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ORIGINAL PAPER

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Podoplanin expression in primary central nervous system germ cell tumors: a useful histological marker for the diagnosis of germinoma

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Abstract Podoplanin, a mucin-like transmembrane sialoglycoprotein, promotes platelet aggregation and may be involved in cancer cell migration, invasion, metastasis, and malignant progression. Podoplanin/aggrus is highly expressed in testicular seminoma, suggesting that it may be a sensitive marker for testicular seminomas. Here we investigated the expression of podoplanin in central nervous system (CNS) germ cell tumors (GCTs) by immunohistochemical staining of tumor samples from 62 patients. In 40 of 41 (98%) germinomas (including germinomatous components in mixed GCTs), podoplanin was diffusely expressed on the surface of germinoma cells; lymphocytes, interstitial cells, and syncytiotrophoblastic giant cells were negative for podoplanin. Except for immature teratomas (12/17; 71%), podoplanin expression was absent in non-germinomatous GCTs, including seven teratomas, seven embryonal carcinomas, seven yolk sac tumors, and seven choriocarcinomas. In immature teratomas, focal podoplanin staining was observed in fewer than 10% of immature squamous and columnar epithelial cells. Thus, podoplanin expression may be a sensitive immunohistochemical marker for germinoma in CNS GCTs. As such, it may be useful for diagnosis, for monitoring the efficacy of treatment, and as a potential target for antibody-based therapy.

Keywords Podoplanin · Germinoma · Germ cell tumor · YM-1 · Tumor marker

Introduction

Germ cell tumors (GCTs) of the central nervous system (CNS) are a heterogeneous group of lesions found in children and young adults. They are classified into five basic histological types—germinoma, teratoma, embryonal carcinoma, yolk sac tumor, and choriocarcinoma—and into mixed tumor types when two or more components are present [14]. The prognosis of GCTs, independent of their location in the CNS, is highly dependent on the histological subtype. In general, germinomas are sensitive to radiotherapy and chemotherapy and have a better prognosis than non-germinomatous GCTs containing highly malignant components (e.g., embryonal carcinoma, yolk sac tumor, or choriocarcinoma). The 5-year survival rates are 95.4 and 17.4%, respectively [11].

Tumor markers can be helpful in diagnosing GCTs and assessing prognosis. Alpha-fetoprotein (AFP) is produced by yolk sac tumors, a part of embryonal carcinomas and immature teratomas, and beta human chorionic gonadotropin (HCG) is produced by syncytiotrophoblasts in choriocarcinomas. Embryonal carcinomas often have both of these components and therefore are associated with both markers [21]. Human placental alkaline phosphatase (PLAP), expressed by

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primodial germ cells, has been widely used for immunochemical diagnosis of germinomas. However, it lacks specificity and its low secretion titer makes detection difficult [21]. Immunohistochemical staining after tumor resection is often important for diagnosis, and the differentiation between germinoma and non-germinomatous GCTs is essential for determining the appropriate treatment of CNS GCTs.

Mouse podoplanin (aggrus), a 44-kDa sialoglycoprotein with platelet aggregation-inducing ability, is expressed on the surface of mouse colon adenocarcinoma cells [22]. Antibody against podoplanin inhibited lung metastasis of NL-17 colon carcinoma cells in vivo. [20]. Cloning of cDNA revealed that human podoplanin is identical to T1α, a separately isolated protein that can also induce mouse and human platelet aggregation [5]. Therefore, podoplanin could be involved in platelet aggregation induced by tumor cells and metastasis. Podoplanin expression was found in lymphatic endothelium and in tumor-associated lymphangiogenesis, and podoplanin deficiency resulted in congenital lymphedema and impaired lymphatic vascular patterning [16]. Furthermore, podoplanin expression has been shown to be upregulated in squamous cell carcinomas, testicular seminomas, and several sarcomas [2, 6, 7, 17].

Recently, Schacht et al. [17] showed that antibody D2-40, originally produced against a glycoprotein named oncofetal M2A antigen, specifically recognizes human podoplanin. They also demonstrated strong expression of podoplanin by ovarian dysgerminomas. D2-40 reacts with fetal gonocytes, testicular seminoma, and dysgerminoma [9]. Roy et al. [15] reported that D2-40 is a useful marker to distinguish hemangioblastoma from metastatic renal cell carcinoma in the brain. In the adult non-neoplastic CNS, D2-40 staining was seen in the subependymal areas, the leptomeninges and Purkinje cells.

In this study, we investigated the expression of podoplanin in primary GCTs of the CNS to evaluate its potential as a diagnostic marker for CNS germinomas.

Materials and methods

Tissue samples

Tumors specimens were obtained at surgery from 62 patients with GCTs of the CNS, ten patients with CNS lymphomas, three patients with central neurocytoma, three patients with pineocytoma, three patients with pineoblastoma, three patients with schwannoma, four patients with meningioma, seven patients with metastatic brain tumors from lung cancer, three from breast cancer, one from colorectal cancer, and two from renal cell carcinoma. There were 27 germinomas, two germinomas with syncytiotrophoblastic giant cells (STGCs), three embryonal carcinomas, three yolk sac tumors, two choriocarcinomas, nine immature teratomas, four teratomas, and 12 mixed tumors (Table 1). Hematoxylin- and eosin-stained slides from these cases were reevaluated to confirm the diagnosis, and the tumors were categorized according to World Health Organization criteria [14]. Informed consent was obtained from all patients.

Antibodies

Anti-human podoplanin (aggrus) monoclonal antibody (YM-1: Medical Biological Laboratories, Nagoya, Japan) was obtained by immunizing rats with the synthetic peptide CEGGVAMPGAEDDVV, corresponding to amino acids 38–51 of human podoplanin plus the N-terminal cysteine [4].

Table 1 Results of podoplanin immunostaining in 62 patients with GCTs

Tumor type	No. of cases	Podoplanin immunostaining				Positive cells
		+++	++	+	–	
Pure GCTs						
Germinoma	27	25	0	1	1	Germinoma cells
Germinoma with STGCs	2	2	0	0	0	Germinoma cells
Non-germinomatous GCTs						
Embryonal carcinoma	3	0	0	0	3	
Yolk sac tumor	3	0	0	0	3	
Choriocarcinoma	2	0	0	0	2	
Immature teratoma	9	0	0	4	5	Squamous and columnar epithelial cells
Teratoma	4	0	0	0	4	
Mixed GCTs						
Germinoma and teratoma	3	3	0	0	0	Germinoma component
Germinoma and choriocarcinoma	1	1	0	0	0	Germinoma component
Immature teratoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma, and germinoma	4	0	0	4	0	Germinoma and immature teratoma
Immature teratoma and germinoma	4	0	0	4	0	Germinoma and immature teratoma

Western blot analysis

The tissues were solubilized with lysis buffer [25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Na deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, and 50 mg/ml aprotinin] and electrophoresed under reducing conditions on 10–20% polyacrylamide gels (DRC, Tokyo, Japan). The separated proteins were transferred to a nitrocellulose membrane. After blocking with 4% skim milk in PBS, the membrane was incubated first with YM-1 or anti-β-actin antibody (Sigma, St. Louis, MO, USA), and then with peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, UK) and developed for 3 min with ECL reagents (Amersham) using Kodak X-Omat AR film.

Immunohistochemistry

For immunohistochemical analysis, specimens were deparaffinized, rehydrated, and incubated first with YM-1 (1:20 dilution of concentrated culture supernatant from Medical Biological Laboratories) at room temperature for 1 h, then with biotin-conjugated secondary anti-rat IgG antibody (DakoCytomation, Glostrup, Denmark) for 1 h, and finally with peroxidase-conjugated avidin-streptavidin complex (Vectastain ABC Kit, Vector Laboratories, Peterborough, UK) for 1 h. Color was developed with 3, 3-diaminobenzidine tetrahydrochloride tablet sets (DakoCytomation) for 3 min. Immunohistochemical staining for tumor markers including beta-HCG, AFP, and PLAP was also used to confirm the classification. Podoplanin expression was semi-quantitatively assessed from the percentage of tumor cells with cytoplasmic/membrane staining: 0, no staining; +, $<10\%$; ++, 10–50%; and +++, $>50\%$.

Results

Immunohistochemical analysis of podoplanin in CNS GCTs

The immunohistochemical findings are summarized in Table 1. Podoplanin immunoreactivity was detected in 26 of 27 (96%) pure germinomas; the staining was graded as +++ in 25 cases and as + in one. One germinoma was negative for podoplanin. Including germinomatous components in mixed GCTs, 40 of 41 (98%) germinomas were stained by YM-1.

Immunostaining for podoplanin showed a diffuse cell-surface pattern in germinoma cells (Fig. 1a). Immunostaining revealed germinoma cells infiltrating into brain parenchyma (Fig. 1b) and in the germinomatous components of mixed tumors (Fig. 1c). Nontumor components such as infiltrated lymphocytes and stromal cells were negative for podoplanin, as were STGCs.

Among the non-germinomatous GCTs and mixed GCTs, 12 of 17 immature teratomas were positive for podoplanin; in all cases, the staining was focal (+) and limited to basal layers of immature squamous epithelium and immature columnar epithelium (Fig. 2a, b). Seven

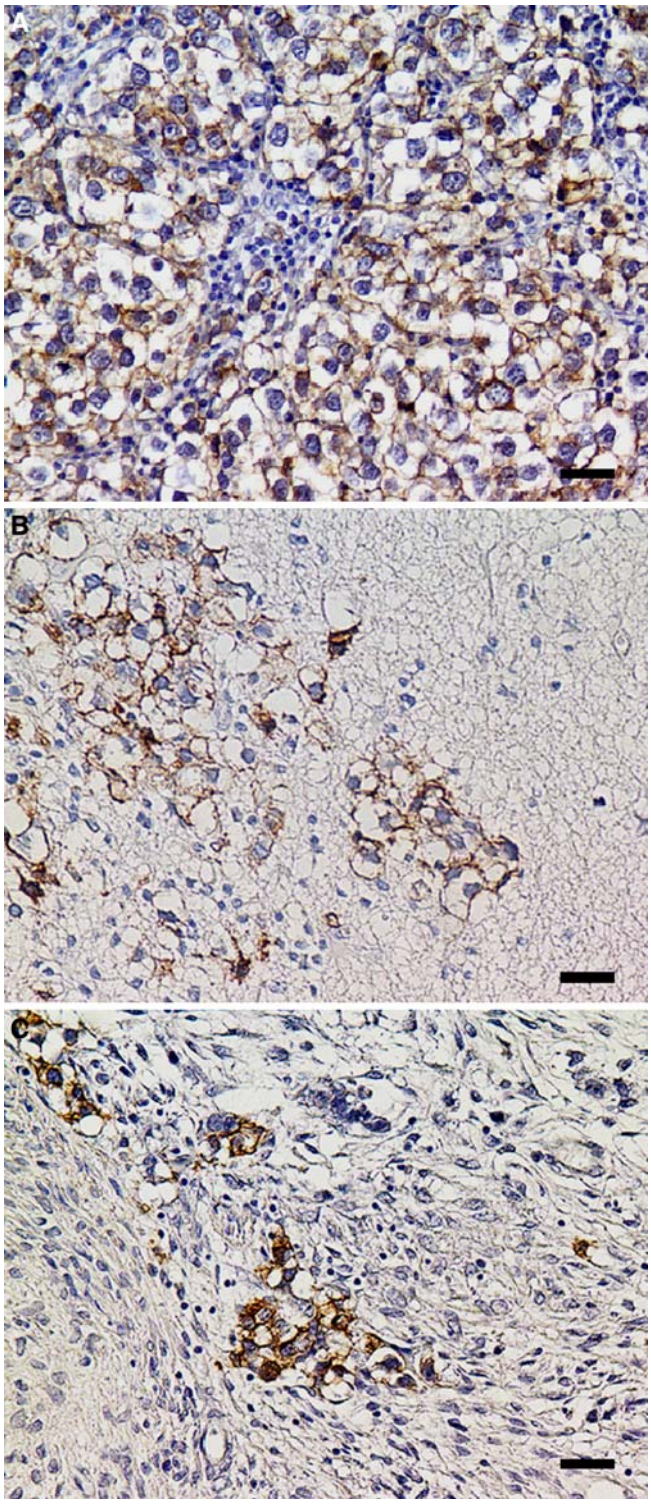

Fig. 1 Immunohistochemical detection of podoplanin in CNS germinomas. **a** The surface of germinoma cells is strongly positive (*brownish color*). Lymphocytes and interstitial cells are negative. Germinoma cells infiltrating into brain parenchyma (**b**) and germinomatous components in mixed GCTs (**c**) are also positive. *Bar* 10 μm

embryonal carcinomas, seven choriocarcinomas, and seven yolk sac tumors were not stained with YM-1 (Fig. 2c–e, Table 1).

In pineal region, GCTs need to be distinguished from other tumors (e.g., metastatic brain tumors, pineocytomas, and pineoblastomas). Therefore, we examined the expression of podoplanin in other CNS tumors to assess the specificity of podoplanin in distinguishing germinomas from certain metastatic brain tumors and some primary brain tumors in pineal region. The immunohistochemical findings are summarized in Table 2. Podoplanin immunoreactivity was detected in one of three (33%) metastatic squamous carcinomas of the lung; the staining was graded as +, although none of the adenocarcinomas of the lung were positive for podoplanin ($n=4$). Other metastatic tumors from colorectal cancer ($n=1$), renal cell carcinomas ($n=2$) and breast cancer ($n=3$) did not express podoplanin. Primary brain tumors in pineal region such as CNS lymphoma ($n=10$), pineocytoma ($n=3$), pineoblastoma ($n=3$), and meningioma ($n=4$) were negative for podoplanin. Other primary brain tumors such as central neurocytoma ($n=3$) and schwannoma ($n=3$) were also negative.

To confirm the immunohistochemical findings from CNS GCTs, lysates of frozen tumor specimens from seven patients were analyzed by western-blot analysis (Fig. 3). Podoplanin protein was overexpressed in germinoma, but not in choriocarcinoma, yolk sac tumor or normal brain tissue. Podoplanin was also detected in two immature teratomas and one mixed tumor containing immature teratoma, germinoma, and embryonal carcinoma.

Discussion

In this study of 62 primary GCTs of the CNS, immunostaining with monoclonal antibody YM-1 demonstrated podoplanin immunoreactivity in 98% (40/41) of pure or mixed germinomas and a limited number of immature teratomas, but not in embryonal carcinoma, yolk sac tumor, choriocarcinoma, or teratoma. In mixed

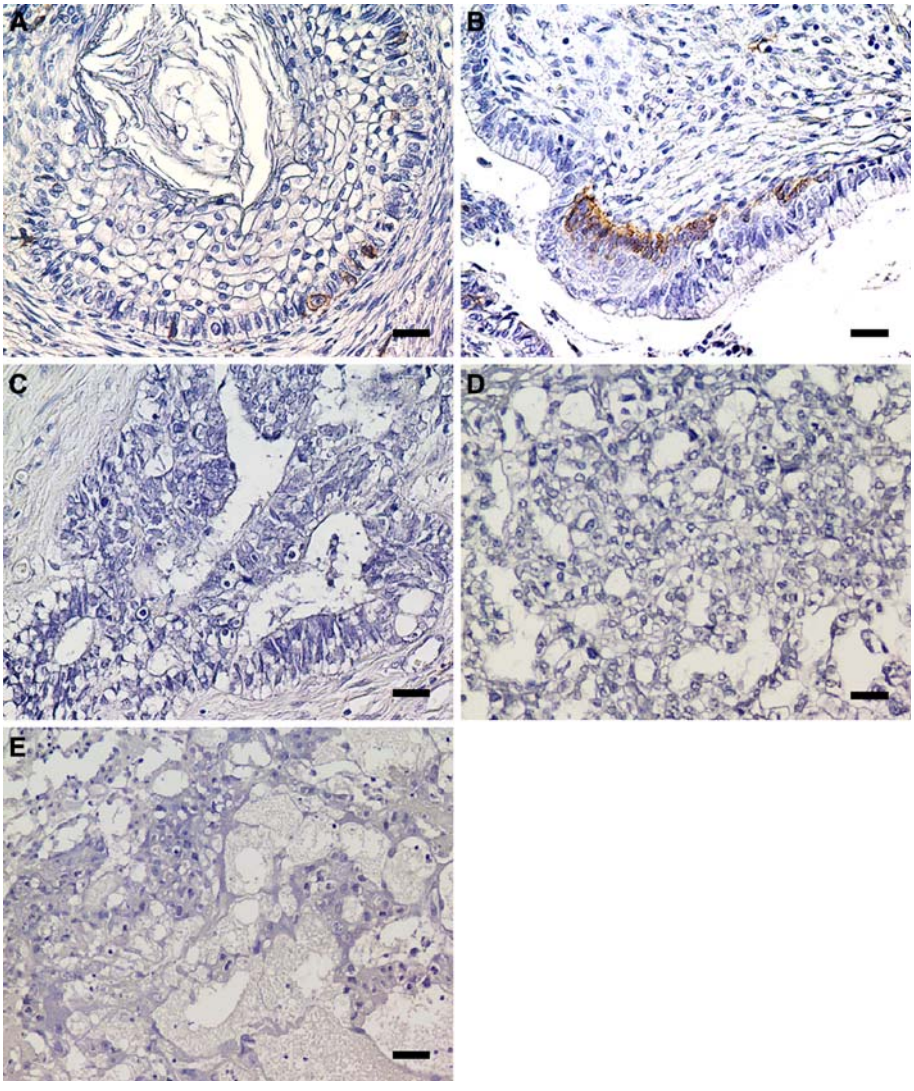

Fig. 2 Photomicrographs showing immunohistochemical detection of podoplanin in non-germinomatous GCTs. In immature teratomas, positive staining for podoplanin (*brownish color*) is limited to basal layers of immature squamous epithelium (**a**) and immature columnar epithelial cells (**b**). Embryonal carcinoma (**c**), yolk sac tumor (**d**), and choriocarcinoma (**e**) are negative for podoplanin. *Bar* 10 μm

Table 2 Results of podoplanin immunostaining in patients with metastatic brain tumors and primary brain tumors

Tumor type	No. of cases	Podoplanin immunostaining			
		+++	++	+	−
Matastatic brain tumors					
Lung					
Adenocarcinoma	4	0	0	0	4
Sqamous cell carcinoma	3	0	0	1	2
Breast	3	0	0	0	3
Renal cell	2	0	0	0	2
Colorectal	1	0	0	0	1
Primary brain tumors					
Lymphoma	10	0	0	0	10
Pineocytoma	3	0	0	0	3
Pineoblastoma	3	0	0	0	3
Meningioma	4	0	0	0	4
Central neurocytoma	3	0	0	0	3
Schwannoma	3	0	0	0	3

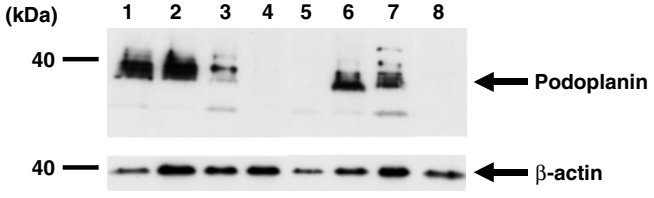

Fig. 3 Western-blot analysis of podoplanin expression in CNS GCTs. Tumor tissues from a germinoma (*lanes 1* and *2*), a mixed tumor (mainly embryonal carcinoma with immature teratoma/germinoma) (*lane 3*), an embryonal carcinoma (*lane 4*), a yolk sac tumor (*lane 5*), an immature teratoma (*lanes 6* and *7*), and normal brain (*lane 8*) were solubilized and immunoblotted with anti-human podoplanin monoclonal antibody YM-1 (*upper panel*) or anti-β-actin antibody (*lower panel*)

GCTs, staining for podoplanin was restricted to germinomatous and immature teratoma components and highlighted germinoma cells. These findings suggest that podoplanin may be a sensitive immunohistochemical marker of CNS germinoma.

Distinguishing germinoma from non-germinomatous GCTs is of paramount importance in patient management. Neuroradiologic findings are not particularly helpful for differentiating between histological subtypes of GCTs [12]. Unlike testicular GCTs, diagnosis of CNS GCTs often requires immunohistochemical analysis because of the small size of the biopsy samples by recent less invasive surgery and the morbidity associated with irradiation of the brain. In addition, a granulomatous inflammatory reaction sometimes overwhelms the tumor cell parenchyma, causing a diagnostic failure at biopsy [8]. Therefore, a specific immunohistochemical marker would be extremely useful for identifying tumor cells in biopsy samples submitted for diagnosis.

Antibodies to PLAP, a cell-surface glycoprotein elaborated by syncytiotrophoblasts and produced by primordial germ cells, have been widely used for immunochemical diagnosis of germinomas and seminomas. About 75–100% of germinomas and 33–86% of non-germinomatous GCTs such as embryonal carcinomas are positive for PLAP [21]. Unfortunately, it lacks specificity and its low secretion titer makes detection difficult.

On the other hand, our findings suggest that podoplanin is highly expressed in germinomas and focally in immature teratomas but not in embryonal carcinomas, choriocarcinomas, and yolk sac tumors and any of these components in mixed GCTs. Indeed, the subtypes of nongerminomatous GCTs (e.g., embryonal carcinoma, yolk sac tumor, and choriocarcinoma) occur rarely as a pure form and are mostly seen as a part of a mixed germ cell tumor in CNS GCTs, therefore the number of non-germinomatous GCTs examined in this study might be small to conclude the specificity of podoplanin expression for germinoma.

The most common site of origin for pineal region metastasis is the lung, followed by the breast. In our study, metastatic brain tumors from lung squamous carcinomas occasionally expressed podoplanin, as the primary lung squamous cell carcinoma expressed podoplanin/aggrus [7]. However, adenocarcinomas from lung and other metastatic brain tumors from colorectal cancer, renal cell carcinoma and breast cancer did not express podoplanin. Other primary brain tumors such as CNS lymphoma, pineocytoma, pineoblastoma, meningioma, central neurocytoma, and schwannoma were also negative for podoplanin. Recently, podoplanin was reported to be expressed in hemangioblastoma in the brain [15]. Thus, podoplanin is not a specific marker of germinoma in brain tumors, but could be a useful diagnostic marker for germinomas in CNS GCTs. We suggest that the combination of several markers, such as podoplanin detected by D2-40 or YM-1, PLAP, beta-HCG, and AFP, provides more precise diagnostic information. Establishing the clinical efficacy of podoplanin as a marker for GSTs will require further investigation.

The biological function of podoplanin is largely unknown. In mice, deficiency in T1α/podoplanin causes defects attributed to disruption of epithelial-mesenchymal signaling [13] and to impairments in cell to substratum adhesion and cell migration [16]. In vascular endothelial cells, overexpression of T1α/podoplanin induces elongated cell extensions and significantly increases cell adhesion, migration, and tube formation by promoting rearrangement of the actin cytoskeleton [16]. PA2.26 antigen/podoplanin was identified as a cell-surface protein induced in epidermal carcinogenesis and skin remodeling. Expression of PA2.26 antigen/podoplanin in pre-malignant keratinocytes induces fully transformed and metastatic phenotype [18, 19]. Human PA2.26 antigen/podoplanin has been found in the invasive front of oral squamous cell carcinomas, consistent with a role in tumor cell migration and invasion [10]. Although germinomas rarely metastasize outside the CNS, they have a predilection for disseminating within the subarachnoid space and for invading sur-

rounding brain tissue [1, 3, 23]. These findings, together with the upregulated expression of podoplanin we observed in CNS germinomas, suggest a potential role of podoplanin in the progression, invasion, and dissemination of CNS germinoma; however, its direct biological function in germinomas remains to be established. We are currently investigating whether enhanced podoplanin expression contributes to the invasive spread of experimental tumors.

In conclusion, podoplanin appears to be a sensitive marker of germinoma and thus may be useful for diagnosis and for monitoring the effects of treatment. Because of its oncogenic potential, high-level expression in germinomas, and localization on the cell surface, podoplanin is a potential target for antibody-based therapy.

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