Usefulness of immunohistochemistry to distinguish between secretory carcinoma and acinic cell carcinoma in the salivary gland

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Abstract

Secretory carcinoma (SC) of the salivary gland is a relatively newly described disease, separate from acinic cell carcinoma (ACC), which frequently displays ETV6-NTRK3 gene fusion. However, the differences between SC and ACC remain unclear. Here, histological reevaluation of 12 formerly diagnosed ACC cases was performed, which yielded a new diagnosis of SC in 4 cases due to a lack of obvious acinar-like cells. Immunohistochemically, phosphorylated signal transducer and activator of transcription 5 (p-STAT5) was expressed in SC but not in ACC, whereas discovered on GIST-1 (DOG1) was expressed in ACC but not in SC. Molecular analysis was possible in three SC cases, of which two showed the ETV6-NTRK3 fusion transcript on reverse-transcription polymerase chain reaction, as well as breaks in the ETV6 gene on fluorescence in situ hybridization. However, the remaining SC cases did not show this fusion transcript. Recently, several reports have suggested that SC might not be adequately diagnosed if the focus is placed solely on the ETV6-NTRK3 fusion gene due to genetic diversity. In this regard, immunohistochemistry of p-STAT5 and DOG1 is expected to be a useful alternative diagnostic tool to discriminate SC from ACC.

Introduction

Salivary gland carcinomas are rare tumors that comprise multiple histologic types, with mucoepidermoid carcinoma being the most frequent, followed by adenoid cystic carcinoma and acinic cell carcinoma (ACC) [1]. Secretory carcinoma (SC) of the salivary gland is a relatively new disease concept separate from ACC. Similar to breast cancer, SC harbors the ETV6-NTRK3 fusion gene, which is not present in other salivary gland tumors and is therefore unique to SC. Microscopically, cases formerly diagnosed with ACC may have a revised diagnosis of SC if tumor cells show a microcystic, follicular, or papillary-cystic pattern [2, 3]. Recently, several studies have suggested that SC might not be adequately diagnosed due to the sole focus on the ETV6-NTRK3 fusion gene for diagnosis, as SC shows greater genetic diversity than previously thought [4-9]. Therefore, immunohistochemistry may be an alternative method to diagnose SC. However, the immunohistochemical profile of SC has not been fully determined. Baghai et al. reported that intense and diffuse positivity for mammaglobin and S100 was detected in SC [10]. SC also tends to have variable expression levels of pan-cytokeratin, STAT5a, and GCDFP15 and is usually negative for DOG-1 and basal and myoepithelial cell markers, such as p63. Bishop et al. [11] and Kawahara et al. [12] reported that mammaglobin is a highly sensitive marker for SC but lacks sufficient specificity.

In the present study, we reviewed 12 cases formerly diagnosed with ACC. Of these, four were given a new diagnosis of SC based on their histological features using immunohistochemistry and genetic techniques to clarify the pathologic differences between SC and ACC.

Materials and methods

Patients

This study enrolled 12 patients formerly diagnosed with ACC during 2005–2014 at Osaka International Cancer Institute and Osaka University Hospital. The clinical findings are shown in Table 1. These 12 patients comprised 7 females and 5 males, and the mean age was 54.1 years (range, 15–85 years). The tumors of all 12 cases occurred in the parotid gland. This study was approved by the Ethical Review Board of Osaka International Cancer Institute (No. 20025) and that of Osaka University Hospital (No. 20038). This study was performed in accordance with both Committee guidelines and regulations.

Immunohistochemistry

All tissue samples were fixed in 10% formalin, embedded in paraffin, cut into 4-μm-thick serial sections, and used for hematoxylin and eosin and immunohistochemical staining. Immunohistochemical staining was performed using the Roche BenchMark ULTRA IHC/ISH Staining Module (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. The primary antigens and dilution ratios used are presented in Table 2. Immunohistochemical staining was scored by two independent pathologists (Y.H. and H.H.).

Reverse-transcription (RT) polymerase chain reaction (RT-PCR)

Formalin-fixed paraffin-embedded specimens were deparaffinized and lysed using proteinase K. Total RNA was extracted using RNAiso Blood extraction reagent (Takara Bio Inc., Kusatsu, Shiga, Japan). Crude RNA was treated with recombinant DNaseI (Roche Applied Science, Penzberg, Upper Bavaria, Germany) and re-purified using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The RT reaction was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd, Osaka, Japan). The RT products were diluted fivefold with Tris-EDTA buffer (pH 8.0). The 10-μL PCR mixture was composed of 1 μL diluted RT product, 5 pmol each of forward and reverse primers, 1× PCR buffer, and 0.2 U KOD -Multi & Epi- DNA polymerase (Toyobo Co., Ltd). The primer sequences (5' to 3') were as follows: ETV6, ACCACATCATGGTCTCTGTCTCCC; and NTRK3, CAGTTCTCGCTTCAGCACGATG. PCR was performed using the T-100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following parameters: 94°C for 2 minutes, 40 cycles of 98°C for 15 seconds, 58.8°C for 15 seconds, and 68°C for 15 seconds. The products were analyzed by microchip electrophoresis using Shimadzu MCE-202 MultiNA (Shimadzu Corporation, Kyoto, Japan) and the DNA-500 Reagent kit for MultiNA. A “gel-like” image was produced based on electropherograms using the MultiNA Viewer software. One case diagnosed as SC from another hospital was used as a positive control.

Fluorescence in situ hybridization analysis (FISH)

FISH analysis was performed on paraffin sections using the Vysis LSI ETV6 Dual Color Break Apart

Rearrangement Probe (Abbott Molecular Inc., Des Plaines, IL, USA), using the BenchMark ULTRA IHC/ISH Staining Module. The “U FISH Open Probe v2 (v1.00.0000)” procedure was used. Briefly, deparaffinization, heat treatment, proteolysis, denaturation, hybridization, and washing were performed.

Results

Microscopic findings

The histology of the 12 formerly diagnosed ACC cases was examined microscopically (Table 1). In 8 of the 12 cases (cases 1–8), a solid (Fig. 1a), microcystic (Fig. 1b), or follicular pattern (Fig. 1c) was seen in tumor cells, with an eosinophilic or basophilic cytoplasm and small round nuclei with slight atypia. Because acinar-like cells were observed [3], cases 1–8 were confirmed to be ACC. The tumors from the remaining four cases (cases 9–12) did not contain obvious acinar-like cells and were composed mainly of cells with microcystic (Fig. 2a), papillary or hobnail (Fig. 2b), and follicular (Fig. 2c) patterns. These tumor cells showed eosinophilic and focally vacuolated cytoplasms with swollen nuclei, apparent nucleoli, and a small number of mitotic figures. Intrafollicular colloid-like secretions were seen in the follicular component (Fig. 2c, 2d). Based on these findings, we considered cases 9–12 to be SC. In case 11, perineural invasion and vascular invasion were present. In case 10, a solid and trabecular pattern was seen adjacent to the conventional papillary pattern (Fig. 2e, 2f, 2g). Moreover, colloid-like secretions were not apparent, and many tumor cells with eosinophilic cytoplasm showed marked swelling and irregularly

shaped nuclei, and mitotic figures were frequent throughout the solid and trabecular pattern. Taken together, these findings suggest that case 10 was in a state of high-grade transformation.

Clinical findings of the ACC and SC cases

The eight ACC cases (cases 1–8) were from six female and two male patients (Table 1), and the mean age of these eight patients was 59.8 years (range 32–85 years). In contrast, the four SC cases (cases 9–12) were from one female and three male patients (Table 1), whose mean age was 42.8 years (range 15–60 years).

Immunohistochemistry

The immunohistochemical results of the 12 cases are shown in Table 3. The four SC cases, but not the eight ACC cases, were positive for p-STAT5. The tumor cells of the high-grade transformed SC case also showed positive staining for p-STAT5 (Fig. 3). Focal and weak p-STAT5 staining was seen in the nucleus of SC regardless of morphological pattern in case 11. In contrast, the eight ACC cases, but not the four SC cases, were positive for DOG1 (Fig. 3). The tumor cells of the ACC cases generally showed strong DOG1 staining intensity on the apical side, but strong cytoplasmic staining was detected in some ACC cases, especially in areas with a solid pattern. In contrast to the p-STAT5 and DOG1 staining patterns, the staining patterns of S100, mammaglobin, GATA3, GCDFP15, and p53 were not different between the SC and ACC cases (Table 3). The MIB-1 labeling index was comparable between the ACC and SC cases, but that in the high-grade transformed SC (case 10) showed a higher index (Table 3 and Fig. 3).

Molecular analyses

The results of RT-PCR and FISH are shown in Table 4. Molecular analysis was possible in three SC cases. Among them, two cases (cases 9 and 12) showed the ETV6-NTRK3 fusion transcript on RT-PCR (Fig. 4) and breaks in the ETV6 gene on FISH (Fig. 5).

Discussion

SC of the salivary gland is a relatively newly described disease, which was initially listed in the fourth edition of the World Health Organization (WHO) classification (2017) [2]. The term “mammary analogue secretory carcinoma (MASC)” was widely used before SC was defined. Originally, MASC referred to a disease spectrum absent of the clear serous acinar-like cells seen in ACC. As it was demonstrated that MASC harbors a chromosomal translocation t(12;15) (p13;q25) resulting in ETV6-NTRK3 fusion, similar to breast cancer, MASC was defined as a separate entity from ACC.

Although the frequency of SC varies among hospitals, SC may account for up to one-third to one-half of the tumors conventionally considered ACC. ACC is one of the major salivary gland carcinomas [1, 13], which are rare tumors, whereas SC is not rarer than expected.

In this study, the ACC cases showed a female predominance (6 female vs. 2 male patients), whereas the

SC cases showed slight male predominance (1 female vs. 3 male patients). The ACC (mean age, 59.8 years) and SC (mean age, 42.8 years) patients were mainly adults. These results accord with those of previous studies [2, 14, 15].

The high-grade SC case (case 10) in our analysis showed a similar morphology to the cases reported by Skalova et al. [16] and to a case reported by Luo et al. [17]. Salivary gland carcinomas with high-grade transformation include areas of high-grade morphology next to conventional carcinoma areas, and there may not be an obvious delineation between these areas [18]. High-grade SC transformation is associated with adverse clinical outcomes [19], but our patient showed no evidence of disease during the first 5 years after surgery. This could be because the lesion was composed of a unilocular cyst with an endophytic pedunculated solid tumor mass, and invasion to surrounding tissues was not significant.

Immunohistochemical positivity for GCDFP15, mammaglobin, and S100 protein, and negativity for DOG1, are usually regarded as useful diagnostic findings for SC [19-21]. In our analysis, all ACC cases were negative and all SC cases positive for p-STAT5. In contrast, all ACC cases were positive and all SC cases negative for DOG1. Thus, p-STAT5 and DOG1 were considered useful markers in terms of differentiating SC from ACC. p-STAT5 shows endogenous levels of STAT5a, and STAT5a has been reported to play a role in mammary gland differentiation [12]. DOG1 is also reported to relate to the acinar differentiation in salivary gland [22]. Due to these, p-STAT5 and DOG1 may be used as a marker for discriminating SC and ACC.

Immunohistochemical analyses of STAT5a and p-STAT5 have been reported previously [12, 16, 20,

23], and p-STAT5 could be an especially useful marker of SC to differentiate salivary gland cancers [12]. Skalova et al. reported that conventional SC and SC with high-grade transformation both showed focal staining of STAT5a on immunohistochemistry [16]. Considering that our SC case with high-grade transformation was also positive for p-STAT5 on immunohistochemistry, STAT5a and p-STAT5 may be retained in SC with high-grade transformation.

As genetic mutation research has progressed in recent years, it has been shown that NTRK3 mutation is not always detected in SC cases, and that the ETV6 gene is rearranged according to FISH. Therefore, the term “ETV6-X” is used to refer to unknown fusion genes involving ETV6 and a gene other than NTRK3 [4, 5]. Recently, it was revealed that “X” corresponds to MET, RET, or MAML3 [6-8]. However, ETV6 gene mutation was absent in only a few SC cases. Therefore, some reports have suggested that genetic analysis is not essential for the diagnosis of SC [14, 24]. Moreover, the fusion gene VIM-RET was detected in a SC case with no ETV6 gene mutation [9]. In the fourth edition of the WHO classification [2], the definition of SC includes the ETV6-NTRK3 fusion gene; thus, its confirmation is essential or desirable for a diagnosis of SC. As a result, in a hospital that cannot perform such an analysis or when the results of genetic analyses are unanalyzable, pathologists cannot make a definite diagnosis of SC. In those cases, immunohistochemical and histological analyses of SC may provide an alternative diagnostic method.

The prognosis of SC patients may improve with molecular targeted therapy, highlighting the need for continuous data accumulation concerning SC gene mutations. As an example of molecular targeted therapy in Japan, entrectinib was listed in the National Health Insurance price list and became available in

September 2019. Entrectinib is a tropomyosin receptor kinase inhibitor used to treat patients with solid cancers harboring NTRK gene fusions [25]. Thus, entrectinib might be applicable for treating SC of the salivary gland. Comprehensive genetic analyses using next-generation sequencing could clarify novel therapeutic targets. Continuous accumulation of SC cases and analyses of their genetic variety are warranted to develop novel molecular targeted therapies. After all, genetic analyses seem to be optional methods on demand.

Conclusion

Although the diagnostic criteria of SC remain unclear, the current study may contribute to gradual acceptance of SC diagnosis without genetic analysis. Useful immunohistochemical markers as alternatives to genetic analysis have become increasingly desirable, and immunohistochemical panels including p-STAT5, which shows both high sensitivity and specificity, provide an alternative. On the other hand, accumulation of SC cases and analysis of their genetic variety are necessary to develop molecular targeted therapies. Practical diagnostic methods and genetic analyses should not be mutually exclusive.

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Disclosure of conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1 Clinical findings

No	Age	Sex	Primary site	Tumor size (cm)	Treatment	Nodal metastasis	Distant metastasis	Follow up (mo)	Outcome
1	57	F	Parotid	7.5	Resection	-	NA	131	NA
2	40	F	Parotid	4.2	Resection	-	NA	23	NA
3	63	F	Parotid	4.0	Resection	-	-	121	Alive (NED)
4	85	M	Parotid	8.0	Resection	+	-	90	Dead (senile decay, NED)
5	32	F	Parotid	1.3	Resection	-	-	89	Alive (NED)
6	69	M	Parotid	2.0	Resection, CRT	-	+	90	Alive with bone metastasis
7	68	F	Parotid	3.0	Resection	Unclear	Unclear	91	Dead (SCC in uterine cervix, NED)
8	64	F	Parotid	2.6	Resection	-	-	112	NA (NED for 9y5mo)
9	60	M	Parotid	1.5	Resection	Unclear	Unclear	9	Dead (adenocarcinoma in stomach, NED)
10	15	M	Parotid	6.0	Resection	-	-	60	NA (NED for 5y)
11	60	M	Parotid	3.2	Resection	+	NA	NA	NA
12	36	F	Parotid	2.3	Resection	-	NA	57	NA

CRT, chemoradiation therapy; NA, not available; NED, no evidence of disease; SCC, squamous cell carcinoma

Table 2 Primary antigens used for immunohistochemical analyses

Antigen	Clone	Dilution	Manufacturer
p-STAT5	C71E5	1:100	Cell Signaling
S100 protein	Polyclonal	RTU	Roche
p63	4A4	RTU	Roche
GCDFP15	EP1582Y	RTU	Roche
Mammaglobin	31A5	RTU	Cell Marque
DOG1	SP31	RTU	Roche
p53	DO-7	RTU	Roche
GATA3	L50-823	RTU	Roche
MIB-1	Mib-1	1:500	Agilent (DAKO)

RTU, ready to use

Table 3 Results of immunohistochemical analyses

No	Diag	p-STAT5	DOG1	S100 protein	GCDFP15	Mammaglobin	GATA3	p53	p63	MIB-1 index (%)
1	ACC	-	+	-	-	+, f, w	-	n	ND	0-1
2	ACC	-	+	+, f	+, f, w	+, f, w	+, f, w	wi	ND	5
3	ACC	-	+	+, f	+, f	+, f	+, f, w	wi	ND	2
4	ACC	-	+	-	+	+, f, w	-	wi	ND	10
5	ACC	-	+	+, f	+	+	+, f	wi	ND	5
6	ACC	-	+	-	-	+, f, w	+, f	wi	ND	10
7	ACC	-	+	-	+	-	+, f, w	wi	ND	7
8	ACC	-	+	+, f	-	+, f, w	+, f, w	wi	ND	7
9	SC	+	-	+	+, f	+	+	wi	-	3
10	SC	+ (+)	- (-)	+ (+, f)	- (+, f)	+ (+)	+, w (+)	wi (wi)	- (+, f)	6 (23)
11	SC	+, f, w	-	+	+, f, w	+	+, f, w	wi	-	1
12	SC	+	-	+, f	-	+, f	+	wi	-	4

In case 10, the symbols on the left refer to conventional SC findings, and the symbols on the right (in parentheses) refer to findings of high-grade transformed SC.

ACC, acinic cell carcinoma; Diag, diagnosis; f, focal (positivity rate < 50%); n, null; ND, no data; SC, secretory carcinoma; w, weak; wi, wild

Table 4 RT-PCR and FISH results

No	RT-PCR (ETV6-NTRK3 fusion gene)	FISH (ETV6 split)
9	+	+
10	- (ND)	UA (UA)
11	ND	UA
12	+	+

In case 10, the symbols on the left refer to conventional SC findings, and the symbols on the right (in parentheses) refer to findings of high-grade transformed SC.

ND, no data; UA, unanalyzable

Figure legends

Fig. 1 Histology of acinic cell carcinoma (ACC). **a** ACC with a solid pattern composed of acinar-like cells (case 1, hematoxylin and eosin staining [H&E], ×400 magnification). **b** ACC composed of a microcystic pattern (case 3, H&E, ×200 magnification). **c** ACC composed of a follicular pattern (case 3, H&E, ×100 magnification).

Fig. 2 Histology of secretory carcinoma (SC). **a** Microcystic pattern (case 9, hematoxylin and eosin staining [H&E], ×400 magnification). **b** Papillary or hobnail pattern (case 11, H&E, ×400 magnification). **c** Follicular pattern with colloid-like and eosinophilic secretions (case 9, H&E, ×200 magnification). **d** Colloid-like and eosinophilic secretions (case 11, periodic acid-Schiff (PAS) staining with diastase, ×400 magnification). **e** Transitional area between the high-grade transformed solid pattern (left) and conventional papillary pattern (right) (case 10, H&E, ×40 magnification). **f** Conventional papillary pattern (case 10, H&E, ×400 magnification). **g** High-grade transformed solid and trabecular pattern with swollen nuclei, prominent nucleoli, and frequent mitotic figures (case 10, H&E, ×400 magnification).

Fig. 3 Immunohistochemical analyses of p-STAT5, DOG1, and MIB-1 (×400 magnification). p-STAT5 staining images of ACC, SC, and SC with high-grade transformation correspond to case 1, case 9, and case 10. DOG1 staining images of ACC, SC, and SC with high-grade transformation correspond to case 2, case

9, and case10. MIB-1 staining images of ACC, SC, and SC with high-grade transformation correspond to case 6, case 10, and case 10.

Fig. 4 RT-PCR detection of the ETV6-NTRK3 fusion transcript (110 bp, arrow). M, marker; PC, positive control.

Fig. 5 FISH detection of a rearranged ETV6 gene in case 9. Green and red signals (split signals) are significant. Yellow signals show unaltered chromosomes.