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REGULAR PAPER

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D2-40, a novel monoclonal antibody against the M2A antigen as a marker to distinguish hemangioblastomas from renal cell carcinomas

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Abstract Hemangioblastomas (HB) are characterized by the presence of vacuolated tumor cells resembling the tumor cells seen in clear cell renal cell carcinomas (CRCC). The distinction between HB and metastatic CRCC in the brain is critical as they have different therapeutic and prognostic ramifications. The issue is further complicated by the possibility of both HB and metastatic CRCC in brains of patients with Von Hippel Lindau (VHL) disease. We studied the expression of a novel monoclonal antibody D2-40, which recognizes an oncofetal antigen (M2A) in HB and CRCC, by immunohistochemistry. The vacuolated tumor cells in all HB were stained positively with D2-40. Nineteen of 23 (83%) HB showed strong, membranous staining in the vacuolated tumor cells, and 4 of 23 (17%) showed weaker staining. No expression was seen in CRCC, either primary in the kidney (0/20), or metastatic CRCC in the brain (0/8). Three of the patients with HB also had VHL disease, and no difference was seen in D2-40 staining of HB in patients with or without VHL disease. Two of these three VHL disease patients had both primary CRCC and HB resected at our institution. In these two patients, strong D2-40 expression was seen in the HB, but no expression was seen in the CRCC, underlying the utility of this marker in distinguishing HB from CRCC in patients with VHL disease in addition to sporadic cases. In summary, the monoclonal antibody D2-40 is a useful marker to distinguish HB from CRCC.

Keywords D2-40 · Hemangioblastoma · Renal cell carcinoma · Lymphatic endothelial marker · Cerebellum

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Introduction

Hemangioblastomas (HB) are brain tumors of uncertain histogenesis characterized by the presence of vacuolated tumor cells and a highly vascular component. These tumors are most commonly seen in middle-aged men and tend to favor the posterior fossa, particularly the cerebellum. However, they are also seen in the spinal cord and, rarely, in supratentorial locations [9]. While the vascular component is thought to be largely supportive in function, the vacuolated cells that comprise the majority of the tumors have received much attention in the literature. Various studies by immunohistochemistry and electron microscopy have attempted to elucidate the nature of the foamy tumor cells. While no consensus has been reached on the origin of these cells, some studies show that these cells exhibit neuronal markers [10, 12, 14], whereas others have suggested fibrohistiocytic [11] as well as endothelial/pericytic origins [18].

Von Hippel Lindau (VHL) disease is characterized by the presence of tumors in various organs, particularly HB in the brain and clear cell renal cell carcinomas (CRCC) in the kidney [9]. These two tumors often coexist in VHL disease patients and there are many reports of patients with VHL disease who present with metastatic CRCC as well as an HB [9]. Furthermore, metastatic CRCC are often the first manifestation of a renal tumor in these patients. In addition, there are documented cases of metastatic CRCC in an HB [5]. Histologically, there is a similarity between the clear, lipidized cells seen in CRCC and the vacuolated cells seen in HB (compare Fig. 1A and E). Thus a cerebellar mass with vacuolated or clear cells can present a diagnostic challenge for the neuropathologist, especially in patients with VHL disease. The importance of distinction between these two tumors cannot be overstated, as HB have a typically benign course following surgery, while metastatic CRCC in the brain are often treated much more aggressively, and often carry a grim prognosis.

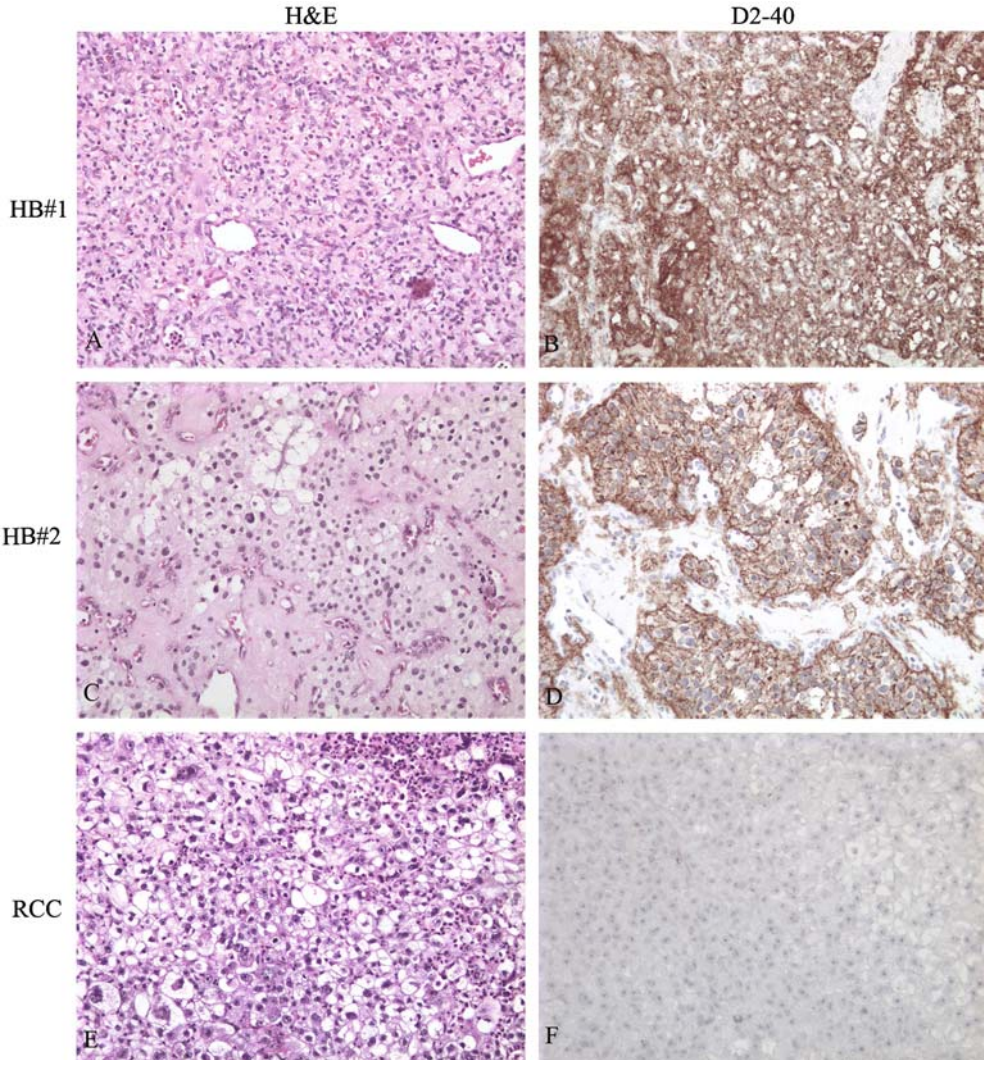

Fig. 1 The monoclonal antibody D2-40 stains HB but not CRCC. **A**–**D** Hematoxylin and eosin and D2-40 staining pattern of HB from two sporadic HB cases as well as a CRCC are shown. Note that D2-40 stains the vacuolated tumor cells but not the vascular endothelial cells within the tumor. **E**, **F** No D2-40 staining is seen in the CRCC (*HB* hemangioblastomas, *CRCC* clear cell renal cell carcinomas). **A**–**F** ×100

D2-40 is a novel monoclonal antibody that was initially raised against a fetal gonadal antigen, the M2A antigen [2]. Subsequently, it was shown that the antigen also recognized germ cell tumors but not adult gonadal tissue [3]. Further studies have shown that the M2A antigen is expressed by certain tumor cell lines including ovarian epithelial carcinomas, but is absent in many other cell lines of extra-gonadal origin [19]. In addition to the above tumors, the M2A antigen is expressed in lymphatic, but not vascular endothelial cells [8, 17]. Biochemical studies have shown that the M2A antigen consists of a 40-kDa sialoglycoprotein with O-linked simple mucin-type carbohydrate structure and this carbohydrate structure is required for the antigenicity of the antibody [19].

We have recently observed D2-40 immunoreactivity in HB. In this study, we evaluated the utility of D2-40 as an immunohistochemical marker to distinguish HB from CRCC. In addition, since little is known about D2-40 expression in brain, we also evaluated D2-40 immunoreactivity in normal brain tissue.

Methods

Paraffin-embedded formalin-fixed tissue was obtained from the archives of the University of Pennsylvania. Most HB were from the cerebellum ($n = 20$), and the rest were from the medulla ($n = 2$) or the cervical spinal cord ($n = 1$). For looking at normal D2-40 staining in the brain, representative sections were taken from the frontal, parietal, occipital lobes, hippocampus, basal ganglia, midbrain, medulla, spinal cord, and cerebellum. Clinical information was obtained from reviewing the patients' charts. Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections. Sections (5 μm thick) were cut and deparaffinized in xylene and rehydrated in graded alcohols. Slides were boiled in 1× EDTA buffer, pH 8.0 (LabVision, Fremont, CA) for 20 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 20 min. Slides were incubated with the D2-40 monoclonal antibody (clone D2-40, 1:25 dilution, Signet

Laboratories, Dedham, MA) for 1 h at room temperature. Immunohistochemical staining was performed on a DAKO Cytomation Autostainer using the EnVision + HRP DAB system (DAKO Cytomaton, Carpinteria, CA), according to the manufacturer's recommendations. D2-40 immunoreactivity was evaluated semiquantitatively based on two parameters: the intensity and the extent of staining. The intensity was graded on a subjective scale of weak and strong, and the extent of staining was defined as the percentage of tumor cells staining positively in any given sample based on a scale of 0–3: 0, < 10% of cells were stained; 1, 11–25% were stained; 2, 26–50% were stained; and 3, > 50% of cells were stained. In addition to HB, various parts of non-neoplastic brain were also evaluated for D2-40 immunostaining.

Recently, inhibin α was described as a useful marker for distinguishing HB from metastatic CRCC [13]. We therefore also stained some of our HB cases with an antibody to inhibin α (cases 10–18, and 22–23, Table 1). We used the same antibody clone R1 (1:60 dilution, DAKO, Carpinteria, CA) used by Hoang and Amirkhan [13]. For inhibin immunostaining, after deparaffinization, the sections were immersed in 1× target retrieval buffer, pH 6.0 (Dako Cytomaton) and steamed for 40 min in a Black and Decker steamer at 95–100°C. The sections were then incubated with primary antibody, and the remainder of the protocol was the same as described above.

Table 1 Table showing clinical features and D2–40 immunohistochemical staining in 23 hemangioblastomas from 22 patients. Note that all cases stained with the D2–40 antibody. The immunoreactivity was graded on a scale of 1+ to 3+ with 3+ representing strong staining intensity, and extent of staining (for a description of the quantitative staining, see results) (*HB* hemangioblastoma, *VHL* von-Hippel Lindau, *CBL* cerebellum)

Case	Age at presentation, sex, location of HB	D2–40 staining of HB
1	47, M, CBL	3+
2	21, M, CBL	3+
3	30, F, CBL	3+
4	42, M, CBL	3+
5	39, M, CBL	3+
6	75, M, CBL	3+
7	56, M, CBL	3+
8	69, F, CBL	3+
9	38, F, CBL	3+
10	41, M, CBL	3+
11	47, M, CBL, VHL	3+
12	27, M, CBL, VHL	3+
13	Second excision of case 12	3+
14	53, M, CBL, VHL	3+
15	65, M, CBL	3+
16	64, F, CBL	3+
17	37, F, CBL	3+
18	64, F, CBL	3+
19	62, M, CBL	2+
20	77, M, Cervical spine	1+
21	51, M, Medulla	1+
22	41, F, Medulla	1+
23	38, F, CBL	1+

Results

Clinical information

Twenty-three HB from 22 patients were evaluated. Most cases were sporadic HB with 3 patients diagnosed with VHL disease. The earliest age at presentation was 21 years and the oldest patient to present was 77 years old. The mean age at presentation was 49 years. The male to female ratio was 1.75:1. Most tumors were located in the cerebellum ($n = 19$, 90%); others were located in the brain stem (medulla) and cervical spinal cord ($n = 3$, 10%). All surgical resections were made with an open craniotomy. Two of the 3 VHL disease patients had both HB and primary CRCC resected at our institution. Eight metastatic and 20 additional primary CRCC were evaluated. All patients with metastatic CRCC were male, and the mean age at presentation was 54 years.

D2-40 immunostaining of HB and CRCC

All HB were stained positively by monoclonal antibody D2-40, but none of the CRCC tested, either primary in the kidney or metastatic to the brain were positive (Fig. 1). No staining was seen in the vascular component of HB, either in the endothelial cells or in the pericytes. In all HB, the vacuolated tumor cells showed membranous staining. Three of the HB patients were also diagnosed with VHL disease. In two of these cases, we had biopsy material from HB as well as primary CRCC resected from the same patient. Whereas D2-40 showed intense staining of the vacuolated cells in the HB from these patients, no staining was seen in their primary CRCC (Fig. 2). Although the staining was strong and uniform in most samples, there was some variability within the tumors in some cases. In all cases, we observed that when the extent of staining was > 50% (Score 3), the staining intensity was also strong. The data shown in Table 1 reflects the extent of staining only.

As shown in Table 1, vacuolated cells in all HB stained positively with D2-40, with 19/23 (83%) of the HB showing strong and uniform staining, whereas no staining was seen in the any of CRCC tested, either primary in the kidney, or metastatic to the brain.

D2-40 immunostaining in normal adult brain

In adult non-neoplastic non-central nervous system (CNS) tissues, the D2-40 antibody stains lymphatic endothelial cells [16]. In our study, no endothelial cell staining was seen in the cerebral vessels, confirming the lack of true lymphatics in the brain. We also looked at the expression of D2-40 in the adult non-neoplastic CNS. We saw strong D2-40 staining in the choroid

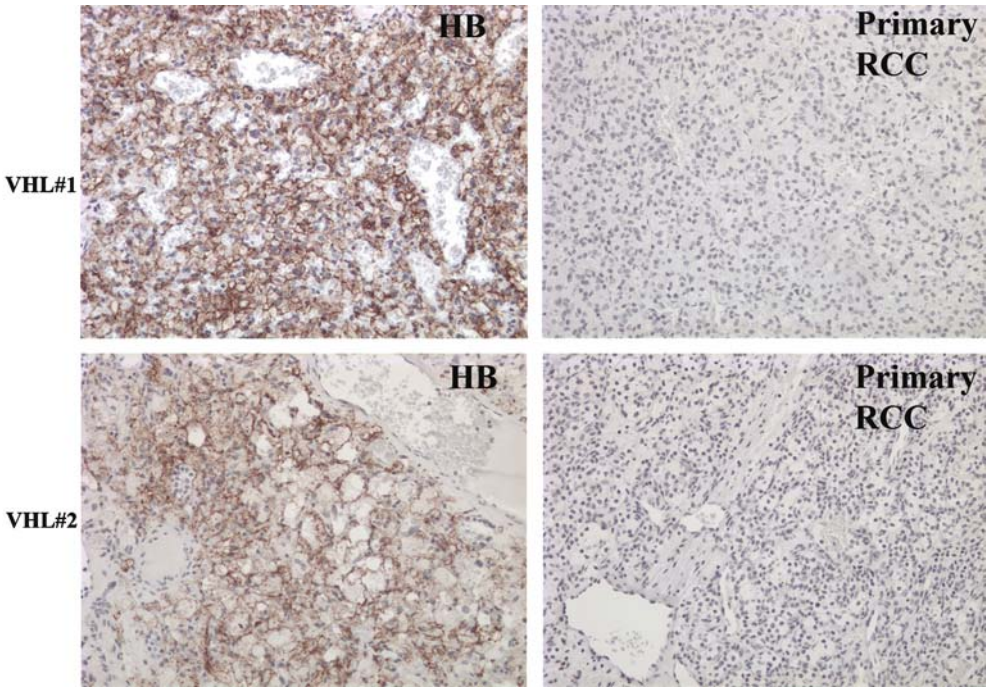

Fig. 2 D2-40 staining in HB and primary CRCC from two patients with VHL disease. Note that D2-40 stains the vacuolated tumor cells in the HB, but not the primary CRCC from the same VHL disease patient (*VHL* von-Hippel Lindau). ×100

plexus epithelium, ependymal cells, subependymal areas and the leptomeninges. The cerebral cortex was devoid of any specific staining. However, in the cerebellar cortex, we saw intense membranous staining of the Purkinje cells and their dendritic processes (Fig. 3). There was some fibrillar staining in the white matter, with no staining of any cell bodies.

In some of our tumor samples, there was adjacent normal cerebellum, and/or some staining in the infiltrative processes between the tumor cells, particularly at the interface between the tumor and non-neoplastic brain parenchyma. For both HB and metastatic CRCC, it was easy to distinguish the fibrillar staining of the infiltrative processes from the membranous staining pattern (or absence thereof) of the clear tumor cells.

Inhibin immunostaining in HB

Inhibin α stained all HB cases, with 9/11 (81%) showing strong positivity in the cytoplasm. However, the pattern of inhibin staining was different from that of D2-40 staining. While D2-40 showed widespread membranous staining of the vacuolated tumor cells, inhibin α usually stained a subset of the tumor cells in a punctuate manner (Fig. 4).

Discussion

In this study, we show that D2-40, a novel monoclonal antibody raised against the M2A antigen labels the vacuolated cells of HB specifically and helps to distinguish HB from metastatic CRCC in brain, in patients with or without VHL disease. Several immunohistochemical markers have been described as useful in differentiating HB from metastatic CRCC [1, 4, 6, 10, 12, 13, 18]. Epithelial markers like epithelial membrane antigen (EMA) and CAM5.2 have been described as being positive in CRCC and negative in HB. However, using EMA and CAM5.2 in this setting has several disadvantages. First, a definite subset (10–30%) of

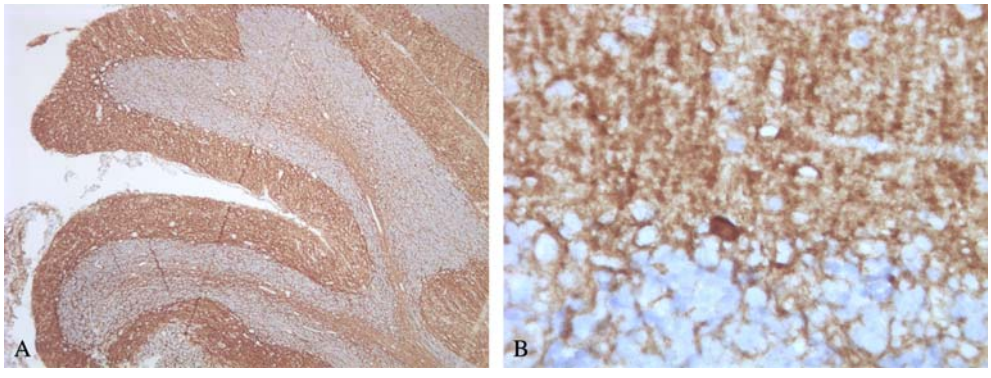

Fig. 3 D2-40 staining of non-neoplastic cerebellar cortex, showing staining of Purkinje neurons in the molecular layer. **A** Note that intense staining is seen in the dendritic processes in the cerebellar molecular layer. **B** Higher magnification shows staining of the cell bodies of the Purkinje neurons. Also note the fibrillary staining of the white matter. **A** ×50, **B** ×400

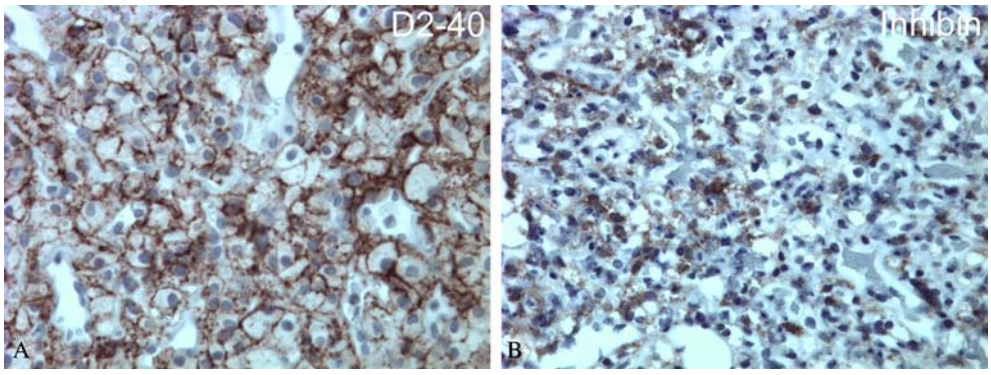

Fig. 4 D2-40 and inhibin α staining patterns in HB. Both D2-40 and inhibin stains the vacuolated tumor cells in HB, note that while D2-40 shows a widespread membranous staining pattern (**A**), the inhibin staining is punctuate and usually focal in our studies (**B**). **A**, **B** ×400

CRCC are negative for EMA and CAM5.2 [21, 24]. Also, tumor diagnostic markers are often most useful when they stain the tumor cells positively, whereas in the case of EMA and CAM5.2, the presence of HB has to be inferred from a negative staining pattern, and thirdly, EMA staining in the CRCC is often focal, with only a small subset of cells staining for the marker, and this can be a limiting factor in small surgical biopsy specimens. Other markers like S-100 protein and neuron-specific enolase (NSE) are largely nonspecific [20]. Recently, an antibody to inhibin was shown to demonstrate specific staining of the vacuolated tumor cells in HB and was reported to be absent in most CRCC [13]. In another study, also published recently, CD10, a known marker for CRCC was shown to be absent in HB [15]. In the same study, however, the authors showed that three of five cases of metastatic CRCC were also positive for inhibin. In our studies, as reported previously, inhibin stained the vacuolated tumor cells of most HB, and was negative in the CRCC tested. The differences in the results of Hoang and Amirkhan [13] and Jung and Kuo [15] may reflect technical differences between the two studies, an unintended bias in case selection, or the subset of CRCC showing positivity for inhibin may be larger than generally believed. Notably, the staining patterns of D2-40 and inhibin were different. Specifically, inhibin usually stained a subset of tumor cells in a punctuate manner, while D2-40 staining was membranous and seen in many of the tumor cells, in almost all cases (Fig. 4). Notably, two cases (nos. 22 and 23, Table 1) with weaker D2-40 staining also showed very weak immunoreactivity with inhibin, suggesting the possibility that there may have been some technical issue in both cases that generally interfered with our immunohistochemical staining procedure. We could not perform inhibin stains in the remaining two cases that showed weaker D2-40 immunoreactivity due to loss of tissue in the deeper sections.

The significance of the specific D2-40 staining of the vacuolated cells in HB is not clear. Preliminary biochemical studies have shown that the M2A antigen recognized by the D2-40 antibody consists of a sialoglycoprotein with a simple O-linked carbohydrate chain that is required for the immunoreactivity of the antibody [19]. However, the specificity of D2-40 for some cells suggests that this antibody may recognize a yet unknown secondary or tertiary structure [19]. Further biochemical and cell biological studies are needed to identify the specific epitope recognized by this antibody in HB and CNS tissue.

In adult non-neoplastic tissue, D2-40 has been shown to be a marker for lymphatic endothelium. Classically, the brain has been described as being free of true lymphatics, with the circulating cerebrospinal fluid being the corollary of the lymphatics. However, until recently, there has been a paucity of definite lymphatic endothelial markers [23]. In our studies with non-CNS tissues, we found that D2-40 specifically stained lymphatic endothelial cells and not vascular endothelial cells, as described. Initially, we were interested in using the D2-40 antibody to evaluate the cerebral vessels. As expected, none of the endothelial cells in the cerebral vessels stained, confirming the absence of true lymphatics in the brain. Interestingly, we saw staining of the choroid plexus, ependymal cells and leptomeninges, structures that are thought to be associated with the secretion, circulation and absorption of the cerebrospinal fluid in the brain [25]. More studies are needed to elaborate these findings.

In summary, the monoclonal antibody D2-40 raised again the membranous antigen M2A is an immunohistochemical marker that helps to distinguish HB from metastatic CRCC in brain in patients with or without VHL disease, and is of potential diagnostic utility in neuropathology specimens.

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