

Specific immunohistochemical pattern of carbonic anhydrase IX is helpful for the diagnosis of CNS hemangioblastoma

Tina Schaller^a, Markus Bode^b, Ansgar Berlis^c, Michael C. Frühwald^d,
Ines Lichtmanegger^a, Katharina Endhardt^a, Bruno Märkl^{a,*}

^a Institute of Pathology, Klinikum Augsburg, Augsburg, Germany

^b Department of Neurosurgery, Klinikum Augsburg, Augsburg, Germany

^c Clinic of Radiology and Neuroradiology, Klinikum Augsburg, Augsburg, Germany

^d Swabian Children's Cancer Centre, Klinikum Augsburg, Augsburg, Germany

Introduction

Hemangioblastomas are vascular neoplasms of the CNS with a characteristic lipid-rich stromal cell component. These tumors are mainly located in the posterior fossa. However, they also occur in the spinal cord and very rarely in the cerebral hemispheres. Hemangioblastomas arise not only as solitary and sporadic tumors, but also in the setting of von Hippel–Lindau (VHL) syndrome [2,17]. Among other lesions, VHL syndrome includes hemangioblastomas, cystic lesions of liver, kidney and pancreas, renal cell carcinomas and pheochromocytomas [13]. Syndromal cases show a mutational inactivation of the *VHL* gene located in chromosome 3p25–26. The VHL protein (pVHL) interacts with hypoxia-inducible factor (HIF), inducing its rapid degradation. Under hypoxic conditions or when pVHL is functionally inhibited, the HIF is stabilized with

expression of hypoxia-induced genes such as vascular endothelial growth factor (VEGF), which is thought to be responsible for the vascular component of hemangioblastomas. Moreover, inactivated pVHL causes overexpression of transmembrane carbonic anhydrases including CA IX. The *VHL* gene is also affected in sporadic hemangioblastomas [10].

VHL is also associated with the oncogenesis of renal clear cell carcinoma [27]. Although most renal clear cell carcinomas are sporadic and not related to von Hippel–Lindau disease, deletion of 3p, the locus for *VHL*, is found in the vast majority of cases. Moreover, renal clear cell carcinomas show remarkable histological similarities with hemangioblastoma. The expression of the hypoxia-associated antigen carbonic anhydrase IX in renal clear cell carcinoma is well known and is used to separate it from other renal cancer subtypes in the differential diagnosis [11]. Because of its usefulness in subtyping renal cancers [4,25], the diagnostic antibody belongs to the routine panel in many pathology laboratories. The morphological and genetic similarities between renal clear cell cancers and hemangioblastomas suggest that carbonic anhydrase IX is expressed in hemangioblastomas as well. This could help establish the diagnosis and distinguish it from other histological and/or

* Corresponding author at: Institute of Pathology, Klinikum Augsburg, Stenglinstrasse 2, 86156 Augsburg, Germany. Tel.: +49 821 4003199; fax: +49 821 400173199.

E-mail address: Bruno.Maerkl@klinikum-augsburg.de (B. Märkl).

Table 1
Clinicopathological data.

	Hemangioblastoma group, n = 20	Control group, n = 46	P-value
Mean age \pm SD	47 \pm 17	52 \pm 26	0.390
Pediatric (age \leq 18)	2	10	0.319
Gender (m:f)	1:1	1:0.92	0.916
Cerebellar location	15	11	<0.001

SD, standard deviation.

radiological mimickers of hemangioblastoma. Besides metastatic clear cell carcinomas of different origins, other mimickers include clear cell and microcystic meningiomas and pilocytic astrocytomas. The latter, in particular, often shows both radiological and histomorphological similarities.

The aim of this retrospective immunohistochemical analysis was to evaluate the expression of carbonic anhydrase IX and its value in the diagnosis and differential diagnosis of hemangioblastomas.

Material and methods

Case collection

The records of the Augsburg Institute of Pathology were screened for hemangioblastomas in the years between 1999 and 2014. In cases with available paraffin blocks, immunohistochemical staining for carbonic anhydrase IX was performed, except in cases for the years 2013–2014 in which this marker already was part of our routine panel in suspicious cases. Keratin staining was performed if not done during the primary evaluation.

The same procedure took place for clear cell and microcystic meningiomas. Because of their considerably higher incidence, pilocytic astrocytomas and gliomas (grades II–IV) were collected only from 2008 to 2014. Metastatic renal clear cell carcinoma cases from the period 2009 to 2014 were included because of their particular differential diagnostic relevance. An angiomatous fibrous histiocytoma and a pleomorphic xanthoastrocytoma were included because of their radiological presentation suspicious for hemangioblastomas.

In addition, cases were also included if carbonic anhydrase IX already had been used for differential diagnostic considerations during routine evaluation of CNS lesions.

Clinical follow-up data and information concerning possible underlying von Hippel–Lindau disease were obtained from the clinical files.

The study has been performed according to the national rules.

Histological examination and immunohistochemistry

All slides were independently re-evaluated by two examiners (T.S. and B.M.). In cases in which immunohistochemical staining was necessary, 3–5 μ m thin sections were cut. All reactions were performed using a Ventana Benchmark Ultra system (Roche Diagnostics, Mannheim, Germany). Diagnostic antibodies against carbonic anhydrase IX (Novocastra, Clone TH-22, 1:100) and cytokeratin (MNF-116, 1:100) were provided by DCS, Hamburg, Germany and Dako, Hamburg, Germany, respectively. All reactions were developed using the Ventana Ultravision detection system (Roche Diagnostics, Mannheim, Germany). Keratin staining was only performed in cases in which no cytokeratin-stained slides were available from the archive.

Only strong and diffuse staining with definitive membranous accentuation of carbonic anhydrase IX was considered positive. An additional negative staining for keratin was deemed diagnostic for hemangioblastomas (CAIXmem-pos/KERneg).

Statistics

The unpaired *T*-test was used to compare normal distribution parameters of two groups. If the normal distribution was not given, then a Mann–Whitney rank sum test was performed instead. Depending on the sample number, the χ^2 test or Fisher's exact test was used to compare dichotomous data. Diagnostic sensitivity, specificity and the positive and negative predictive values were calculated for the diagnostic combination CAIXmem-pos/KERneg.

A *P*-value of 0.05 was considered significant. All calculations were performed using the Sigma Plot 11.0 software package (Systat, Richmond, VA, USA).

Results

Case collection

The clinicopathological data are summarized in Table 1. A total of 20 hemangioblastomas in 19 patients were identified in our files. One case was supratentorial in location, 16 cases were cerebellar lesions and three tumors were found in the spinal cord. The von Hippel–Lindau disease was confirmed in three cases (Table 2).

A collection of 46 cases served as the control. This collection consists of 12 pilocytic astrocytomas, seven gliomas (grades II–IV), 11 meningiomas, one pleomorphic xanthoastrocytoma, one angiomatous fibrous histiocytoma and 14 carcinoma metastases (including eight cases outside the CNS) (Table 3).

Expression of carbonic anhydrase IX

Hemangioblastomas

Nineteen out of 20 (95%) hemangioblastomas showed the typical immunohistochemical pattern of strong and diffuse expression with definitive membranous accentuation throughout the tumor (Fig. 1c and d). Only one single case expressed carbonic anhydrase IX somewhat weaker but still in a distinct manner in all tumor cells. Nuclear staining varied from case to case and within the tumors.

Pilocytic astrocytomas

Three out of 12 (25%) pilocytic astrocytomas showed a moderate-to-strong expression of carbonic anhydrase IX. This expression, however, did not show membranous accentuation and was often more granular and patchy with broad areas of completely negative reaction (Fig. 2b). In nine cases, no expression at all was found.

Gliomas (grades II–IV)

Two diffuse and one anaplastic astrocytoma did not show any expression of carbonic anhydrase IX. In another grade III glioma, a patchy staining with membranous accentuation especially at biopsy margins was found. However, broad areas reacted completely negative. Strong positive staining was readily observed in all three glioblastoma cases. Nevertheless, this staining was restricted to parts of the samples. Mainly, areas in neighborhood of vessels and necrosis showed positive expression, while coherent highly cellular areas were spared out (Fig. 2j–l).

Table 2
Cases of the hemangioblastoma group.

Case no.	Gender	Age	Localization	von Hippel–Lindau disease	CA IX staining intensity	CA IX staining pattern	Keratin staining
HB1	F	40	Cerebellar right	No	Strong	Diffus; memb. acc.	Negative
HB2	M	27	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB3	M	24	Cerebellar left	No	Strong	Diffus; memb. acc.	Negative
HB4	M	57	Cerebellar right	No	Strong	Diffus; memb. acc.	Negative
HB5	M	34	T12 spinal	No	Strong	Diffus; memb. acc.	Moderate; focal MNF116
HB6	F	67	Cerebellar left and C7/T1	Yes	Moderate to strong	Diffus; memb. acc.	Negative
HB7	M	60	Posterior fossa	Yes	Strong	Diffus; memb. acc.	Negative
HB8	F	56	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB9	F	18	Sphenoid ridge	No	Strong	Diffus; memb. acc.	Negative
HB10	F	72	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB11		61	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB12	F	73	C4/5 spinal	No	Strong	Diffus; memb. acc.	Negative
HB13	M	62	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB14	M	37	Posterior fossa	No	Strong	Diffus; memb. acc.	Negative
HB15	F	38	T2 spinal	No	Strong	Diffus; memb. acc.	Negative
HB16	M	46	Supratentorial frontal	No	Strong	Diffus; memb. acc.	Negative
HB17	F	48	Cerebellar right	No	Strong	Diffus; memb. acc.	Negative
HB18	F	16	Cerebellar right	Yes	Strong	Diffus; memb. acc.	Negative
HB19	F	49	Cerebellar right	No	Strong	Diffus; memb. acc.	Negative
HB20	M	50	Cerebellar right	No	Strong	Diffus; memb. acc.	Negative

CA IX, carbonic anhydrase IX.

Table 3
Cases of the control group with CNS involvement.

Case no.	Gender	Age	Diagnosis	Localization	CA IX staining intensity	CA IX staining pattern	Keratin staining
nHB1	f	10	Pilocytic astrocytoma	Optical nerve	Strong	Focal; nuclear and processes	Negative
nHB2	m	5	Pilocytic astrocytoma	Posterior fossa	Negative		Negative
nHB3	m	3	Pilocytic astrocytoma	Cerebellar left	Negative		Negative
nHB4	m	11	Pilocytic astrocytoma	Optical nerve	Negative		Negative
nHB5	f	15	Pilocytic astrocytoma	Cerebellar	Negative		Negative
nHB6	f	60	Pilocytic astrocytoma	Basal ganglia	Negative		Negative
nHB7	f	73	Pilocytic astrocytoma	Optical nerve	Negative		Negative
nHB8	m	9	Pilocytic astrocytoma	Cerebellar	Negative		Negative
nHB9	f	65	Pilocytic astrocytoma	T11/12 spinal	Moderate	Focal; matrix	Negative
nHB10	m	5	Pilocytic astrocytoma	Cerebellar	Negative		Negative
nHB11	m	16	Pilocytic astrocytoma	Cerebellar right	Strong	Focal; processes	Negative
nHB12	f	8	Pilocytic astrocytoma	Cerebellar right	Moderate	Diffus, no memb. acc.	Negative
nHB13	m	68	Angiomatous meningioma	Temporal right	Moderate to strong	Mainly vascular structures	Negative
nHB14	m	80	Angiomatous meningioma	Parietal right	Moderate	Focal	Negative
nHB15	f	77	Clear cell meningioma	Frontal right	Negative		Negative
nHB16	f	43	Clear cell meningioma	Temporobasal left	Negative		Negative
nHB17	f	53	Clear cell meningioma	Convexity	Negative		Negative
nHB18	f	77	Meningioma transitional type	Parietal right	Negative		Negative
nHB19	f	47	Microcystic meningioma	Falx cerebri	Moderate	Diffus; no clear memb. acc.	Negative
nHB20	m	55	Microcystic meningioma	Frontobasal right	Moderate	Focal; no clear memb. acc.	Negative
nHB21	f	82	Microcystic meningioma	Parasagittal left	Weak	Very focal at the tissue margin	Negative
nHB22	m	77	Microcystic meningioma	Frontal right	Weak	Very focal, matrix	Negative
nHB23	f	52	Microcystic meningioma	Convexity	Strong	Diffus; no clear memb. acc.	Negative
nHB24	f	74	Metastasis CCRC	Frontal right	Strong	Diffus; clear memb. acc.	Strong CK18
nHB25	f	76	Metastasis CCRC	Parieto-occipital	Strong	Diffus; clear memb. acc.	Strong CK18
nHB26	m	74	Metastasis CCRC	Parietal	Strong	Diffus; clear memb. acc.	Strong CK18
nHB27	m	62	Metastasis NSCLC	Cerebellar right	Moderate	Very focal, stromal, cytoplasmic, membranous	Strong CK7
nHB28	m	60	Metastasis NSCLC	Infra- and supratentorial	Moderate	Nuclear	Strong CK7
nHB29	f	71	Metastasis thyroid carcinoma	intraspinal	Strong	Nuclear	Strong CK7
nHB30	f	32	Pleomorphic xanthoastrocytoma	Temporoparietal	Strong	Very focal at the tissue margin	Negative
nHB31	m	13	Angiomatous fibrous histiocytoma	Cerebellar right	Negative		Negative
nHB40	m	34	Diffuse astrocytoma	Brain stem			Negative
nHB41	w	77	Diffuse astrocytoma	Frontotemporal left			Negative
nHB42	w	26	Anaplastic astrocytoma	Temporal left			Negative
nHB43	w	55	Anaplastic astrocytoma	Occipital left			Negative
nHB44	m	60	Glioblastoma	Frontal left			Negative
nHB45	m	59	Glioblastoma	Frontal left			Negative
nHB46	m	74	Glioblastoma	Right hemisphere			Negative

CCRC, clear cell renal carcinoma; NSCLC, non-small cell lung cancer; CA IX, carbonic anhydrase IX; CK, cytokeratin; memb. acc., membranous accentuation.

Note: The cases of metastasis CCRC outside the CNS are not listed here.

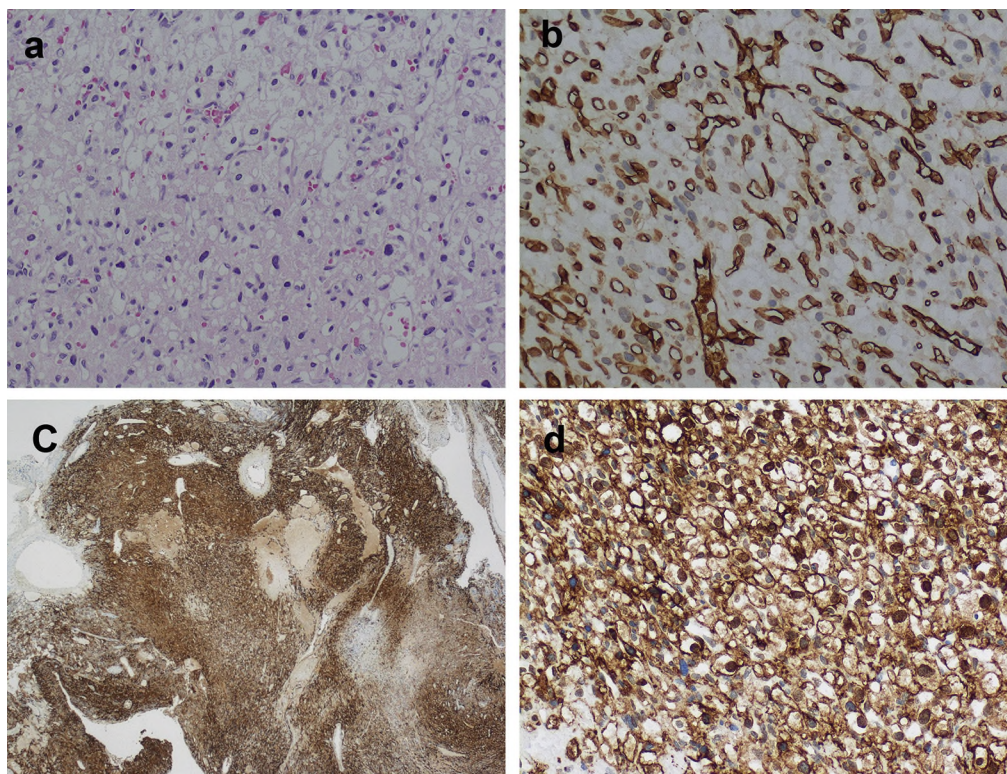


Fig. 1. Typical histological picture of a hemangioblastoma with many capillaries and prominent stromal cells with clear cytoplasm and considerable nuclear pleomorphism (case HB1; original magnification: $\times 200$, H&E stain) (a). The very dense vascular network is highlighted by an endothelial marker (case HB1; original magnification: $\times 200$, CD31) (b). Carbonic anhydrase IX staining with a very strong and diffuse reaction with a clear membranous accentuation throughout the tumor. This pattern was considered as positive for hemangioblastoma (case HB1; original magnifications: $\times 50$ and $\times 200$) (c and d).

Meningiomas

The control group included two angiomatous, three clear cell, five microcystic and one transitional meningioma.

Seven out of 11 (63%) meningiomas showed a weak (two), moderate (three) or strong (two) positivity for carbonic anhydrase IX. This expression, however, was found only focally or in a patchy manner in five cases. Only two cases showed a diffuse expression. Importantly, none of these tumors showed the definitive membranous accentuation that is typically seen in hemangioblastomas (Fig. 2d). The remaining four cases were consistently negative for carbonic anhydrase IX. The meningioma subtypes did not differ with regard to their expression pattern of carbonic anhydrase IX.

Carcinoma metastases

All 14 metastases expressed carbonic anhydrase IX. However, as expected, diffuse and strong expression with membranous accentuation was exclusively appreciable in the renal clear cell carcinoma (Fig. 2h). One non-small cell lung cancer (adenocarcinoma) metastasis showed a very focal and strong membranous and cytoplasmic expression. Another adenocarcinoma of the lung showed a very distinct nuclear reaction. The same was appreciated in a metastasis of a follicular thyroid carcinoma (Fig. 2f).

Other tumors

An angiomatous fibrous histiocytoma, initially misdiagnosed as hemangioblastoma, showed no expression of carbonic anhydrase IX. A focal circumscription and a strong expression were seen in a pleomorphic xanthoastrocytoma. The remaining neoplastic tissue reacted completely negative.

Expression of keratin markers

Hemangioblastomas

One out of 20 hemangioblastoma cases showed a weak-to-moderate expression of pan-cytokeratin (MNF-116) in about 30% of the tumor. There was no clinical evidence for an underlying carcinoma, especially not renal cancer. PAX2 and PAX8 as renal cancer markers were not expressed.

Control cases

None of the 32 primary CNS tumors showed expression of keratin. As expected, the four carcinoma metastases were strongly positive for keratin (cytokeratin 7 or 18 or MNF-116) (Fig. 2i).

Diagnostic value

We defined the immunohistochemical pattern of a strong, diffuse expression of carbonic anhydrase IX with membranous accentuation in combination with keratin negativity as diagnostic for hemangioblastomas. This pattern was found in 18 out of the 20 hemangioblastoma cases and in none of the 46 control cases ($P < 0.001$). All other possible phenotypic expression patterns were considered negative. Using this definition, we calculated a sensitivity of 90% and a specificity of 100%. The positive and negative predictive values were 100% and 96%, respectively.

Discussion

Hemangioblastomas are uncommon WHO grade I tumors of the central nervous system [2]. Most of these are located in the posterior fossa. Only a very small proportion occurs outside the CNS [30]. The tumors consist of a prominent vascular and

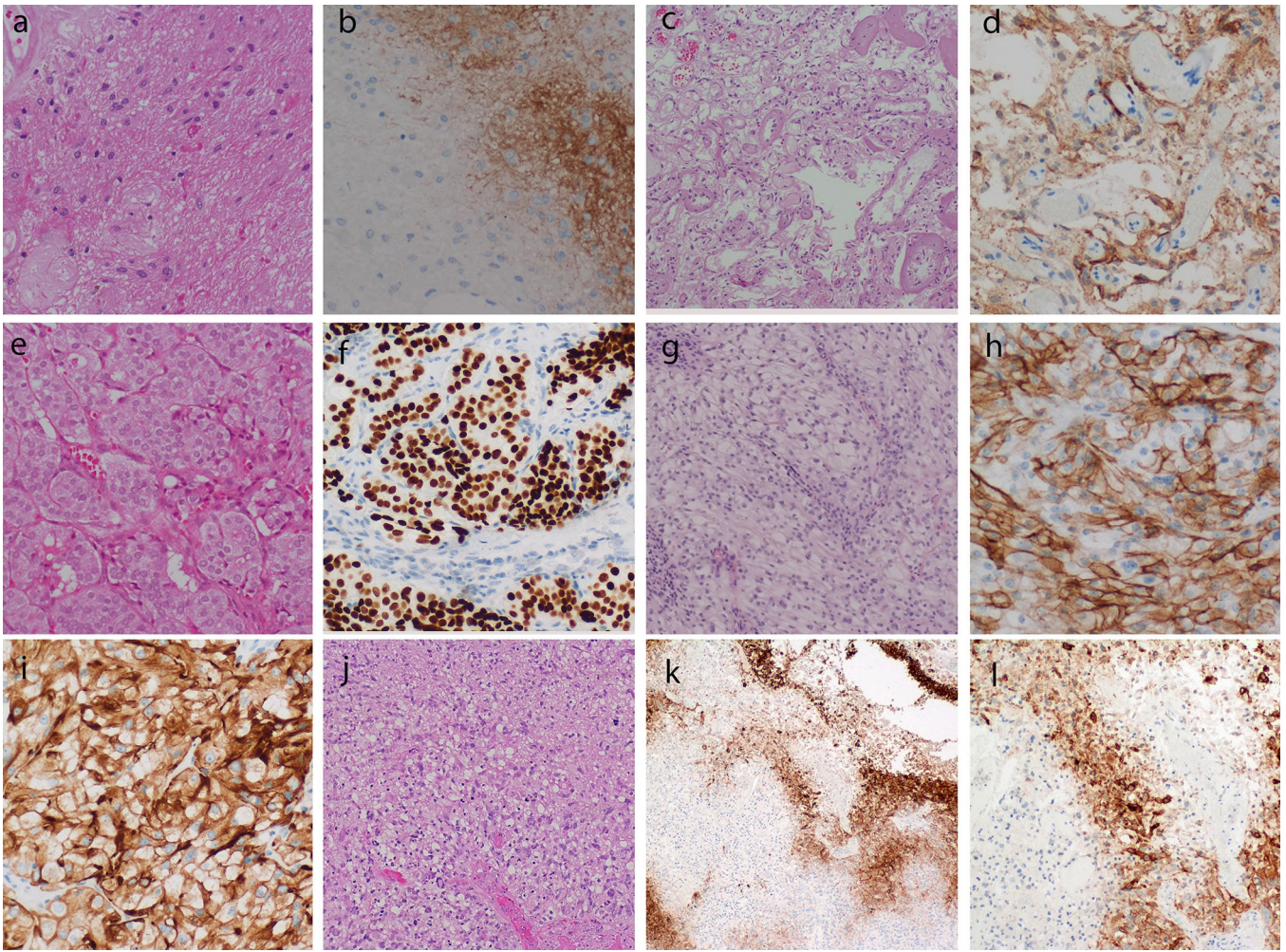


Fig. 2. Pilocytic astrocytoma with moderate cellularity, prominent vessels, some Rosenthal fibers and eosinophilic granular bodies (case nHB9; original magnification: $\times 200$, H&E stain) (a). Carbonic anhydrase IX expression in this case was patchy and without membranous accentuation (case nHB9; original magnification: $\times 200$) (b). Angiomatous meningioma with a predominance of thin- and thick-walled vessels of different sizes (case nHB13; original magnification: $\times 100$; H&E stain) (c). Moderate-to-strong carbonic anhydrase IX expression is mainly restricted to the vascular structures (case nHB13; original magnification: $\times 200$) (d). Metastases of a follicular thyroid carcinoma inhere with insular pattern, eosinophilic cytoplasm and moderate nuclear atypia (case nHB27; original magnification: $\times 200$, H&E stain) (e). The tumor expressed carbonic anhydrase IX with strong intensity but opposite to hemangioblastoma, not membranous but nuclear (case nHB29; original magnification: $\times 200$) (f). Metastasis of typical clear cell renal carcinoma Fuhrman grade 2 (case nHB24; original magnification: $\times 200$; H&E stain) (g). The tumor expressed carbonic anhydrase IX in a diffuse strong manner throughout with membranous accentuation. Similar to the expression pattern, which is typically found in hemangioblastomas (case nHB24; original magnification: $\times 200$) (h). Cytokeratin 18 is coexpressed in strong intensity which discriminates it from hemangioblastomas (case nHB24; original magnification: $\times 200$) (i). Glioblastoma with high cellularity, high-grade atypia and atypical vascular proliferations (case nHB45; original magnification: $\times 100$, H&E stain) (j). The tumor expressed carbonic anhydrase IX in a patchy manner mainly in perivascular and perinecrotic areas (original magnifications: $\times 40$ and $\times 100$) (k and l).

stromal component. The latter is considered to be the true neoplastic part, while the often impressive vascular proliferation is very likely caused by the overexpression of vascular endothelial growth factor of the stromal cells and therefore is only an epiphenomenon [14,17]. The histogenesis of these neoplastic stromal cells still remains unclear. However, data from more recent investigations suggest embryonic progenitor cells as a possible origin [5,12,16]. Meanwhile, the underlying genetic alterations—at least in cases associated with von Hippel–Lindau disease—are well known. Germline mutations of the *VHL* gene in the short arm of chromosome 3 induce different manifestations of the von Hippel–Lindau disease including syndromal hemangioblastomas. Moreover, Muscarella et al. [15] recently described somatic mutations of *VHL* in 52% of the sporadic cases. The authors concluded, therefore, that mutations of *VHL* play a key role in the tumourigenesis of not only syndromal but also sporadic hemangioblastomas. As mentioned before, mutation of *VHL* is found in the vast majority of

clear cell renal cancer cases. Consequently, it is very likely that a marker-like carbonic anhydrase IX, which is overexpressed due to stabilization of hypoxia-inducible factor caused by *VHL* inactivation, will play a diagnostic role in both entities. The usefulness of carbonic anhydrase IX in renal cancer has been thoroughly investigated [4]. Recently, Luong-Player *et al.* [11] published data of a tissue microarray study enrolling samples from 1551 specimens. They detected overexpression of carbonic anhydrase IX in 88% of clear cell renal carcinomas and demonstrated its usefulness in discriminating clear cell carcinomas from other renal or extrarenal tumor entities. These similarities between renal clear cell carcinoma and hemangioblastoma seem to further suggest that carbonic anhydrase IX is also overexpressed in hemangioblastoma. There are only a few studies that have reported expression of carbonic anhydrase IX in brain tumors, especially in hemangioblastomas [14,18]. In our study, we found expression in all 20 (100%) cases of the hemangioblastoma group. It has to be emphasized that, with the

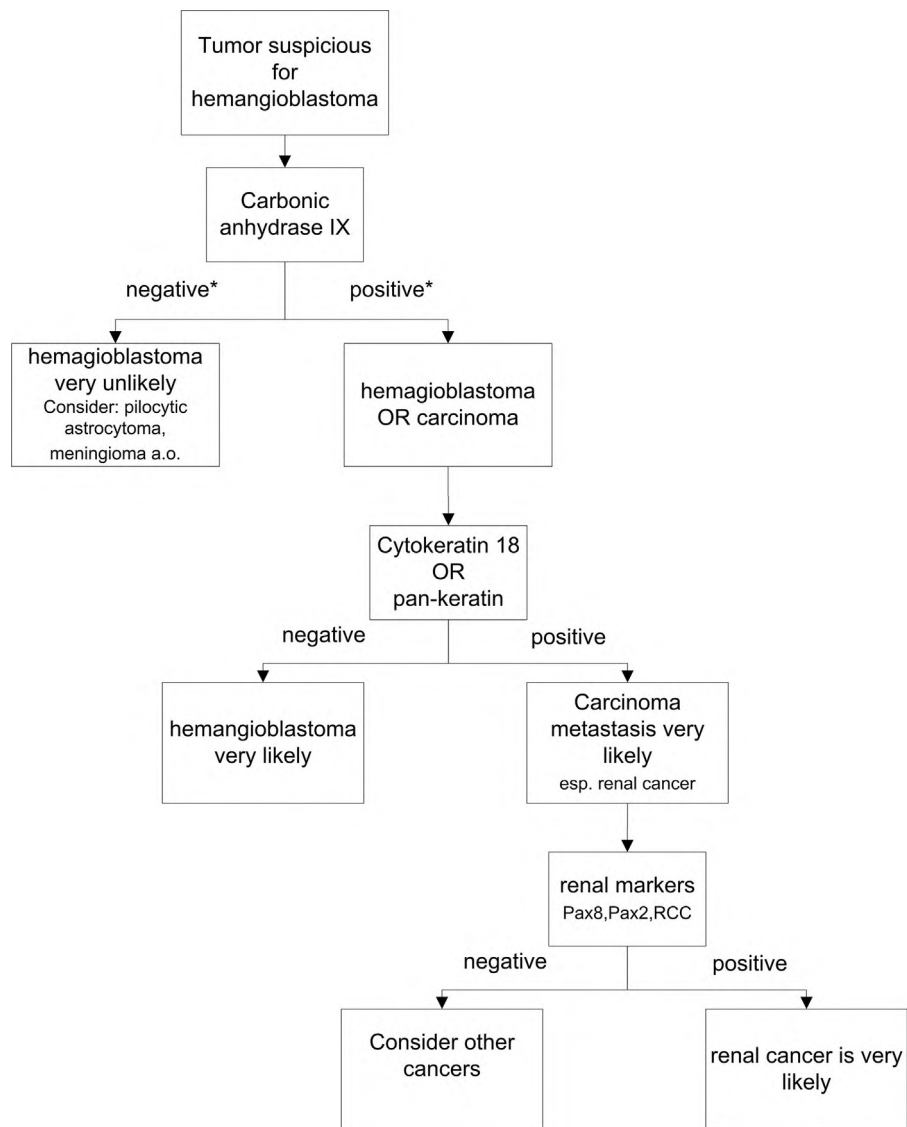


Fig. 3. Recommended workflow for CNS lesions suspicious for hemangioblastoma.

exception of one single case, this expression was always strong with definitive membranous accentuation throughout the tumor tissue (Fig. 1c and d). This stands in concordance with the published literature about renal cancers and hemangioblastomas.

Several further markers have been suggested as being useful for the diagnosis and differential diagnosis of hemangioblastomas. In this context, it seems reasonable to differentiate between markers that are present in hemangioblastomas and markers that are present in entities relevant to the differential diagnosis. Inhibin- α , D2-40, aquaporin-1 and brachyury are expressed in hemangioblastomas [3,7,20,21,29]. Brachyury, a very recently published marker, might be the most promising one. It shows high specificity, sensitivity and positive and negative predictive values in hemangioblastomas [3]. Assuming that the promising data of this single study can be confirmed, it would enable pathologists to establish the diagnosis of hemangioblastoma and to rule out other entities with a single antibody. Moreover, it is an excellent marker for the diagnosis of chordomas [28]. In contrast, this antibody to our knowledge is not in widespread usage in diagnostic pathology yet. The same is true for aquaporin-1 [29]. The data about inhibin- α and D2-40 are conflicting. While the initial reports showed promising

results, other studies were not able to confirm them [7,20,21,29]. The differentiation of metastatic renal cell carcinomas from primary CNS tumors is investigated in many studies and summarized in several recently published reviews [20,22,25,26]. Frequently used markers in renal neoplasms are CD10, Vimentin, Pax2, Pax8 and RCC. Cytokeratins seem especially helpful when tumors located in the CNS are under consideration. Cytokeratin 18, a marker for simple epithelia, is reported to be positive in virtually all renal carcinomas [23]. Of note, the broad-spectrum antibody AE1/AE3 lacks sensitivity for cytokeratin 18.

Pilocytic astrocytomas lack expression of specific immunohistochemical markers to confirm the diagnosis, which is based on morphological criteria. Molecular investigations can close this gap in the majority of cases. BRAF-KIAA fusions are specific for this entity and are found in 70% of the cases. Cerebellar tumors show an even higher frequency [8,19].

IDH-1 and -2 mutations are found in high frequency in grades II and III and secondary glioblastomas (70–75%). Primary glioblastomas, however, only rarely show these genetic alterations [9]. Especially cellular hemangioblastomas show morphological similarities with glioblastomas [6]. Strong carbonic anhydrase IX

staining was found in all three cases included in our control group. However, this positivity is mainly restricted to perivascular and perinecrotic areas. Identical findings were reported by Proescholdt et al. [18]. Therefore, diagnostic problems can arise when only very small bioptic samples are available.

Meningiomas are known to express EMA, claudin-1 and the progesterone receptor [24]. Very recently, Agaimy et al. [1] analysed a five-marker panel (SSTR2, PR, EMA, Claudin-1 and GLUT-1) in 68 meningiomas in comparison to soft tissue perineuromas. Coexpression of progesterone receptor and somatostatin receptor 2 was found to be a specific phenotype in 70% of the meningiomas. The other markers were considered to have either low specificity or low sensitivity.

In this retrospective study, we focused on the immunohistochemical expression of carbonic anhydrase IX in hemangioblastoma and its mimickers. Therefore, except keratin staining, all other relevant staining was performed during the initial routine evaluation. In cases in which no keratin-stained slides were available, MNF116 staining (pan-cytokeratin) was performed. As mentioned before, the question of how to distinguish renal neoplasms from hemangioblastomas has been addressed by several authors. Because carbonic anhydrase IX is expressed in 90% of the clear cell renal carcinomas [4,11] in the same manner as in hemangioblastoma, this antibody can solve the diagnostic problem only in combination with a second immunohistochemical marker. In our study, we included 11 metastatic clear cell renal carcinomas. In these cases, coexpression of carbonic anhydrase IX and cytokeratin 18 was appreciable as expected (Fig. 2h and i). Based on the findings of this study and the published literature, we recommend this panel for the diagnosis of CNS tumors suspicious for hemangioblastoma (Fig. 3).

Carbonic anhydrase IX has gained importance in subtyping renal neoplasms [11,25] and is therefore already part of the routine antibody panel in many pathology departments. This seems to be favorable in comparison to other hemangioblastoma markers mentioned before. In combination with cytokeratin markers, we determined an excellent sensitivity and specificity for the diagnosis of hemangioblastomas. Keeping in mind the limitations of a single non-consultant center study with retrospective design, these very promising results will need to be confirmed by a larger investigation.

Disclosure/conflict of interest

The authors declare no conflicts of interest.

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