Histopathological and Immunohistochemical Characteristics of the Progressive Front of Ameloblastoma

（エナメル上皮腫の進展先端における病理組織学的および免疫組織化学的特徴）

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Abstract

Ameloblastoma is a slow-developing benign odontogenic tumor, but it has relatively large number of local recurrence. Ameloblastoma was classified on the basis of the morphology that predominates in the tumor itself into plexiform type, follicular type, and other subtypes, and there have been comparative histological and clinical studies that include prognosis predictions for the different types. However, there are no conclusions regarding histological patterns and tumor activities have been reached.

The purpose of the present study was to perform a detailed investigation of the progressive front of solid/multicystic type ameloblastoma in order to search for possible prognostic factors.

For this study, 22 cases were chosen, in whom solid/multicystic type of a ameloblastoma. Progressive fronts of the solid/multicystic type ameloblastoma were morphologically classified into six types; plexiform, follicular, basaloid cell, sheet, trabecular and polycystic types, and immunohistochemical and morphometrical comparative studies were performed.

Proliferative activity of columnar cells was highest in basaloid cell type at 1.9%. Periostin showed moderate to strong positive reaction in columnar cells of plexiform and basaloid cell types. Columnar and stellate-reticulum-like cells of basaloid cell type showed strong positive reaction for VEGF. The highest microvessel density and microvessel area using CD105 in basaloid cell type were 48.2±24.2 and 11.4±8.6%, respectively.

The result of this study suggested with resection of solid/multicystic type ameloblastoma, it is important the prognosis observation to base judgment on the morphology of cells in the progressive front or in the vicinity of the resected surface.

Introduction

Odontogenic tumors are neoplastic lesions occurring in the jaw bone that originate in the cells involved in tooth formation. Most of odontogenic tumors are benign but similar to malignant tumors for relatively large number of local recurrence and high invasiveness toward surrounding tissues. Such tumors include ameloblastoma, which is known to progress by invading surrounding tissue as the tumor grows and develops.

Ameloblastoma was classified on the basis of the tissue structure of the tumor cells into plexiform type, follicular type, and other subtypes (1), and there have been comparative histological and clinical studies that include prognosis predictions for the different types (2, 3). However, it was pointed out that multiple tumor tissue types are often mixed together in a single ameloblastoma case (4). As there was little clinical evidence for the classification into plexiform type and follicular type, the WHO subsequently reclassified ameloblastoma into solid/multicystic type, unicystic type, extraosseous/peripheral type, and desmoplastic type (5).

However, among the histopathological studies of ameloblastoma, there are few papers that use only the updated 2005 WHO classification (6). Also, while there have been reports of immunohistochemical studies into the ability of ameloblastoma to induce stroma (7, 8) and the proliferative activity of ameloblastoma (9, 10), no conclusions regarding histological patterns and tumor activity have been reached.

The degree of malignancy of oral squamous cell carcinoma has been used grade classification by evaluation of the degree of differentiation of tumour cell nests (11, 12). There are reports that this grading is only of merit when used to evaluate deeply invasive margin (13-16).

Prognosis predictions of oral squamous cell carcinoma have therefore placed greater importance on the Jakobsson or Y-K (Yamamoto-Kohama) classification, which divides the pattern of invasion of the invasion front tumor cells into four types, and the relationship of this classification with prognosis has been reported (17-20). From this perspective, there is a need for research into the progressive front of ameloblastoma, the nature of the progression resembles that of malignant tumors, in order to predict relapse as part of the prognosis management following ameloblastoma resection. However, to date no such studies can be found.

The tumor cells of ameloblastoma histologically mimic enamel organs. Periostin is abundantly expressed in the tooth germ of mice during the growth period (21), and

the possibility of periostin being involved in tumor development has been suggested (22).

Consequently, a relationship with periostin expression may be conjectured. However, there has been very little research into periostin in relation to human odontogenic tissue or odontogenic tumors (23).

The progressive front in solid/multicystic type ameloblastoma were histopathologically classified into six types for purpose of considering a prognostic factor of the tumor. Those cell proliferation, angiogenesis, and periostin distribution pattern were immunohistochemically compared, and the significance of the original classification was inspected.

Materials and Methods

1) Clinicopathological examination

Table 1 shows the clinicopathological data of cases. For this study, 22 cases were chosen, in whom an removal and non-decalcification case of solid/multicystic type from 86 people who were given a definitive diagnosis of a benign ameloblastoma after biopsy or an removal surgery at the Nihon University Hospital at Matsudo from April, 2006 to April, 2014.

2) Histopathological examination

Specimens were immediately fixed in 10% neutral formalin solution for 24-72 h at room temperature, and then paraffin blocks were prepared according to conventional methods. The specimens used were 4-μm thin-tissue sections from paraffin blocks that were prepared at the time of histopathological examination after surgery and were stored at the Nihon University Hospital at Matsudo. The specimens were prepared using standard techniques. After deparaffinization in a xylene-alcohol series, the specimens were stained hematoxylin and eosin (HE). The histologic form of the progressive front of the solid/multicystic type ameloblastoma was reexamined by microscopic examination of HE-stained specimens of each patient. The observation point was progressive front (tumor nest closest to the normal part). The findings were classified into the original following six types: plexiform, follicular, basaloid cell, sheet, trabecular and polycystic (Table2).

3) Immunohistochemical examination

Immunohistochemical staining was performed for all specimens which preparing by the same method with histopathological examination. The specimens were prepared using standard techniques. After deparaffinization in a xylene-alcohol series, the specimens were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. The primary antibodies used to analyze proliferative activity, the matricellular protein factor, an angiogenic factor and neovascular proliferation potential were Anti-Ki-67 antibody (Ki-67), Anti-periostin antibody (periostin), Anti-vascular endothelial growth factor receptor antibody (VEGF), and Anti-CD105 antibody (CD105), respectively. Details of the antibodies and retrieval methods used are shown in Table 3. After antigen activation, each primary antibody was allowed to react at room temperature for 1 hour. The secondary antibody was ChemMATE En-

vision (DakoCytomation, Glostrup, Denmark). The chromogenic substrate was liquid DAB+ (DakoCytomation, Glostrup, Denmark). Counterstaining was performed by using Mayer's hematoxylin. After dehydration and clearing in a xylene-alcohol series, the slides were mounted. Positive findings of Ki-67, periostin and VEGF were estimated each of columnar cells and stellate reticulum-like cells in parenchyma.

In addition, oral squamous cell carcinoma, which includes a normal epithelium, was used as a positive control for each of the Ki-67, VEGF and CD105 antigens. Dental follicle was used as a positive control for periostin. For the negative control, mouse IgG1 (dilution: 1: 200, DakoCytomation, Glostrup, Denmark) was used in place of the primary anti-Ki-67, anti-VEGF or anti-CD105 antibodies, while immunoglobulin fraction (Normal Rabbit) (dilution: 1:200, DakoCytomation, Glostrup, Denmark) was used in place of the primary anti-periostin antibody. This study was conducted after obtaining patient consent, and attention was paid to maintaining patient privacy (Ethics Committee Approval No. EC14-018).

4) Quantitative morphological examination

For determination of the Ki-67 lavelling index (Ki-67 L.I.), an arbitrary field was photographed under an optical microscope at 200× magnification, and cells with nuclei that had stained dark reddish-brown with the anti-Ki-67 antibody were considered positive and counted less than 20, with image analysis software (Win ROOF Version 3.4, Mitani Corporation) . Ki-67 L.I. was calculated as the percentage of positive cells in a total of 1,000 or more analyzed cells. For determination of new blood vessels, the cell membrane of vascular endothelial cells was stained a dark reddish-brown using the anti-CD105 antibody, and vessels forming a luminal structure were examined under an optical microscope at 200× magnification. Quantitative analysis of the microvessel density (MVD) and microvessel area (MVA) was performed based on the criteria of Weidner et al. (24) using an optical microscope at 200× magnification. To determine the MVD and MVA, slides were screened and three areas with the highest number of stained microvessels (hotspots) were selected. Calculations based on the analysis of CD105-positive blood vessels were performed using Image J software (US NIH, Bethesda, MD, ver.1.48). MVD counted the number of the CD105-positive blood vessels in a microscopic field; MVA calculated it [Total area of CD105-positive blood vessels of the total area / Total area of stroma (%)] in a microscopic field.

Immunohistochemical results were assessed by combining quantitative concentration analysis, which was performed using Win ROOF Version 3.4 (Mitani Corporation) image analysis software, with microscopic observation. The protein expression level of VEGF, periostin and Ki-67 was judged as follows, +++: strong positive (20 or less), ++: moderately positive (21-50), +: weak positive (51-136), ±: very week (137-178), and -: negative (179 or more).

5) Statistical examination

Statistical analysis was performed by using SPSS 11.0J software. For Ki-67 L.I., MVD and MVA, a Kruskal-Wallis test was performed to check for significant differences in mean values between types, and a multiple comparison using the Bonferroni method was performed. The level of statistical significance was set as less than 5%.

Results

1. Clinicopathological results

The age distribution of the 22 cases of solid/multicystic type ameloblastoma used in this study was 9-71 years (mean age 43.4 years), and there were 14 males and 8 females by gender. The tumor occurred in the mandible in 19 cases and the maxilla in 3 cases.

2. Histopathological results

Figs.1 (a1, b1, c1, d1, e1, f1) shows typical HE-stained histopathological findings of six types in progressive front indicated in Table2. Details of observed six types in the progressive front of all cases were shown in Table4. In 10 cases the progressive front showed one type of histopathological characteristic, and in 12 cases the progressive front showed multiple types. The types observed in the progressive front of each of the 22 cases were plexiform type in 12 cases, follicular type in 10 cases, basaloid cell type in 7 cases, sheet type in 6 cases, trabecular type in 5 cases, and polycystic type in 5 cases. In any case, tumor cells contacting with stroma were columnar cells in all six types of the progressive front.

3. Immunohistochemical and quantitative morphological results

The immunohistochemical results are shown in Table 5 and Fig. 1.

(1) Proliferative activity shown by Ki-67

Proliferation of columnar cells was highest in basaloid cell type at 1.9% (Fig. 1-c2), followed by plexiform type (1.4%, Fig. 1-a2) and polycystic type (1.3%, Fig. 1-f2). The differences between basaloid cell type and other types; follicular, sheet, and trabecular types were significant ($p<0.05$). Proliferative activity of stellate reticulum-like cells was lower than that of columnar cells in all types of progressive front of solid/multicystic type ameloblastoma (Table5).

(2) Periostin

In plexiform type, periostin was weak to moderate positive in columnar cells and very weak to weak positive in stellate reticulum-like cells (Fig. 1-a3). In follicular type, very weak to weak positive reactions were seen in both columnar cells and stellate reticulum-like cells (Fig. 1-b3). In basaloid cell type, columnar cells were strong positive and stellate reticulum-like cells were very weak to weak positive (Fig. 1-c3).

In sheet type and trabecular type, almost all columnar cells and stellate reticulum-like cells were negative, with only a very few very weak positive reactions observed (Figs. 1-d3, e3). In polycystic type, both columnar cells and stellate reticulum-like cells showed some weak positive places (Fig. 1-f3).

(3) VEGF

In plexiform type, VEGF was moderate positive in columnar cells and weak positive in stellate reticulum-like cells (Fig. 1-a4). In follicular type, very weak positive reactions were seen in columnar cells, while stellate reticulum-like cells showed negative (Fig. 1-b4). In basaloid cell type, both columnar cells and stellate reticulum-like cells were strong positive (Fig. 1-c4). In sheet type, very weak to weak positive reactions were seen in both columnar cells and stellate reticulum-like cells (Fig. 1-d4). In trabecular type, very weak positive reactions were seen in columnar cells, while stellate reticulum-like cells were negative (Fig. 1-e4). In polycystic type, very weak to weak positive reactions were seen in columnar cells, while stellate reticulum-like cells were negative (Fig. 1-f4).

(4) Evaluation of stromal blood vessels

Immunohistochemical analysis showed that positive reactions for CD105 were observed immunohistochemically in blood vessels and also in cell cytoplasm and neoplastic odontogenic epithelial cells (Figs. 1-a5, b5, c5, d5, f5). The highest MVD value was in basaloid cell type (48.2±24.2, Fig. 1-c5), followed by plexiform type (40.4±14.1, Fig. 1-a5). Statistical testing showed that MVD was significantly greater in plexiform type than in sheet type and trabecular type, and was significantly greater in basaloid cell type than in sheet type ($p<0.05$)(Fig. 2).

The highest MVA value was also found in basaloid cell type (11.4±8.6%, Fig. 1-c5), followed by plexiform type (5.1±2.3%, Fig. 1-a5). Statistical testing showed that MVA was significantly greater in plexiform type than in follicular type, trabecular type and polycystic type, and was significantly greater in basaloid cell type than in follicular type and polycystic type ($p<0.05$) (Fig. 3).

Discussion

Ameloblastoma is a slow-developing benign epithelial tumor in which metastasis is not found. However, as it has relatively large number of recurrence, resection that includes a sufficient margin of healthy tissue is considered necessary (5). While this method conforms to the methods used with malignant tumors, there has been almost no research into the progressive front of ameloblastoma.

Classification to four types by mode of invasion in oral squamous cell carcinoma was firstly advocated by Jakobsson et al. and revised by Yamamoto et al., and reported a relationship with prognosis (17-19). Therefore, we classified the progressive front of solid/multicystic type ameloblastoma with relatively large number of local recurrence into six original types. In case of the difference was recognized by tumor cell activities among six types, this classification surmised that there was significance as a prognostic factor. The purpose of the present study was to perform a detailed investigation of the progressive front of solid/multicystic ameloblastoma.

The mean age of the 22 cases of solid/multicystic type ameloblastoma in the present study was 43.4 years (Table 1), which is consistent with the description of Joseph et al. (25). In the present study there were more male cases (14 cases, 63.6%) than female cases (8 cases, 36.4%). There are reports of no gender difference in ameloblastoma (6, 26), on the other hand, Gorlin described that the number of occurrence of ameloblastoma tends to be slightly higher in the case of males (27) . Conclusively, gender difference in ameloblastoma was under controversial. The site of lesion formation is reported to be the mandible in 80–85% of cases and the maxilla in 15–20% of cases (28); in this study, 19 cases (86.4%) were in the mandible and 3 cases (13.6%) in the maxilla, which is a similar result.

Six types (plexiform, follicular, basaloid cell, sheet, trabecular, and polycystic types) were observed histopathologically in the progressive front of the tumor of the cases in this study. Different types were found mixed together in over half of the cases, as has previously been reported (4, 29, 30) . All of the progressive front were consisted of columnar cells. Analyzes of the feature of columnar cells which composes each types were important.

Tumor cell proliferation markers are used for diagnostic and prognostic purposes (6). Ki-67 is present during the active G_1, S, G_2, and M phases of the cell cycle, and is not found during the G_0-phase (31). It is thus a valid marker for tumor proliferation (32). In the present study, tumor cell growth potential in the progressive front was

compared among six types of solid/multicystic type ameloblastoma (Table 5). Only columnar cells showed significant differences in Ki-67 L.I. among the six types of solid/multicystic type ameloblastoma ($p<0.05$), and high proliferative activity was particularly seen in columnar cells of basaloid cell type, and next was plexiform type. With all progressive front types of solid/multicystic type ameloblastoma, the proliferative activity of stellate reticulum-like cells was lower than that of columnar cells, and was consistent with prior reports (33, 34). At the same time, Hegab et al. found high positive reaction for Ki-67 in the plexiform type, and also reported that Ki-67 correlates with ameloblastoma recurrence (10). Migaldi et al. found a mean incidence of Ki-67 positive cells in ameloblastoma of 1.05% (0.4-5.8%), and reported that the period until ameloblastoma recurrence was shorter with higher Ki-67 positive rates (26).

Periostin was first confirmed as an osteoblast-specific factor 2 by Takeshita et al. in 1993 (35). Abundant expression is found in mouse tooth germ at the cap stage and the bell stage, and it disappears as the tooth develops (21). Norris et al. described periostin as an extracellular matrix protein and also proposed qualification as a matricellular protein in 2008 (36). Many types of cells are unable to proliferate if not established in the extracellular matrix (37). Also, odontogenic tumors are believed to occur due to interaction between the epithelium and the mesenchyme (5). However, there has been very little research into periostin in relation to human odontogenic tissue and odontogenic tumors (23). The present study therefore used immunohistochemical staining to clarify the expression behavior of periostin in solid/multicystic type ameloblastoma. Moderate or strong positive reaction for periostin was found in columnar cells of plexiform type and basaloid cell type. Sheet and trabecular types with little expression of periostin should be scarce in the character of ameloblasts. This means that within the tumor cell nests, periostin was probably expressed mainly in the tumor cells corresponding to ameloblasts. Chau et al. studied periostin in human tissue, and reported positive reactions in normal human pre-ameloblast and ameloblast cells, and also in the basal membrane zone of tumor nests of ameloblastic fibroma and in tumorous pre-ameloblast-like and ameloblast-like cells (23). The utility was suggested by periostin as an index of turmor cell activity in ameloblastoma because Ki-67 index were high in plexiform and basaloid cell types with periostin positiveness.

Stromal environment changes such as angiogenesis play an important part in inducing epithelium and maintaining epithelial cells. Mesenchymal cells will partici-

pate at the establishment of the epithelial stroma and maintain a lifelong relationship with the epithelial cells (38). The tumor cells themselves induce angiogenesis, as they need oxygen and nutrients in order to grow and survive. Recent discoveries have shown that hypoxia activates hypoxiainducible transcription factors (HIFs), which function as master switches to induce expression of several angiogenic factors including VEGF, nitric oxide synthase (NOS), platelet-derived growth factor(PDGF), Ang2 and others (39). Vascular endothelial growth factor (VEGF) is a growth factor that acts specifically on vascular endothelial cells, and it has the effect of promoting the angiogenesis mechanism and accelerating vascular permeability (40, 41). In the present study, columnar and stellate-reticulum-like cells of basaloid cell type showed strong positive reaction for VEGF, and columnar cells of plexiform type showed moderate positive reaction. These results resemble those of Kumamoto et al. (2). VEGF production from odontogenic epithelial cells is believed to be up-regulated by neoplastic change in epithelial cells (2). VEGF expression is in particular localized mainly in tumor cells, and is conjectured to act on vascular endothelial cells through a paracrine mechanism (40, 41).

When tumor cells proliferate, they are unable to grow beyond a diameter in the range of 2 to 3 mm if there are no new microvessels (42). Microvessel density (MVD) is an expression of angiogenesis in the tumor tissue, and can be observed by immunohistochemical staining of the endothelial cells of new blood vessels (9, 43). CD105 is a cell membrane glycoprotein that is specifically expressed in newly formed tumor vessels (44, 45). In the analysis of stromal angiogenesis by MVD and MVA using CD105, significant differences were found between different progressive front types of solid/multicystic type ameloblastoma (Fig. 2, 3). Both MVD and MVA showed the highest values in basaloid cell type, followed by plexiform type. This result correlates with the expression level of VEGF, and was consistent with prior report (9). In addition, the same trends were observed in the cell proliferation ability and in the expression level of periostin. This implies the possibility that periostin may be a valid index of tumor activity in solid/multicystic type ameloblastoma. Moreover, basaloid cell type showed significantly greater expansion of new tumor vessels than the other types of progressive front (Fig. 1-c5). The mechanism of action of VEGF is believed to vary according to the configuration of the tumor tissue (2). It may be conjectured that through the action of VEGF, basaloid cell type has a tendency toward proliferation of ameloblastoma-like tumor cells while ensuring abundant blood flow

by producing new blood vessels. The result of this study suggested that basaloid cell type was the most active in angiogenesis.

The results of the present study showed that there were differences in the proliferative activity of cells, the expression level of periostin, and angiogenesis among six types of the progressive front of solid/multicystic type ameloblastoma. Therefore, the significance which classifies the progressive front into six types was indicated to predict the prognosis after solid/multicystic type ameloblastoma resection. The careful follow up was necessary for the basaloid cell type or plexiform type existing in the progressive front of solid/multicystic type ameloblastoma. In particular, basaloid cell type suspected to have the highest tumor cell activity, because these factors showed most conspicuously. From this, it may be conjectured that with resection of solid/multicystic type ameloblastoma, it is important the prognosis observation to base judgment on the morphology of cells in the progressive front or in the vicinity of the resected surface, rather than on the morphology of cells that predominate in the tumor itself.

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2006.

Figure legend

Fig. 1. Histopathological findings of six types of the progressive front of the solid/multicystic type ameloblastoma

Plexiform type (Fig.1-a1), follicular type (Fig.1-b1), basaloid cell type (Fig.1-c1), sheet type (Fig.1-d1), trabecular type (Fig.1-e1), polycystic type (Fig.1-f1) (Original magnification: ×100).

Immunohistochemical labeling for Ki-67 (Figs.1- a2, b2, c2, d2, e2, f2), periostin (Figs.1- a3, b3, c3, d3, e3, f3), VEGF (Figs.1- a4, b4, c4, d4, e4, f4), CD105 (Figs.1- a5, b5, c5, d5, e5, f5).

Value for Ki-67 positive in columnar cells was highest in basaloid cell type (Fig.1-c2), followed by plexiform type (Fig.1-a2). Ki-67 L.I. of stellate reticulum-like cells was lower than that of columnar cells in all types (Figs.1-a2, b2, c2, d2, e2, f2) (Original magnification: ×200).

Positive periostin reaction for columnar cells was weak to moderate in plexiform type (Fig.1-a3) and strong positive in basaloid cell type (Fig.1-c3) (Original magnification: ×400).

Columnar cells showed moderate positive reaction for VEGF in plexiform type (Fig.1-a4), and both columnar and stellate reticulum-like cells were strong positive in basaloid cell type (Fig.1-c4) (Original magnification: ×400).

Lots of microvessels were observed in plexiform and basalid cell types using CD105 (Figs.1-a5, c5). Moreover, basaloid cell type showed greater expansion of new tumor vessels than the other types (Fig.1-c5) (Original magnification: ×200).

Fig. 2. Statistical result of microvessel density (MVD) in six types of the progressive front of the solid/multicystic type ameloblastoma

The highest MVD value was in basaloid cell type, followed by plexiform type. Statistical testing showed that MVD was significantly greater in plexiform type than in sheet type and trabecular type, and was significantly greater in basaloid cell type than in sheet type ($p<0.05$).

Fig. 3. Statistical result of microvessel area (MVA) in six types of the progressive front of the solid/multicystic type ameloblastoma

The highest MVA value was also found in basaloid cell type, followed by plexiform type. MVA was significantly higher in plexiform type than in follicular type, trabecular type and polycystic type, and was significantly greater in basaloid cell type than in follicular type and polycystic type ($p<0.05$), statistically.

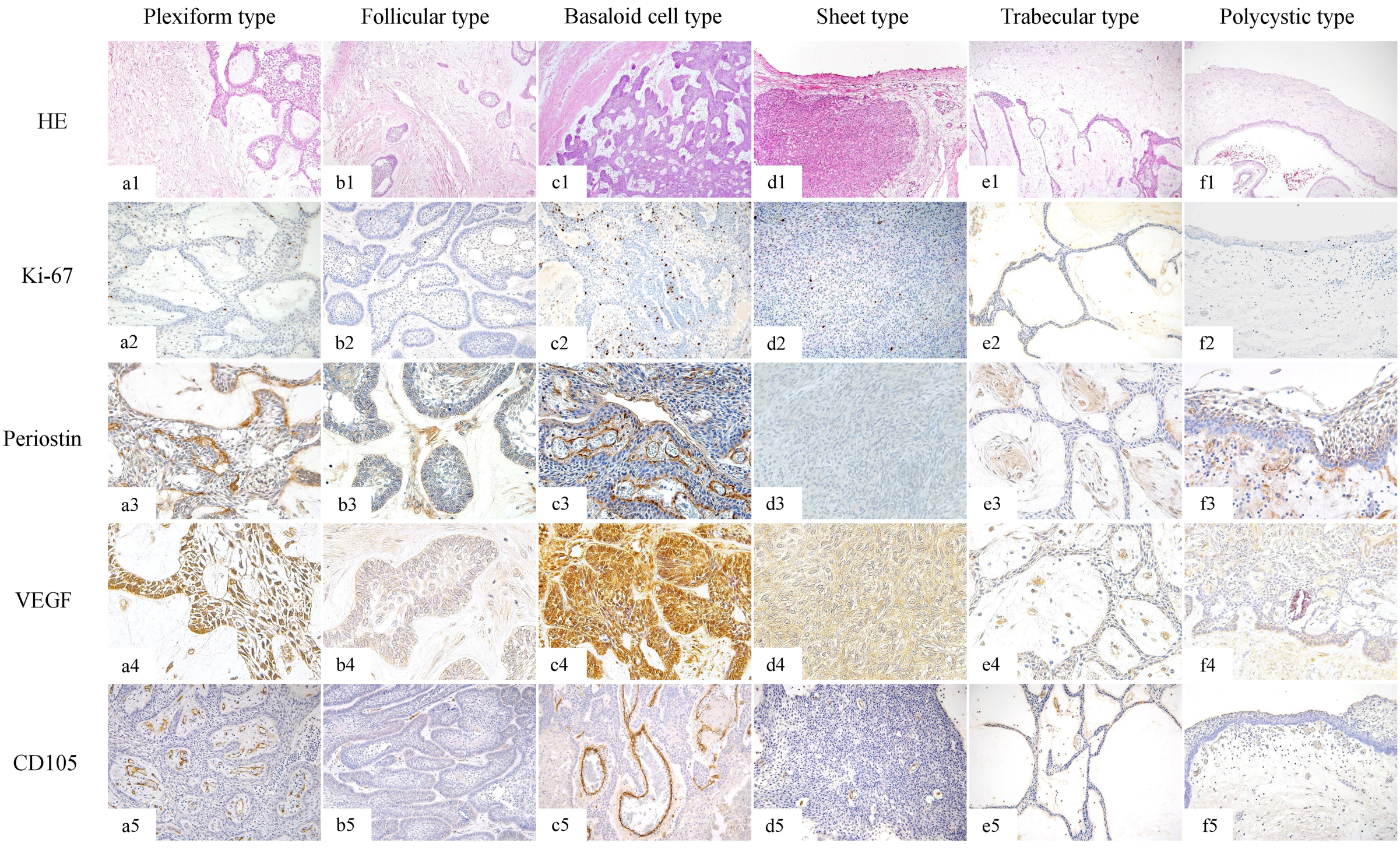
Plexiform type
Follicular type
Basaloid cell type
Sheet type
Trabecular type
Polycystic type
HE
Ki-67
Periostin
VEGF
CD105
a1
b1
c1
d1
e1
f1
a2
b2
c2
d2
e2
f2
a3
b3
c3
d3
e3
f3
a4
b4
c4
d4
e4
f4
a5
b5
c5
d5
e5
f5

Fig. 1

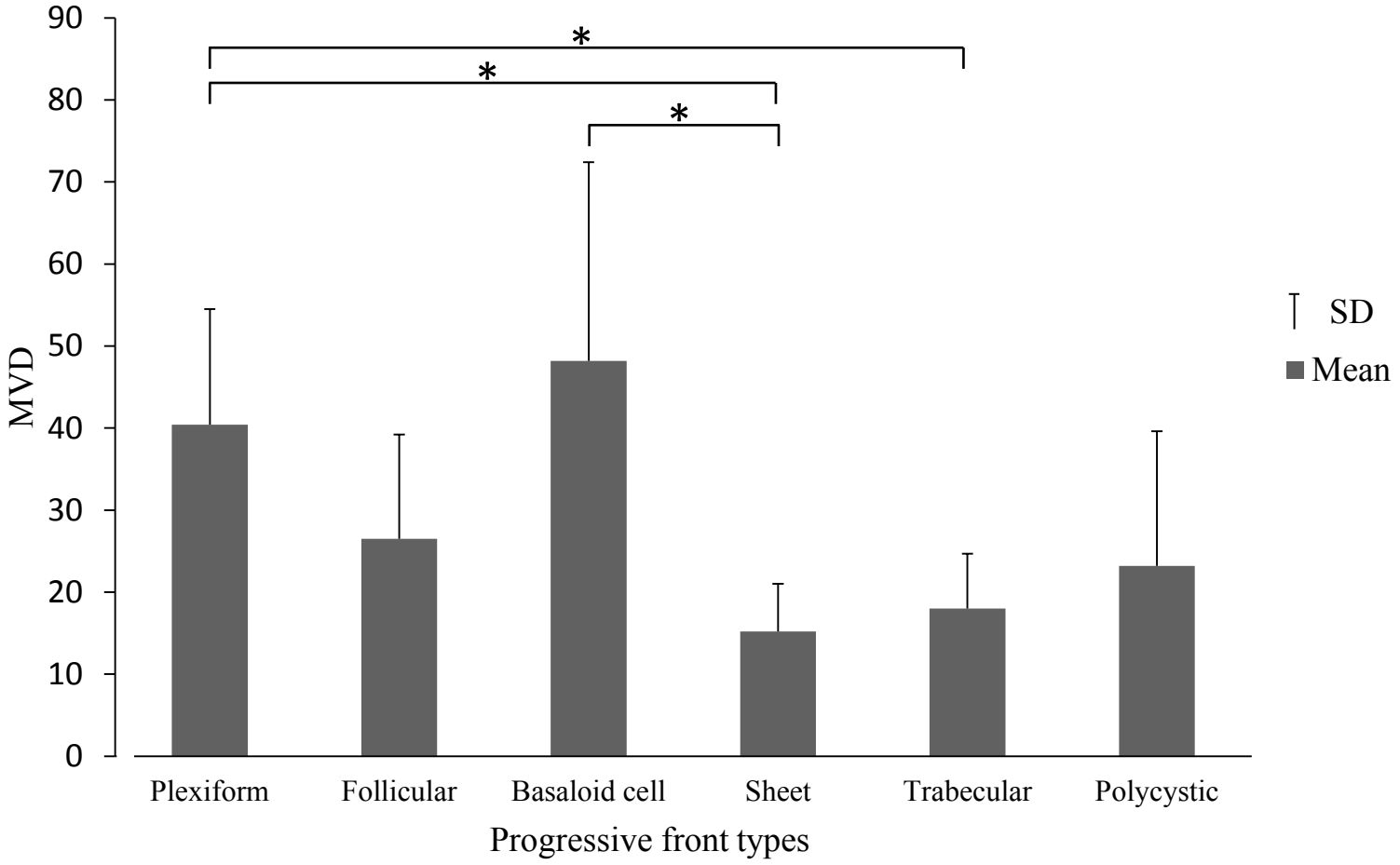
90
80
70
60
50
40
30
20
10
0
MVD
*
*
*
SD
Mean
Plexiform
Follicular
Basaloid cell
Sheet
Trabecular
Polycystic
Progressive front types

Fig. 2

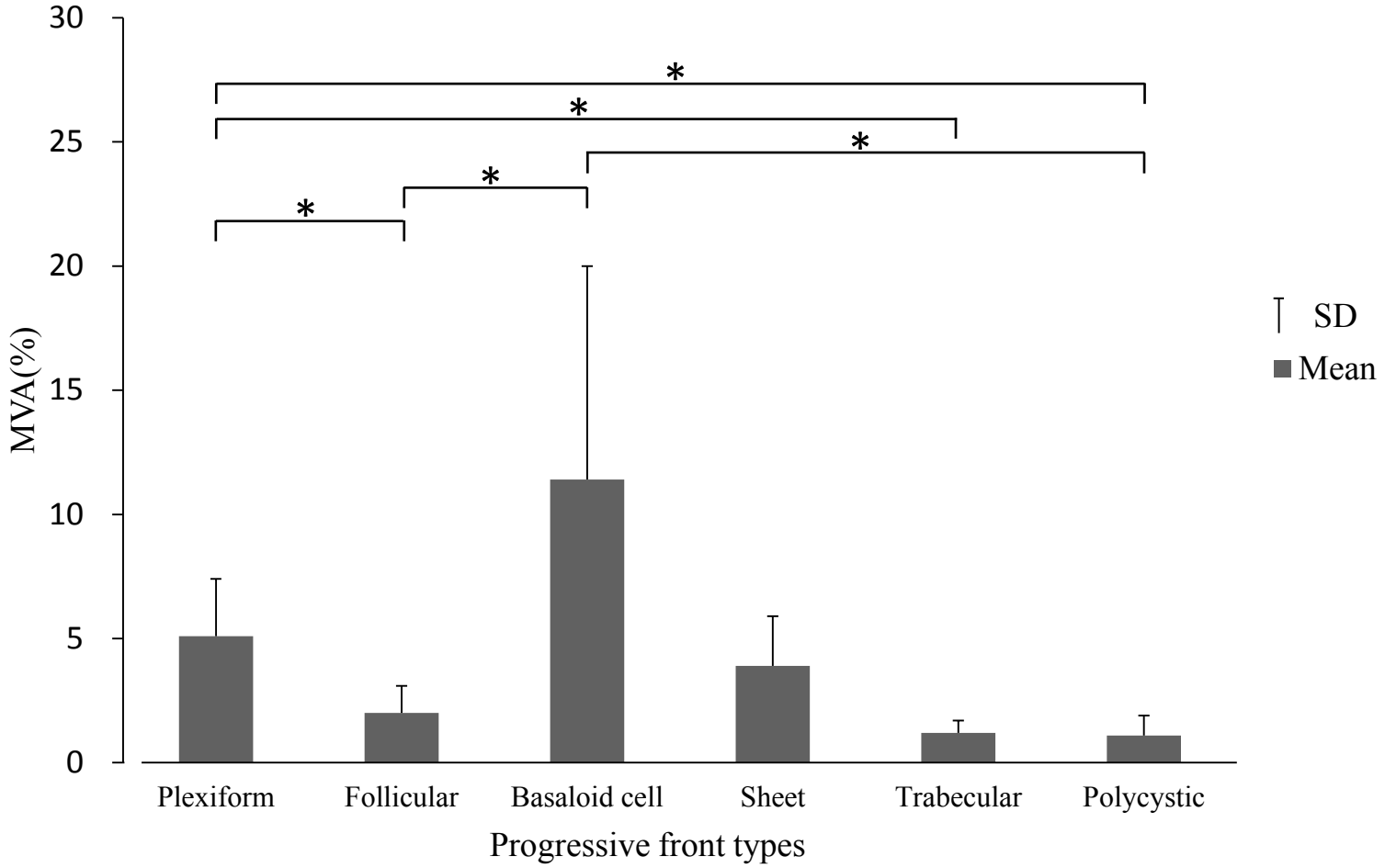
30
25
20
15
10
5
0
MVA(%)
*
*
*
*
*
SD
Mean
Plexiform
Follicular
Basaloid cell
Sheet
Trabecular
Polycystic
Progressive front types

Fig. 3

Table 1 Clinicopathological data of this study

Case No.	Age (years)	Sex	Location
1	63	M	Md
2	65	M	Md
3	47	F	Md
4	22	F	Md
5	23	F	Mx
6	22	M	Md
7	71	M	Md
8	39	F	Md
9	56	M	Md
10	67	M	Mx
11	39	F	Md
12	57	M	Md
13	24	M	Md
14	57	M	Md
15	25	F	Md
16	41	M	Md
17	9	M	Md
18	40	F	Md
19	59	M	Md
20	55	M	Mx
21	54	M	Md
22	19	F	Md

No. : number

M: male, F: female

Mx: maxilla, Md: mandible

Table 2 Histopathological characteristics of the six types in the progressive front

Progressive front types	Histopathological characteristics
Plexiform	The tumor cells proliferate to form an irregular network, and the parenchyma shows a construction similar to enamel organs
Follicular	The tumor comprises tumor nests of different sizes; where they are in contact with stroma, columnar cells are arranged in a relatively regular fashion, while the inner side comprises a sparse arrangement of stellate reticulum-like cells
Basaloid cell	Relatively small columnar cells and cuboidal cells resembling basal cells bud to proliferate into dense reticular and trabecular structures
Sheet	Columnar cells and stellate reticulum-like cells proliferate into sheets
Trabecular	The parenchyma proliferates showing a fine trabecular structure
Polycystic	Similar to the follicular type, but cyst formation is found inside all the nests

Table 3 Antibodies and retrieval methods used in the present study

Purpose	Antibody	Clone	Dilution	Company	Retrieval methods
Proliferative activity	Anti-Ki-67 antibody	MIB-1	1 : 100	Dako	Heat121℃, 5min (10mM Tris-1mM EDTA buffer pH9 .0)
Matricellular protein marker	Anti-periostin antibody	—	1 : 500	abcam	—
Angiogenic factor	Anti-VEGF antibody	VG-1	1 : 500	abcam	—
Neovascular marker	Anti-CD105 antibody	SN6h	1 : 20	Dako	Proteinase K, 5min

VEGF: vascular endothelial growth factor

Table 4 Progressive front types data of this study

Case No.	Progressive front types					
	Plexiform	Follicular	Basaloid cell	Sheet	Trabecular	Polycystic
1	○	—	—	—	—	—
2	○	—	—	—	—	—
3	—	○	—	—	—	—
4	—	—	—	○	—	—
5	○	○	—	○	—	—
6	—	○	—	—	—	—
7	—	○	—	—	—	—
8	—	○	—	—	—	○
9	—	—	○	—	○	—
10	○	○	○	—	—	—
11	—	○	—	—	—	—
12	○	—	—	○	○	—
13	○	—	○	○	○	—
14	○	—	—	—	—	—
15	○	—	○	○	○	○
16	—	—	—	—	—	○
17	○	—	○	—	—	—
18	—	—	○	○	○	○
19	○	—	—	—	—	—
20	○	○	—	—	—	—
21	—	○	○	—	—	○
22	○	○	—	—	—	—

No. : number
○: observed
—: not observed

Table 5 Results of immunohistochemical study

Progressive front types (No. of case)	Types of tumor cell	Ki-67 L. I. (%)		Periostin expression	VEGF expression	CD105			
						MVD		MVA(%)	
		Mean	SD			Mean	SD	Mean	SD
Plexiform (12)	Columnar cell	1.4*	±1.9	＋～＋＋	＋＋	40.4	±14.1	5.1	±2.3
	Stellate reticulum like cell	0.3	±0.4	±～＋	＋				
Follicular (10)	Columnar cell	0.2	±0.3	±～＋	±	26.5	±12.7	2.0	±1.1
	Stellate reticulum like cell	0.1	±0.2	±～＋	－				
Basaloid cell (7)	Columnar cell	1.9**	±1.1	＋＋＋	＋＋＋	48.2	±24.2	11.4	±8.6
	Stellate reticulum like cell	0.2	±0.6	±～＋	＋＋＋				
Sheet (6)	Columnar cell	0.1	±0.2	－～±	±～＋	15.2	±5.8	3.9	±2.0
	Stellate reticulum like cell	0.0	±0.0	－～±	±～＋				
Trabecular (5)	Columnar cell	0.2	±0.1	－～±	±	18.0	±6.7	1.2	±0.5
	Stellate reticulum like cell	0.1	±0.3	－～±	－				
Polycystic (5)	Columnar cell	1.3	±2.3	±～＋	±～＋	23.2	±16.4	1.1	±0.8
	Stellate reticulum like cell	0.7	±1.7	±～＋	－				

No. : number, SD: standard deviation, Ki-67 L. I. : Ki-67 lavelling index, VEGF: vascular endothelial growth factor, MVD: microvessel density, MVA: microvessel area
periostin and VEGF expression: (+++) strong positive, (++) moderately positive, (+) weak positive, (±) very weak, (－) negative
*: The difference between plexiform type and other types; follicular, sheet and trabecular types was significant ($P<0.05$)
**: The difference between basaloid cell type and other types; follicular, sheet and trabecular types was significant ($P<0.05$)