Clinical Cancer Research

Neuroblastoma Progression Correlates with Downregulation of the Lymphangiogenesis Inhibitor sVEGFR-2

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Abstract

Purpose: Tumor progression correlates with the induction of a dense supply of blood vessels and the formation of peritumoral lymphatics. Hemangiogenesis and lymphangiogenesis are potently regulated by members of the vascular endothelial growth factor (VEGF) family. Previous studies have indicated the upregulation of VEGF-A and -C in progressed neuroblastoma, however, quantification was performed using semiquantitative methods, or patients who had received radiotherapy or chemotherapy were studied.

Experimental Design: We have analyzed primary neuroblastoma from 49 patients using real-time reverse transcription-PCR and quantified VEGF-A, -C, and -D and VEGF receptors (VEGFR)-1, 2, 3, as well as the soluble form of VEGFR2 (sVEGFR-2), which has recently been characterized as an endogenous inhibitor of lymphangiogenesis. None of the patients had received radiotherapy or chemotherapy before tumor resection.

Results: We did not observe upregulation of VEGF-A, -C, and -D in metastatic neuroblastoma, but found significant downregulation of the lymphangiogenesis inhibitor sVEGFR-2 in metastatic stages III, IV, and IVs. In stage IV neuroblastoma, there were tendencies for the upregulation of VEGF-A and -D and the downregulation of the hemangiogenesis/lymphangiogenesis inhibitors VEGFR-1 and sVEGFR-2 in *MYCN*-amplified tumors. Similarly, *MYCN* transfection of the neuroblastoma cell line SH-EP induced the upregulation of VEGF-A and -D and the switching-off of sVEGFR-2.

Conclusion: We provide evidence for the downregulation of the lymphangiogenesis inhibitor sVEGFR-2 in metastatic neuroblastoma stages, which may promote lymphogenic metastases. Downregulation of hemangiogenesis and lymphangiogenesis inhibitors VEGFR-1 and sVEGFR-2, and upregulation of angiogenic activators VEGF-A and VEGF-D in *MYCN*-amplified stage IV neuroblastoma supports the crucial effect of this oncogene on neuroblastoma progression. *Clin Cancer Res; 16(5); 1431–41. ©2010 AACR.*

A key step in the malignant progression of tumors in adults is the angiogenic switch, the induction of blood vessels by hypoxic tumors (1, 2). Additionally, tumor-induced lymphangiogenesis correlates frequently with the dissemination of tumor cells via lymphatic vessels. Hemangiogenesis and lymphangiogenesis are robustly regulated by members of the vascular endothelial growth factor (VEGF) family: VEGF-A being (predominantly) hemangiogenic, and VEGF-C and -D are lymphangiogenic (3–5). In adults, hemangiogenesis and lymphangiogenesis are associated with progressed tumor stages. Tumors of infants present a much broader spectrum of heterogeneity than those of adults, especially exemplified by the biology of neuroblastoma.

Neuroblastoma is derived from sympatho-adrenal progenitor cells that migrate from the neural crest into target regions of the embryo. Neuroblastoma is mostly located along the sympathetic trunk ganglia and in the adrenal medulla. The spectrum of disease ranges from complete spontaneous regression, which can be immediately observed in the "special" neuroblastoma stage IVs—but may as well occur in early stages—to malignant progression into stage IV, with 5-year survival rates of <30%. Partial differentiation into ganglioneuroblastma is another developmental pathway (6). The most critical molecular predictor for the behavior and treatment of neuroblastoma is the *MYCN* proto-oncogene. Amplification (up to 150×) of *MYCN* characterizes highly aggressive tumors and poor outcome despite intensive treatment (7). Staging of neuroblastoma is performed according to the

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Translational Relevance

It has been shown that growth and metastatic spread of tumors in adults are closely linked to the development of blood and lymphatic vessels. Hemangiogenesis and lymphangiogenesis are specifically regulated by vascular endothelial growth factors (VEGF). The use of antiangiogenic drugs in tumors of infants, like neuroblastoma, has been suggested. Neuroblastoma develops from sympathoadrenal progenitor cells, and dense vascular supply is found in progressed neuroblastoma. However, studies on the expression of VEGFs in neuroblastoma are very sparse. We show downregulation of the lymphangiogenesis inhibitor, sVEGFR-2, in neuroblastoma stages III, IV, and IVs, which are characterized by metastases into regional and distant lymph nodes. We found tendencies for the upregulation of hemangiogenesis and lymphangiogenic factors (VEGF-A and VEGF-D) and downregulation of hemangiogenesis and lymphangiogenesis inhibitors (VEGFR-1 and sVEGFR-2) in *MYCN*-amplified stage IV neuroblastoma. Our data confirm the negative effect of the *MYCN* proto-oncogene and indicate a function for lymphangiogenesis inhibitors in neuroblastoma progression.

International Neuroblastoma Staging System. Stage I and II neuroblastomas are localized tumors, which have grown across the midline in stage II. Metastasis to regional and systemic lymph nodes characterizes stages III and IV, respectively (8), indicating active interactions with the lymphovascular system. Additionally, high vascularity is characteristic for the progressed tumor stages (9, 10), indicating an influence of blood capillaries on neuroblastoma cell behavior and their typical dissemination into the bone marrow.

The effect of VEGFs on tumor hemangiogenesis and lymphangiogenesis has been shown in numerous studies of adult tumors, and several investigations in recent years have postulated a similar function for VEGFs in neuroblastoma. Blood vessels and lymphatics are present in neuroblastoma, and expression of the ligands VEGF-A, -C, and -D as well as their receptors has been found (11–13). Increased expression of VEGF-A has been described in neuroblastoma stages III and IV (14), whereas VEGF-C has been identified as a risk factor in stage IV neuroblastoma (15). High levels of VEGF-A have been found in stage IV neuroblastoma, but neither VEGF-A nor VEGF-C correlated with age, *MYCN* copy number or lymph node metastasis (16). Moreover, it has been observed that *MYCN* amplification correlates strongly with dense vascular supply, tumor dissemination, and poor survival (9). According to Kang et al. (17), *MYCN* upregulates VEGF-A and *MYCN*-amplified neuroblastomas exert changes in the vascular pattern of the chick chorioallantoic membrane (18). Some authors have suggested anti–VEGF-A treatment with bevacizumab for high-risk neuroblastoma (19). There are, however, results that challenge the unequivocal functions of VEGFs for the progression of neuroblastoma. Vessel density was not predictive of survival in a cohort of patients with neuroblastoma (20). Anti–VEGF-A treatment did not result in any reduction of experimental neuroblastoma growth in mice (21, 22), and some authors have emphasized the heterogeneity of angiogenesis stimulators and inhibitors in neuroblastoma (23, 24). We have therefore reinvestigated the expression of VEGFs and their receptors in a cohort of 49 neuroblastoma patients and in 24 neuroblastoma cell lines. Additionally, we studied the effect of *MYCN* on the expression of VEGFs in primary neuroblastomas and in neuroblastoma cell lines. In contrast to previous studies, we did not observe a positive correlation between tumor progression and the expression of VEGF-A and -C. Of note, we found significant downregulation of the VEGF-C inhibitor sVEGFR-2 in the progressed stages III, IV, and IVs, indicating a positive correlation between lymphangiogenesis and lymph node metastases. Additionally, we observed increased expression of VEGF-A and -D, as well as reduced expression of the inhibitors VEGFR-1 and sVEGFR-2 in *MYCN*-amplified stage IV neuroblastoma, a finding which was recapitulated by *MYCN*-transfected SH-EP cells, but not generally observed in *MYCN*-amplified neuroblastoma cell lines. Our data show that upregulation of the hemangiogenesis and lymphangiogenesis activators VEGF-A and VEGF-D, and downregulation of the hemangiogenesis and lymphangiogenesis inhibitors VEGFR-1 and sVEGFR-2, act in concert during neuroblastoma progression. Cell lines do not necessarily reflect the *in vivo* situation. In addition to the downregulation of hemangiogenesis inhibitors (25, 26), downregulation of lymphangiogenesis inhibitors is an alternative mechanism for the increased vascularization and metastasis formation of progressed neuroblastoma.

Materials and Methods

Primary neuroblastomas. RNA samples of 50 primary, untreated tumors, were kindly provided by the Tumorbank of the German Neuroblastoma Studies Group, Drs. F. Berthold, B. Hero, and J. Theissen, Children's Hospital University of Cologne, Cologne, Germany. Tumor specimens were prepared according to a standard protocol. Two representative areas were dissected out of the tumor and each was divided into four parts. One part was fixed in formalin and three parts were snap-frozen in liquid nitrogen. Snap-frozen specimens were used in our study. RNA was isolated with Trizol (Invitrogen). The samples were tested with Bioanalyzer 2100 (Agilent Technologies). One sample failed the test and was discarded. The remaining 49 samples were allocated to the neuroblastoma stages as follows: stage I (n = 8), stage II (n = 6), stage III (n = 5; 2 were *MYCN* amplified), stage IV (n = 20; 10 were

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MYCN amplified), and stage IVs (n = 10; 1 was *MYCN* amplified).

Cell culture. All 24 human neuroblastoma cells lines (Table 1; see ref. 27 for a well-arranged review of 113 neuroblastoma cell lines) were maintained in a humidified incubator at 37°C and 5% CO_2 atmosphere using RPMI 1640 (Lonza) with 10% fetal bovine serum (Biochrome) and 1% penicillin/streptomycin (Invitrogen). The neuroblastoma cell line SH-EP was stably transfected to overexpress the *MYCN* oncogene as described previously (28). The transfected cell line was designated WAC2. Transfected cells were continuously selected by adding G418 (100 μg/mL; Invitrogen) to the medium. PC-3 human prostate carcinoma cells were purchased from DSMZ, and used as reference for VEGF-A and VEGF-C ELISAs.

RNA isolation from cultured neuroblastoma cells. Cells were rinsed twice with PBS and RNA was isolated directly from the culture plate using Trizol (Invitrogen) as recommended by the supplier. Quality of RNA samples was analyzed with NanoDrop spectrophotometer (NanoDrop Products) and ethidium bromide staining on agarose gels.

Real-time reverse transcription-PCR. We prepared cDNA from 2 μg total RNA using Omniscript reverse transcriptase (Qiagen). Real-time PCR was performed with Opticon2 thermal cycler (MJ Research), using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). Primers used are listed in Table 2. All primers were designed to produce fragments, which span exon-intron boundaries, to exclude amplification of genomic DNA. The primers were also designed to detect all known splice variants of the respective gene of interest. For sVEGFR-2, the reverse primer recognizes the intron 13 motif, which is specific for the truncated transcript variant of this secreted form of VEGFR-2 (29). The probe was measured against two different β-actin probes. Both measurements revealed significant downregulation in metastatic neuroblastoma (only one is shown in Fig. 1G).

Sandwich ELISA. Sandwich ELISA was performed to measure VEGF-A and VEGF-C protein in cell culture supernatants of neuroblastoma cell lines SH-EP and SH-IN in comparison with the human prostate carcinoma PC-3 cell line, using methods and tools described recently (30, 31). Cells were cultured for 4 d in RPMI 1640. Supernatant was collected and compared with control (day 0) supernatant of PC-3 cells. Experiments were performed twice.

Statistical analyses. Statistical analyses were performed using SAS Software v. 9.1 (SAS Institute).

Results

Primary neuroblastomas. We have studied primary neuroblastoma from a cohort of 49 patients by real-time reverse transcription-PCR (RT-PCR) using SYBR Green and the ΔΔCt method for relative quantification of VEGFs and VEGF receptors. None of the patients had received radiotherapy or chemotherapy before tumor resection. Staging of neuroblastoma was performed according to the International Neuroblastoma Staging System and the specimens were allocated to the stages as follows: stage I (n = 8), stage II (n = 6), stage III (n = 5; 2 of which were *MYCN* amplified), stage IV (n = 20; 10 of which were *MYCN* amplified), and stage IVs (n = 10; 1 of which was *MYCN* amplified). We studied expression of VEGF-A, VEGF-C, VEGF-D, VEGFR-1 (FLT1), VEGFR-2 (KDR), and VEGFR-3 (FLT4), as well as a soluble splice variant of VEGFR-2 (sVEGFR-2), which has very recently been shown to act as an endogenous inhibitor of lymphangiogenesis (29). Expression of all VEGFs and their receptors varied greatly. We did not observe a statistically significant difference between locoregional tumors (stages I and II) and metastasized neuroblastoma (stages III, IV, and IVs) for VEGF-A, -C, and -D (Fig. 1A–C). Also, VEGFR-1, -2, and -3 were not significantly regulated (Fig. 1D–F); however, we observed significant downregulation of the lymphangiogenesis inhibitor sVEGFR-2 in metastatic stages III, IV, and IVs (Fig. 1G).

We have then compared the expression levels of VEGFs in *MYCN*-amplified stage IV neuroblastoma with those that had normal *MYCN* status (each 10 per group). We did not find statistically significant differences; however, there were tendencies for an increase in the expression of VEGF-A and -D and for downregulation of VEGFR-1 and sVEGFR-2 in *MYCN*-amplified stage IV tumors (Fig. 2). This suggests increased hemangiogenic and lymphangiogenic potential.

Neuroblastoma cell lines. In a further approach, we isolated RNA from 24 human neuroblastoma cell lines and studied the expression of VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2 and VEGFR-3, and sVEGFR-2. Relative expression in neuroblastoma cell line CHLA20 was used as a reference and was set as 1 (Fig. 3). Only a few neuroblastoma cell lines showed robust expression of VEGF-A. We observed 15-fold expression in CHLA90 and almost 40-fold expression in SH-IN (Fig. 3A). To analyze the biological significance of this observation, we inoculated SH-IN (high VEGF-A), GI-MEN and CHP134 (low VEGF-A) on the chorioallantoic membrane of chick embryos. The three cell lines produced solid tumors after 7 days, but only SH-IN were densely vascularized, whereas GI-MEN and CHP134 produced almost avascular tumors (data not shown). VEGF-C was highly expressed in SK-N-SH and SH-EP (Fig. 3B), and VEGF-D was high in IMR5, KCN, NLF, SH-IN, IMR32, and SK-N-AS (Fig. 3C). Expression of VEGF receptors was low, and for VEGFR-3, was almost undetectable (data not shown). We found very weak expression of VEGFR-1 in various neuroblastoma cell lines (Fig. 3D), elevated expression of VEGFR-2 only in NB 69 (Fig. 3E), but considerable expression of sVEGFR-2 in a number of cell lines, most prominently in NLF, LAN6, SK-N-AS, LAN1, and LAN2 (Fig. 3F). For two cell lines, SH-EP and SH-IN, we measured VEGF-A and VEGF-C at protein level with sandwich ELISA (Table 3). The data show that RNA and protein expression correlate very well,

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Table 1. Human neuroblastoma cell lines: references and sources

Name	Reference	Commercial source
CHLA 20	Keshelava N, Seeger RC, Groshen S, and Reynolds CP. Drug resistance patterns of human neuroblastoma cell lines derived from patients at different phases of therapy. Cancer Res, 58:5396–5405, 1998	
CHLA 90	Keshelava N, Seeger RC, Groshen S, and Reynolds CP. Drug resistance patterns of human neuroblastoma cell lines derived from patients at different phases of therapy. Cancer Res, 58:5396–5405, 1998	
CHP 100	Schlesinger HR, Gerson JM, Moorhead PS, Maguire H, and Hummeler K. Establishment and characterization of human neuroblastoma cell lines. Cancer Res, 36:3094–3100, 1976	
CHP 134	Schlesinger HR, Gerson JM, Moorhead PS, Maguire H, and Hummeler K. Establishment and characterization of human neuroblastoma cell lines. Cancer Res, 36:3094–3100, 1976	RIKEN
GI MEN	Cornaglia-Ferraris P, Ponsoni M, Montaldo P, Mariottini G, Donti E, Di Martino D, and Tonini G. A new human highly tumoigenic neuroblastoma cell line with undetectable expression of N-myc. Ped Res, 27:1–6, 1990	CLS
IMR 32	Tumilowicz JJ, Nichols WW, Cholon JJ, and Greene AE. Definition of a continuous human cell line derived from neuroblastoma. Cancer Res, 30:2110–2118, 1970	CLS RIKEN
IMR5	Tumilowicz JJ, Nichols WW, Cholon JJ, and Greene AE. Cancer Res, 30:2110–2118, 1979	
Kelly	Schwab M, Alitalo K, Klempnauer K, Varmus H, Bishop J, Gilbert F, Brodeur G, Goldstein M, Trent J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature, 305:245–248, 1983	DSMZ
Lan 1	Seeger RC, Rayner SA, Banerjee A, Chung H, Laug WE, Neustein HB, Benedict WF. Morphology, growth, chromosomal pattern, and fibrinolytic activity of two new human neuroblastoma cell lines. Cancer Res, 37:1364–1371, 1977	RIKEN
Lan 2	Seeger RC, Rayner SA, Banerjee A, Chung H, Laug WE, Neustein HB, Benedict WF. Morphology, growth, chromosomal pattern, and fibrinolytic activity of two new human neuroblastoma cell lines. Cancer Res, 37:1364–1371, 1977	RIKEN
Lan 5	Dr. Seeger, Robert C. Children's Hospital Los Angeles, 4546 West Sunset Boulevard, Mailstop no. 57, Smith Research Tower no. 509, Los Angeles, CA 90027	RIKEN
Lan 6	Wada RK, Seeger RC, Brodeur GM, Einhorn PA, Rayner SA, Tomayko MM, and Reynolds CP. Human neuroblastoma cell lines that express N-myc without gene amplification. Cancer, 72:3346–3354, 1993	
NB 69	Brodeur GM and Goldstein MN. Histochemical demonstration of an increase in acetylcholinesterase in established lines of human and mouse neuroblastomas by nerve growth factor. Cytobios, 16:133–138, 1976	RIKEN
NB-LS	Cohn S, Salwen H, Quasney M, Ikegaki N, Cowan J, Herst C, Kennett R, Rosen S, DiGiuseppe J, and Brodeur G. Prolonged N-myc protein half-life in a neuroblastoma cell line lacking N-myc amplification. Oncogene, 5:1821–1827, 1990	
NGP	Brodeur GM, Goldstein MN. Histochemical demonstration of an increase in acetylcholinesterase in established lines of human and mouse neuroblastomas by nerve growth factor. Cytobios, 16:133–138, 1976	
NLF	Schwab M, Alitalo K, Klempnauer K, Varmus H, Bishop J, Gilbert F, Brodeur G, Goldstein M, Trent J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature, 305:245–248, 1983	
NMB	Brodeur GM, Sekhon GS, Godstein MN. Chromosomal aberrations in human neuroblastomas. Cancer, 40:2256–2263, 1977	

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Table 1. Human neuroblastoma cell lines: references and sources (Cont'd)

Name	Reference	Commercial source
SH-EP	Ross RA, Spengler BA, Biedler JL. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst, 77:741–749, 1983	
SH-IN	Ross RA, Spengler BA, Biedler JL. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst, 77:741–749, 1983	
SH-SY5Y	Ross RA, Spengler BA, Biedler JL. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst, 77:741–749, 1983	CLS ATCC
SK-N-AS	Sugimoto T, et al. Determination of cell surface membrane antigens common to both human neuroblastoma and leukemia-lymphoma cell lines by a panel of 38 monoclonal antibodies. J Natl Cancer Inst, 73:51–57, 1984	ATCC
SK-N-SH	Biedler, JL, Helson L, Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res, 33:2643–2652, 1973	ATCC RIKEN
SMS-Kan	Reynolds CP, Biedler JL, Spengler BA, Reynolds DA, Ross RA, Frenkel EP, Smith RG. Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst, 76:375–387, 1986	
SMS-KCN	Reynolds CP, Biedler JL, Spengler BA, Reynolds DA, Ross RA, Frenkel EP, Smith RG. Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst, 76:375–387, 1986	

NOTE: Sources: ATCC: American Type Culture Collection, P.O. Box 1549 Manassas, VA.
CLS: Cell Lines Service, Justus-von-Liebig-Strasse 14, 69214 Eppelheim, Germany.
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraâe 7B, 38124 Braunschweig, Germany.
RIKEN: Cell Bank, RIKEN BioResource Center 3-1-1 Koyadai, Tsukuba, Ibaraki, 305-0074, Japan.

e.g., SH-IN, which has the highest VEGF-A mRNA expression, secretes high amounts of VEGF-A (2,005 pg/mL), whereas VEGF-C protein is not measurable (compare Fig. 3A and B). SH-EP secretes VEGF-C (140 pg/mL), which is comparable to the prostate carcinoma cell line PC-3, and has considerably high levels of VEGF-C mRNA.

It has been shown that *MYCN* inhibition by short interfering RNA blocks VEGF-A secretion in *MYCN*-amplified IMR-32 cells (17). To test the effects of *MYCN*, we studied stable overexpression of *MYCN* in SH-EP, which has regular *MYCN* expression. The stably transfected cell line was designated WAC2, because the cells form colonies in soft agar (28). For VEGF-C, VEGFR-1, -2 and -3, expression in WAC2 was not different from that of the parental cell line SH-EP (Fig. 3B, D, and E); however, VEGF-A and -D were upregulated, and sVEGFR-2 was significantly downregulated, so that it was no longer detectable in WAC2 (Fig. 3A, C, and F). Notably, these effects may promote hemangiogenesis and lymphangiogenesis.

According to published data, we also divided the 24 cell lines into two groups with normal versus amplified *MYCN* expression and compared VEGF ligands and receptors (Fig. 4A–F). However, the results did not reflect any of the results measured in primary tumors, and underline that *in vitro* data have to be interpreted with great caution.

In summary, our data show that there is significant downregulation of the lymphangiogenesis inhibitor sVEGFR-2 in metastatic neuroblastoma as compared with localized neuroblastoma stages I and II. In *MYCN*-amplified stage IV neuroblastoma, there is a tendency for the upregulation of VEGF-A and -D, and downregulation

Table 2. Primers used for real-time RT-PCR

Primer	Sequence
β-Actin1 fwd	5′-GCATCCCCCAAAGTTCACAA-3′
β-Actin1 rev	5′-AGGACTGGGCCATTCTCCTT-3′
β-Actin2 fwd	5′-TCGTGCGTGACATTAAGGAG-3′
β-Actin2 rev	5′-CCATCTCTTGCTCGAAGTCC-3′
VEGF-A fwd	5′-AAGGAGGAGGGCAGAATCAT-3′
VEGF-A rev	5′-GCAGTAGCTGCGCTGATAGA-3′
VEGF-C fwd	5′-TGAACACCAGCACGAGCTAC-3′
VEGF-C rev	5′-GCCTTGAGAGAGAGGCACTG-3′
VEGF-D fwd	5′-TGGAACAGAAGACCACTCTCATCT-3′
VEGF-D rev	5′-GCAACGATCTTCGTCAAACATC-3′
VEGFR-1 fwd	5′-TCCAAGAAGTGACACCGAGA-3′
VEGFR-1 rev	5′-TTGTGGGCTAGGAAACAAGG-3′
VEGFR-2 fwd	5′-GACTTGGCCTCGGTCATTTA-3′
VEGFR-2 rev	5′-ACACGACTCCATGTTGGTCA-3′
VEGFR-3 fwd	5′-CAGCTCCTACGTGTTCGTGA-3′
VEGFR-3 rev	5′-GTTGACCAAGAGCGTGTCAG-3′
sVEGFR-2 fwd	5′-GCCTTGCTCAAGACAGGAAG-3′
sVEGFR-2 rev	5′-CAACTGCCTCTGCACAATGA-3′

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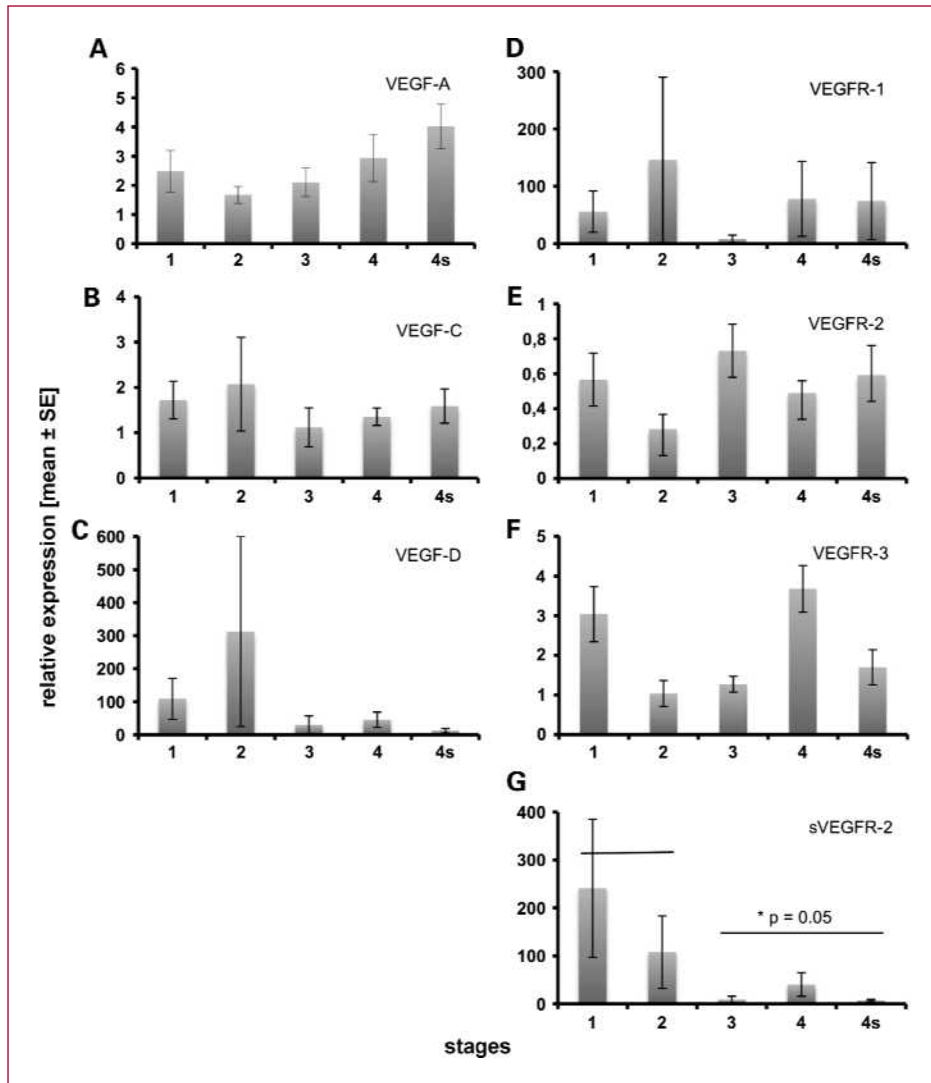

Fig. 1. Expression of VEGF ligands and receptors in 49 primary neuroblastoma specimens as measured by real-time RT-PCR. A, VEGF-A; B, VEGF-C; C, VEGF-D; D, VEGFR-1; E, VEGFR-2; F, VEGFR-3; G, sVEGFR-2. Note statistically significant downregulation of sVEGFR-2 in metastatic stages III, IV, and IVs. None of the other molecules exhibits any obvious stage-specific regulation. Columns, mean relative expression; bars, SE.

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of VEGFR-1 and sVEGFR-2. Similar results could be observed in *MYCN*-transfected SH-EP cells. Together, upregulation of hemangiogenesis and lymphangiogenesis activators (VEGF-A and VEGF-D, respectively) and downregulation of hemangiogenesis and lymphangiogenesis inhibitors (VEGFR-1 and sVEGFR-2, respectively) may represent cooperative mechanisms during neuroblastoma progression.

Discussion

VEGFs and neuroblastoma vascularity. Quite some time ago, the importance of VEGF-A for hemangiogenesis and of VEGF-C and VEGF-D for lymphangiogenesis was revealed (32–39). The essential role of VEGF-A in tumor hemangiogenesis is the basis for its targeting in anti-angiogenesis therapy (40). Tumor-induced lymphangiogenesis by VEGF-C and -D, and the positive correlation with the lymphogenic spread of tumor cells had been described several years ago (41–44). Dense vascularization is a key feature of malignant tumor progression (1). This holds true for numerous tumor types in the adult, and has also been observed in tumors of infants, such as neuroblastoma.

A vascular index of neuroblastoma specimens (total number of vessels per mm^2) has been measured in a cohort of 50 patients, and it was found that an index of >4 correlates strongly with widely disseminated disease, poor survival, and *MYCN* amplification (9). A similar correlation with angiogenic (integrin $\alpha_v\beta_3$-positive and $\alpha_v\beta_5$-positive) endothelium has been described by Erdreich-Epstein et al. (45). A number of studies have revealed a positive correlation between neuroblastoma progression and VEGFs, however, most of these studies were performed *in vitro* or in experimental tumors in nude mice, and there

are only few data available on primary tumors. Among these, Pavlakovic et al. (46) have shown that VEGF-A levels are not increased (rather slightly reduced) in the serum of neuroblastoma patients as compared with healthy controls. In contrast, elevated levels of VEGF-A, -B, and -C have been measured by RT-PCR (quantified by densitometric analysis, normalized against glyceraldehyde-3-phosphate dehydrogenase expression in a cohort of 37 patients) in neuroblastoma stages III and IV as compared with stages I, II, and IVs (11). By means of ELISA, VEGF-A protein has been measured in five primary neuroblastoma and revealed values of 150 to 2,400 pg/g total protein with the highest value in a stage II neuroblastoma (13). Fakhari et al. (14) studied the expression of VEGF-A, -B, and -C in 37 patients with neuroblastoma (patients that underwent routine radiologic and medical program before surgery) using real-time RT-PCR and VEGF-A in serum with ELISA. They observed a significant upregulation of VEGF-A and -C in stages III and IV, as compared with adrenal control tissue, and elevated serum VEGF-A levels in stage III only. Additionally, they found upregulation of VEGFR-1 and -2 in stage III neuroblastoma. In summary, the published data do not yet provide a consistent picture of VEGFs in neuroblastoma and we have therefore reinvestigated their expression in a cohort of 49 untreated, primary tumors, and in 24 cell lines using real-time RT-PCR. Thereby we also included the soluble splice variant of VEGFR-2 (sVEGFR-2), an endogenous inhibitor of lymphangiogenesis (29), and studied the effect of MYCN on the respective expression patterns.

Regulation of hemangiogenesis and lymphangiogenesis in neuroblastoma. In contrast with previous studies, we have not detected elevated expression of VEGF-A and -C in stage III and IV neuroblastoma. In fact, there is no significant difference between localized stages I and II and the metastatic stages III, IV, and IVs with regards to the expression of VEGF-A, -C, and -D. VEGFR-1 is expressed at almost equal amounts in all stages, and VEGFR-2 and -3 are also detectable in all stages. A significant difference between localized and metastatic stages was found for sVEGFR-2, a secreted endogenous inhibitor of lymphangiogenesis (29). This inhibitor is highly expressed in stages I and II, and barely detectable in stages III, IV, and IVs. Because the regulation of lymphangiogenesis by tumors has an influence on their metastatic behavior (3), our data for the first time indicates that downregulation of an inhibitor

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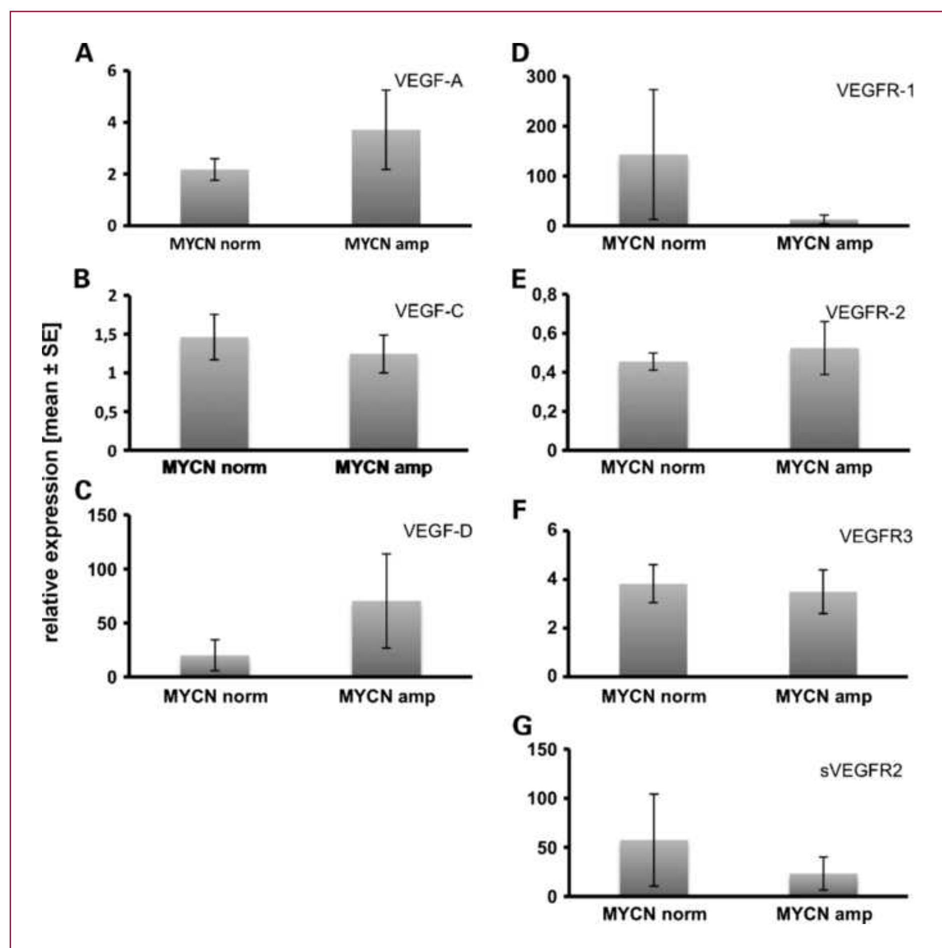

Fig. 2. Comparison of the expression of VEGF ligands and receptors in stage IV neuroblastoma with normal versus amplified *MYCN* expression. A, VEGF-A; B, VEGF-C; C, VEGF-D; D, VEGFR-1; E, VEGFR-2; F, VEGFR-3; G, sVEGFR-2. There are no statistically significant differences, but tendencies for the upregulation of VEGF-A and VEGF-D, and the downregulation of VEGFR-1 and sVEGFR-2 in *MYCN*-amplified tumors. Columns, mean relative expression; bars, SE.

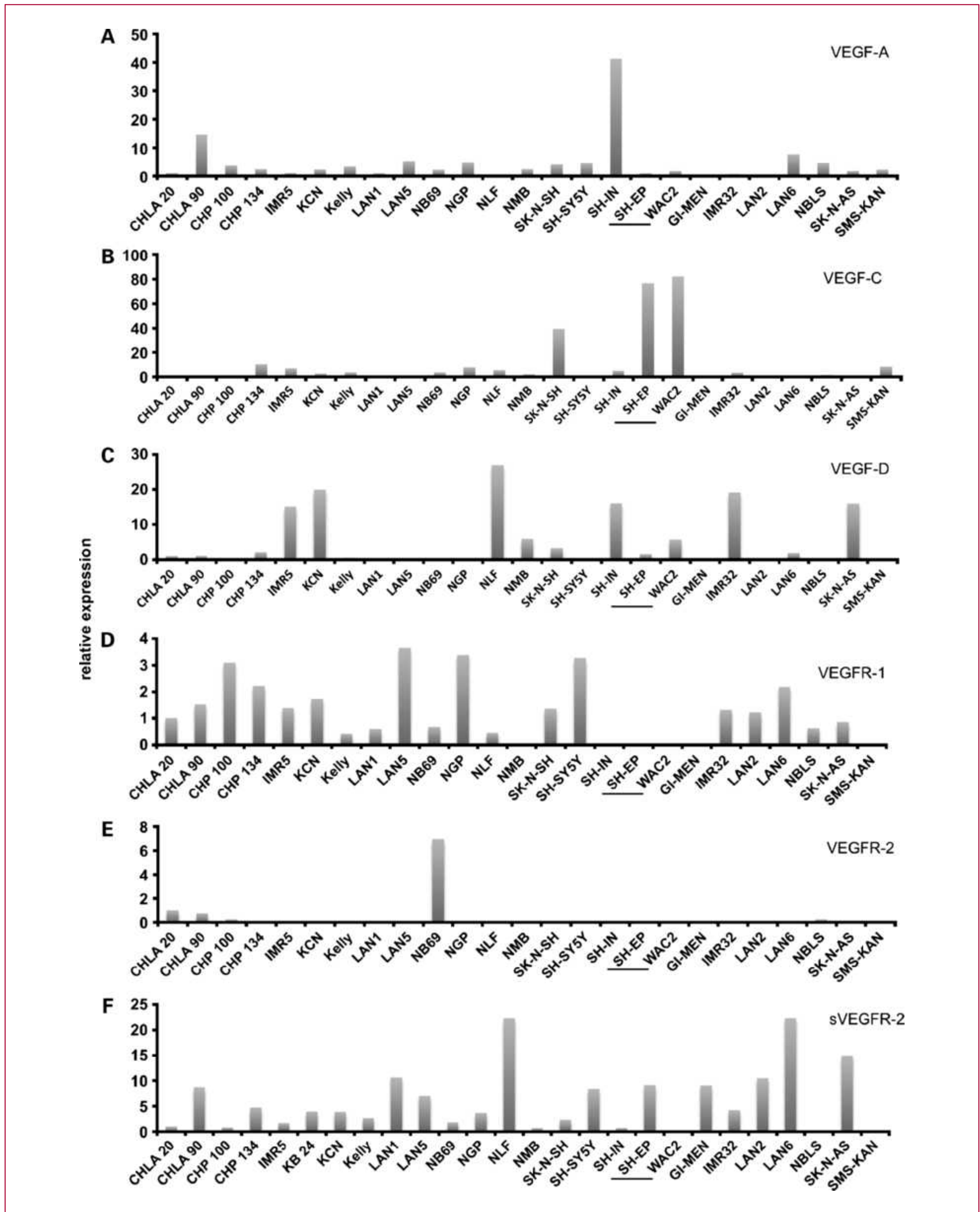

Fig. 3. Expression of VEGF ligands and receptors in 24 neuroblastoma cell lines as measured by real-time RT-PCR. A, VEGF-A; B, VEGF-C; C, VEGF-D; D, VEGFR-1; E, VEGFR-2; F, sVEGFR-2. Data for VEGFR-3 are not shown, because this receptor was almost undetectable. Expression of CHLA20 cells was used as a reference and set as 1. Note that WAC2 are stably *MYCN*-transfected SH-EP cells. There is upregulation of VEGF-A and VEGF-D, and a switch-off of sVEGFR-2 in WAC2.

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Table 3. Concentration (pg/mL) of VEGF-A and VEGF-C in supernatants of two neuroblastoma cell lines and PC-3 cells measured by sandwich ELISA

Cell type	VEGF-A (pg/mL)	VEGF-C (pg/mL)
PC-3 (day 0)	n.d.	n.d.
PC-3 (day 4)	146	172
SH-EP	192	140
SH-IN	2005	n.d.

NOTE: The VEGF-A ELISA measures total VEGF-A and the VEGF-C ELISA detects the fully processed form of VEGF-C. Indicated are the mean values of two measurements. n.d., not detectable (detection limit is ~60 pg/mL).

of lymphangiogenesis may expedite the formation of lymph node metastases. Neuroblastoma stages III, IV, and IVs are characterized by the spread of tumor cells to local, distant, and dermal lymph nodes, respectively. However, we have to be aware that our measurements, performed at the RNA level, do not necessarily reflect the amount of secreted protein. Downregulation of sVEGFR-2 may be enhanced by the *MYCN* oncogene, which is a critical clinical predictor for poor outcome. In stage IV neuroblastoma, *MYCN* amplification does not only downregulate sVEGFR-2, but also the hemangiogenesis inhibitor VEGFR-1. At the same time, the hemangiogenesis and lymphangiogenic factors VEGF-A and -D are upregulated. Identical regulation patterns could be observed after *MYCN* transfection of SH-EP cells. In these cells, we even observed complete switching-off of sVEGFR-2 expression. Our data support the observation that *MYCN* amplification promotes the progression of neuroblastoma, and it obviously does so by both the upregulation of activators and the downregulation of inhibitors. Downregulation of other inhibitors of hemangiogenesis in neuroblastoma has been described previously (25, 26).

The concept of high vascularity of progressed tumors has been challenged recently, as a number of data have pointed to a more aggressive behavior and the upregulation of genes associated with poor survival in avascular glioblastomas (47) and hypoxic neuroblastoma (48). In mice, the inhibition of tumor angiogenesis either by targeting the VEGF-A or the VEGFR/PDGFR kinase pathways have induced progression to greater malignancy and increased invasiveness, and decreased overall survival (49, 50). However, our observations in general support the classic view, although differences in the expression of angiogenic factors may not become immediately evident by comparing tumor stages. The proportion between proangiogenic and antiangiogenic factors has to be taken into account, as well as the fact that tumor cell lines do not necessarily reflect the *in vivo* behavior in the human.

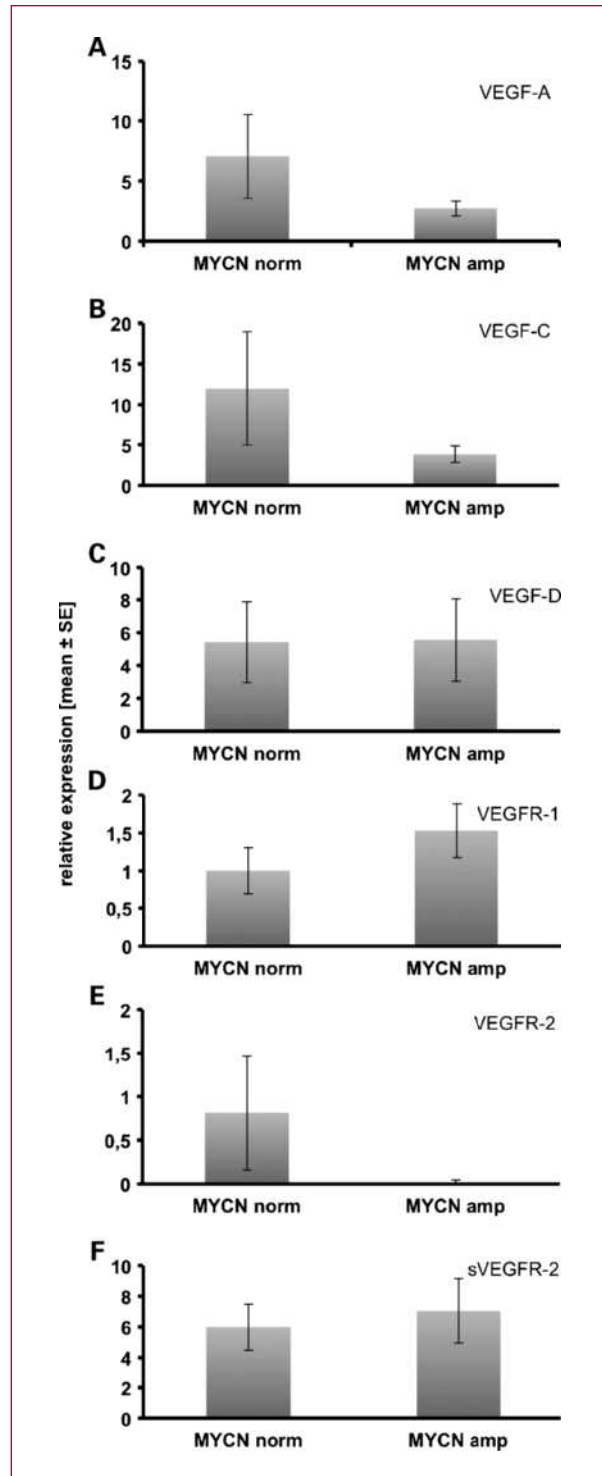

Fig. 4. Comparison of the expression of VEGF ligands and receptors in 24 neuroblastoma cell lines, sorted by their *MYCN* copy numbers (normal versus amplified). A, VEGF-A; B, VEGF-C; C, VEGF-D; D, VEGFR-1; E, VEGFR-2; F, sVEGFR-2. There are no statistically significant differences, but tendencies for the downregulation of VEGF-A, VEGF-C, and VEGFR-2 in *MYCN*-amplified cell lines. However, this does not reflect our observations on primary neuroblastomas. Columns, mean relative expression; bars, SE.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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