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ORIGINAL ARTICLE

Immunophenotypic features of immaturity of neural elements in ovarian teratoma

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Abstract Neural components in mature teratomas are common and the general assumption is that they are quite similar to those in the mature central nervous system (CNS). We investigated 44 ovarian teratomas by immunohistochemistry to determine cellular and structural immaturity of neural elements. Most teratomas contained cells differentiating into astrocytes positive for nestin, a neural stem cell marker. These nestin-positive astrocytes generally co-expressed glial fibrillary acidic protein-delta, an immature astrocyte marker. Olig2-positive cells were randomly scattered. Areas comprising cells that differentiated into neurons were positive for NeuN and synaptophysin. The border between white and gray matter was ill-defined and more NeuN-positive cells were distributed in areas that were positive for myelin basic protein, indicating that the distribution of neurons and glial cells was disturbed. Peripheral nerve bundles positive for Schwann/2E, an antigen specific for myelinating Schwann cells, were mixed within CNS-like tissues. These results show that apparently mature teratomas are not in fact mature, at least in terms of neural elements, as they harbor immature cells and structural abnormalities. The neural elements of surgically resected teratomas might represent a premature state of the human CNS, and thus be potentially useful for studies of developmental neurobiology as well as gliomagenesis.

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Introduction

Rudolf Virchow initially described a monstrous intraspinal tumor as a *teratoma* based on the Greek words *teras* meaning monster, and *onkoma* meaning swelling or tumor [1]. Almost a century later, Rupert A. Willis defined teratoma as "a true tumor or neoplasm composed of multiple tissues of kinds foreign to the part in which it arises" [2].

Mature teratomas are common ovarian tumors composed exclusively of mature tissues derived from two or three germ layers (ectoderm, mesoderm, and endoderm) and they constitute about 20 % of all ovarian tumors [3, 4]. The most abundant components of ectodermal derivatives are squamous epithelium and brain tissue (glia, ependymal tubules, and cerebellum). Mesodermal derivatives contain bone, cartilage, smooth muscle, and adipose tissue. Gastrointestinal and respiratory/bronchial epithelium, thyroid, and salivary glands are endodermal derivatives. Caruso et al. found ectodermal derivatives, mesodermal structures, and endodermal derivatives in 99.3, 73.3 and 31.9 % of mature teratomas, respectively, and neural elements 32.3 % of them [5]. Another study found a 33.3 % frequency of nervous tissue in mature teratomas [6].

On the other hand, immature teratomas are composed of variable amounts of immature embryonal-type tissues, mostly in the form of neuroectodermal tubules and rosettes, admixed with mature tissues [4, 7]. Immature teratomas have been classified into three grades based on relative amounts of immature neuroectodermal components [4, 7]. Thus, the differentiation of neural components in teratomas has been a good indicator of the overall status of tumor maturity [8]. However, little is known about the status of cellular and structural

immaturity of neural elements in mature teratomas other than neural tube formation [9, 10].

In the present study, we searched for and immunohistochemically characterized neural elements in ovarian teratomas, using a panel of antibodies including those that preferentially label glial precursor cells, such as nestin and glial fibrillary acidic protein-delta (GFAP-δ). Disorganized structures and immature cells in the neural elements were identified even in mature teratomas.

Materials and methods

We reviewed 1618 surgically resected ovarian tumors archived between 1999 and 2013 at Gunma University Hospital. All tissue samples were routinely formalin-fixed and paraffin-embedded, and for histological and immunohistochemical studies, 2.5-μm-thick sections were cut. Teratomas were diagnosed based on hematoxylin and eosin (H & E)-stained preparations using the World Health Organization classification [4]. Mature teratomas accounted for 368 (22.7 %) of the 1618 ovarian tumors and 176 (47.8 %) of the 368 had neural components. Only seven immature teratomas were identified. We selected 37 mature teratomas, with a sufficient amount of neural elements, and all seven immature teratomas for this study. All 37 mature teratomas were cystic. Four immature teratomas were grade 1, and three were grade 2. Patient age ranged from 5 to 72 (mean = 26.0; median = 24) years.

A panel of primary antibodies to the following antigens was applied for immunohistochemical assessment: GFAP (1:5000) [11], nestin (10C2; 1:200; Immuno-Biological Laboratories, Takasaki, Japan), GFAP-δ (polyclonal; 1:1000; Abcam, Cambrige, UK), Olig2 (polyclonal; 1:100; Immuno-Biological Laboratories) [12], myelin basic protein (MBP) (polyclonal; 1:2000; Dako, Glostrup, Denmark), NeuN (A60; 1:1000; Chemicon International, Temecula, California, USA), synaptophysin (27G12; 1:200; Novocastra, Newcastle upon Tyne, UK), Schwann cell/peripheral myelin antibody (Schwann/2E; 1:10,000; Cosmo Bio, Tokyo, Japan) [13], Ki-67 (MIB-1; 1:50; Dako,) and LIN28A (A177; 1:50; Cell Signaling Technology, Boston, MA, USA). Cells were visualized using biotin-streptavidin immunoperoxidase kits (Histofine, Nichirei, Tokyo, Japan) with diaminobenzidine as chromogen. Staining intensity was evaluated as ratio (%) of positive cells relative to the total number of cells and scored as follows: −, <5 %; 1+, 5–25 %; 2+, 26–50 %; 3+, 51–75 %; 4+, >75 %; 5+.

Cells were visualized by double immunofluorescent staining for nestin and GFAP-δ using Alexa Fluor 568 (Molecular Probes, Eugene, Oregon, USA) or Alexa Fluor 488 (Molecular Probes)-labeled secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

The Ethics Committee at Gunma University approved the present study on June 30, 2015.

Results

All teratomas had neural elements with the appearance of tissues of a mature central nervous system (CNS). Mitotically inactive astrocytes possessed round nuclei and eosinophilic cytoplasm with fine processes (Fig. 1a). Other areas

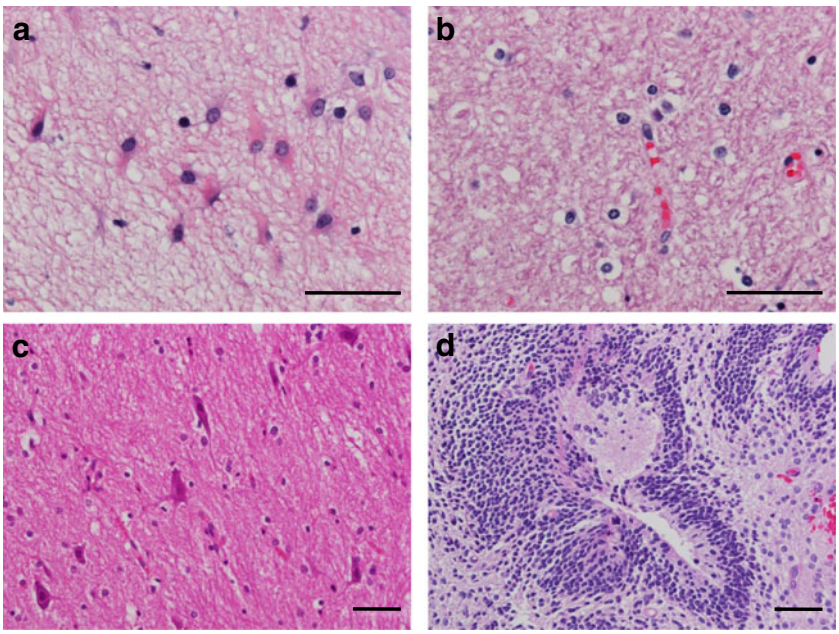

Fig. 1 Microscopic appearance of the neural tissues in teratomas. **a** Cells differentiating into astrocytes have round nuclei and eosinophilic cytoplasm with processes. Area is mostly filled with the astrocytes. **b** Cells differentiating into oligodendrocytes have round nuclei and clear cytoplasm. Although oligodendrocyte distribution is disorganized, area is quite similar to white matter. **c** Cells differentiating into neurons are randomly scattered and border between white and gray matter is ill-defined. **d** Neuroectodermal tubules in apparently mature central nervous system (CNS)-like tissues. Nuclei are multistratified. *Bar* = 50 μm

Table 1 Immunohistochemical findings of apparently mature central nervous system-like tissues in teratomas

Patient no.	Grade[a]	Year	Size (mm)	GFAP	Nestin	GFAP-δ	Olig2	MBP	NeuN	Synaptophysin	Schwann/2E	LIN28A
Mature teratoma												
1		25	75 × 69 × 69	5+	2+	3+	1+	–	–	–	–	–
2		5	49 × 37 × 36	5+	4+	4+	3+	5+	3+	4+	–	–
3		29	63 × 52 × 40	5+	4+	3+	3+	3+	1+	2+	–	–
4		14	65 × 59 × 58	5+	4+	4+	2+	2+	2+	3+	–	–
5		42	86 × 55 × 38	5+	3+	1+	3+	4+	1+	3+	–	–
6		33	52 × 50 × 44	5+	–	–	4+	4+	1+	3+	2+	–
7		15	90 × 84 × 74	5+	3+	3+	2+	3+	2+	3+	2+	–
8		35	75 × 60 × 57	5+	3+	4+	3+	3+	4+	3+	–	–
9		72	71 × 63 × 56	5+	1+	1+	–	2+	1+	3+	–	–
10		24	124 × 106 × 80	5+	2+	3+	4+	4+	4+	4+	1+	–
11		20	213 × 159 × 70	5+	2+	3+	3+	3+	3+	4+	2+	–
12		26	35 × 30 × 24	5+	2+	2+	–	1+	1+	1+	–	–
13		7	95 × 83 × 58	5+	1+	1+	1+	–	–	–	–	–
14		42	63 × 55 × 53	5+	4+	4+	1+	–	3+	–	–	–
15		20	190 × 178 × 116	5+	2+	2+	3+	4+	2+	4+	–	–
16		17	85 × 72 × 60	5+	–	–	1+	2+	1+	2+	–	–
17		40	63 × 55 × 47	4+	1+	1+	3+	4+	3+	4+	1+	–
18		26	83 × 80 × 56	5+	5+	5+	1+	2+	2+	1+	1+	–
19		17	75 × 67 × 55	5+	1+	1+	1+	4+	1+	4+	–	–
20		17	77 × 48 × 46	5+	4+	4+	1+	2+	3+	3+	–	–
21		38	62 × 51 × 48	5+	3+	3+	1+	3+	2+	3+	1+	–
22		22	51 × 46 × 41	5+	2+	1+	1+	3+	1+	2+	–	–
23		32	73 × 56 × 52	5+	1+	1+	1+	1+	2+	1+	–	–
24		25	77 × 70 × 64	5+	1+	1+	1+	4+	1+	3+	2+	–
25		24	73 × 50 × 43	5+	2+	2+	1+	–	–	2+	–	–
26		42	72 × 45 × 36	5+	2+	3+	–	–	–	–	–	–
27		24	75 × 56 × 55	5+	2+	2+	3+	3+	3+	2+	–	–
28		42	41 × 32 × 23	5+	3+	4+	1+	4+	1+	4+	–	–
29		20	43 × 40 × 33	5+	5+	5+	1+	–	2+	1+	–	–
30		21	76 × 60 × 57	5+	5+	5+	1+	1+	3+	2+	1+	–
31		37	92 × 63 × 56	5+	5+	5+	1+	4+	3+	3+	–	–
32		24	160 × 123 × 97	5+	3+	2+	2+	3+	2+	2+	–	–
33		25	64 × 40 × 20	5+	3+	3+	2+	2+	3+	3+	1+	–
34		38	47 × 47 × 45	5+	1+	1+	1+	3+	2+	2+	2+	–
35		21	85 × 60 × 45	5+	4+	3+	1+	2+	1+	1+	1+	–
36		13	152 × 118 × 113	5+	4+	4+	1+	2+	2+	3+	–	–
37		28	91 × 58 × 54	5+	4+	4+	1+	–	3+	–	–	–
Immature teratoma												
1	1	24	110 × 105 × 83	5+	5+	5+	1+	–	2+	4+	–	–
2	2	19	252 × 213 × 129	5+	5+	4+	1+	1+	1+	2+	1+	–
3	1	25	200 × 181 × 91	5+	5+	5+	2+	–	3+	1+	–	–
4	1	24	143 × 126 × 91	5+	5+	5+	2+	2+	2+	3+	1+	–
5	2	9	no data	2+	5+	2+	2+	–	4+	4+	–	–
6	1	27	no data	3+	5+	2+	2+	–	4+	4+	–	1+[b]
7	2	12	165 × 160 × 100	5+	4+	4+	1+	3+	3+	4+	2+	–

Ratios (%) of positive cells: −, < 5 %; 1+, 5–25 %; 2+, 26–50 %; 3+, 51–75 %; 4+, >75 %; 5+

[a] Immature teratoma only

[b] Only neuroectodermal tubules were positive

contained oligodendrocyte-like cells with small round nuclei and clear cytoplasm (Fig. 1b), and cells simulating neurons with round nuclei as well as a slightly basophilic cytoplasm with dendritic processes (Fig. 1c). However, the polarity of neural fibers was disorganized, unlike normal mature CNS tissues. The border between white and gray matter was ill-defined. All immature teratomas had neuroectodermal tubules and rosettes encompassed by apparently more mature neural tissues (Fig. 1d).

Table 1 summarizes the immunohistochemical findings of the mature-appearing CNS-like tissues. All neural areas in teratomas were diffusely positive for conventional GFAP (Fig. 2a). Cells differentiating into astrocytes in most teratomas were positive for nestin (Fig. 2b). Cells immunolabeled with GFAP-δ were generally distributed like the nestin-positive cells (Fig. 2c). Double immunofluorescent staining showed mostly overlapping signals for nestin (red) and GFAP-δ (green) (Fig. 2d–g). Though cells differentiating into glial cells were partly stained with Olig2, expression of this factor was irregular (data not shown). The cells that differentiated into neurons were positive for NeuN (Fig. 2h). Synaptophysin expression overlapped NeuN-positive areas in general (Fig. 2i). NeuN-positive cells were likely to be buried in MBP-positive regions (Fig. 2j). The distribution of neurons and glial cells was disorganized. Notably, peripheral nerve structures indicated by Schwann/2E staining were scattered in CNS-like tissues (Fig. 2k). Clusters of Schwann cells were barely identified by detailed examination of adjacent H & E specimens (Fig. 2k inset). All tissues with a mature CNS appearance were negative for LIN28A (data not shown). MIB-1-labeling index of mature CNS-like areas <1 % indicated lack of proliferative activity (data not shown).

We also assessed neuroepithelial tubules and surrounding neural tissues in immature teratomas. Conventional GFAP was positive to a variable degree in neural tube-like tissues (Fig. 3a), but negative for NeuN, synaptophysin, MBP, and Schwann/2E (data not shown). Nestin-positive cells were distributed in all layers of neuroectodermal tubules and surrounding nervous tissues in all immature teratomas (Fig. 3b), but the tubular structures were negative for GFAP-δ, unlike the other neural tissues (Fig. 3c). Neuroectodermal tubules were partly stained with Olig2 but its expression was irregular (data not shown). The immunohistochemical profile of the mature-appearing neural components in all immature teratomas was generally similar to that in mature teratomas (Table 1). Neuroectodermal tubules of only one immature teratoma were positive for LIN28A (Fig. 3d).

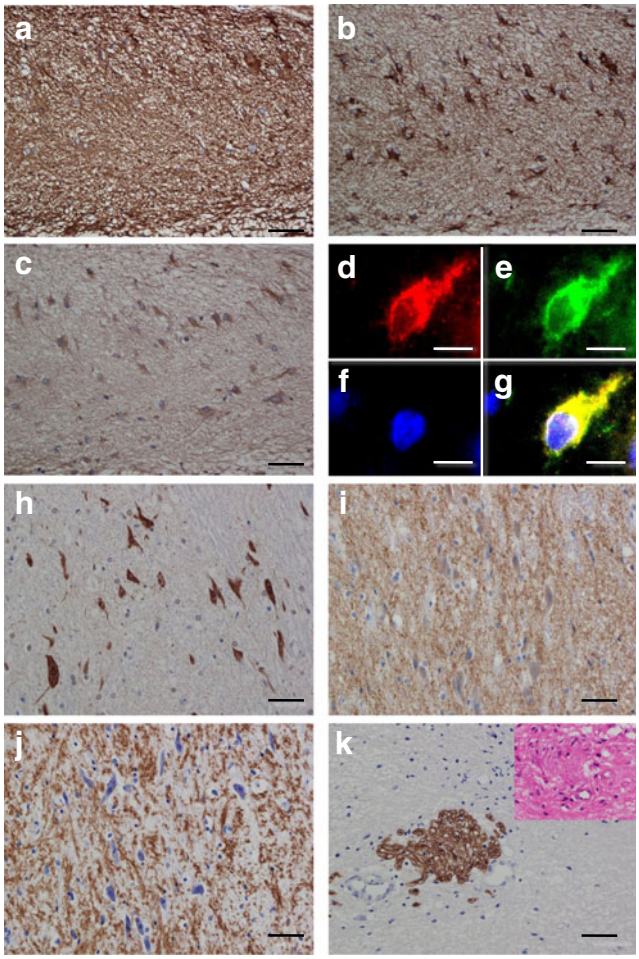

Fig. 2 Microscopic appearance of immunostained and double immunofluorescent stained apparently mature CNS-like tissues of teratomas. **a** Glial fibrillary acidic protein (GFAP) is diffusely positive in neural areas. GFAP-positive areas are larger than GFAP-δ positive areas. **b** Nestin is positive in cells differentiating into astrocytes (same area as **a**). Nestin-positive cells are morphologically indistinguishable from reactive astrocytes of various brain lesions. **c** GFAP-δ-positive cells are distributed like nestin-positive cells (same area as **a**). **d**–**g** Immunofluorescent staining. **d** Nestin-positive cells (*red*). **e** GFAP-δ-positive cells (*green*). **f** The nuclei are counterstained with 4′,6-diamidino-2-phenylindole (*blue*). **g** Merged image. **h** NeuN is positive in cells differentiating into neurons. Border between white and gray matter is ill-defined. **i** Synaptophysin positivity is overlapped on NeuN-positive areas on the whole (same area as **h**). **j** Unlike normal mature CNS, myelin basic protein immunostaining is patchy around neurons (same area as **h**). **k** Schwann/2E staining reveals peripheral nerve bundles in CNS-like areas. *Inset* shows same peripheral nerve bundles in adjacent section. *Black and white bars* = 50 and 10 μm, respectively

Discussion

Pathologists often encounter ovarian teratomas in daily practice. In many cases, mature and immature teratomas are relatively easy to distinguish by H & E staining because the presence of neuroectodermal tubules is a good indicator of immature teratomas [8]. Neural elements other than tubular structures have long been considered to be mature. Trojanowski et al. [9] and Sangruchi et al. [10] used several neural antibodies to characterize neural elements in teratomas. Although two studies showed that most teratomas contain neural elements, cellular and structural (im)maturity of the

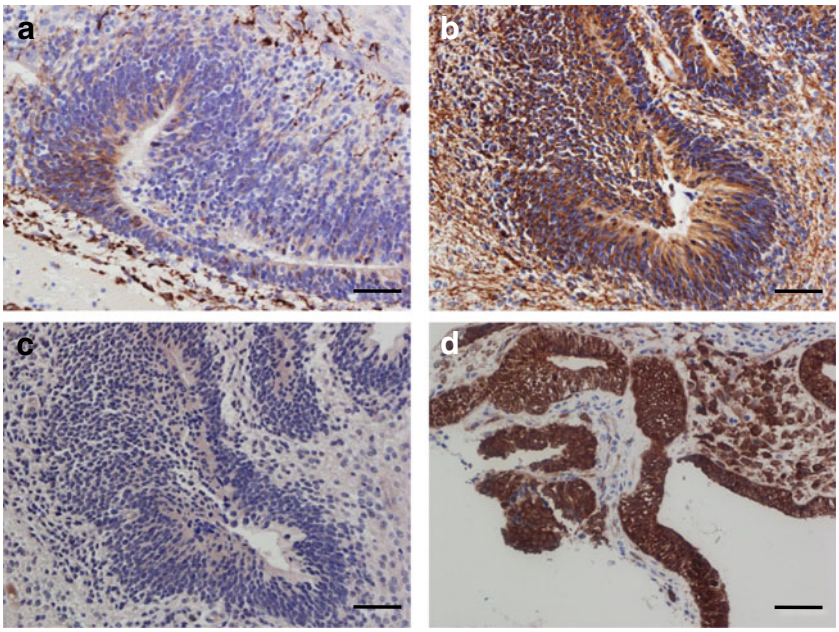

Fig. 3 Microscopic appearance of immunostained neuroectodermal tissues of immature teratomas. **a** GFAP is positive to varying degrees in neuroepithelial tissues. **b** Nestin-positive cells are distributed over all layers of neuroectodermal tubules and surrounding apparently mature nervous tissues. **c** GFAP-δ. Neuroepithelial tissues in immature teratomas are negative (same area as **b**). **d** All layers of neuroectodermal tubules are positive for LIN28A. *Bar* = 50 μm

neural elements has not been investigated in detail. Nogales et al. studied expression of SOX2, a neural stem cell marker, in immature teratomas and glial peritoneal implants [14, 15]. García-Galvis et al. [16] used antibodies specific for neural elements such as GFAP, nestin, NeuN, and synaptophysin, to characterize neural components of teratoid carcinosarcoma. The studies documented expression of neural stem cell markers, which suggests immaturity of neural components. In the present study, we expanded the immunohistochemical analysis to 44 ovarian teratomas, using mature and immature neural markers.

Nestin is an intermediate filament expressed in undifferentiated CNS cells [17–19], the subventricular zone of the normal adult human brain [17] and in vascular endothelial cells [20, 21]. GFAP-δ is expressed in particular in the subventricular zone and specifically marks the population of astrocytes containing neural stem cells in the adult human brain [22]. Our immunostaining of normal adult brains with antibodies to nestin and GFAP-δ provided results similar to those of previous reports (data not shown). GFAP-δ plays a role in regulation of the size and motility of astrocytes and a subpopulation of GFAP-δ-positive glia might constitute multipotent stem cells [23]. Previous studies have suggested the GFAP-δ-positive cells co-express nestin [23–25]. Nestin and GFAP-δ differ in that nestin is positive [26] and GFAP-δ is negative [22] in reactive astrocytes. We found that nestin and GFAP-δ are mostly co-expressed in differentiated astrocytes, which supports the notion that the cells of neural elements in mature teratomas are immature. However, why neural tube-like structures were positive for nestin and conventional GFAP, and negative for GFAP-δ remains unknown.

Mature CNS is usually divided into regions of white and gray matter, whereas such regions were randomly mixed in teratomas. We also identified insular foci of Schwann cells identified by the novel anti-Schwann/2E antibody in CNS-like tissues of teratomas. We conclude that neural elements of mature and immature teratomas harbor structural abnormalities.

A previous study demonstrated immunostained LIN28A in 12 of 14 immature teratomas [27], whereas only one of seven was positive in the present study. In contrast, immature neuroectodermal tissues including tubular formations were positive for nestin in all seven immature teratomas. This confirms that immature neuroectodermal tissues are positive for nestin and/or LIN28A. Currently, the identification of neural tubular structures is obligatory for a diagnosis of histologically immature teratoma, but similar tubular/glandular structures might appear in teratomas and intermingled with immature neuroectodermal tissues. To facilitate their recognition, the use of nestin and/or LIN28A immunostaining might help to distinguish neural tubular structures from similar tubular/glandular structures.

Rare neuroepithelial tumors such as astrocytoma [28, 29], neuroblastoma [30], glioblastoma [31], central neurocytoma [32], and oligodendroglioma [33, 34] can arise in mature teratomas. The mechanism through which mature teratomas yield CNS-type neoplasms remains unknown, but immature neural elements might serve as precursors of such tumors.

Neural progenitor cells have become one focus of studies in developmental neurobiology. As opportunities for surgical pathologists to handle nascent human brain tissues are very limited and ethical barriers are high, neural elements in teratomas might be a valuable research surrogate. Even if the components are pathological, analyzing the expression of neural progenitor cell markers might provide new insight. Ovarian teratomas are common and neural elements in

teratomas are frequent. In addition, examination of tissues from surgical specimens poses less ethical barriers. When new neural progenitor cell markers emerge, preliminary investigation of ovarian teratomas might be useful.

In summary, we show that neural elements in ovarian teratomas have immunophenotypic features of immaturity. The implication of this finding is that what we call "mature" teratoma is not really mature. Prognosis of patients with a "mature" teratoma with immunohistochemically immature elements remains excellent. In the future, the classical definition of mature teratoma in terms of its neural elements might need to be revised. Further studies of "maturity status" of tissue components of teratomas might lead to modified definitions and marker applications.

Compliance with ethical standards The Ethics Committee at Gunma University approved the present study on June 30, 2015.

Conflict of interest The authors declare that they have no competing interests.

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