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Molecular analysis of a mammary analog secretory carcinoma in the upper lip
Novel search for genetic and epigenetic abnormalities in MASC

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ABSTRACT

INTRODUCTION: Mammary analog secretory carcinoma (MASC) is a newly described rare malignancy of the salivary glands characterized by an ETS variant 6 (*ETV6*)–neurotrophic tyrosine kinase receptor type 3 (*NTRK3*) fusion gene (*EN* fusion gene).
PRESENTATION OF CASE: We present a case of MASC derived from the left upper lip in a 61-year-old woman. *ETV6* rearrangement was detected by fluorescence in situ hybridization (FISH). The presence of *EN* fusion transcripts was verified by reverse-transcriptase polymerase chain reaction (RT-PCR) and sequencing of the PCR products. Accordingly, this tumor was diagnosed as MASC. Moreover, we performed mutation analysis of the 50 known cancer-related genes using next-generation sequencing. No mutation of cancer-related genes was identified here. Subsequently, the methylation status in promoter region of tumor-suppressor genes, *RASSF1A* and *RARB2*, was examined. Both genes have been reported to be methylated in malignant salivary gland tumors, but they were found to be unmethylated.
DISCUSSION: Recent studies have demonstrated that distinct types of malignant salivary gland carcinomas are driven by specific, highly recurrent genetic alterations. Detection of molecular abnormalities could be powerful diagnostic tools in the field of salivary gland tumors in near future.
CONCLUSION: We experienced a rare malignant salivary gland carcinoma, MASC. We diagnosed this tumor by molecular approach and subsequently tried to identify novel molecular abnormalities. Although no novel molecular alteration except for EN fusion gene was identified, this result might represent the favorable prognosis of patients with MASC.

1. Introduction

MASC is a recently described salivary gland carcinoma that is characterized by its morphologic and molecular similarities to breast secretory carcinoma (SC), which is a rare but distinct subtype of breast carcinoma, with a characteristic histomorphology and generally favorable prognosis [1–4].

MASC is reported to be morphologically close to ACC in salivary gland carcinoma. It can be distinguished from ACC by the absence of zymogen granules, and by its strong S-100, vimentin, and mammaglobin immunoexpression. However, the most definitive characterization of diagnosis for MASC is a balanced chromosomal translocation, *t*(12;15)(*p13*;*q25*), resulting in the formation of the *EN* fusion gene that encodes a chimeric oncoprotein tyrosin kinase [3].

We encountered a salivary gland tumor that was suspected to be MASC and tried to diagnose the tumor by immunostaining and a molecular approach. Furthermore, genetic and epigenetic background except for *EN* gene have not yet been elucidated in MASC. Accordingly, we looked for any genetic and/or epigenetic alterations in this tumor.

2. Case report

A 61-year-old Japanese female presented in the department of oral and maxillofacial surgery with a soft and tender mass in the left upper lip (Fig. 1a). The major axis of the mass was 8 mm. She had been aware of the painless mass for 1 year. However, she recently discovered bleeding from the mass, which brought her to the hospital. A biopsy was performed and the pathologic evaluation of the specimen identified that the tumor was analogous to ACC with mammillary cellular proliferation. The tumor was composed of tubular structures or papillary architecture, with eosinophilic

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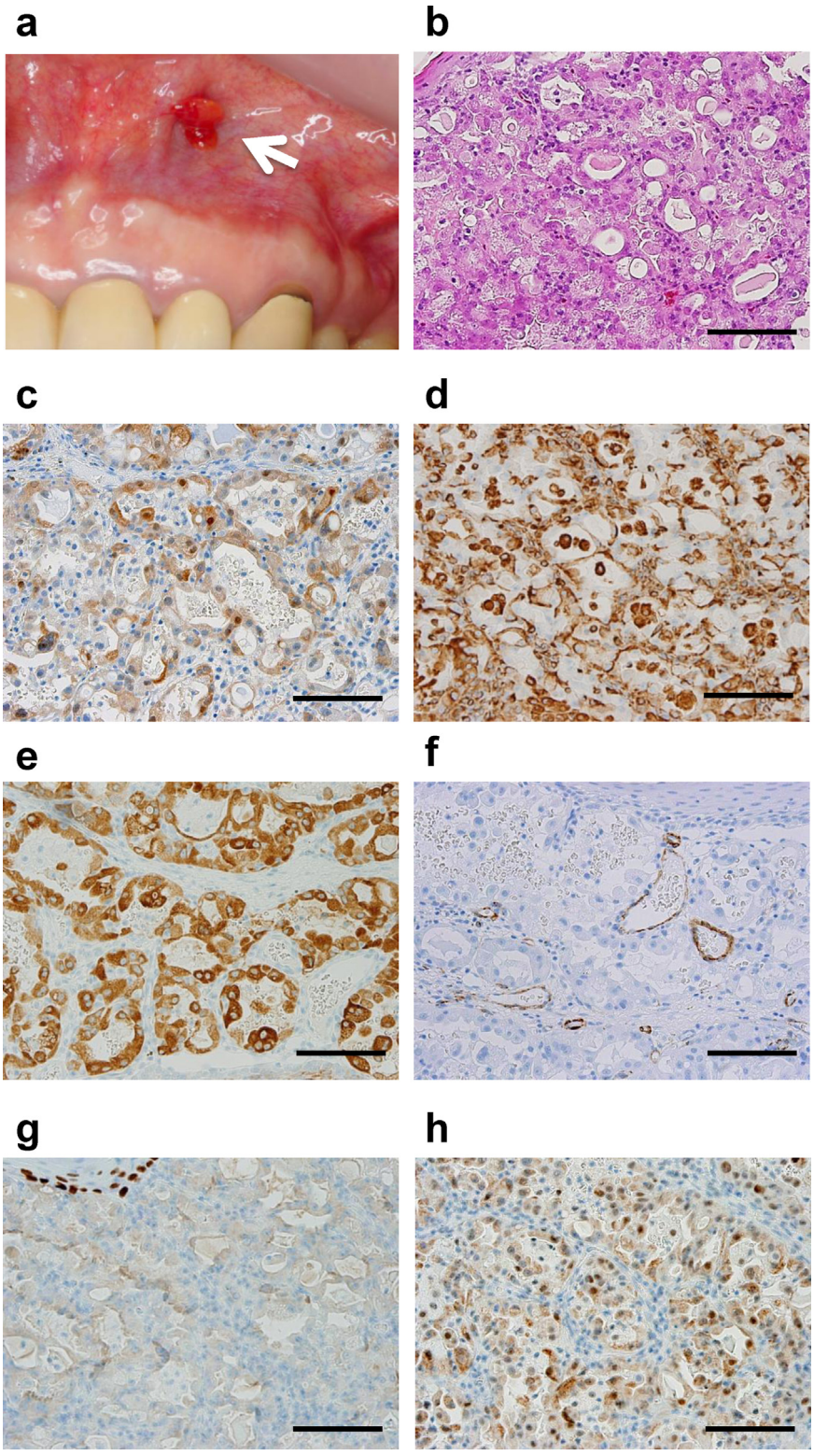

Fig. 1. Clinical and histopathological findings.
The arrowhead showed a soft and tender mass in the left upper lip (a). Microscopic findings are as follows (b–h). Hematoxylin-eosin staining showed that the tumor was composed of tubular structures or papillary architecture, with eosinophilic secretory fluid within the lumens of the tubules (b). Strong immunostaining was observed for the S-100 protein (c), vimentin (d) and EMA (e). Weak immunostaining was observed for α-SMA (f) and *p63* (g). Strong immunoreactivity was observed for *p53* (h).

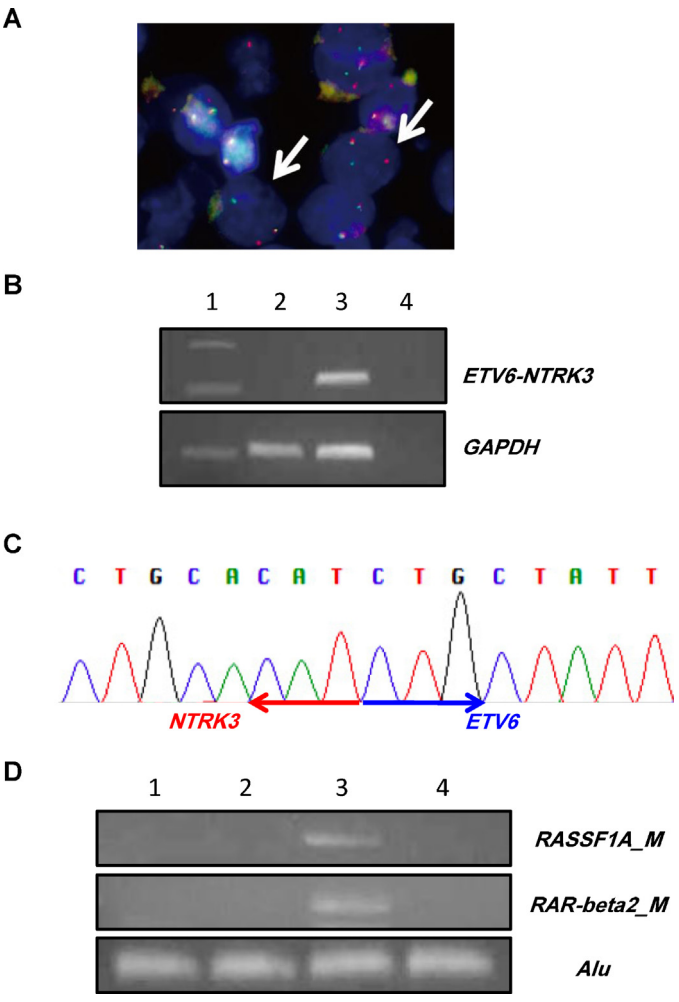

Fig. 2. Molecular findings.
(A) FISH analysis of *ETV6* gene rearrangement using Vysis® LSI® *ETV6* Break Apart Rearrangement Probe (Abbott Molecular/Vysis). The arrowheads show the cells which have *ETV6* rearrangement. A yellow (red/green fusion) signal indicates an intact chromosome while separate red and green signals indicate an *ETV6* gene break.
(B) Expression of *ETV6-NTRK3* fusion transcript in the MASC and noncancerous oral mucosa by RT-PCR. (1) DNA marker; (2) noncancerous oral mucosa; (3) cancerous lesion; (4) distilled water.
(C) Validation of *ETV6-NTRK3* fusion transcript by direct sequencing. Arrows show translocation breakpoint.
(D) Methylation analysis of promoter CGIs of *RASSF1A* and *RARB2* in MASC and non-cancerous specimens. M, primers specific to methylated DNA; *Alu*, primers that target the *Alu* repeat sequence were used as a control of the amount of bisulfite-treated DNA. Promoter CGIs of *RASSF1A* and *RARB2* were unmethylated in both the non-cancerous specimen (1) and MASC (2). Fully methylated DNA was prepared by methylating genomic DNA using *Sss*I-methylase (3). Fully unmethylated DNA was prepared by amplifying genomic DNA with ø29 DNA polymerase (4).

secretory fluid within the lumens of the tubules (Fig. 1b). Immunohistochemically, tumor cells showed diffusely positive staining of the S-100 protein, vimentin, EMA and *p53* (Fig. 1c–e,h). On the other hand, tumor cells show negative staining of α-SMA and *p63* (Fig. 1f,g).

The chromosomal translocation, *t*(12;15)(*p13*;*q25*), was analyzed by fluorescence in situ hybridization (FISH) using Vysis® LSI® *ETV6* (TEL) (12p13) Dual Color, Break Apart Rearrangement Probe (Abbott Molecular/Vysis, Des Plaines, IA) in formalin-fixed and paraffin-embedded tissue. Obvious rearrangement of the *ETV6* gene was detected in the cancerous lesion (Fig. 2A). The *ETV6* rearrangement was identified in 30% of the tumor cells (data not shown). The presence of *EN* fusion transcripts in this tumor was analyzed by RT-PCR and sequencing of the PCR products. RT-PCR revealed the *EN* fusion transcripts (Fig. 2B). Furthermore, the PCR products were cloned to T-Vector pMD20 (Takara Bio Inc., Shiga, Japan), and the sequences of the PCR products were determined. The result matched the sequencing of EN fusion transcripts (Fig. 2C). Accordingly, this tumor was in full accordance with the concept of MASC [2].

Mutational analysis of known cancer-related genes was performed using Ion AmpliSeq™ Cancer Hotspot Panel v2 (Ion Torrent), which covers 2800 COSMIC mutations from 50 cancer genes, *MPL*, *NRAS*, ALK, *IDH1*, *ERBB4*, *VHL*, *MLH1*, *CTNNB1*, *PIK3CA*, *FGFR3*, *PDGFRA*, *KIT*, *KDR*, *FBXW7*, *APC*, *CSF1R*, *NPM1*, *EGFR*, *MET*, *SMO*, *BRAF*, *EZH2*, *FGFR1*, *JAK2*, *CDKN2A*, *GNAQ*, *ABL1*, *NOTCH1*, *RET*, *PTEN*, *FGFR2*, *HRAS*, *ATM*, *KRAS*, *PTPN11*, *HNF1A*, *LT3*, *RB1*, *AKT1*, *IDH2*, *CDH1*, *TP53*, *ERBB2*, *SMAD4*, *STK11*, *GNA11*, *JAK3*, *SRC*, *GNAS* and *SMARCB1*, by next-generation sequencing, using the Ion Torrent Personal Genome Machine (PGM™) sequencer (Ion Torrent). The result showed that the MASC specimen did not harbor these genetic abnormalities (data not shown).

Abnormalities of DNA methylation have not been analyzed yet in MASC. However, the methylation status of promoter CGIs of *RASSF1A* and *RARB2*, [5] whose aberrant methylation has been reported in malignant salivary gland tumors, [6] were determined by methylation-specific PCR (MSP) in MASC and a non-cancerous specimens. Sodium bisulfite treatment was performed as described previously. For MSP, 1 μl of the solution was used for PCR with primers specific to methylated DNA sequences (M) or primers for *Alu* repeat sequences as a control of the amount of bisulfite-treated DNA [7,8]. Fully methylated DNA and fully unmethylated DNA were prepared respectively according to the previous report [5]. The results showed no methylation of the promoter CpG islands (CGIs) of the two tumor-suppressor genes, *RASSF1A* and *RARB2*, in either the MASC or non-cancerous specimen (Fig. 2D).

3. Discussion

MASC is a recently described salivary gland carcinoma characterized by the *EN* fusion gene encoding a chimeric tyrosine kinase. However, the molecular details of this rare tumor have not been elucidated yet. Here, FISH analysis demonstrated that this salivary gland tumor was positive for *ETV6* disruption. Accordingly, we could diagnose this tumor as a MASC and subsequently searched for unknown genetic and epigenetic abnormalities in this MASC case.

The most common primary site of MASC is the parotid gland, followed by the oral cavity, submandibular gland and accessory parotid gland. In the oral cavity, the lip, soft palate, and buccal mucosa are the most commonly affected subsites [2]. Recently, it was reported that most non-parotid ACCs could be MASCs [9]. The mean size of MASC tumors was reported to be 21 mm (range 7–55 mm) [2]. The size of the tumor in this case (major axis of the mass) was 8 mm, which is close to the smallest documented size of this tumor [2,4].

MASC most often presents as a slow-growing, painless mass. The important differential diagnostic considerations of MASC are low-grade adenocarcinoma not otherwise specified (NOS), cystadenocarcinoma, and ACC. These tumors may share an overlapping morphology with MASC. Especially, MASC is known to be morphologically close to ACC in salivary gland carcinomas and characterized by strong S-100 protein mammaglobin and vimentin, whereas ACC is reported to have moderate or weak staining for S-100 protein and vimentin, and negative staining for mammaglobin [3]. In this case, the tumor cells showed relatively strong staining for S-100 protein and vimentin. Subsequently, MASC cells were found positive for the glandular epithelial marker, EMA, and negative for the myoepithelial markers, α-SMA and *p63*. Transcripts of

a tumor-suppressor gene *p53* were strongly stained in and around the glandular epithelial cells. These results were in accordance with previous reports [4].

Mutations of the 50 known cancer-related genes were analyzed using Ion AmpliSeq™ Cancer Hotspot Panel v2 and Ion Torrent PGM sequencer. No mutation of cancer-related genes was identified in this analysis. The result of immunostaining demonstrated overexpression of *p53* in this MASC case; however, no mutation of the *p53* gene was detected. This result is in accordance with the previous studies [4,9].

No methylation analysis has previously been performed in MASC. Williams et al. demonstrated that *RASSF1A* and *RARB2* were highly methylated in malignant salivary gland tumors, but did not examine MASC. *RASSF1A* is frequently methylated, especially in ACC and salivary duct carcinomas (SDC). *RARB2* can be highly methylated, especially in mucoepidermoid carcinomas (MEC) and salivary duct carcinomas (SDC) [6]. In our analysis, no aberrant methylation of *RASSF1A* and *RARB2* was observed in either the MASC or non-cancerous specimen.

Recent studies have demonstrated that distinct types of malignant salivary gland carcinomas are driven by specific, highly recurrent genetic alterations. In addition to MASC, mucoepidermoid carcinoma and hyalinizing clear cell carcinoma are driven by fusion genes, *CTRC1-MAML2* and *EWSR1-ATF1*, respectively. Polymorphous low-grade adenocarcinoma (PLGA) is driven by hot-spot mutation of the *PRKD1* gene [10].

Here, we experienced a rare malignant salivary gland carcinoma, MASC. We diagnosed this tumor by molecular approach and subsequently tried to identify novel molecular abnormalities. Although no novel molecular alteration was identified in this tumor, this result might represent the favorable prognosis of patients with MASC.

Conflicts of interest

Nothing to declare.

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Consent

Written informed consent was obtained from the patient. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

Ethical approval

This research was approved by the institutional review boards.

Author contribution

Masanobu Abe: study design, data collections, experiments, data analysis, writing; Ryoko Inaki: study design, data collections, experiments, data analysis, writing; Yuki Kanno: data collections; Kazuto Hoshi: data collections; and Tsuyoshi Takato: data collections.

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