly developed perivascular mural architecture (Fig. 1F), and SMA^- perivascular sheaths. Their structure is highly reminiscent of the spongiform mesh work, often observed in early developmentally immature vessels such as the embryonal primitive vascular plexus (Fig. 1D), and the variability of vessel size is in keeping with ''sprouting angiogenesis''[28] and with the typical finding of ''mother'' versus ''daughter vessels'' in the process of activated angiogenesis.[43]

In the early descriptions of paracrine growth factor loops in tumor-associated angiogenesis, it was thought that the source of VEGF is the tumor cell derived. Over the past decade, it has been appreciated that many other cells such as macrophages, tumor-associated fibroblasts, white blood cells, and platelets contribute to VEGF production. Our individually matched specimens indicate that the proangiogenic phenotype development involves not only the upregulation of VEGF in the tumor tissue, but also the ectopic expression of VEGFR2/Flk-1 and VEGFR1/Flt-1 receptors on the tumor cell, tumor-associated fibroblasts, and inflammatory cells.

There exists a controversy concerning the methods used to assess and quantify blood vessels in angiogenesis research to satisfy statistical standards of quantitative assessment.[44–46] At present, no standard method antigenic phenotype assessment exists. Many investigators use mean vascular density or vessel maturation index (VMI) by counting the highest density of vascular structures or ''hot spots'' as a prognosticator. It must be recognized, however, that it provides no guidance as to therapeutic response in antiangiogenic therapy, and can only be used as prognostic indicator of an aggressive phenotype. We have not used such an immuno-histologic approach in this study to evaluate the degree of angiogenesis associated with a phenotypic change during the progression of PNF to PMNST in children with NF1. Our immuno-stainings corroborate the likelihood that the striking differences between PNF and PMNST are owing to an ''angiogenic switch'' in these tumors, and even though our ability to quantify the differences was limited, the comparison of these changes across the 5 available patients was very consistent and reproducible. Owing to the nature of our surgical specimens, we could not compare any differences in the microvasculature in the periphery and central regions of tumors and we limited ourselves to the comparison of the underlying intratumoral microvasulature of PNF and PMNST.

In earlier studies, the vascularity of human neurofibromas was assessed by transmission electron microscopy, and the conclusion was made, that the disorganized proliferation of vasculature vessels are a generalized malformative process of blood vessels caused by the shape of neurofibromas rather than a consequence of the action of a tumor angiogenesis factor.[47] With the novel finding herein supporting the later concept, we may now be able to reinterpret these findings concerning the generation of angiogenic phenotype in solid tumors. We know that the ras oncogenic transformation within a tumor cell leads to the upregulation of proangiogenic factors such as VEGF and bFGF[29,30] and subsequent increase in angiogenesis, and that the endothelial-specific inactivation of neurofibromin (NF1) in mice results in midembryonic lethality, an elevated level of ras signaling.[30] This strongly supports our hypothesis that NF-1 related malignant transformation and tumorigenesis is angiogenesis dependent, and is in line with an earlier finding that ras signaling contributes to the proangiogenic phenotype.[29,30] A recent literature review on antiangiogenesis in NF1[39,40,48] discusses the potential mechanisms of angiogenesis in NF1, but the malignant progression of PNF to PMNST has yet to be associated with specific, histologically detectable differences in patient tissues.

With growing evidence of the role of angiogenesis in NF-1 related malignancies and benign, but life-threatening invasive tumors, therapies diminishing the effects of VEGF may provide an effective treatment alternative. In the past, treatment of these patients was delayed until significant morbidity occurred mainly because of the heightened toxicity of radiation and chemotherapy. With a nontoxic and effective alternative such as antiangiogenic therapy, a more prophylactic strategy may be developed for patients with progressive PNF, hence a therapy representing a milestone in the care of NF1 patients.

To furthermore corroborate the clinical application of our histologic findings, it would be pertinent to undertake studies in the controlled environment of animal experimentation and analyze the response of xenografts of progressive PNF and PMNST to antiangiogenic agents, or similar nontoxic treatment modalities. Recently, a new mouse model has become available specifically for NF1 research providing new insights about the structure and behavior of NF tumors.[48] For future clinical research, many additional angiogenesis-specific immuno-histochemical techniques will be used and in situ hybridization, 3-dimensional reconstruction of the tumor vasculature, perfusion-specific imaging, urine, and plasma quantification of angiogenesis markers all may assist to improve the diagnostic and prognosticcapabilities for children with NF1 and progressive PNF or PMNST.

Overall, despite the site-specific clinical differences among the various tumors that can develop in children with NF1, a marked conformity was noted in the angiogenic phenotypes.[49,50] In all tested patients, the phenotype usually associated with increased angiogenesis was displayed exclusively by the malignant counterpart, suggesting that an early antiangiogenic intervention may prevent a malignant transformation.

ACKNOWLEDGMENTS

The authors thank Michael Ho, DPLM for his outstanding help with immuno-staining. For measuring the tumor size in Table 1 authors thank Dr. Paul Babyn, Radiologist-in-Chief, both at The Hospital for Sick Children, Toronto, Canada.

REFERENCES

1. Von Recklinghausen F. *Ueber Die Miltiplen Fibrome der Haut und Ihre Beziehung zu den Multiplen Neuronen*. Berlin: Hirschwald; 1882.
2. Matsui I, Tanimura M, Kobayashi N, et al. Neurofibromatosis type 1 and childhood cancer. *Cancer*. 1993;72:2746–2754.

3. American Academy of Pediatrics Commitee on Genetics. Health supervision for children with neurofibromatosis. *Pediatrics*. 1995;96:368–372.
4. Korf BR. Malignancy in neurofibromatosis type 1. *Oncologist*. 2000;5:477–485.
5. Korf BR. Neurocutaneous syndromes: neurofibromatosis 1, neurofibromatosis 2, and tuberous sclerosis. *Curr Opin Neurol*. 1997;10:131–136.
6. Gutmann DH, Aylsworth A, Carey JC, et al. The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. *JAMA*. 1997;278:51–57.
7. Mulvihill JJ, Parry DM, Sherman JL, et al. NIH conference. Neurofibromatosis 1 (Recklinghausen disease) and neurofibromatosis 2 (bilateral acoustic neurofibromatosis). An update. *Ann Intern Med*. 1990;113:39–52.
8. Zvulunov A, Barak Y, Metzker A. Juvenile xanthogranuloma, neurofibromatosis, and juvenile chronic myelogenous leukemia. World statistical analysis. *Arch Dermatol*. 1995;131:904–908.
9. Zachos M, Parkin PC, Babyn PS, et al. Neurofibromatosis type 1 vasculopathy associated with lower limb hypoplasia. *Pediatrics*. 1997;100:395–398.
10. Szudek J, Birch P, Riccardi VM, et al. Associations of clinical features in neurofibromatosis 1 (NF1). *Genet Epidemiol*. 2000;19:429–439.
11. Korf BR. Diagnosis and management of neurofibromatosis type 1. *Curr Neurol Neurosci Rep*. 2001;1:162–167.
12. Cutting LE, Koth CW, Denckla MB. How children with neurofibromatosis type 1 differ from ''typical'' learning disabled clinic attenders: nonverbal learning disabilities revisited. *Dev Neuropsychol*. 2000;17:29–47.
13. Listernick R, Louis DN, Packer RJ, et al. Optic pathway gliomas in children with neurofibromatosis 1: consensus statement from the NF1 optic pathway glioma task force. *Ann Neurol*. 1997;41:143–149.
14. Bader JL, Miller RW. Neurofibromatosis and childhood leukemia. *J Pediatr*. 1978;92:925–929.
15. Hope DG, Mulvihill JJ. Malignancy in neurofibromatosis. *Adv Neurol*. 1981;29:33–56.
16. Kimura N, Watanabe T, Fukase M, et al. Neurofibromin and NF1 gene analysis in composite pheochromocytoma and tumors associated with von Recklinghausen's disease. *Mod Pathol*. 2002;15:183–188.
17. Schneider M, Obringer AC, Zackai E, et al. Childhood neurofibromatosis: risk factors for malignant disease. *Cancer Genet Cytogenet*. 1986;21:347–354.
18. Obringer AC, Meadows AT, Zackai EH. The diagnosis of neurofibromatosis-1 in the child under the age of 6 years. *Am J Dis Child*. 1989;143:717–719.
19. Coleman BG, Arger PH, Dalinka MK, et al. CT of sarcomatous degeneration in neurofibromatosis. *AJR Am J Roentgenol*. 1983;140:383–387.
20. Raney B, Schnaufer L, Ziegler M, et al. Treatment of children with neurogenic sarcoma. Experience at the children's hospital of Philadelphia, 1958-1984. *Cancer*. 1987;59:1–5.
21. Angelov L, Davis A, O'Sullivan B, et al. Neurogenic sarcomas: experience at the university of Toronto. *Neurosurgery*. 1998; 43:56–64.
22. Korf BR. Plexiform neurofibromas. *Am J Med Genet*. 1999; 89:31–37.
23. Packer RJ, Prados M, Phillips P, et al. Treatment of children with newly diagnosed brain stem gliomas with intravenous recombinant beta-interferon and hyperfractionated radiation therapy: a children's cancer group phase I/II study. *Cancer*. 1996;77:2150–2156.
24. Needle MN, Packer RJ. A phase II randomized trial of 13-cis retinoic acid (CRA) and Interferon alpha-2a in the treatment of PNF in patients with NF1. *Pediatr Res*. 1995;37:889. Abstract
25. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182–1186.
26. Folkman J. New perspectives in clinical oncology from angiogenesis research. *Eur J Cancer*. 1996;32A:2534–2539.
27. Folkman J. Fighting cancer by attacking its blood supply. *Sci Am*. 1996;275:150–154.
28. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86:353–364.
29. Rak J, Mitsuhashi Y, Bayko L, et al. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res*. 1995;55: 4575–4580.
30. Rak J, Filmus J, Finkenzeller G, et al. Oncogenes as inducers of tumor angiogenesis. *Cancer Metastasis Rev*. 1995;14:263–277.
31. Folkman J. Angiogenesis and angiogenesis inhibition: an overview. *EXS*. 1997;79:1–8.
32. Fukumura D, Xavier R, Sugiura T, et al. Tumor induction of VEGF promoter activity in stromal cells1. *Cell*. 1998;94: 715–725.
33. Nguyen M, Watanabe H, Budson AE, et al. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. *J Natl Cancer Inst*. 1994;86:356–361.
34. Ezekowitz RA, Mulliken JB, Folkman J. Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med*. 1992;326:1456–1463.
35. Marler JJ, Rubin JB, Trede NS, et al. Successful antiangiogenic therapy of giant cell angioblastoma with interferon alfa 2b: report of 2 cases. *Pediatrics*. 2002;109:E37.
36. Dinney CP, Bielenberg DR, Perrotte P, et al. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration2. *Cancer Res*. 1998;58:808–814.
37. Angelov L, Salhia B, Roncari L, et al. Inhibition of angiogenesis by blocking activation of the vascular endothelial growth factor receptor 2 leads to decreased growth of neurogenic sarcomas. *Cancer Res*. 1999;59:5536–5541.
38. Kawachi Y, Xu X, Ichikawa E, et al. Expression of angiogenic factors in neurofibromas. *Exp Dermatol*. 2003;12:412–417.
39. Kurtz A, Martuza RL. Antiangiogenesis in neurofibromatosis 1. *J Child Neurol*. 2002;17:578–584.
40. Mashour GA, Ratner N, Khan GA, et al. The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. *Oncogene*. 2001;20:97–105.
41. Gupta A, Cohen BH, Ruggieri P, et al. Phase I study of thalidomide for the treatment of plexiform neurofibroma in neurofibromatosis 1. *Neurology*. 2003;60:130–132.
42. Benjamin LE, Golijanin D, Itin A, et al. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest*. 1999;103:159–165.
43. Pettersson A, Nagy JA, Brown LF, et al. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. *Lab Invest*. 2000;80:99–115.
44. Auerbach R, Auerbach W, Polakowski I. Assays for angiogenesis: a review. *Pharmacol Ther*. 1991;51:1–11.
45. Jain RK, Schlenger K, Hockel M, et al. Quantitative angio genesis assays: progress and problems. *Nat Med*. 1997;3: 1203–1208.
46. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst*. 2002;94:883–893.
47. Teixeira F, Martinez-Palomo A, Riccardi VM, et al. Vascular changes in cutaneous neurofibromas. *Neurofibromatosis*. 1988;1:5–16.
48. Zhu Y, Ghosh P, Charnay P, et al. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. *Science*. 2002;296:920–922.
49. Gitler AD, Zhu Y, Ismat FA, et al. Nf1 has an essential role in endothelial cells. *Nat Genet*. 2003;33:75–79.
50. Mashour GA, Wang HL, Cabal-Manzano R, et al. Aberrant cutaneous expression of the angiogenic factor midkine is associated with neurofibromatosis type-1. *J Invest Dermatol*. 1999;113:398–402.