Immunoexpression patterns for Hypoxia-inducible Factor-1α and von Hippel-Lindau protein, in relation to Hsp90, of human brain tumors

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Summary. The pathogenesis of many tumors, including brain tumors, has been associated with hypoxia, which induces the transcriptional activity of Hypoxia-inducible Factor-1α (HIF-1α). HIF-1α is normally degradated by the von Hippel-Lindau protein (pVHL) but, in hypoxia, pVHL/HIF-1α interaction is inhibited resulting in the nuclear accumulation of HIF-1α. Hsp90 (Heat shock protein 90), as a chaperone protein, plays a critical role for both stabilization of HIF-1α and degradation of pVHL. The aim of this study was to estimate immunohistochemically the expression levels of HIF-1α and pVHL, in relation to Hsp90, in different types of human brain tumors (42 gliomas, 9 medulloblastomas, and 38 meningiomas) using specific antibodies. The tumors were further divided into two groups according to the age of patients (≥19 years old or <19 years old). Nuclear, for HIF-1α, and cytoplasmic, for pVHL and Hsp90, localization was detected in a high percentage of tumor cells in the majority of tumors. In astrocytomas, a significant, grade-dependent relationship for HIF-1α immunoexpression was observed (p<0.05). Furthermore, there was a significant correlation between pVHL and Hsp90 immunoexpression (p<0.01). The group of ≥19 years old patients with glioblastomas (WHO grade IV) demonstrated significantly increased immunoexpression for HIF-1α compared to pVHL (p<0.0001) and Hsp90 expression (p<0.01). In medulloblastomas, a significant

correlation of HIF-1α with Hsp90 immunoexpression (p<0.05) was found. In meningiomas, no significant correlation for the expression of the three proteins was detected (p≥0.05). These results indicate that HIF-1α/pVHL/Hsp90 interactions may be implicated in biology of different types of brain tumors through different signaling mechanisms.

Key words: HIF-1α, pVHL, Hsp90, Human brain tumors

Introduction

Intratumoral hypoxia is involved in many aspects of cancer cell biology including reprogramming of metabolism (Masson and Ratcliffe, 2014), genetic instability (Luoto et al., 2013), acquisition of stemness properties (Heddleston et al., 2010; Mathieu et al., 2011), and production of angiogenic factors (Semenza, 2013a) through induction of transcriptional activity of hypoxia-inducible factors (HIFs) and especially HIF-1 (Semenza, 2013b). HIF-1 is a heterodimeric protein complex composed of two subunits: a stable and constitutively expressed HIF-1β, and an inducible, O_2- and growth factor-regulated HIF-1α-subunit (Wenger, 2002). Under normoxia, HIF-1α protein binds with the von Hippel Lindau protein (pVHL) (Lisztwan et al., 1999), a tumor suppressor protein (Frew and Krek, 2007), and is rapidly degraded (Bruick and McKnight, 2001). Under hypoxic conditions, the pVHL/HIF-1α interaction is inhibited, resulting in HIF-1α

accumulation in the nucleus and subsequent promotion of the transcription of its target genes (Semenza, 2013b).

Brain tumors, particularly the highly aggressive glioblastoma with its necrotic areas, are among the tumors affected by hypoxic mechanisms (Kaur et al., 2005; Jensen, 2009). Enhanced expression of HIF-1α has been described in astrocytomas (Zhong et al., 1999; Zagzag et al., 2000; Søndergaard et al., 2002; Korkolopoulou et al., 2004; Giannopoulou et al., 2006; Jensen et al., 2006; Korkolopoulou et al., 2007; Flynn et al., 2008; Kaynar et al., 2008; Dreyfuss et al., 2009; Liu et al., 2009; Mashiko et al, 2011; Mayer et al., 2012; Ji et al., 2013; Reszec et al., 2013b), oligodendrogliomas (Birner et al., 2001, 2004; Abraham et al., 2012), and meningiomas (Jensen et al., 2002; Kaynar et al. 2008; Jensen and Lee, 2012; Preusser et al, 2012; Reszec et al., 2013a,b). Furthermore, HIF-1α is an independent factor for both shorter overall survival and progression-free survival in glioblastoma (Mashiko et al., 2011; Ji et al., 2013). Similarly, higher expression of HIF-1α was associated with shorter overall survival in oligodendrogliomas (Birner et al., 2001, 2004; Abraham et al., 2012), as well as in meningiomas (Jensen and Lee, 2012). Accumulating data highlight the critical role of HIF-1α in glioblastoma resistance to antiangiogenic therapy (Hu et al., 2012) and chemotherapy (Oliver et al., 2009; Kolenda et al., 2011).

Heat shock protein (Hsp90) is a central participant in a multistep chaperoning process that folds and stabilizes a wide range of cellular clients (Wandinger et al., 2008). Tumor cells express high levels of Hsp90 and have increased reliance on the Hsp90 chaperoning pathway compared with normal cells. Moreover, the activities of a multitude of key oncogenic proteins and, especially, transcription factors that promote tumorigenesis, are supported by Hsp90 (Whitesell and Lindquist, 2005; Chiosis and Neckers, 2006; Brown et al., 2007; Koga et al, 2009; Trepel et al., 2010). Thus, Hsp90 has attracted considerable attention as a potential cancer therapy target, and multiple Hsp90 inhibitors are now in clinical trials worldwide (Koga et al., 2009; Trepel et al., 2010).

Experimental data demonstrate that Hsp90 correlates with HIF-1α (Gradin et al., 1996; Isaacs et al., 2002) and the disruption of the interaction between Hsp90/HIF-1α induces the degradation of HIF-1α, in a way that is independent of oxygen and pVHL (Mabjeesh et al., 2002; Liu et al., 2007; Kim et al., 2009). Furthermore, according to Mcclellan et al. (2005), Hsp90 is essential for the degradation of pVHL, although it neither participates in pVHL folding nor is required to maintain misfolded VHL solubility. High expression of Hsp90 has been reported in human astrocytomas (Kato et al., 1995; Hermisson et al., 2000; Strik et al., 2000; Shervington et al., 2008), oligodendrogliomas (Strik et al., 2000), meningiomas (Kato et al., 1995), and medulloblastomas (Hauser et al., 2006; Alexiou et al., 2013). The objective of this study was to investigate the co-expression patterns of transcription factor HIF-1α and von Hippel Lindau protein (pVHL) in different human brain tumors and normal brain. The expression of Hsp90, compared to the expression levels of HIF-1α and pVHL, was also included. Furthermore, correlation analysis was assessed between the expression of HIF-1α, pVHL, Hsp90 and EpoR - the receptor of the glycoprotein hormone erythropoietin (Epo), which is upregulated by hypoxia, and as we have shown in a previous study, demonstrates increased expression in gliomas and meningiomas but not in medulloblastomas (Kondyli et al., 2010).

Materials and methods

Tissue samples

A total of 89 patients with brain tumors (34 astrocytomas (age range 2-74); four oligodendrogliomas (age range 46-59); four ependymomas (age range 41-63); nine medulloblastomas (age range 3-31); and 38 meningiomas (age range 28-87), who underwent surgery at the Neurosurgery Department of Patras University School of Medicine, during a 12-year period, were included in this study. We further divided the group of patients, based on their age. Patients younger than 19

Table 1. Tumor histopathology and demographic characteristics for patients with brain tumors included in the current study.

Histology/grading	Gender (no. of cases)		Age (no. of cases)		Total
	Male	Female	≥19 years	<19 years	
Gliomas					
Pilocytic astrocytomas (WHO grade I)	2	1	0	3	3
Diffuse fibrillary astrocytomas (WHO grade II)	1	3	3	1	4
Anaplastic astrocytomas (WHO grade III)	3	1	2	2	4
Glioblastomas (primary) (WHO grade IV)	14	9	21	2	23
Oligodendrogliomas	2	2	4	0	4
Ependymomas	2	2	4	0	4
Medulloblastomas	7	2	3	6	9
Meningiomas	21	17	38	0	38
Total	52	37	75	14	89

WHO, World Health Organization.

years old compromised the group of pediatric tumors and patients older than or equal to 19 years old compromised the group of adult tumors (Fangusaro, 2012). Clinicopathologic features regarding the study population are presented in Table 1. Normal human brain tissue was obtained postmortem (two males, 56 and 28 years). The use of human specimens was in accordance with the University Ethics Commission. The formalin-fixed, paraffin-embedded archival tissue blocks were retrieved from the archives of the Department of Pathology of the same Hospital and matching hematoxylin and eosin (H&E)-stained slides were reviewed and screened for representative tumor regions by a pathologist. Tumors were evaluated by routine methods for histopathology, including immunohisto-chemical staining for glial fibrillary acidic protein (GFAP) and Ki-67 index as proliferation marker, and graded according to the diagnostic criteria of the WHO classification system (Louis et al., 2007).

Immunohistochemistry

Immunohistochemical expression of proteins was detected on 4 μm thick semi-serial sections using the EnVision System (DakoCytomation, DAKO, Carpinteria, CA) and the specific primary antibodies: a rabbit monoclonal antibody directed against Hsp90 (C45G5) (dilution, 1:200) (Cell Signaling, Beverly, MA)
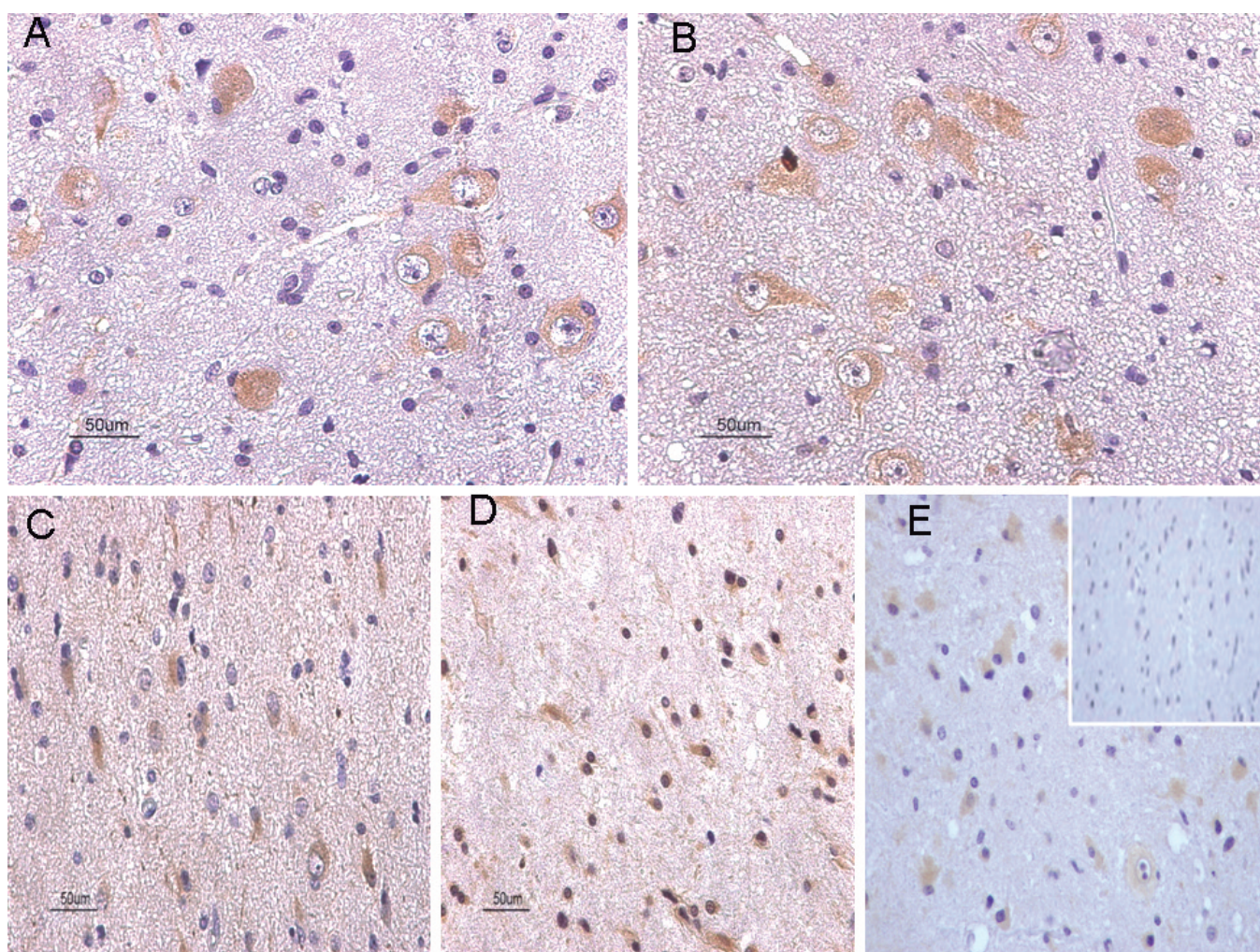

Fig. 1. Expression of HIF-1α, pVHL, and Hsp90 in diffuse fibrillary astrocytomas (WHO grade II). **A, B.** Photomicrographs from homologous fields of immediately adjacent sections of a diffuse fibrillary astrocytoma demonstrating Hsp90 **(A)**, and pVHL **(B)** cytoplasmic staining in neurons and scattered tumor cells. This case has Hsp90 LI 1% and pVHL LI 1%. **C, D, and E.** A high percentage of tumor cells demonstrate cytoplasmic immunoreactivity for Hsp90 **(C)** and pVHL **(D)** proteins (LI=80) in another case of fibrillary astrocytoma. HIF-1α immunoreactivity is detected in the cytoplasm and not in the nucleus of tumor cells (LI=0) **(E)**. Immunostaining is absent in sections incubated with control IgG **(E, insert)**. Dako Envision Plus detection System with hematoxylin counterstain. Scale bar: 50 μm.

which detects endogenous levels of total Hsp90 protein, a mouse monoclonal antibody against pVHL (clone 52A11) (dilution 1:100) (Acris Antibodies, Herford), and a mouse monoclonal antibody against HIF-1α (clone H1alpha67) (dilution 1:30) (Novus Biologicals, Littleton). Briefly, antigen retrieval was performed either by microwaving the slides in 0.01 M citrate buffer (pH 6) for the antibodies against pVHL and Hsp90 or incubating with pepsin at 37°C for 15min for the antibody against HIF-1α. Endogenous peroxidase activity was quenched by treatment with 1% hydrogen peroxide for 20 min. Incubation with an appropriate protein blocking solution was performed. Sections were subsequently incubated with primary antibodies overnight at 4°C. 3,3'-diaminobenzidine (DAB) was used as a chromogen (which yielded brown reaction products). Sections were counterstained with Mayer's hematoxylin solution. To ensure antibody specificity, negative controls included the omission of primary antibody and substitution with non-immune serum. Control slides were invariably negative for immuno-staining. As positive controls, cancer specimens known to express HIF-1α, pVHL, and Hsp90 were used.

Analysis of staining, and statistical methods

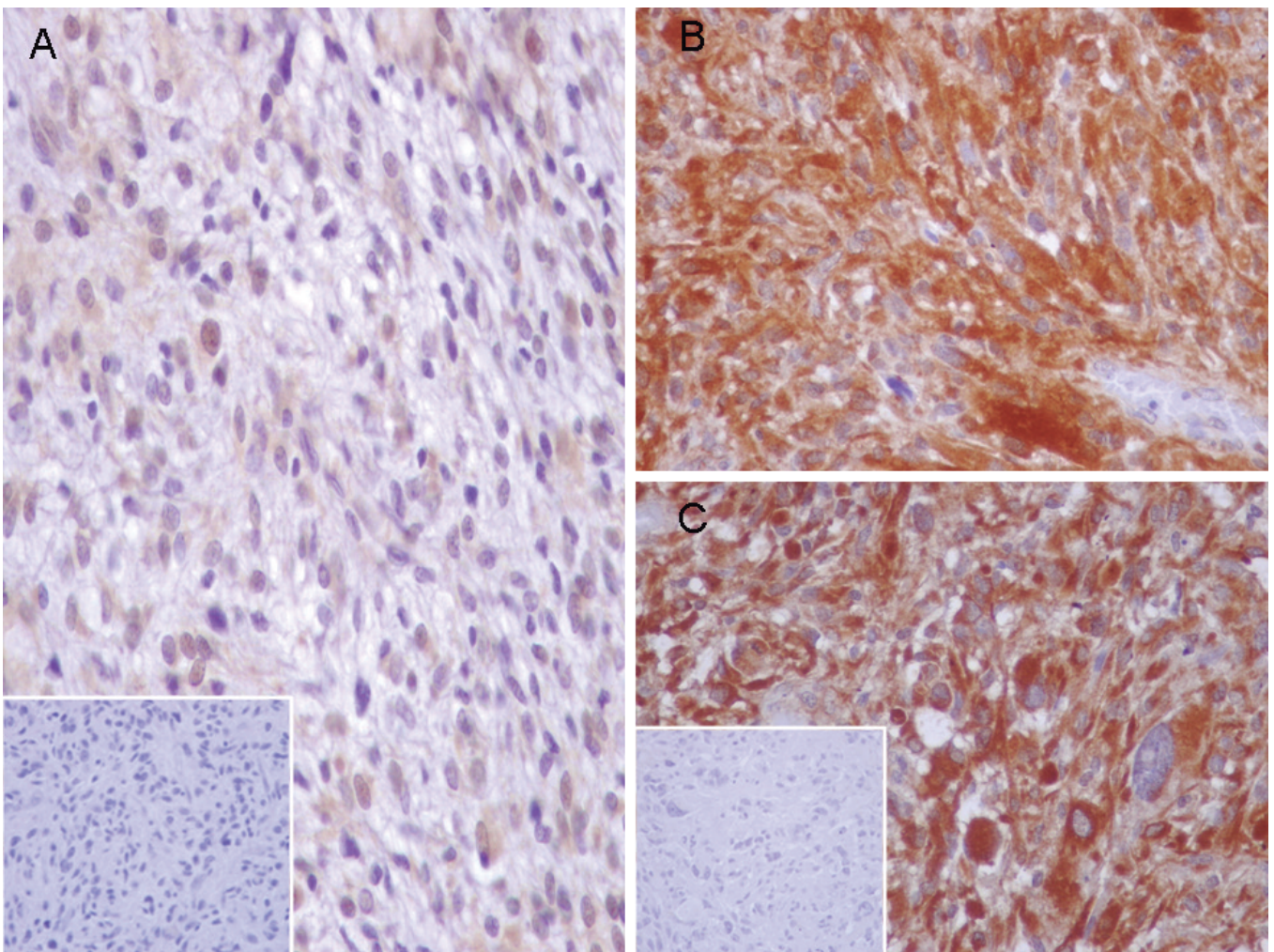Expression of proteins was examined only in cases where adjacent (semi-serial) sections were provided

Fig. 2. Expression of HIF-1α, pVHL, and Hsp90 in glioblastomas (WHO grade IV). **A.** HIF-1α immunolocalization is distributed in the nucleus in the majority of tumor cells and in the cytoplasm of some cells. This case has a labeling index (LI) of 70% for HIF-1α. The LIs for both pVHL and Hsp90 is 0% (**A**, insert). **B, C.** The 2 photomicrographs are from different areas of another tumor depicting the strong cytoplasmic immunoreactivity in a high percentage of tumor cells (LI=70) for both Hsp90 **(B)** and pVHL **(C)** proteins. Note that giant and multinucleated cells show conspicuous immunoreactivity for Hsp90 and pVHL. Immunostaining is absent in sections incubated with control IgG (**C**, insert). Dako Envision Plus detection System with hematoxylin counterstain. x 400

(Kudo et al., 1996). To determine the labeling index (LI) (% labelled cells) for each antibody, two observers independently assessed ten non-overlapping, random fields (x 400 total magnification) for each case and manually counted 100 tumor cells in each field with the aid of an ocular grid. Immunopositive endothelial cells were excluded from the cell counts. In rare cases where there was disagreement, both observers re-examined the slides together and agreed on the final percentage. In this study, a tumor was designated as immunopositive if the score was ≥10.

Nonparametric methods were used for statistical analysis of the results. Median comparisons were performed with Wilcoxon's Rank-Sum test (equivalent to the Mann-Whitney U test) and the Kruskal-Wallis test. Spearman correlation was used to assess the significance of relationships between different LIs. To investigate the existence of a relationship between HIF-1α, pVHL, and Hsp90 LIs and an ascending degree of malignancy in astrocytomas, linear regression analysis was performed between the WHO tumor grades and the LIs for each of these proteins. P values <0.05 were considered significant. Statistical analyses were carried out using the SPSS package (version 17.0).

Results

HIF-1α, pVHL, and Hsp90 immunoexpression in normal brain tissue

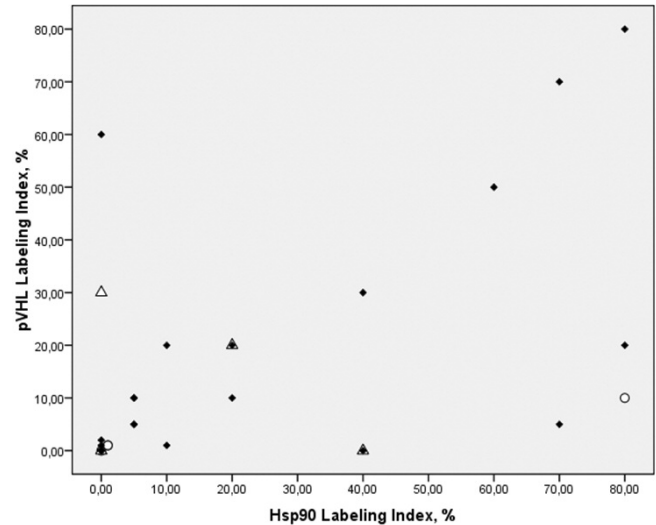In normal brain sections and in the apparently

Fig. 3. Scatter plot graph comparing the labeling indices (LIs) for Hsp90 and pVHL in a sample of 31 astrocytomas representing tumor grades II-IV. Various grades of astrocytoma are represented by different symbols: diffuse fibrillary astrocytomas (WHO grade II), ○; anaplastic astrocytomas (WHO grade III), △; and glioblastoma (WHO grade IV), ♦. Spearman correlation analysis revealed the existence of a highly significant relationship between Hsp90 and pVHL labeling indices (r^2=0.515, p=0.004).

Table 2. Immunohistochemical expression of HIF-1α, pVHL, and Hsp90, in correlation to the age, for human brain tumors.

Histology/grading	HIF-1α Mean±SD, % (range)		pVHL Mean±SD, % (range)		Hsp90 Mean±SD, % (range)	
	≥19 years	<19 years	≥19 years	<19 years	≥19 years	<19 years
Gliomas						
Pilocytic astrocytomas (WHO grade I)	-	30.66±25.32 (2-50)	-	33.33±20.81 (10-50)	-	3.33±2.88 (0-5)
Diffuse fibrillary astrocytomas (WHO grade II)	0.33±0.57 (0-1)	1#	3.66±5.50 (0-10)	1#	27.00±45.90[c] (0-80)	1#
Anaplastic astrocytomas (WHO grade III)	10.00±14.14 (0-20)	0.00±0.00 (0-0)	10.00±14.14 (0-20)	15.00±21.21 (0-30)	10.00±14.14[c] (0-20)	20.00±28.28 (0-40)
Glioblastomas (WHO grade IV)	51.50±33.43[a,b] (0-90)	6.00±5.65 (2-10)	18.40±25.34[a] (0-80)	15.50±20.50 (1-30)	23.33±29.63[b,c] (0-80)	20.00±28.28 (0-40)
Oligodendrogliomas	35.00±28.86 (10-60)	-	12.50±18.92 (0-40)	-	37.50±47.87 (0-100)	-
Ependymomas	47.00±39.83 (1-70)	-	37.50±43.93 (0-80)	-	67.50±22.17 (40-90)	-
Medulloblastomas	7.67±10.69 (1-20)	36.20±37.57 (0-80)	10.00±17.32 (0-30)	20.00±21.90 (0-60)	56.66±49.32 (0-90)	31.66±39.20 (0-80)
Meningiomas	23.35±28.12 (0-70)	-	29.05±31.09 (0-90)	-	42.83±36.94[c] (0-90)	-

Labeling Index (LI), the percentage of positively (labeled) cells out of the total number of epithelial cells counted; Mean, mean labeling index; SD, standard deviation; # LI for one tumor specimen; [a], indicates significantly increased expression of HIF-1α compared to pVHL expression in glioblastomas (WHO grade IV) for patients ≥19 years old (p<0.0001); [b], indicates significantly increased expression of HIF-1α compared to Hsp90 expression in glioblastomas (WHO grade IV) for patients ≥19 years old (p=0.006); [c], indicates significantly increased expression of Hsp90 in meningiomas compared to astrocytomas (WHO grades II-IV) for patients ≥19 years old (p=0.02).

"normal tissue" of the peritumoral region found in some cases, Hsp90 and pVHL immunoreactivity was detected in the cytoplasm of neurons (Fig. 1A,B) whereas HIF-1α cytoplasmic and/or nuclear immunoreactivity was observed in a few glial cells.

HIF-1α, pVHL, and Hsp90 immunoexpression in gliomas

Nuclear and/or cytoplasmic immunoreactivity for HIF-1α was detectable in tumor cells. Only the nuclear immunoreactivity was taken into consideration during the evaluation of staining. In glioblastomas (WHO grade IV), HIF-1α strong nuclear immunoreactivity was noted in tumor cells around microvessels and glomeruloid vascular structures. In pilocytic astrocytomas (WHO grade I), the nuclear HIF-1α immunostaining was weak or absent. pVHL immunoreactivity was localized in the cytoplasm of tumor cells and, occasionally, in endothelial cells of tumor vessels. Moderate to strong cytoplasmic immunoreactivity was observed for Hsp90. In glioblastomas (WHO grade IV), giant, multinucleated tumor cells and elongated, bipolar cells were intensely stained (Figs. 1C-E, 2A-C). In some astrocytomas [10/34 (29.4%)], the endothelium of tumor capillaries and florid angioproliferative changes showed conspicuous immunoreactivity for Hsp90. Immunopositivity (LIs≥10) for HIF-1α, pVHL, and Hsp90 displayed 60%, 46.6%, and 48.3%, respectively, of astrocytomas (WHO grades II-IV).

A significant correlation with histological grade of malignancy for HIF-1α immunoexpression (p=0.03) and not for pVHL or Hsp90 immunoexpression (p≥0.05) was
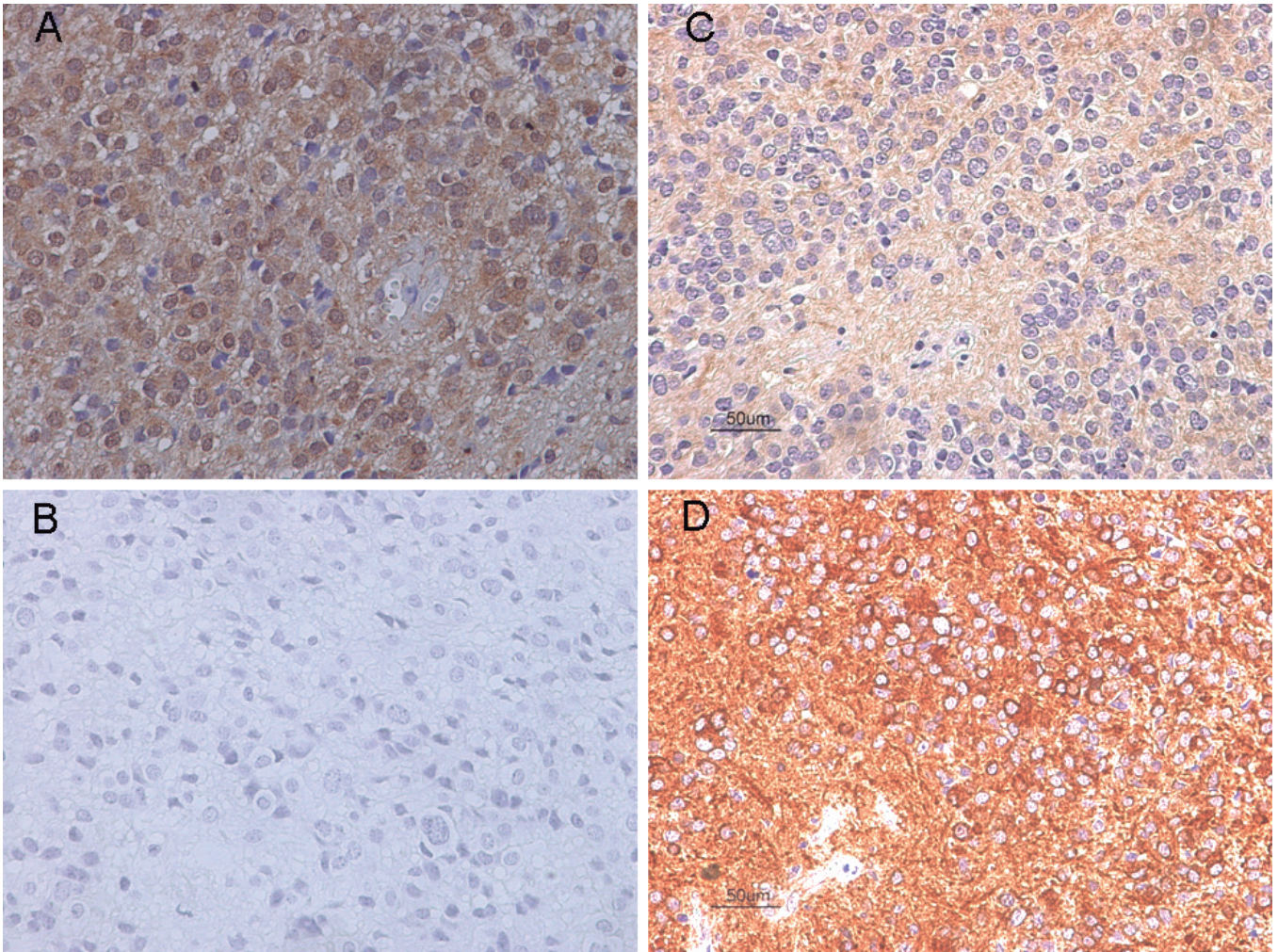

Fig. 4. Expression of HIF-1α, pVHL, and Hsp90 in oligodendrogliomas. **A.** Immunostaining of an oligodendroglioma with a HIF-1α LI of 60%. Immunostaining is absent in sections incubated with control IgG **(B)**. The same oligodendroglioma demonstrates weak to moderate immunoreactivity for pVHL (LI=10) **(C)**, and strong immunoreactivity for Hsp90 (LI=100) **(D)**. Dako Envision Plus detection System with hematoxylin counterstain. x 400; Scale bar: 50 μm.

detected in astrocytomas. Furthermore, in patients ≥19 years old with glioblastoma (WHO grade IV), the distribution of HIF-1α was significantly increased compared to pVHL (p<0.0001) and Hsp90 (p=0.006) (Table 2). pVHL immunoexpression was significantly correlated with Hsp90 immunoexpression in WHO grades II-IV (Spearman correlation=0.515, p=0.004) (Fig. 3) (Figs. 1C,D, 2B,C). However, there was considerable staining heterogeneity for HIF-1α, pVHL, and Hsp90 immunoreactivity among different tumors. Variability of positive cells was also common within individual tumors (intratumoral heterogeneity). Quantitative analyses of the immunohistochemical indices (LIs) showed no significant differences based on gender of the patients (p≥0.05).

The ependymomas included in the present study showed nuclear immunoreactivity for HIF-1α in three out of four (75%) samples. pVHL was detected in the cytoplasm of 50% (2/4) of samples and assessment of Hsp90 expression revealed an abundant cytoplasmic expression in all ependymomas (4/4). Some vessels were Hsp90 immunopositive. Oligodendrogliomas displayed an aberrant nuclear localization for HIF-1α expression (4/4) (two cases with LI=10, and two cases with LI=60). The oligodendrogliomas with lower HIF-1α immunoexpression (LI=10) were pVHL and Hsp90 immunonegative (LI=0). A representative oligodendroglioma with high immunoexpression for HIF-1α is presented in Fig. 4.

HIF-1α, pVHL, and Hsp90 immunoexpression in medulloblastomas

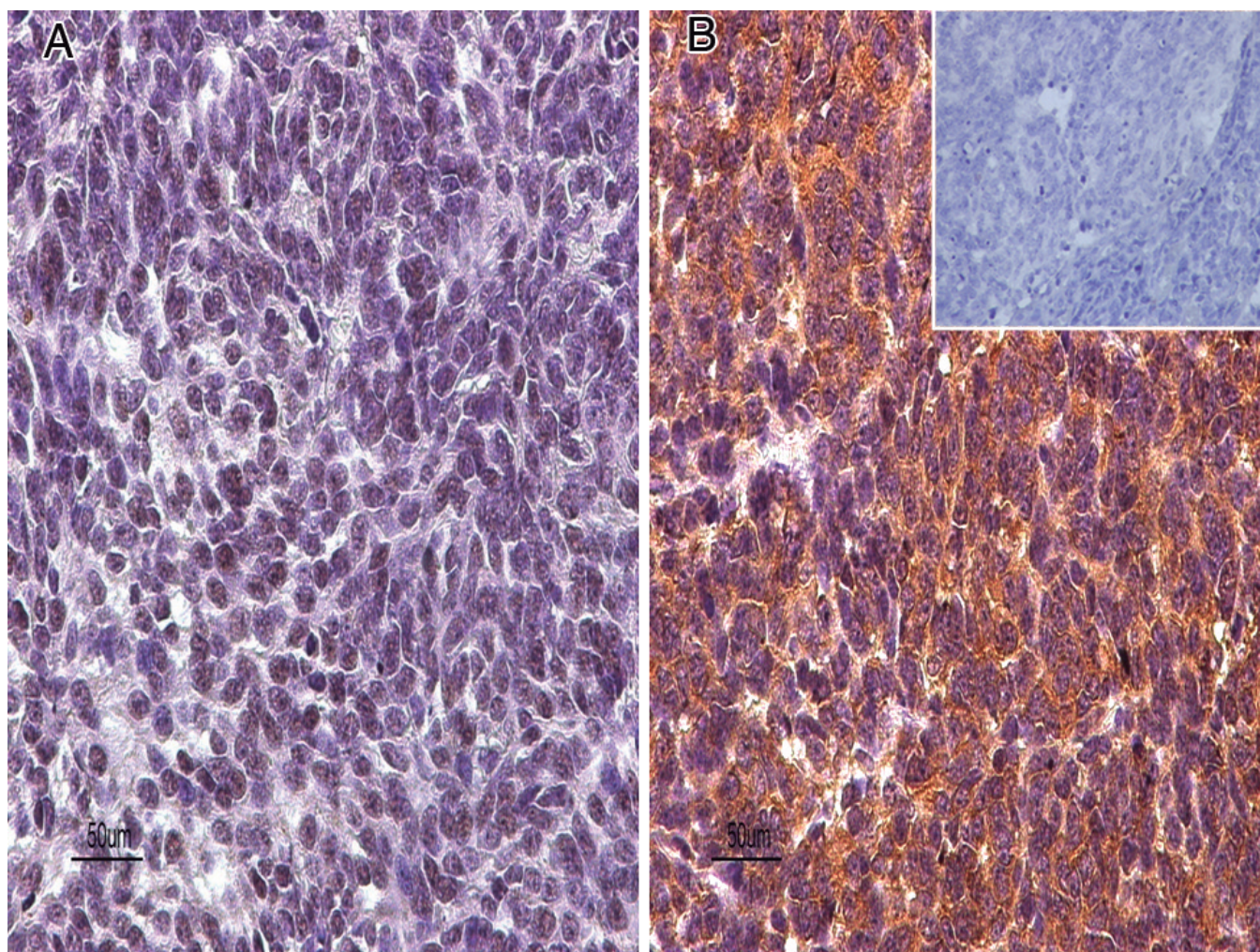Medulloblastomas demonstrated strong nuclear

Fig. 5. Expression of HIF-1α, pVHL, and Hsp90 in medulloblastomas. Homologous fields of immediately adjacent sections from a medulloblastoma of a 3.5 year old child, depicting strong nuclear HIF-1α **(A)**, and cytoplasmic Hsp90 immunoreactivity **(B)** in a high percentage of tumor cells (HIF-1α LI=80, Hsp90 LI=80). This tumor has a pVHL labeling index (LI) of 20% (not shown). Immunostaining is absent in sections incubated with control IgG (**B**, insert). Dako Envision Plus detection System with hematoxylin counterstain. Scale bar: 50 μm.

immunoreactivity for HIF-1α, strong cytoplasmic immunoreactivity for Hsp90 and/or weak cytoplasmic immunoreactivity for pVHL (Fig. 5). In one specimen some vessels were pVHL immunopositive. HIF-1α (50%), pVHL (55.5%), and Hsp90 (55.5%) immuno-positive tumors (LIs≥10) were recorded for the medulloblastomas. There was a significant correlation between the LIs for HIF-1α and Hsp90 (Spearman correlation=0.72, p=0.02). In patients <19 years old, 60% of tumors were immunopositive for HIF-1α (LIs≥10). Furthermore, a negative relationship between HIF-1α expression and age was detected in these patients (Spearman correlation=-0.99, p=0.03) (Fig. 6). No significant correlation between HIF-1α, pVHL, and Hsp90 expression and gender of the patients (p≥0.05) was found.

HIF-1α, pVHL, and Hsp90 immunoexpression in meningiomas

Nuclear and/or cytoplasmic HIF-1α immuno-reactivity, moderate to strong cytoplasmic pVHL immunoreactivity, and strong Hsp90 cytoplasmic immunoreactivity was detected in meningiomas (Fig. 7). In a few cases, endothelial cells exhibited pVHL, and/or Hsp90 immunoexpression. Whorl formations always showed conspicuous pVHL and Hsp90 cytoplasmic immunoreactivity. Immunopositivity (LIs≥10) for HIF-1α, pVHL, and Hsp90 was detected in 50%, 54%, and 64.8%, respectively, of meningiomas. Hsp90 expression was significantly increased compared to astrocytic gliomas (WHO grades II-IV, ≥19 years old) (p=0.02) (Table 2). Quantitative analyses of the immunohisto-chemical labeling indices (LIs) showed no significant correlations based on age or gender of the patients. Furthermore, there were no significant correlations between the LIs for the three proteins studied (p≥0.05).

Co-expression of HIF-1α, pVHL, and Hsp90 in astrocytomas, medulloblastomas, and meningiomas

The co-expression analysis was assessed for immunopositive tumors (LIs≥10). HIF-1α/pVHL co-expression was detected in 28% of astrocytomas (WHO grades II-IV) mainly glioblastomas (WHO grade IV), 25% of medulloblastomas, and 18.5% of meningiomas. HIF-1α/Hsp90 co-expression demonstrated 28% of astrocytomas (WHO grades II-IV) mainly glioblastomas (WHO grade IV), 50% of medulloblastomas, and 25.9% of meningiomas. pVHL/Hsp90 co-expression was found in 34.4% of astrocytomas (WHO grades II-IV), 33.3% of medulloblastomas, and 38.8% of meningiomas. A small percentage of glioblastomas (WHO grade IV) (11.1%), medulloblastomas (25%), and meningiomas (15.3%) were pVHL immunonegative and HIF-1α/Hsp90 immunopositive.

Finally, there was no significant relationship between HIF-1α, pVHL, Hsp90, and EpoR immuno-expression in astrocytomas (WHO grades II-IV), medulloblastomas, and meningiomas included in this study (p≥0.05).

Discussion

Accumulating experimental data implicate Hsp90 in stabilization and activation of HIF-1α enhancing its oncogenic role (Gradin et al., 1996; Isaacs et al., 2002; Mabjeesh et al., 2002; Liu et al., 2007; Kim et al., 2009). However, an early and prerequisite step in tumor development is the inactivation or loss of pVHL function (Frew and Krek, 2007). In the present study, a significant correlation of HIF-1α nuclear immunoexpression with tumor grade and proliferative potential was found in astrocytomas confirming previous studies which have shown that hypoxia-related markers are a pivotal element of the glioblastoma phenotype (Korkolopoulou et al., 2004; Flynn et al., 2008; Dreyfuss et al., 2009; Mayer et al., 2012). Moreover, HIF-1α expression in ≥19 year old patients with glioblastoma (WHO grade IV) was independent from pVHL and Hsp90 expression (Table 2). There was no significant difference for HIF-1α expression between astrocytomas and meningiomas. Kaynar et al. (2008) also studied the expression of HIF-1α in tumors of patients with glioblastoma and transitional meningioma and demonstrated that the protein is elevated in both tumors. Recently, Reszec et al. (2013a) correlated HIF-1α expression with the presence of mast cells and peritumoral brain edema, including HIF-1α in pathogenesis of meningiomas.
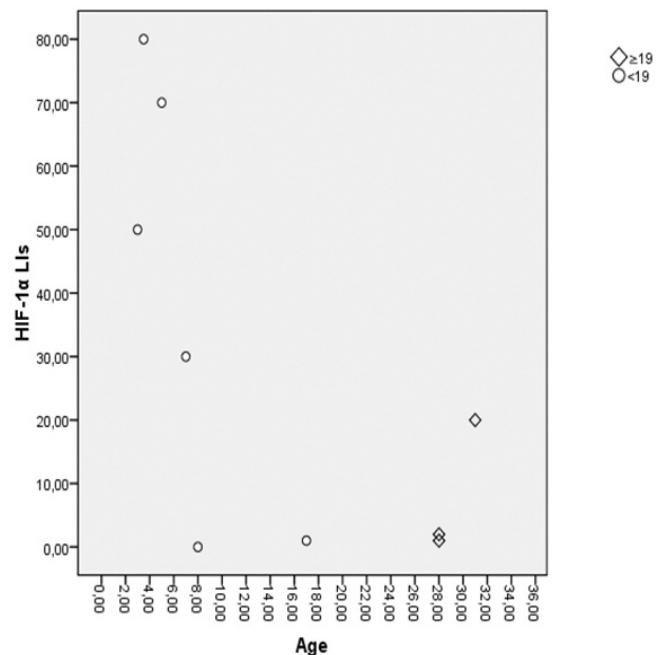

Fig. 6. Scatter plot graph presenting the HIF-1α labeling indices (LIs), %, for medulloblastomas, in relation to the age of patients. ≥19 years, rhombus; <19 years, circle.

Furthermore, the same as for the gliomas, HIF-1α is associated with worse prognosis and more aggressive phenotype (Jensen and Lee, 2012; Reszec et al., 2013b) in meningiomas. Consequently, the increased levels of HIF-1α in both tumors, which are different in their origin and biologic behavior, indicate that apart from hypoxia, a possible oncogenic mechanism, implicated in the HIF-1α activation triggers the malignant progression of these tumors.

Interestingly, the majority (60%) of HIF-1α immunopositive medulloblastomas was from patients <19 years old. Furthermore, in this group of patients,

HIF-1α expression seems to be negatively correlated with age (p<0.05). Medulloblastomas, the most common brain tumors of childhood, are thought to arise from undifferentiated neural stem cells in the cerebellum (Manoranjan et al., 2012). Recent data indicate a hypoxic microenvironment, through HIF-1α, as a key regulator in preservation of cancer stem cells' viability and expansion, in medulloblastomas (Pistollato et al., 2010). Besides, HIF-1α is involved in mechanisms controlling brain development (Mutoh et al., 2012). These data lend support to the hypothesis that HIF-1α immunopositive cells involved in the development of
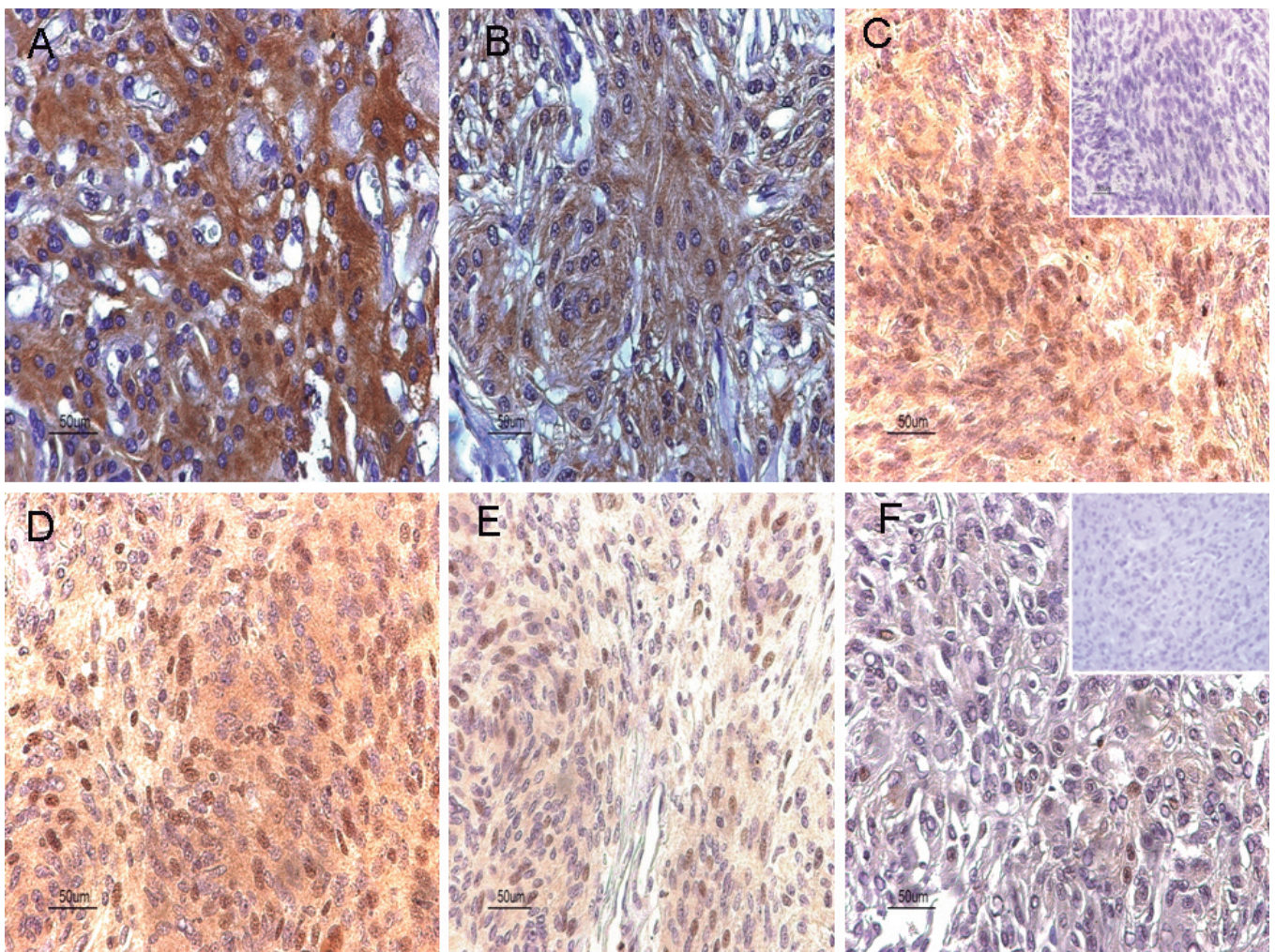

Fig. 7. Expression of HIF-1α, pVHL, and Hsp90 in meningiomas. **A, B.** The 2 adjacent photomicrographs are from homologous fields of adjacent sections of the same meningioma demonstrating strong cytoplasmic Hsp90 (LI=60) **(A)**, and pVHL (LI=80) **(B)** immunoexpression. **C-F.** Panel comparing HIF-1α immunostaining among different cases of meningiomas. **C.** A meningioma sample with abundant nuclear and cytoplasmic HIF-1α immunoexpression (LI=70). This case has a labeling index (LI) of 90% for Hsp90 and 0% for pVHL immunoexpression (not shown). Immunostaining is absent in sections incubated with control IgG (**C**, insert). **D, E.** The 2 adjacent photomicrographs are from different areas of the same meningioma, highlighting the intratumoral staining heterogeneity. This case has a labeling index (LI) of 60% for HIF-1α. The LIs for both Hsp90 and pVHL immunoexpression is 0% (not shown). **F.** Scattered tumor cells display strong HIF-1α nuclear immunoreactivity in this meningioma (LI=1). This case has a pVHL LI of 80% and Hsp90 LI of 80% (not shown). Immunostaining is absent in sections incubated with control IgG (**F**, insert). Dako Envision Plus detection System with hematoxylin counterstain. Scale bar: 50 μm.

cerebellum maintain their stem properties in later stages of embryogenesis under specific conditions of microenvironment. Furthermore, correlation analysis revealed a statistically significant relationship between HIF-1α and Hsp90 immunoexpression (p<0.05), indicating a possible mechanism for HIF-1α stabilization and upregulation through molecular chaperone activity of Hsp90 in medulloblastomas. Our recent findings in pterygium, which is a stem cell disorder, present also co-expression of HIF-1α and Hsp90 (Pagoulatos et al., 2014). However, medulloblastomas with no or very low HIF-1α immunoreactivity were also detected, the biological behavior of which needs to be further clarified.

Cytoplasmic localization of pVHL was detected in a high percentage of brain tumors included in this study. Sun et al. (2006), have demonstrated that engineered overexpression of VHL by C6 gliomas downregulates the expression of HIF-1α and VEGF, which consequently leads to delayed tumorigenesis. Furthermore, recent data show that activated pVHL results in the inhibition of glioma invasion (Chen et al., 2012) and self-renewal ability (Kanno et al., 2013). Thus, it is logical to speculate that pVHL immuno-positive brain tumors might have a better prognosis. However, in astrocytomas (WHO grades II-IV) a significant relationship of pVHL immunoexpression with Hsp90 immunoexpression (Fig. 3), and not with HIF-1α, was found. Recent knowledge provides evidence of involvement of chaperones, specifically of Hsp90, in reduction of pVHL function due to rapid degradation (Yang et al., 2013) so the above results may indicate that Hsp90 plays a critical role in the stabilization of HIF-1α in astrocytomas (WHO grades II-IV) through binding to the pVHL. In contrast, increased expression for both HIF-1α and pVHL was noticed in pilocytic astrocytomas (WHO grade I) (Table 2), although the number of pilocytic astrocytomas was very low (n=3), in this study, and further experiments with larger series of pilocytic astrocytomas are necessary. Notably, some tumors (gliomas, meningiomas, medulloblastomas) were pVHL immuno-negative but HIF-1α and/or Hsp90 immunopositive. Isaacs et al. (2002) used renal carcinoma cell (RCC) lines that lack functional VHL and express stable HIF-1 protein under normoxia and showed that disruption of Hsp90 function: 1) promotes HIF-1 degradation via a novel, oxygen-independent E3 ubiquitin ligase, and 2) diminishes HIF-1 transcriptional activity. Functional studies would provide the therapeutic utility of Hsp90 inhibitors in these pVHL immunonegative/HIF-1α-Hsp90 immunopositive brain tumors.

In summary, in astrocytic tumors, Hsp90 seems to be implicated in HIF-1α activation, through binding to pVHL. On the other hand, in medulloblastomas, HIF-1α is probably regulated by Hsp90, independently from pVHL. As a key-molecule induced under specific conditions in a fraction of cells, the HIF-1α attenuates multiple signaling pathways controlling self-renewal

ability, survival, altered energy metabolism, invasion, and angiogenesis which may lead to tumor growth (Heddleston et al., 2010; Mathieu et al., 2011; Semenza, 2013a,b). The decoding of interactions in which HIF-1α participates will provide further knowledge for the biology and individualized therapeutic approach of human brain tumors.

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