Abstract Purpose: Hypoxia is considered to be a major driving force behind tumor angiogenesis. The stabilization and activation at hypoxia of the hypoxia-inducible factors HIF-1α and HIF-2α and the concomitant induction of expression of vascular endothelial growth factor (VEGF) and other proangiogenic factors provide a molecular frame for hypoxia-driven tumor angiogenesis. This study has investigated how HIF and VEGF protein levels relate to each other with regard to vascularization, tumor stage, and overall survival in neuroblastoma.

Experimental Design: Tissue cores taken from tumor specimens representing 93 children with neuroblastoma were arranged on a microarray and stained for HIF-1α, HIF-2α, VEGF, and CD31 proteins. Both fraction of positive cells and staining intensity were evaluated and protein levels were correlated with each other and with clinical variables.

Results: Although high levels of both HIF-1α (P < 0.001) and HIF-2α (P < 0.001) correlated positively to VEGF expression, they did not fully correlate with each other. Moreover, HIF-1α (P = 0.002) and VEGF (P < 0.001), but not HIF-2α, correlated negatively to vascularization as determined by CD31 staining abundance. VEGF expression or degree of vascularization did not correlate with tumor stage or overall survival. High HIF-1α levels correlated with low tumor stage (P < 0.001) and were associated with a favorable patient prognosis (P = 0.08).

Conclusions: The discordant results on expression of HIF-1α and HIF-2α suggest that these two proteins are differentially regulated in vivo, thus reflecting distinctive protein expression/stabilization mechanisms. The association between HIF-1α and favorable outcome stresses the importance of discriminating HIF-2α from HIF-1α expression and has implications for using HIFs as treatment targets. (Clin Cancer Res 2009; 15(23):7130–6)

Oxygen shortage, hypoxia, is a common state in solid tumors and a hallmark of aggressive tumor cells in that they have acquired the capacity to both survive and proliferate at conditions of low oxygen tensions (1). Tumor cells seem to use the same adaptation mechanisms to hypoxia as nontransformed cells in which stabilization and activation of the hypoxia-inducible factor-1 (HIF-1) and HIF-2 are central components. Each of these two heterodimeric transcription factors consists of one oxygen-sensitive α-subunit (HIF-1α and HIF-2α, respectively) in complex with one β-subunit (ARNT), which is expressed and transcribed independent of the cellular oxygen status (2). Once stabilized and activated, the HIFs transcribe genes that are involved in cellular and physiologic adaptation to hypoxia, which include shifts toward anaerobic metabolism with increased glycolysis and downregulation of energy-consuming processes such as DNA repair. An important effect of HIF activation is the increased transcription of genes encoding...
Proteins with systemic effects on the oxygen supply such as VEGF and other proangiogenic genes (3). In tumors, hypoxia and subsequent activation of HIFs is considered to be one of the main tumor angiogenesiss–promoting mechanisms (reviewed in ref. 4).

The childhood tumor neuroblastoma is derived from precursor cells of the sympathetic nervous system; is characterized by extensive genetic, morphologic, and clinical heterogeneity; and presents as benign to highly aggressive forms. Generally, tumor cells of low- and intermediate-risk neuroblastomas are more mature, i.e., neuronally differentiated, than cells of high-risk neuroblastoma and HIF-2α seems to reflect different stabilization/expression mechanisms in relation to the microenvironment and tumor-initiating capacity. In summary, our findings highlight the importance of discriminating between HIF-1α– and HIF-2α–associated effects on outcome when investigating the hypoxic phenotype as a putative treatment target.

In the present study, we have investigated how HIF-1α and HIF-2α protein levels in a clinical neuroblastoma material relate to each other and to VEGF protein with regard to vascularization and patient outcome. We show that the expression of HIF-1α and HIF-2α protein in neuroblastoma specimens does not fully correlate. Although high HIF levels of either protein correlate positively to VEGF expression, only HIF-1α correlated negatively to vascularization, determined as CD31 expression. Contrary to our previously published results on HIF-2α (7), high HIF-1α protein levels did not correlate with adverse outcome, but rather associated with low-stage disease and favorable outcome. As both HIF-1α and HIF-2α are suggested as cancer treatment targets, our findings highlight the importance of discriminating between HIF-1α– and HIF-2α–associated effects on outcome.

Materials and Methods

Patient material. All analyzed samples were from the Spanish Reference Center for Biological Studies of Neuroblastoma, Department of Pathology, University of Valencia. The material, specimens from 93 children corresponding to more than 1 year of neuroblastoma turnover in Spain, was routinely processed to obtain paraffin blocks. The tumor material and its relation to patient and genetic data are described in Table 1. To construct the tissue microarrays (TMA), one pathologist chose representative tumor areas on H&E-stained sections. Necrotic, hemorrhagic, and nontumorous areas were avoided. Using a Beecher Instrument, TMAs were constructed, introducing two cores of 1-mm diameter of the selected tumor regions in a recipient paraffin block. Normal kidney tissue was included in the TMAs and used as control normal tissue. The samples included in the study were assembled in two TMAs: TMA1 contains 50 MYCN (+), MYCN myelocytomatosis viral oncogene, neuroblastoma derived) – amplified cases diagnosed between 1999 and 2004, whereas TMA2 contains 43 cases diagnosed between 1998 and 1999.

Genetic markers. The status of different genetic markers was analyzed by fluorescence in situ hybridization (FISH) using the following DNA probes: MYCN (2p24) red/LAF (2q11) control probe green (Kreatech Biotechnology); chromosome 1 satellite probe red (Q-Biogene, Kreatech)/chromosome 1p36 misisatellite probe green (MP Biomedicals). The criteria used to identify cells with genetic alterations are based on the guidelines of the European Neuroblastoma Quality Assessment Group (11). A minimum of 100 nuclei per core was scored, taking into consideration the selection of nonoverlapping nuclei and the analysis of multiple focal planes to ensure the detection of all signals (12). A control tissue (normal kidney) incorporated in the TMAs was used to define the distribution of interphase FISH signals. Following previous studies (13), we applied the scoring scheme to the tumor samples, and genetic alteration was considered when the percentage of cells with genetic alteration was higher than 15%. The status of MYCN and the 1p36 region has been previously studied in touch preparations from fresh material in all samples by FISH and there was no discordance between these and MYCN/1p36 TMA FISH results.

Immunohistochemistry. Neuroblastoma specimens were collected and analyzed for gene expression according to ethical approval 59CI8ABR2002, Valencia, Spain, and LU 389-98, Lund University, Sweden. Immunoreactivity was detected in 4-μm-thick freshly cut sections of paraffin-embedded tissues after antigen retrieval using the Envision system and DAKO Techmate 500. The following antibodies and dilutions were used: anti-CD31 (diluted 1/100; Dako), HIF-1α (1/10,000; Novus Biologicals), HIF-2α (1/1,000; Novus), and VEGF (1/250; Santa Cruz Biotechnology). All specimens were stained simultaneously. HIF immunospecificity was verified in sections of fixed and embedded cultured normoxic and hypoxic neuroblastoma cells (Supplementary Fig S1) and in normoxic/hypoxic breast cancer cells transfected with control siRNA or HIF-1a and HIF-2a siRNA, respectively, as previously described (9). Immunoreactivity was independently scored by two
pathologists (RN and SN) and classified according to fractions of positive cells (0, 0-10%; 1, 10-25%; 2, 26-75%; 3, 76-100%) and general intensity of positive cells (0, none; 1, mild; 2, moderate; 3, intense). Conflicting results (<10%) were revised and a consensus was reached.

Vascular density was measured based on a combination of CD31 staining and morphologic analysis. Capillary structures were defined as CD31-positive capillary endothelial cells with a capillary-lumen diameter of 5 to 10 μm.

**Statistics.** Spearman’s correlation test was used for assessing relationships between variables. Kaplan-Meier analysis and the log-rank test were used to illustrate differences between overall survival to parameters assessed by immunohistochemistry. Overall survival was defined as time from diagnosis until neuroblastoma-related death. For survival analysis, groups 0 and 1 (low) and 2 and 3 (high) were pooled, respectively. All statistical tests were two-sided and P values <0.05 were considered significant. Calculations were done using the R statistical programming language.3

**Results**

**HIF-1α and HIF-2α protein levels in neuroblastoma specimens correlate poorly.** In a previous study, we have analyzed HIF-2α protein levels in the patient material studied here and found that the presence of intensely HIF-2α–staining tumor cells correlated positively to poor outcome (7) and, as shown here, high clinical tumor stage (Fig. 1A; Supplementary Table S1). These tumor cells were frequently found to be located in association with blood vessels and tumor stroma (8), suggesting that the HIF-2α protein is abundant for reasons other than hypoxia-induced protein stabilization. We therefore asked whether the HIF-1α protein is present in the same vascularized tumor areas in which HIF-2α protein is high. As exemplified in Fig. 2, and as a rule, the HIF-1α protein was undetectable or very low in regions with strongly HIF-2α–positive cells adjacent to blood vessels (identified by CD31 positivity; Fig. 2), in agreement with our assumption that the local tumor microenvironment surrounding these cells are reasonably well oxygenated. To

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**Table 1. Patient data for the two tissue microarrays**

| Array 1 | Array 2 | Sum total | Percentage of total number of cases (%) |
|---------|---------|-----------|----------------------------------------|
| Cases with amplification and gain of MYCN locus | 50 | 43 | 93 | 100 |
| Type of patients on array | Population based | |
| No. patients | | |
| Male | 25 | 25 | 50 | 54 |
| Female | 25 | 18 | 43 | 46 |
| MYCN amplification | | |
| MYCN gain | 13 | 1 | 14 | 15 |
| ltp36 deletion | 45 | 8 | 53 | 57 |
| Mean age (mo) | 32 | 24 | 31 | |
| Median age (mo) | 23 | 11.5 | 22 | |
| Clinical follow-up available | 46 | 33 | 79 | 85 |
| Mean follow-up time (mo) | 20 | 48 | 31 | |
| Median follow-up time (mo) | 17 | 67 | 23 | |
| Outcome | | |
| Favorable | 24 | 26 | 50 | 54 |
| Deceased | 22 | 6 | 28 | 30 |
| Remission | 0 | 1 | 1 | 1 |
| Stage | | |
| 1, 2, 4S | 5 | 26 | 31 | 5 |
| 3 | 9 | 4 | 13 | 82 |
| 4 | 33 | 6 | 39 | 14 |

Abbreviation: 4S, stage 4 “special” according to the International Neuroblastoma Staging System.

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3 http://www.r-project.org

**Fig. 1.** A, HIF-2α protein staining intensity shows a weak significant correlation (ρ = 0.27, P = 0.02) to high clinical INSS (International Neuroblastoma Staging System) stage, as determined by three groups containing the less aggressive INSS 1, 2, and 4S tumors versus the increasingly spreading INSS stage 3 and 4 tumors. B, fraction of HIF-1α– and HIF-2α–positive cells correlates significantly with each other in neuroblastoma tumor tissue (ρ = 0.34, P = 0.002). HIF protein levels were assayed using immunohistochemical analyses on two tissue microarrays representing a total of 93 neuroblastoma cases.
extend our studies on the relation between HIF-1α and HIF-2α protein expression, we also stained two neuroblastoma TMAs, in total representing 93 children (see Table 1), for HIF-1α. As indicated in Fig. 1B, there was a positive correlation between HIF-1α and HIF-2α (ρ = 0.34, P = 0.002) when scoring for the number of positive cells (fraction). However, when assaying staining intensity, there was no correlation (ρ = 0.18, P = 0.11), clearly indicating that the two HIFs are expressed and/or stabilized by different conditions and mechanisms in neuroblastoma specimens.

**HIF-1α, HIF-2α, and VEGF expression in relation to vascularization in neuroblastoma.** The observation that the HIF proteins are differently regulated and previous data suggesting that VEGF expression is driven by HIF-1α during acute hypoxia and by HIF-2α at prolonged hypoxia prompted us to investigate how HIF protein expression in neuroblastoma specimens relates to VEGF expression and to vascularization. Staining the neuroblastoma TMAs for VEGF protein showed that both HIF-1α (fraction: ρ = 0.58, P < 0.001; intensity ρ = 0.47, P < 0.001; Fig. 3A-B; Supplementary Table S1) and HIF-2α (P < 0.001; see ref. 7) proteins correlated positively to VEGF both when assaying fraction of positive cells and staining intensity. To visualize blood vessels, we stained the TMA for CD31 and scored stained cells with apparent vascular morphology, which revealed that the presence of VEGF protein correlated negatively with CD31 staining (ρ = -0.47, P < 0.001) and, presumably, vascularization (Fig. 3C), a result in agreement with the general observation that VEGF expression is driven by hypoxia and HIFs and the assumption that well-vascularized tumor areas are not hypoxic. This conclusion inferred that the expression

![Image](image-url)

**Fig. 2.** Strong HIF-2α but weak HIF-1α staining in a vascularized area of a neuroblastoma specimen. Consecutive sections of a fixed and paraffin-embedded neuroblastoma specimen were stained for HIF-1α, HIF-2α, and CD31 by immunohistochemistry. Note that the HIF-2α-positive cells (brown stain) close to blood vessels (CD31-positive endothelial cells) are negative for HIF-1α.

![Image](image-url)

**Fig. 3.** Correlative analyses of hypoxia-inducible factors, VEGF, and vascularization. Protein levels were assayed using immunohistochemical analyses on two tissue microarrays representing 93 neuroblastoma cases. Vascularization was estimated by scoring tumor samples for abundance of CD31 staining. Fraction (A), as well as staining intensity (B), of HIF-1α- and VEGF-positive cells shows a strong and significant correlation in neuroblastoma (fraction: ρ = 0.58, P < 0.001; intensity: ρ = 0.47, P < 0.001). C, fraction of VEGF-positive cells is lower in well-vascularized compared with poorly vascularized tumor samples (ρ = -0.47, P < 0.001). D, fraction of HIF-1α-positive cells shows a significant negative correlation to tumor vascularization status (ρ = -0.35, P < 0.001). E, fraction of HIF-2α-positive tumor cells shows no significant correlation to vascularization status (ρ = -0.05); however, a positive trend toward high HIF-2α levels in well-vascularized tumor areas can be seen.
of HIF proteins and the presence of blood vessels will be negatively correlated. Indeed, we show that HIF-1α correlates negatively to CD31 quantified either as fraction of positive cells (\(\rho = -0.35, P = 0.002\); Fig. 3D) or staining intensity (\(\rho = -0.30, P = 0.006\)). In contrast, HIF-2α does not correlate with CD31 (fraction: \(\rho = -0.06\); intensity: \(\rho = -0.11\); Fig. 3E). The combined CD31 and HIF results again suggest that HIF-1α and HIF-2α are differentially regulated in vivo. For instance, when examining cores from the tissue microarray in detail, we could find examples of an inverse relationship between HIF-1α staining and CD31-positive vessels as exemplified in Fig. 4. No such direct discordant staining patterns were found when HIF-2α and CD31 staining was compared.

Highly vascularized and highly VEGF–expressing neuroblastomas have been associated with poor outcome and stage 4 disease, respectively, although the picture from published data is not consistent. For instance, VEGF staining intensity was scored in a four-grade scale on a tissue microarray representing 93 neuroblastoma patients. A, no significant correlation was found between VEGF staining intensity and clinical stage (\(\rho = 0.05\)) as determined by three groups of increasing stage (INSS 1, 2, 4S; INSS 3; INSS 4). B, for Kaplan-Meier survival analysis, VEGF staining scores were pooled into two groups: low (none and mild) and high (moderate and high) immunoreactivity. There was no significant correlation between VEGF staining intensity and patient outcome (\(P = 0.14\), log-rank test). C, a strong and significant correlation was found between high HIF-1α staining intensity and low clinical stage (\(\rho = -0.47, P < 0.001\)). D, Kaplan-Meier analysis, using HIF-1α staining scores pooled into two groups of high or low immunoreactivity, showed a trend between high HIF-1α intensity and favorable patient outcome (\(P = 0.08\), log-rank test).
reports is far from clear (14–19). Interestingly, analysis of our VEGF data revealed no correlation between fraction or staining intensity of VEGF-positive cells and clinical stage (fraction, \( \rho = 0.001 \); intensity, \( \rho = 0.05 \); Fig. 5A and Supplementary Table S1) and only a nonsignificant trend toward an association between high fraction of VEGF-positive cells and poor outcome (\( P = 0.15 \), log-rank test; Fig. 5B). Similarly, the number of CD31-positive endothelial cells in vascular structures, i.e., a surrogate marker for blood vessel, did not correlate with outcome or clinical stage (data not shown).

**High HIF-1α protein levels do not correlate with poor outcome.** Given that high HIF-2α staining intensity and number of HIF-2α-positive neuroblastoma cells correlate with poor prognosis (7) and the weak correlation between HIF protein levels shown here, we finally asked how HIF-1α protein levels relate to neuroblastoma patient outcome. As shown in Fig. 5C and D, high HIF-1α staining intensity correlates negatively with clinical stage (\( \rho = 0.47, P < 0.001 \); Fig. 5C) and there is a strong trend toward a negative correlation between poor patient outcome and occurrence of intensely staining HIF-1α cells (\( P = 0.08 \), log-rank test; Fig. 5D). We conclude that the stabilization/activation of the two HIF proteins in neuroblastoma tissues are not only differentially regulated but also have different impact on tumor behavior in relation to patient outcome, an observation in agreement with our previous findings in breast cancer (9). The staining patterns we find can generally be exemplified by staining tumor sections from bad and good prognosis tumors for HIF-1α, HIF-2α, CD31, and VEGF as shown in Supplementary Figs. S2 and S3.

**Discussion**

Our recent observations in neuroblastoma that HIF-2α is expressed in apparently well-vascularized tumor areas and that the presence of intensely staining HIF-2α-positive cells correlates with poor patient outcome (7, 8) led us to further investigate the association between expression of HIFs and outcome, tumor stage, vascularization, and VEGF expression in neuroblastoma. Although we found that expression of both HIFs correlated with VEGF expression, the presence of HIF proteins in tumor specimens as analyzed by immunohistochemistry was not strongly related to each other, implicating that they are not coherently regulated. This conclusion is further supported by our finding that high protein expression of the two HIFs associated differently with patient outcome. Interestingly, high HIF-2α, but not HIF-1α protein levels, correlate with poor outcome and disseminated disease—findings that are in agreement with our recent observations that HIF-2α, but not HIF-1α protein expression in breast carcinoma, is associated with breast cancer-specific death and distant metastasis (9).

The structurally related HIF-1α and HIF-2α were initially regarded as functionally similar; however, this view is gradually becoming more complex as suggested also by the findings in the present study. First of all, in vitro data show that HIF-1α and HIF-2α are regulated differently in a time- and oxygen-dependent fashion and that HIF-1 activation is primarily associated with an acute hypoxic response, whereas HIF-2 activation is associated with a prolonged hypoxic response (7, 9, 20, 21). Second, the HIFs are not only regulated by hypoxia; both HIF-1α and HIF-2α can be induced by nonhypoxic mechanisms involving growth factor–induced or oncogenic activation of translational and transcriptional pathways (reviewed in refs. 2, 4), and one can assume that these activation mechanisms differ for the two HIFs. As HIF-1α levels in the present study correlated negatively with vascular abundance (as estimated by CD31 positivity), both when assaying intensity and fraction of positive cells, our data strongly suggest that the HIF-1α protein detected is primarily present in poorly vascularized tumor areas and thus regulated by hypoxia-induced stabilization. HIF-2α, on the other hand, shows no correlation to CD31 staining and only weak association to HIF-1α (fraction of positive cells correlate, but not intensity). As we know that HIF-2α is hypoxia regulated in neuroblastoma (6), we suggest that the positive correlation with HIF-1α when scored as number of positive cells (fraction) is related to hypoxia-mediated induction of both HIFs, whereas the lack of overlap when assaying intensity at least in part is explained by the existence of intensely HIF-2α–positive stem-like or neuronally immature cells as previously reported (8). The coexpression of HIF-1α and HIF-2α proteins has not been studied or reported to any large extent; however, we recently showed a lack of correlation between these proteins in breast cancer (9) and Giatromanolaki et al. (22) found a similar pattern in malignant melanoma. The links between HIFs, VEGF, vascular density, and outcome have, to our knowledge, not previously been coherently studied. Thus, a discordant regulation of the HIFs and vascularization in solid tumors and the clinical consequences thereof seems to be an overlooked phenomenon. Our results strongly suggest that future studies on pharmacologic HIF modulation have to consider the possibility that the two HIFs in a given tumor form can have opposing effects on tumor aggressiveness.

A direct and positive role of hypoxia and HIFs in the vascularization of solid tumors through HIF-induced expression of proangiogenic factors like VEGF has been established for many years (23, 24), and we show a strong correlation between either of the HIFs (7) and VEGF. Furthermore, VEGF is more strongly expressed in tumor areas with low vascular abundance, indicating that HIF stimulates angiogenesis in hypoxic areas through induction of VEGF. Despite the known correlation and functional connection between HIF-2α and VEGF and the correlation of high HIF-2α levels with poor outcome, we did not detect a significant correlation between high number of VEGF-positive cells or VEGF staining intensity and negative neuroblastoma patient outcome. Our results are, however, not contradicted by published data. In three different reports (15–17), slightly increased VEGF mRNA levels are seen in clinical stage 3 and 4 neuroblastomas compared with low-stage tumors. In none of these investigations was a correlation between high VEGF expression levels and poor outcome, event-free survival, or MYCN amplification seen or reported, although a weak correlation between high VEGF mRNA and unfavorable tumor histology was noticed. In short, our VEGF protein results fit very well with published mRNA data and suggest a modest impact of high VEGF expression on patient outcome, as recently reported also by Ootsuca et al. (25).

The relation between tumor vascularization status and patient outcome is a matter of some controversy in neuroblastoma. In two studies, Cohn and coworkers show in neuroblastoma that high vascular index (number of vessels/mm²) and disorganized vasculature (glomeruloid microvascular proliferation), respectively, are indicators of poor outcome (18, 19). The correlation between high vascular index and poor outcome has been challenged (14) and a published discussion between the authors of the
two reports with contradicting data suggests that there could be methodologic differences contributing to the disparate observations (26). Our data, although lacking a detailed analysis of tumor vasculature beyond that of CD31 positivity and vascular endothelial cell morphology, show no correlation between level of vascularization and patient outcome or clinical stage, thus supporting the conclusions drawn in Cañete et al. (14). In contrast to a high degree of (organized) vascularization, one would intuitively believe that disorganized vasculature is associated with hypoxic and aggressive tumor growth, and future studies should also focus on putative roles of HIF-1 and/or HIF-2 in the organization of tumor vasculature—a question not addressed in our study.

The HIF proteins transcribe genes with multiple roles related to tumor aggressiveness and consequently expression of HIF's correlate with outcome in several tumor forms, including breast cancer, colon carcinoma, non–small cell lung cancer, and neuroblastoma (2–4). As published and as shown here, HIF-2α but not HIF-1α associates with poor outcome in at least some tumor types (9, 22, 27). We have no clear-cut explanation to this observation; however, we know from in vitro studies and indirectly from in vivo observations that HIF-2 in neuroblastoma and breast carcinoma can be active at near-physiologic oxygen tensions. Furthermore, high HIF-2α, but not HIF-1α, protein levels associate with distant metastasis in breast cancer and neuroblastoma (7, 9), and an immature, neural crest–, stem cell–like phenotype in neuroblastoma (8). If these HIF-2α–positive neuroblastoma cells indeed are tumor-initiating or stem cells, the capacity to produce VEGF and attract blood vessel formation under physiologic oxygen tensions might be crucial during an early phase of establishment of solid tumors.

References

1. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 2004;4: 437–46.
2. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
3. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38–47.
4. Löfstedt T, Fredlund E, Holmqvist-Mengelbier L, et al. Hypoxia inducible factor-2α in cancer. Cell Cycle 2007;6:919–26.
5. Fredlund E, Ringnér M, Maris JM, Påhlman S. High Myc pathway activity and low stage of neuronal differentiation associate with poor outcome in neuroblastoma. Proc Natl Acad Sci U S A 2008;105:14094–9.
6. Nilsson H, Jögi A, Beckman S, Harris AL, Poellinger L, Påhlman S. HIF-2α expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. Exp Cell Res 2005;303:447–56.
7. Holmqvist-Mengelbier L, Fredlund E, Löfstedt T, et al. Recruitment of HIF-1α and HIF-2α to common target genes is differentially regulated in neuroblastoma: HIF-2α promotes an aggressive phenotype. Cancer Cell 2006;10:413–23.
8. Pietras A, Gisselsson D, Örå I, et al. High levels of HIF-2α highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. J Pathol 2008;214:482–8.
9. Helcynska K, Larsson AM, Holmqvist Mengelbier L, et al. Hypoxia-inducible factor-2α correlates to distant recurrence and poor outcome in invasive breast cancer. Cancer Res 2008;68:9212–20.
10. Jögi A, Örå I, Nilsson H, et al. Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A 2002;99:7021–6.
11. Ambros IM, Benard J, Boavida M, et al. Quality assessment of genetic markers used for therapy stratification. J Clin Oncol 2003;21:2077–84.
12. Brown LA, Huntsman D. Fluorescent in situ hybridization on tissue microarrays: challenges and solutions. J Mol Histol 2007;38:151–7.
13. Piqueras M, Navarro S, Castell V, Cañete A, Llombart-Bosch A, Noguera R. Analysis of biological prognostic factors using tissue microarrays in neuroblastic tumors. Pediatr Blood Cancer 2009;52:209–14.
14. Cañete A, Navarro S, Bermudez J, Pellin A, Castell V, Llombart-Bosch A. Angiogenesis in neuroblastomas: relationship to survival and other prognostic factors in a cohort of neuroblastoma patients. J Clin Oncol 2000;18:27–34.
15. Eggert A, Ikegaki N, Kwiatkowski J, Zhao H, Brodeur GM, Himelstein BP. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. J Clin Oncol 2000;18:1900–8.
16. Fukuzawa M, Sugiyura H, Koshinaga T, Ikeda T, Hagiwara N, Sawada T. Expression of vascular endothelial growth factor and its receptor Fk-1 in human neuroblastoma using in situ hybridization. J Pediatr Surg 2002;37:1747–50.
17. Komuro H, Kaneko M, Nakanishi Y, Katzenstein HM, Cohn SL, Crawford S, Meitar D, Crawford WE, et al. Inducibility of angiogenic factors in neuroblastoma patients. J Clin Oncol 2000;18:27–34.
18. Ootsuka S, Asami S, Sasaki T, et al. Analyses of novel prognostic factors in neuroblastoma. J Clin Oncol 2003;21:3024–30.
19. Meitl D, Crawford SE, Rademaker AW, Cohn SL. Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma. J Clin Oncol 1996;14:405–14.
20. Yoshimura H, Dhar DK, Kohno H, et al. Prognostic impact of hypoxia-inducible factors 1α and 2α in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin Cancer Res 2004;10:8554–60.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.