Poorly differentiated clusters predict a poor prognosis for external auditory canal carcinoma

Running head: Indicator of external auditory canal cancer

Masaru Miyazaki, MD[1,2], Mikiko Aoki, MD, Ph.D[1], Yasuko Okado, MD, Ph.D[1,2], Kaori Koga, MD, Ph.D[1], Makoto Hamasaki, MD, Ph.D[1], Fumiaki Kiyomi, Ph.D[4], Toshifumi Sakata, MD, Ph.D [2], Takashi Nakagawa, MD, Ph.D [3], Kazuki Nabeshima, MD, Ph.D [1]

[1]Department of Pathology, Fukuoka University Hospital and School of Medicine

[2]Department of Otorhinolaryngology, Fukuoka University Hospital and School of Medicine

[3]Department of Otorhinolaryngology, Kyushu University Hospital and School of Medicine

[4]Academia, Industry and Government Collaborative Research Institute of Translational Medicine for Life Innovation, Fukuoka University

Corresponding author: Kazuki Nabeshima, MD, Ph.D

Department of Pathology, Fukuoka University School of Medicine, 7-45-1 Nanakuma,

Jonan-ku Fukuoka-shi, Fukuoka, 814-0180, Japan

Postal codes: 814-0180

Tel: +81 (92) 801-1011; Fax: +81 (92) 863-8383

E-mail: kaznabes@fukuoka-u.ac.jp

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The significance conclusion or message (two concise sentences):

PDCs can be used to indicate prognostic subdivisions for SCC-EAC.

Multivariate analysis results indicated that high-grade PDCs were an independent poor prognostic factor.

Abstract

Background: Squamous cell carcinoma (SCC) of the external auditory canal (EAC) is rare and offers a poor prognosis; more accurate prognostic biomarkers are required. Our laboratory recently demonstrated that tumor budding, characterized by tumor cell clusters (<5 cells), and laminin 5-γ2 staining of SCC of the EAC are associated with shorter survival. However, clusters composed of ≥5 tumor cells are also found in the stroma. Previous reports of colorectal cancer suggest that poorly differentiated clusters (PDCs) are a negative prognostic indicator. Here, we report on the association between PDCs and prognosis in SCC of the EAC.

Methods: PDCs and tumor budding were histopathologically and immunohistochemically (cytokeratin AE1/AE3) analyzed in 31 cases of pre-treatment biopsy SCC of the EAC. Clusters in the stroma composed of < or ≥5 cancer cells were defined as tumor budding or PDCs, respectively. Entire tumors were initially scanned to identify greatest PDC density. Tumors with low or high PDC density were classified as low- and high-grade, respectively.

Results: Patients with high-grade PDCs had a significantly poorer outcome than those with low-grade. Even in cases of low-grade tumor budding, those with high-grade PDCs had a poor prognosis. Multivariate analysis results indicated that high-grade PDCs

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were associated with poor prognosis.

Conclusion: PDC grade can provide a more accurate prognosis than tumor budding in SCC of the EAC.

Introduction

The external auditory canal (EAC) is a small area including an outer cartilaginous portion, comprising one-third to one-half of its length, and an inner bony or osseous portion [1]. Stratified squamous epithelium on the EAC and tympanic membrane is known to be unique in its ability to migrate from the tympanic membrane outward, since this migratory phenomenon has not been observed in the squamous epithelium of other organs including skin, oral cavity, esophagus, and the uterine cervix [2, 4]. Squamous cell carcinoma (SCC) arising from the unique epithelium of the EAC is a rare, aggressive cancer with a poor prognosis [5, 6]. The current 5-year disease-specific survival of stage I, II, III, and IV are 94.8%, 78.9%, 68.3%, and 22.9%, respectively [7]. Aggressive surgery is needed for patients with advanced disease, but it also causes severe complications that impair patient quality of life [5, 8]. In order to improve these prognoses and patient quality of life, an accurate staging system that can formulate treatment strategy is needed. The revised University of Pittsburgh TNM staging system is commonly used during prognosis [9]. However, there are some patients with tumors that recur locally despite their categorization as low-risk and, conversely, some patients have longer survival times despite their categorization as high-risk [8]. For more accurate assessment of these patients with intermediate-stage tumors, a search for morphologic or

molecular biomarkers that reflect the biological characteristics of SCC-EAC and patient

outcome prediction is needed [10].

For head and neck SCC, studies have shown that it is not the differentiation grade

but the invasion pattern that is important for assessment of clinical aggressiveness and

prognosis [11, 12]. The invasion pattern classified as Grade or Score 4 represents marked

and widespread cellular dissociation in small groups and/or in single cells. More recently,

tumor budding, a specific type of invasive growth in carcinomas characterized by

invading single or small clusters of tumor cells (<5 cells) at the invasive front, has been

introduced and assessed in SCC of the oral cavity, tongue, and larynx and nasopharyngeal

carcinoma [13]. Tumor budding is only evaluated in <5 tumor cells, although clusters

composed of ≥5 tumor cells are also found. Poorly differentiated clusters (PDCs) are a

histological feature of tumor stroma defined as tumor cell clusters composed of ≥5 tumor

cells and lacking a gland-like structure. PDCs are an established negative prognostic

factor in colorectal cancer [14] and invasive breast cancer [15]. Furthermore, a high

incidence of PDCs is associated with poor prognosis and recurrence in liver, lung,

node/local recurrence, and peritoneal dissemination [14].

Previous SCC-EAC studies in the current laboratory recently demonstrated that

tumor budding and laminin 5-γ2 are associated with a shorter survival [16]. Moreover,

tumor budding helped stratify patients into more meaningful risk groups than TNM staging alone. Thereafter, however, some patients with shorter survival periods were identified in the low-grade tumor budding group. Thus, in the current study, we examined whether morphological assessment of pre-treatment biopsy samples using a grading system based on PDCs was more useful than using an observation of tumor budding for the interpretation of prognosis in SCC-EAC.

Patients and methods

Patient selection

We retrospectively reviewed clinicopathological data for 31 patients with primary SCC-EAC, for whom pretreatment tissue specimens were available, and who were treated with the same strategy [17] at the Department of Otorhinolaryngology Fukuoka University Hospital (Fukuoka, Japan) from April 2006 to December 2015. Patients who underwent chemotherapy or radiotherapy prior to biopsy, patients whose cancer specimen had no stroma, or patients who failed to follow our treatment plan, were excluded from the study. Assuming patient anonymity, biopsy samples can be used for research purposes through a standard treatment agreement with Fukuoka University Hospitals. The study protocol was approved by the Institutional Review Board (The Ethics Committee) of

Fukuoka University (No. 12-7-13) Hospital. Clinical stage was determined using the University of Pittsburgh TNM staging system modified by Hirch, 2000 [9]. Before treatment, patient disease stage was estimated by physical examination and imaging studies: computed tomography (CT) and magnetic resonance imaging (MRI). CT and MRI scans were routinely conducted every 6 months, for 3 years after therapy, and annually thereafter. The absence of any signs of enlargement of a space-occupying lesion was designated as "no evidence of disease." The follow-up period for the complete series ranged from 4 to 66 months (median, 31 months). We assessed the effect of therapy using surgical tissue specimens, according to the General Rules for Clinical Studies on Head and Neck Cancer [18].

Tissue samples and immunohistochemistry (IHC)

Biopsy specimens were fixed in 10% formalin, processed into paraffin blocks, sectioned (4-μm thickness), deparaffinized, and hydrated in descending alcohol dilutions. For anti-cytokeratin antibody staining, sections were immersed in 3% hydrogen peroxide in water for 10 min at room temperature (RT) to block endogenous peroxidase activity, and heated in 10 mM ethylenediamine-tetraacetic acid (EDTA) buffer (pH 8.0) in a microwave (700 W) for 10 min to retrieve epitopes before staining. The sections were

incubated with anti-human cytokeratin (CK) monoclonal antibody (AE1/AE3, Dako; 1:200) for 1 h at RT. Sections were then washed in Tris-buffered saline (TBS) and incubated for 30 min at RT with EnVision reagent conjugated horseradish peroxidase (Dako). Immunoreactive proteins were visualized with 3,3'-diamino-benzidine (Dako), followed by counterstaining with Mayer's hematoxylin. The stained sections were evaluated semiquantitatively by two independent pathologists who were blinded to the clinical data.

PDCs grade

In SCC-EAC, clusters surrounded by stroma and composed of ≥5 cancer cells were defined as PDCs. To quantify PDCs [14], the whole tumor was first scanned under low magnification to identify the area with most PDCs. Next, the clusters were counted within the microscopic field of a ×20 objective lens (WHK 10× ocular lens; Olympus, Tokyo, Japan). Tumors with <5, 5–9, or ≥10 PDCs were classified as grade 1, grade 2, and grade 3, respectively. Although Ueno et al. discuss that the counting of clusters composed of ≥5 cancer cells on hematoxylin and eosin (H&E)-stained sections is a sufficiently easy process [14], IHC enabled us to evaluate PDCs more accurately. For example, it was easier and more accurate in CK immunostained sections to count the number of cells

comprising tumor cell clusters and to discriminate a tumor cell cluster from adjacent single cells or clusters (Fig. 1a, b). PDCs were found in the blue-colored areas in Fig. 1c. The degree of PDCs was classified as low-grade or high-grade, corresponding to 0–4 (grade 1) or >5 clusters (grades 2, 3) in one field, respectively.

Tumor budding grade

We also evaluated tumor budding as a cancer cell nest consisting of <5 cells that infiltrated the stroma of the cancer [16, 19, 20]. After selecting an area in which tumor budding was most intensive, the buds were counted in a field under a ×20 objective lens by using both H&E stained and CK immunostained sections as described previously [16, 19] (Fig 1a, b). Tumor budding was present in the orange-colored areas of Fig. 1d. Depending on the amount of tumor budding, we defined: grade 0, no buds; grade 1, 1–4 buds; grade 2, 5–9 buds; grade 3, ≥10 buds. The degree of tumor budding was classified as low-grade or high-grade, corresponding to 0–9 (grade 0, 1, and 2) or ≥10 budding foci (grade 3) in one field, respectively.

Agreement on tumor grading based on PDCs

To determine interobserver agreement, tumor grading based on PDCs (grade 1, 2,

3) was independently judged by two pathologists (M.M., M.A.) who examined PDCs in

H&E and CK AE1/AE3 IHC for 31 cases of SCC-EAC.

Statistical analysis

The relationships between several clinicopathological parameters and the results of

PDCs assessments were evaluated using the Student t-test for a continuous variable,

Fisher's exact test for nominal variables, and the Wilcoxon test for ordinal categorical

variables. The weighted κ coefficient was used for assessing interobserver agreement in

tumor grading of PDCs. Progression-free survival curves were plotted using the Kaplan-

Meier method, and low-grade PDCs and high-grade PDCs were compared using the log-

rank test. Univariate analyses were performed for clinicopathological parameters using

the Cox regression model; then hazard ratios and their 95% confidence intervals were

estimated. Multivariate analysis (Cox proportional-hazard model) was used to determine

independent prognostic factors. A P value <0.05 was considered statistically significant.

The weighted κ coefficient was conducted with SAS Version 9.4 (SAS Institute, Cary,

North Carolina, USA); other analyses including Cox proportion-hazard model were

conducted using JMP10.0.2 for Windows.

Results

Clinicopathological parameters

The clinicopathological characteristics of the present cohort are given below: n = 32 patients (14 male, 17 female); mean age = 63 y (range: 38–86 y). The median follow-up period was 31 months (range 4–66 months). The TNM stage for the majority of tumors was stage IV [15 (48.4%)]. The proportions of patients with lymph node and distant metastases were 19.4% and 3.3%, respectively (Table 1). Lateral temporal bone resection was achieved in 11 cases, subtotal temporal bone resection was achieved in 10 cases, and 10 cases received only chemo-radiotherapy. Of the 21 (67.7%) patients who underwent surgery, 6 (28.6%) patients had postoperative local recurrence. Of these 21 patients, 18 (85.7%) had received neoadjuvant chemo-radiotherapy. The effect of therapy was classified as one case (5.6%) of grade 0, one (5.6%) of grade 1, 9 (50.0%) of grade 2, and 7 (38.9%) of grade 3.

PDCs and tumor budding in pre-treatment biopsy samples

PDCs results indicated that 7 (22.5%) patients were grade 1, 8 (25.8%) were grade 2, and 16 (51.6%) were grade 3. Seven (22.5%) patients were judged to be low-grade and 24 (77.4%) high-grade. Tumor budding results indicated that 25 (80.6%) patients were

low-grade and 6 (19.4%) high-grade. Fig. 1a–d shows examples with both a high budding

grade and high-grade PDCs. The histopathological sections (Fig. 1e–h) show examples

with low budding grade and high-grade PDCs.

Association between PDCs and clinicopathological parameters

Table 2 summarizes the association between PDCs and clinicopathological

parameters. High-grade PDCs were significantly associated with mean age ($P = 0.0262$),

T factor ($P = 0.007$), TNM stage ($P = 0.0017$), middle ear invasion ($P = 0.0124$), and

effect of therapy ($P = 0.0474$). All low-grade PDCs cases revealed no lymph node

metastasis, distant metastasis, poor differentiation, parotid gland invasion,

temporomandibular joint invasion, otic capsule invasion, pterygoid muscle invasion,

parapharyngeal space invasion, internal jugular invasion, petrous apex invasion, facial

nerve palsy, common carotid artery invasion, or middle cranial fossa invasion.

Agreement in tumor grading based on PDCs

Interobserver agreement in tumor grading was good ($\kappa = 0.888$) in the evaluation of PDCs

using CK AE1/AE3 immunostaining.

Patient survival

The high-grade PDCs group had significantly shorter survival times ($P = 0.0317$; Fig. 2a). All 6 cases with high-grade tumor budding also belonged to the high-grade PDCs group (Fig. 2b). Since the cases with low budding grade (25 cases) could be separated into low- (7 cases) and high- (18 cases) grade PDCs (Fig. 2b), survival times were compared between these two groups; high-grade PDCs were found to have a significantly shorter survival time ($P = 0.0494$; Fig. 2c). On the other hand, high tumor budding grade had shorter survival times than low tumor budding grade, but the difference was not statistically significant ($P = 0.0805$, Fig. 2d)

Univariate and multivariate analyses of clinicopathological predictors of progression-free survival (PFS) in these SCC-EAC cases were performed. Results indicated that poorly differentiated type and high PDCs grade predicted poorer PFS in the univariate analysis ($P = 0.0188$, 0.0317, respectively; Table 3). High PDCs grade was an independent prognostic factor for shorter PFS by multivariate analysis ($P = 0.0046$, Table 3).

Discussion

PDCs can be used to indicate prognostic subdivisions for SCC-EAC. Cases with

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high-grade PDCs showed significantly poorer prognosis. Multivariate analysis results

indicated that high-grade PDCs were an independent poor prognostic factor.

In the present study, the number of low-grade tumor budding cases was 25 (80.6%)

out of 31 cases in the cohort. Because this group also contained some poor prognostic

cases, we searched for a new prognostic indicator. PDCs subdivided these cases into low-

grade and high-grade PDCs cases; the high-grade PDCs had a significantly shorter PFS.

The patients with both low-grade tumor budding and low-grade PDCs had significantly

longer survival, with no progression during the follow up time (Fig. 2c). All these cases

revealed no lymph node metastasis, distant metastasis, or invasion of the parotid gland,

temporomandibular joint, otic capsule, pterygoid muscle, parapharyngeal space, internal

jugular vein, petrous apex, facial nerve, common carotid artery, and middle cranial fossa

(Table 2). We previously reported that low tumor budding grade enabled us to sort patient

groups into those with a better prognosis, even in cases of advanced stage disease (stage

III and IV). Thus, we examined whether PDCs could be used to further subdivide the

advanced stage patient group with low tumor budding grade into those with low- and

high-grade PDCs; differences were not statistically significant, but those patients with a

more favorable prognosis could be selected (Fig. 2e). PDCs can be a more sensitive and

useful prognostic factor for SCC-EAC than tumor budding, although both are significant

prognostic factors. Particularly with low-grade budding, PDCs grade can separate

prognoses with greater precision, although both PDCs and tumor budding are significant

prognostic factors for SCC-EAC. Fig. 3 demonstrates the association between budding

and PDCs grade using simple illustrations. Fig. 3a highlights the cases with both a high

budding grade and high-grade PDCs. In the present study, our cohort included many cases

with both a low budding grade and high-grade PDCs (Fig. 3b, Fig. 1e–f) and we

demonstrated the importance of evaluating both types.

Patterns of cancer cell invasion include single-cell migration, multicellular

streaming, collective cell migration, tissue folding, and expansive growth [14]. Collective

cell migration occurs when 2 or more cells that retain their cell-cell junctions move

together through a three-dimensional interstitial tissue scaffold [22,26]. Strong cell-cell

junctions allow direct force transmission between cells and facilitate supracellular

coordination of the cytoskeleton to maintain cohesion and collective invasion [21,27,28].

Collective cell migration takes place either when cell movement remains connected to the

primary tumor or when cells detach in groups or clusters [29-31]. We surmised that PDCs

may be associated with collective cell migration. A previous report, based on electron

microscopy and immunohistochemistry, demonstrates that tumor budding in isolation in

one plane of a section can be traced to the neighboring neoplasm at the invasive margin

[32]. Furthermore, tumor budding and PDCs may form a sequential structure. The ability to evaluate morphological features associated with prognosis in one tissue section is simple and effective. Therefore, the observation of PDCs may allow such prognoses to be applied in a clinical setting.

As for the invasive mechanisms of PDCs, we statistically analyzed the association between PDCs and laminin 5-ɤ2 because laminin 5-ɤ2 was associated with tumor budding in our previous report [16]. However, no association was found with these indicators (data not shown).

Associations between morphological tumor grading and prognoses have been investigated in the field of head and neck SCC. Broder's system is based on the proportion of differentiated cells to undifferentiated or anaplastic cells [33-35], and this classification has been used in evaluation of prognosis and choice of treatment. A grading system concerning the histologic relationship of the neoplasm to the surrounding host tissue was developed for laryngeal carcinoma [36]. PDCs rate similarly to grade 2 (cords, less marked borderline) or grade 3 (groups of cells; no distinct borderline) in the latter manuscript in mode of invasion. Moreover, the grading system is modified for application with oral squamous cell carcinoma [37]. PDCs may correspond predominantly to score 2 (infiltrating, solid cords, bands and/or strands) or score 3 [small groups or cords of

infiltrating cells (n>15)] in patterns of invasion in the grading system. These grading

systems are associated with poor prognosis [38]. The category of having a cohesive or

non-cohesive invasive edge of auricular squamous cell carcinoma has also been reported

as a prognostic factor for the development of lymph node metastasis [39]. Compared with

these grading systems, the evaluation methods for tumor budding or PDCs is more

concrete and simple. More recently, tumor budding activity (BA) and cell nest size (CNS)

have been shown to determine patient outcome in oral SCC [40]. The latter authors

proposed a grading system by combining both a score for BA and one for CNS. CNS was

classified based on the size of the smallest invasive cell nest, which is quite different from

the evaluation method for PDCs in our study. In evaluations for PDCs, the number of

invading tumor cell nests composed of ≥5 cells was evaluated even in the presence of

single invading cells. It is suggested that the evaluation of PDCs is simpler and easier and

is significantly correlated with prognosis in SCC-EAC.

Reproducibility is important for evaluation methods regarding the mode of invasion

described above. We compared the agreement coefficient of the evaluation methods based

on past reports. Broder's system does not give a stable agreement (κ coefficient: 0.38–

0.69) [41]. A modification of Jakobbson's malignancy grading system was created for use

with carcinoma of the uterine cervix. Agreement of this grading system is also poor (κ

coefficient：0.54) [42]. The grading system for the invasive parts of oral SCC as defined

by Bryne et al. has good interobserver agreement (κ coefficient: 0.63) [37], although its

initial agreement had a low value (κ coefficient: 0.44) [43]. Compared to these evaluation

methods regarding the invasive morphology of tumors, the agreement of the evaluation

of tumor budding using IHC is high (κ coefficient: 0.874) [44]. The κ coefficient for

interobserver agreement of tumor grading based on PDCs may be as high as 0.735 [45].

In the present study, interobserver agreement in PDCs grading was excellent (κ = 0.888)

when using IHC for CK AE1/AE3. Thus, the grading of PDCs in SCC-EAC biopsies by

using CK AE1/AE3 can offer improved objectivity and higher reproducibility.

This study was not free of limitations. Due to the rarity of SCC-EAC the analyzed

sample included only 31 cases. Surgical specimens were not available for our use because

preoperative chemoradiotherapy is necessary in external auditory canal carcinoma

patients. For the study, we had access to only a limited number of pretreatment biopsy

samples; these were small in size and collected from the narrow site of the external

auditory canal. Greater reliability would be achieved from the study of a greater number

of biopsy samples. Despite these limitations, this study had two noteworthy strengths.

Firstly, the study was solely conducted by one surgeon, T. Nakagawa, and thus eliminated

bias due to operational procedure. Secondly, histopathological findings from pretreatment

biopsy samples were associated with prognoses in SCC-EAC.

In conclusion, high-grade PDCs in biopsy sample were an independent poor

prognostic factor for SCC of the EAC. PDCs grade can provide a more accurate prognosis

than budding grade, and can be an indicator that allows subdivision of prognosis in SCC-

EAC.

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differentiated clusters in preoperative biopsy predicts nodal involvement and pTNM

stage in colorectal cancer patients. Hum Pathol. 2014;45: 268-275.

Figure legends

Fig. 1 Histopathology using hematoxylin and eosin (H&E) stains (**a, e**), and immunostaining of cytokeratin AE1/AE3 (**b, c, d, f, g, h**) at the stroma of squamous cell carcinoma of the external auditory canal. **c, g** PDCs are shown in blue. **d, h** Tumor budding is shown in orange

Fig. 2 Patient survival data analyses (**a, c, d**) and the categories for budding and poorly differentiated clusters (PDCs) grades (**b**) in this study: **a** The patient group with high-grade PDCs had significantly shorter survival times ($P = 0.0317$). **b** Six cases with high-grade tumor budding were included in the high-grade poorly differentiated clusters (PDCs) group. Low- and high-grade PDCs were also compared with 25 cases of low-grade tumor budding. **c** Low- and high-grade PDCs were also compared with 25 cases of low-grade tumor budding. High-grade PDCs had significantly shorter survival times ($P = 0.0494$). **d** The patient group with high-grade tumor budding had shorter survival times than that with low-grade tumor budding; the difference was not statistically significant ($P = 0.0805$). **e** Low- and high-grade poorly differentiated clusters (PDCs) were compared with 16 advanced cases (stage III and IV) with low-grade tumor budding. Any observed differences were not statistically significant, but patients with more

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favorable prognoses could be selected.

Fig. 3 Illustrations of poorly differentiated clusters (PDCs) and budding grade: **a** an example of high-grade budding and high-grade PDCs. PDCs are shown in blue. Tumor budding is shown in orange. **b** An example of low-grade budding and high-grade PDCs. **c** An example of low-grade budding and low-grade PDCs
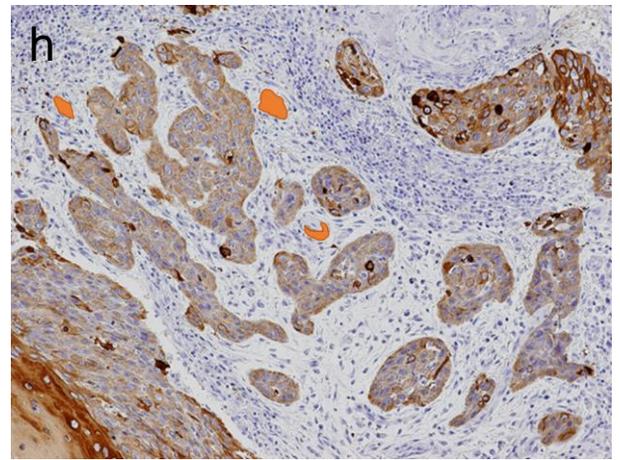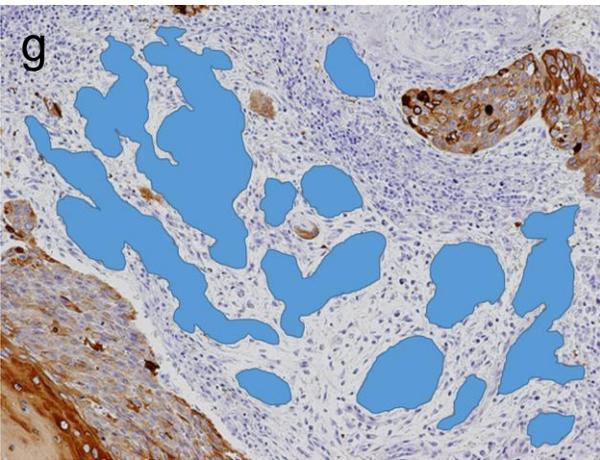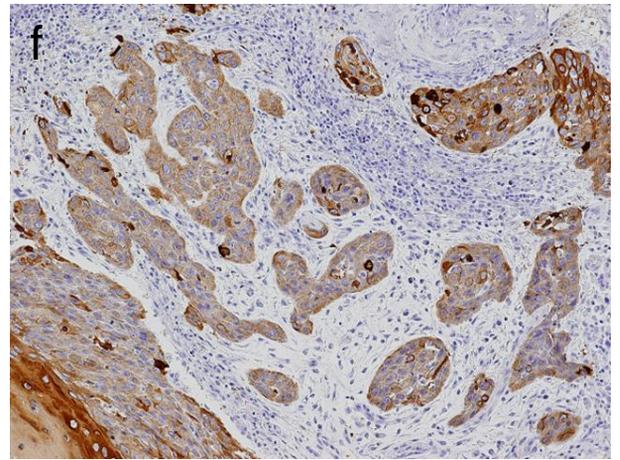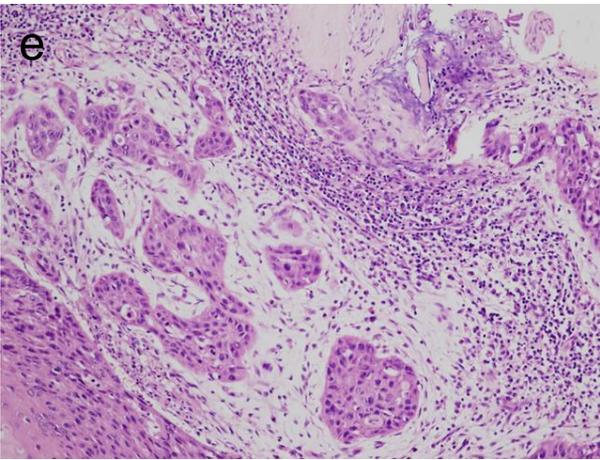
Fig. 1
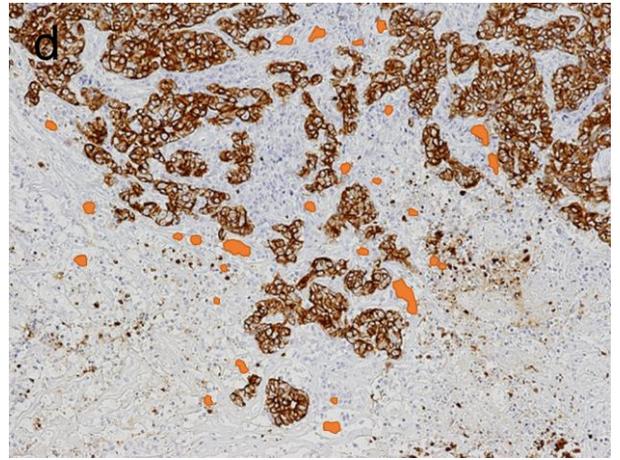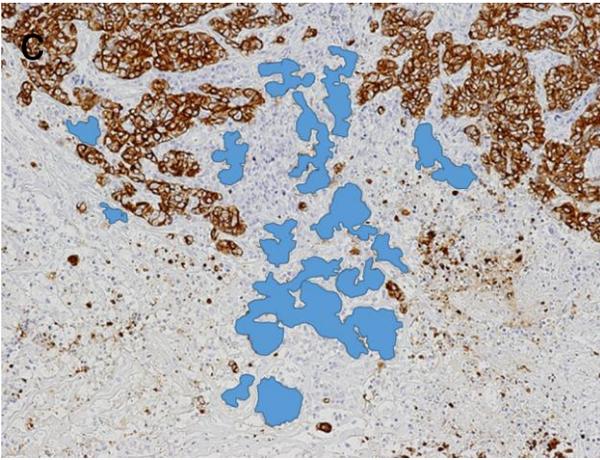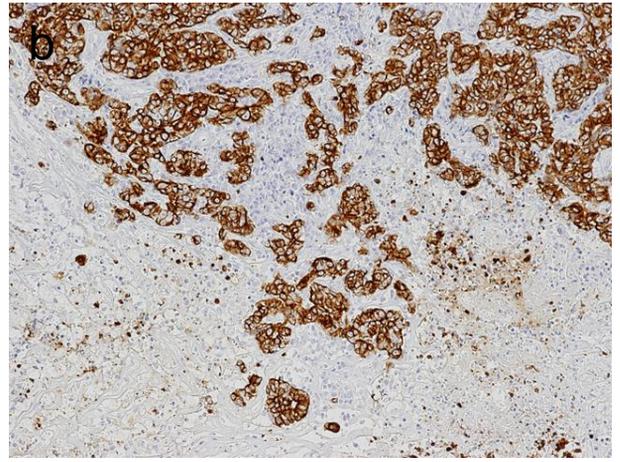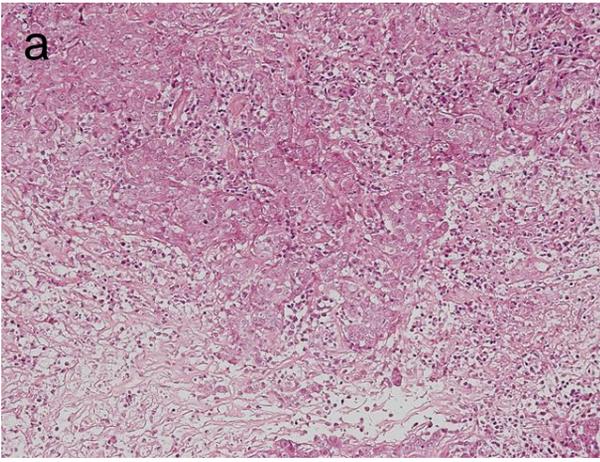
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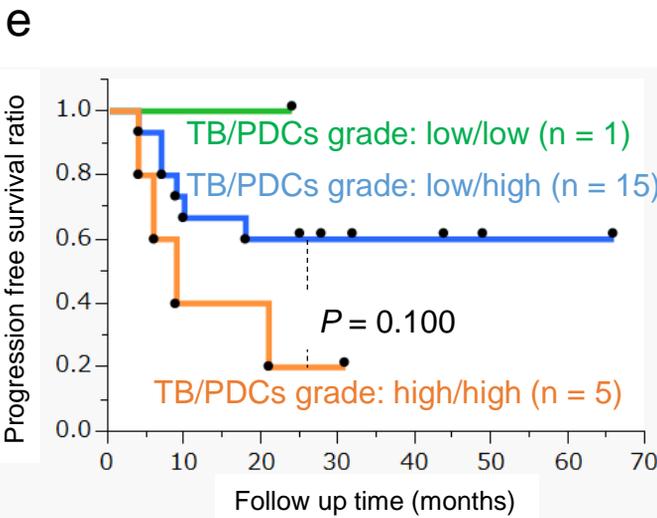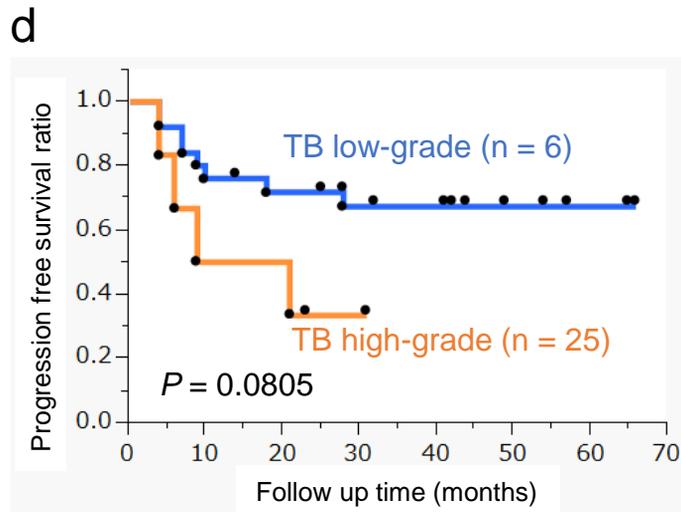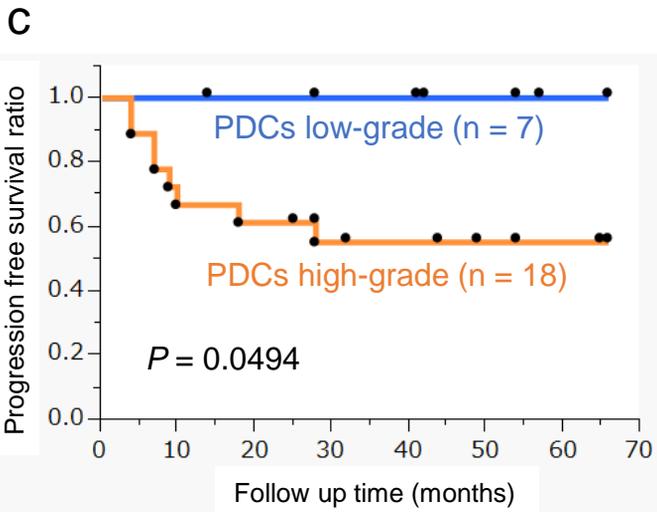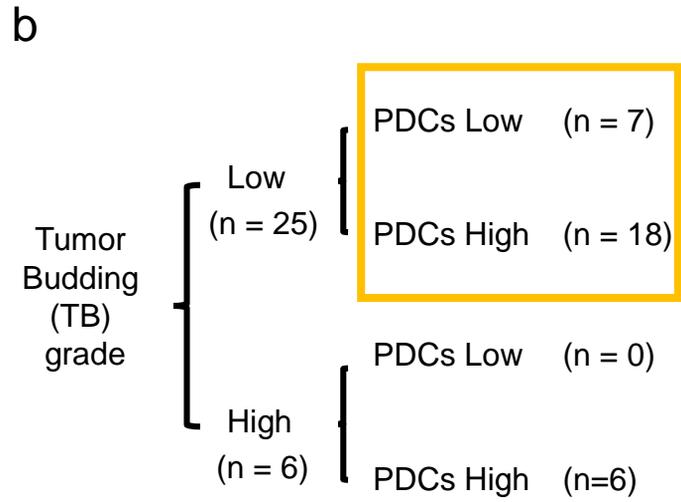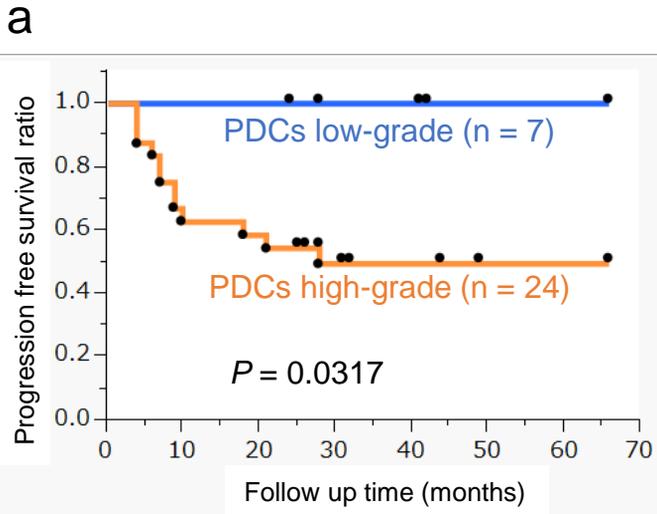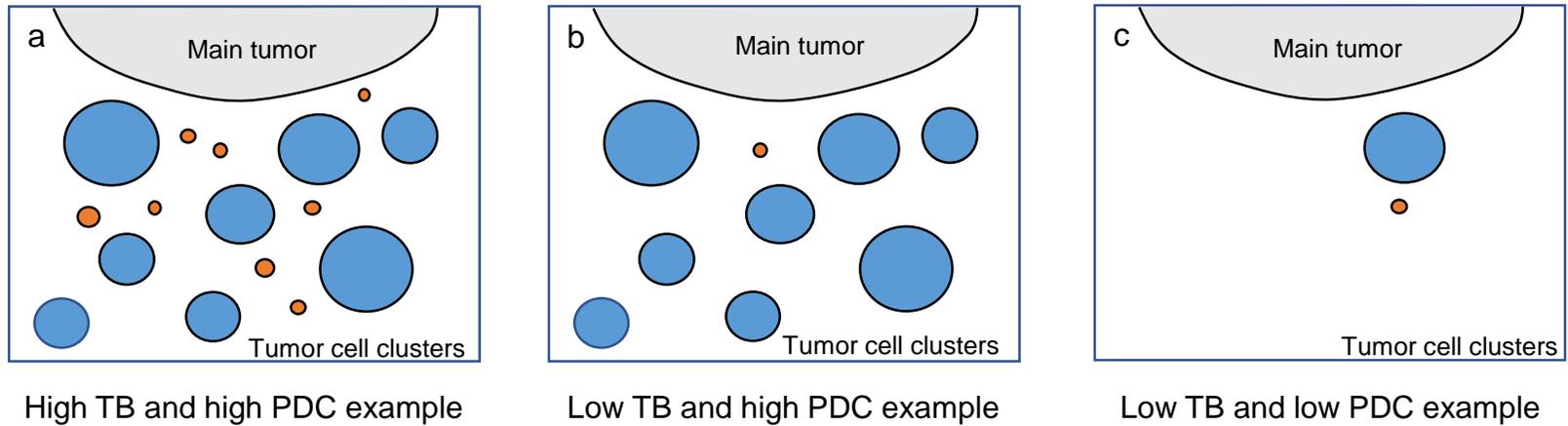
a

b

c

d

e

Fig. 3

Table 1 Patient clinicopathological details

Clinicopathological details	No. of cases or year	Percentage of cases (%)
Patients	31	
Gender		
Female	17	54.8
Male	14	45.2
Age (year)		
Mean	63	
Range	38–86	
Tumor differentiation		
Well	21	67.7
Moderate	7	22.6
Poor	3	9.7
T (primary tumor) *		
T1	1	3.2
T2	11	35.5
T3	4	12.9
T4	15	48.4
N (regional lymph nodes) *		
N0	25	80.6
N1	6	19.4
M (distant metastasis) *		
M0	30	96.7
M1	1	3.3
TNM stage *		
I	1	3.2
II	9	29.0
III	6	19.4
IV	15	48.4

*The staging protocol proposed by the University of Pittsburgh (modified by Hirsh, 2000)

Table 2 Clinicopathological features in relation to poorly differentiated clusters

		Poorly differentiated clusters		
		Low-grade	High-grade	*P*-value
		7 (22.5%)	24 (77.4%)	
Mean age [year, (s. d.)]		71.1 (± 6.5)	60.6 (± 11.2)	0.0262*
M:F		4:3	10: 14	0.671
T	T1			
	T2	6 (85.7%)	6 (25.0%)	
	T3			
	T4	1 (14.3%)	18 (75.0%)	0.007*
N	N0	7 (100%)	18 (75.0%)	
	N+	0 (0%)	6 (25.0%)	0.293
M	M0	7 (100%)	23 (95.8%)	
	M+	0 (0%)	1 (4.2%)	1.000
TNM stage				
	Stage I			
	Stage II	6 (85.7%)	4 (16.7%)	
	Stage III			
	Stage IV	1 (14.3%)	20 (83.3%)	0.0017*
Differentiation				
	Well	6 (85.7%)	15 (62.5%)	
	Moderate	1 (14.3%)	6 (25.0%)	
	Poor	0 (0%)	3 (12.5%)	0.239
Middle ear invasion				
	Absent	6 (85.7%)	7 (29.2%)	
	Present	1 (14.3%)	17 (70.8%)	0.0124*
Parotid gland invasion				
	Absent	7 (100%)	14 (58.3%)	
	Present	0 (0%)	10 (41.7%)	0.0661
Temporomandibular joint invasion				
	Absent	7 (100%)	15 (62.5%)	
	Present	0 (0%)	9 (37.5%)	0.0766
Otic capsule invasion				
	Absent	7 (100%)	14 (58.3%)	
	Present	0 (0%)	10 (41.7%)	0.0661
Pterygoid muscle invasion				

	Absent	7 (100%)	22 (91.7%)	
	Present	0 (0%)	2 (8.3%)	1.000
Parapharyngeal space invasion				
	Absent	7 (100%)	16 (66.7%)	
	Present	0 (0%)	8 (33.3%)	0.146
Internal jugular invasion				
	Absent	7 (100%)	20 (83.3%)	
	Present	0 (0%)	4 (16.7%)	0.550
Petrous apex invasion				
	Absent	7 (100%)	19 (79.2%)	
	Present	0 (0%)	5 (20.8%)	0.562
Facial nerve palsy				
	Absent	7 (100%)	21 (87.5%)	
	Present	0 (0%)	3 (12.5%)	1.000
Common carotid artery				
	Absent	7 (100)	20 (83.3%)	
	Present	0 (0%)	4 (16.7%)	0.550
Middle cranial fossa invasion				
	Absent	7 (100%)	15 (62.5%)	
	Present	0 (0%)	9 (37.5%)	0.0766
Posterior cranial fossa invasion				
	Absent	4 (80.0%)	22 (84.6%)	
	Present	1 (20.0%)	4 (15.4%)	1.000
Recurrence				
	Free	7 (100%)	8 (57.1%)	
	Recurrence	0 (0%)	6 (42.9%)	0.0609
Effect of therapy				
	Grade 0-2	1 (20%)	10 (76.9%)	
	Grade 3	4 (80%)	3 (23.1%)	0.0474*

P-value: Student t-test for a continuous variable, Fisher's exact test for nominal variables, and the Wilcoxon test for ordinal categorical variables

Table 3 Univariate and multivariate analysis of factors affecting progression-free survival (PFS)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio [1]	95% Cl [1]	P-value [2]	Hazard ratio[1]	95% Cl[1]	P-value[1]
Age≧70 years	1.78	0.525 - 5.57	0.313	4.19	0.970 - 18.2	0.0547
Gender (female)	1.37	0.427 - 4.37	0.582	1.12	0.303 - 4.00	0.861
Poorly differentiated type	4.23	0.922 - 14.7	0.0188	1.73	0.365 - 6.40	0.454
Tumor budding high grade	2.77	0.733 - 8.92	0.0805	2.71	0.639 - 10.5	0.166
PDCs high grade[3]	$2.24 \times e^9$	2.15 - -	0.0317	$1.74 \times e^9$	$2.60 - 1.68 \times e^{20}$	0.0046

PDCs, poorly differentiated clusters

[1]: Estimated using Cox regression based on likelihood ratio test.

[2]: Log-rank test

[3]: Upper limited CI and hazard ratios were large figures and could not be estimated appropriately/numerically, because there were no fatal cases in low grade PDC.

The final publication is available at link.springer.com.