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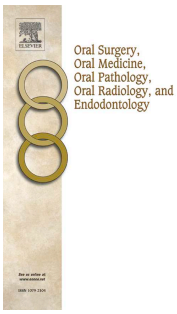

Cytogenetic and immunohistochemical characterization of Mammary Analogue Secretory Carcinoma of salivary glands

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Cytogenetic and immunohistochemical characterization of Mammary Analogue Secretory Carcinoma of salivary glands

Running title- Immunohistochemical signature of salivary mammary analogue secretory carcinoma

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ABSTRACT

Objectives: Mammary analogue secretory carcinoma (MASC), initially considered a sub-set of acinic cell carcinoma (ACC) harbours an *ETV6* translocation [t(12:15)(p13:25q)] and is now regarded as a distinct entity. Several putative markers to differentiate MASC from ACC have been reported however, the immunohistochemical profile is still being explored and updated. The purpose of this study was to further explore the cytogenetic and immunohistochemical profile of MASC.

Study design: Cases were analysed for *ETV6* translocation using fluorescent in situ hybridisation(FISH) and stained for CK8, amylase, mammaglobin, GCDFP□15, MUC1, MUC4, STAT5a, Ki□67 (n=37), CK7, Cam5.2, CK14, SMA, p63, S100, vimentin and DOG-1 (n=42). Histochemical stains for mucins were also performed and data collected for age, gender and site.

Results: FISH showed nine cases with *ETV6* rearrangement and two with increased *ETV6* copies. These eleven cases showed absence of PAS-D resistant granules with 10/11 showing strong S100, mammaglobin and Stat5a staining. All ACCs showed diffuse DOG-1 staining whereas 8/11 MASCs were negative and three showed only focal DOG1 staining.

Conclusion: DOG-1 can be used in conjunction with PAS-D, S100 and mammaglobin to identify MASCs. Cases with increased *ETV6* copies are a novel finding with a similar immunostaining profile and should be considered as MASCs.

Abstract word count- 200

INTRODUCTION

Mammary analogue secretory carcinoma (MASC) was first described in 2010 as a distinct salivary gland neoplasm, showing close resemblance to secretory carcinoma of the breast (SCB) [1,2]. As well as the histological similarity with SCB, MASC was also shown to harbour the *t(12;15) (p13;q25)* translocation and the resulting *ETV6-NTRK3* fusion gene [1,3,4,].

MASC has a similar morphological spectrum to acinic cell carcinoma(ACC) but with minimal zymogen granules and, in the past, has probably been diagnosed as a 'granule-poor' ACC [5]. Overlapping features are seen with other salivary tumours including cystadenocarcinomas and mucoepidermoid carcinoma. Meticulous histological analysis coupled with immunohistochemistry (IHC) and fluorescent in situ hybridisation (FISH) may be employed to aid diagnosis. FISH for the *ETV6* rearrangement is regarded as the diagnostic gold standard, but it is relatively expensive and not universally available, resulting in referrals to specialist units with associated delay and cost. IHC has been employed to narrow down the provisional diagnosis but to date no markers have been identified which can be regarded as specific to MASC. Recently, it was suggested that co-expression of mammaglobin and S100 is sufficient for a diagnosis of MASC [6]. However, this study investigated nineteen cases of MASC, but included only one ACC making the staining specificity somewhat uncertain. One suggested criterion is that S100 expression must be strong and diffuse [7], but others have shown that S100 can be variably expressed in ACC and other tumours may show strong diffuse expression [8]. The correct diagnosis may have implications with respect to clinical behaviour and the presence of a specific chromosomal translocation offers a potential target for future biological therapy.

Recently, DOG-1 (discovered on GIST1) or ANO1 has been reported as a marker for acinar differentiation with variable expression patterns and localisation between tumours [9]. It was first described as a calcium-activated chloride channel in 2008 and is routinely used in the diagnosis of gastrointestinal stromal tumours (GIST) [10-16]. Its expression pattern in salivary gland neoplasms is not well established although a recent study showed expression in acinar lumens in both normal glands and ACC [13].

The aim of this study was to identify a specific IHC signature and compare it with FISH and clinical information, to further help differentiate MASC from ACC.

MATERIALS AND METHODS

Case selection

The pathology archives were searched for all cases diagnosed as ACC between 1984 and 2013. Cases were reviewed to confirm the diagnosis and ensure sufficient tissue was available. Thirty seven cases diagnosed as ACC were identified as suitable. Subsequently five further cases of confirmed MASC were diagnosed (between 2013 and 2015) and were added to the study. The study was approved by the local research ethics committee.

Routine microscopy and histochemical analysis

4μm sections were obtained from paraffin blocks and stained for haematoxylin and eosin (H&E), mucicarmine, and periodic acid-schiff with (PAS-D) and without (PAS) diastase digestion. Relevant clinical data including site, gender and age were also obtained.

Tissue microarray (TMA) construction

Tissue micro-arrays (TMAs) were constructed from the original 37 cases, using a manual TMA machine (Surgipath, Richmond, USA). Using the H&E stained sections as a guide at

least two representative cores were taken from each lesion. Representative sections from normal salivary glands were also included in the TMAs for comparison. For the five additional cases IHC and FISH were performed on conventional paraffin sections.

Fluorescent in situ hybridisation

FISH analysis for *ETV6* rearrangement was undertaken at the Diagnostic Genetics Service, Sheffield Children's Hospital. A Dual-colour break apart rearrangement probe for *ETV6* (12p13) (Catalogue No. 07J77□001, Abbott, UK) was used for this purpose.

Unless stated otherwise, procedures were performed at room temperature (RT). Sections (4µm thick) were dewaxed, dehydrated and washed prior to heat pre-treatment in 50ml Zymed (San Francisco, California, USA) solution at 95˚C for 180 minutes. Slides were washed followed by application of 60µl of Zymed digestion enzyme and incubation at 38˚C in a wet box for 2 x 30 minutes. Sections were dehydrated using ethanol and air dried before probe application. *ETV6* probe was prepared immediately prior to use. The sample and probe DNA were co-denatured at 72˚C for five minutes and hybridised at 37˚C overnight on a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts, USA). The slides were washed in 50ml of 0.4x saline sodium citrate/Tween20 at 73˚C for two minutes and transferred to 50ml 2x saline sodium citrate/Tween20 for 30 seconds. Ethanol series dehydration was performed as before and the slides air-dried in the dark. Slides were counterstained with DAPI (Vector Laboratories, Burlingame, California, USA) and coverslipped.

At least 50 nuclei for each tissue case were analysed. The normal *ETV6* gene is represented by a co-localised green and orange signal i.e. fusion signal. Rearrangement of *ETV6* is indicated by a separation of the green and orange signals. A normal cell would therefore show two fusion signals, whereas a cell with an *ETV6* rearrangement would show one fusion

signal, a green signal and an orange signal. The presence of more than two fusion signals indicates an increased copy number of the *ETV6* gene.

Immunohistochemistry

4μm serial sections were deparaffinised in xylene and dehydrated in 100% ethanol followed by incubation in 3% methanolic H_2O_2 for 20minutes to block endogenous peroxidase. Antigen retrieval was carried out by microwaving in 0.01M sodium citrate buffer for 8 minutes. For EDTA retrieval, the buffer comprised 1mM EDTA, 0.05% Tween20 and 1000 ml distilled water (pH 9.0). Slides were incubated in the buffer at 95ºC for 20 minutes and then washed in PBS, blocked with serum for 30minutes and incubated with the primary antibodies (Table 1) at 4ºC overnight in a humidified container. Omission of primary antibody served as negative control.

After overnight incubation, unbound primary antibody was washed off. Vectastain Elite kits were used for secondary antibody and Avidin-Biotin Complex (ABC) at RT in accordance with the manufacturer's instructions (Vector laboratories). Secondary antibody antibody was added for 30 minutes followed by a wash and incubation with ABC for another 30 minutes. Vector NovaRED kit (Vector laboratories) was used to stain slides for 5-8 minutes and colouring reaction stopped using distilled water. Slides were counterstained with haematoxylin, dehydrated in graded alcohols and mounted in DPX.

Not all cases were stained with all the antibodies, the original cohort of 37 cases was stained with all the antibodies listed in Table 1. The five subsequently diagnosed cases of MASC were stained with a more limited range of antibodies used for diagnostic purposes. The number of cases stained for each antibody is indicated in the results.

RESULTS

FISH for *ETV6* rearrangement

ETV6 rearrangement was identified in four of the 37 (11%) retrieved cases and in all five of the additionally diagnosed MASC cases (n=9/42). These nine cases exhibited the rearrangement in a widespread manner (Figure 1). Two additional cases showed an increased number of *ETV6* copies (56% and 94% of analysed cells respectively) suggesting a close association to MASC (Table 2).

Using FISH as the criteria for selecting cases of MASC, the cases for further analysis were divided into 31 cases of ACC and 11 cases of MASC (Nine with *ETV6* rearrangement and two cases with increased *ETV6* copies).

Histological features (H&E)

Majority of MASCs showed a microcystic architecture (n=6) followed by a papillary-cystic pattern (n=5) (Figure 2). None of the MASCs exhibited a solid or follicular pattern. Both cases with increased *ETV6* copy numbers showed a papillary cystic pattern. The predominant histological pattern in the ACCs was microcystic (n=13; 42%) followed by the papillary cystic variant (n=8) (Figure 2). Six cases were of the solid type and four showed a follicular pattern. Extracapsular extension (ECE) was seen in 6/11 and perineural infiltration (PNI) in 3/11 including one case with increased *ETV6* copies.

Histochemical stains

PAS-D resistant granules were seen in all cases of ACC (n=31). The staining was focal in cases with a papillary-cystic pattern or clear cell change. The luminal secretory material was also PAS-D positive, as well as psammomatoid bodies seen in one ACC case. Mucicarmine showed a similar staining pattern but a lower intensity.

All eleven cases of MASC were negative for PAS-D positive granules but all showed a characteristic strong globular staining pattern in microcyst lumens and intercellular spaces (Figure 3).

Immunohistochemistry

All tumours (n=42) showed strong diffuse staining for CK7, which was also seen in adjacent normal salivary gland tissue. CK8 staining was restricted to ducts in normal glands with scattered staining in a proportion of the tumours (n=24/37). Abluminal cells stained for Cam5.2 in all cases. Four ACCs showed diffuse αSMA and CK14 expression in myoepithelial cells (4/31; 13%), and diffuse p63 staining was seen in only 2/31 (6.4%) cases. Staining for myoepithelial cells was presumed positive in the correct morphological context to ensure that stromal staining was excluded. MASCs showed only limited focal staining for CK14 in two and αSMA in three of the tested cases whereas p63 was negative. However one case with increased *ETV6* copies showed diffuse staining for all three (Figure 4). Insufficient tissue was available for examination for one case.

Staining for MUC4, MUC1, amylase and GCDFP15 was done on the initial cohort of 37 cases (including 6 MASCs). MUC4 staining was seen in ducts and secretory material and variably in luminal cells in all cases (n=37, not shown). Variable staining was seen for vimentin and MUC1. Luminal and secretory material staining for MUC1 was seen in 21/37 cases, but was observed in 6/6 MASCs. Staining for amylase and GCDFP15 in the secretory material appeared more restricted (6/37 and 10/37 respectively). Luminal GCDFP15 staining was seen in only 3/31 ACCs, but in 3/6 MASCs including both cases with increased *ETV6* copies.

S100 staining was carried out on the whole cohort and was seen in 22/42 cases. Ten MASCs (10/11; 90%) including both cases with increased *ETV6* copies showed strong and diffuse

S100 staining throughout the tumours. Twenty ACCs (20/31; 65%) showed variable S100 staining, but this was not as strong or diffuse as in MASCs (Figure 5). Weak cytoplasmic staining of acinar, and ductal cells was seen in ACC.

Eight of the eleven MASC cases (73%) including both cases with increased *ETV6* copy numbers were completely negative for DOG-1. The remaining three cases (3/11) showed only weak and focal luminal DOG-1 staining (Figure 6). All cases of ACC (31/31) were diffusely DOG1 positive with strong apical/luminal and lateral membranous staining of acinar cells, and luminal staining in tumours with a microcystic pattern (Figure 7). Occasional small ductal structures also showed positive luminal staining.

All cases in the original cohort of 37 cases were stained for mammaglobin and Stat5a. Only one ACC (1/31; 3%) showed mammaglobin staining and Stat5a was positive in only 8/31 ACCs. Mammaglobin and Stat5a were positive in 5/6 cases of MASCs tested in the initial cohort of 37 with one case negative for each. Both cases with increased *ETV6* copy number were positive for mammaglobin and Stat5a.

Age, gender and site distribution

A wide age distribution from 12 to 95 years was noted with the mean age in the 5th decade. The median age for MASC was 51 years (range 12-80) with a much lower median age in females (33 years) compared to males (71.5 years). Both cases with increased *ETV6* copies were in male patients (aged 62.25 and 84 years). The median age for ACC was 46 years which was not significantly different compared to MASC (p=0.19, Student's T-test).

There was an almost equal gender distribution between males (n=20; 47.6%) and females (n=22; 52.4%) in the cohort overall as well as for MASC (6 males and 5 females). The predominant tumour site for the entire cohort was the parotid gland accounting for 61.9%

(n=26/42) followed by the submandibular gland (13.15%, n=5/42), soft palate and upper lip (7.89%, n=3/42 each). The lower lip and buccal mucosa were involved in 5.26% of cases (n=2/42 each). The floor of mouth, tongue and parapharynx were the least prevalent sites at 2.63% (n=1/42 each).

The parotid gland was the most commonly involved site for MASC (n=6/11, 55%) with two cases seen in the submandibular gland (n=2/11, 18%) and one case each in lower lip, soft palate and buccal mucosa (9% each). Both cases with increased *ETV6* copies involved the parotid gland.

DISCUSSION

Malignant salivary gland neoplasms can exhibit overlapping histological features making diagnosis challenging. This is particularly true for ACC with multiple variants that may be seen in conjunction with each other. PAS-D resistant granules in acinic cells are an important diagnostic criteria for ACC, however a granule-poor variant of this tumour has been known to exist [5]. A distinct subset of ACC resembling SCB was first reported in 2002 lacking the usual zymogen granules and containing bubbly eosinophilic material in variably sized cystic spaces [3]. This 'granule-poor' variant was established as MASC in 2010 when it was shown that it not only resembles SCB histologically but also harbours the same chromosomal translocation [1,17].

The *ETV6–NTRK3* gene fusion has been shown in other tumours including congenital mesoblastic nephroma, congenital fibrosarcoma and acute myeloid leukaemia [18]. This translocation facilitates fusion of the transcriptional regulator (*ETV6*) with membrane receptor kinase (*NTRK3*) leading to activation of the Ras-MAP kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)-AKT pathways, subsequently promoting survival and proliferation of neoplastic cells.

Nine cases within our cohort showed the *ETV6* rearrangement confirming a diagnosis of MASC. These cases also showed a distinct immunoprofile with strong and diffuse staining for S100, mammaglobin and Stat5a consistent with previous reports. However, one case of MASC was negative for S100 and variable S100 and Stat5a staining was seen in some ACCs. Interestingly, most of the MASCs were also negative for DOG-1with three cases showing only weak focal positivity. In contrast all ACCs showed diffuse strong luminal DOG-1 positivity in the acini and some ducts. This suggests that DOG-1, in conjunction with S100 can potentially distinguish between ACC and MASC. DOG-1 is particularly attractive as it is readily available and routinely used in laboratories for diagnosis of GIST.

Two further cases showed an increase in *ETV6* copy number. This is a novel finding and may represent a simple polysomy for chromosome 12 or segmental chromosome imbalance. These two cases exhibited a similar morphology and immunophenotype to the confirmed MASCs suggesting that increased *ETV6* copy number may be associated with the translocation representing an '*in situ*' or early stage. Copy number genome aberrations have been shown to be associated with patient outcome and treatment response in childhood *ETV6/RUNX1*-positive acute lymphoblastic leukaemia [19,20]. Alteration in *ETV6* copy number has also been reported in infantile fibrosarcoma and cellular type of congenital mesoblastic nephroma [21]. Further work is required to establish the significance of increased *ETV6* copies in the context of MASC. However, given the similarity in morphology and immunophenotype, we would propose that increased *ETV6* copy number can also be used as a criterion for diagnosis of MASC.

S100 staining was seen in 11/31 ACCs but appeared much weaker and restricted compared to MASCs. This is in agreement with previous reports showing that diffuse and strong S100 expression may distinguish MASCs from ACCs [1,7]. Mammaglobin and S100 have also been suggested as proxy markers for MASC [22]. However, both can be variably expressed

in other salivary gland neoplasms [8, 22]. Furthermore, a recent study has shown that mammaglobin expression in MASC can be variable with complete lack of expression in a small subset [23]. This is further illustrated by the fact that one of our cases with rearranged *ETV6* showed no mammaglobin expression.

The eleven cases proposed to be MASC showed a predominantly microcystic pattern (7/11) with the remaining four cases being papillary cystic similar to previous studies [17,22,24,25]. PAS-D positive granules were absent in all 11 MASCs indicating its utility to triage cases. Shah et al. examined 19 cases negative for PAS-D positive granules and showed that all were strongly positive for S100 with 18 exhibiting mammaglobin staining and the *ETV6* fusion transcript [6]. This suggests that morphological examination along with PAS-D staining and appropriate immunohistochemistry might be sufficient to diagnose MASC. Our study suggests that absence of DOG1 staining may add further veracity to the use of immunocytochemistry in the absence of FISH facilities.

In agreement with existing knowledge, there was an almost equal gender distribution for MASC between males (n=6) and females (n= 5) [1,4,26,27]. A recent systematic review reports a slight male predominance for MASC (55%) and an average age of 44.2 years (range 14-77) [2]. In our study, the median age for MASC was 51 years (range 12-84) with a lower age in females (33 years) compared to males (71.5 years). Both cases with increased *ETV6* copies were in male patients (aged 60 and 84 years).

The most commonly reported site for MASC is the parotid gland (71%) followed by the submandibular gland (7%) and other sites including soft palate, buccal mucosa, base of tongue and lips [2]. Recently, two cases involving the upper and lower lip have also been reported [28]. Bishop et al., suggested that most non-parotid ACCs represent misclassified MASCs as 11/14 of their non-parotid cases harboured the *ETV6* rearrangement [22].

However, the remaining three MASCs in their study were from the parotid gland and a significant proportion of our non-parotid tumours were negative for the *ETV6* rearrangement suggesting that neither ACC nor MASCs are site restricted.

There have been 11 reported cases in patients under 18 years of age [6,13,26,29-36]. One of our cases with rearranged *ETV6* involved a 12-year-old female making this the youngest reported MASC in literature. MASC involving the parotid gland in a 13-year-old Taiwanese male has been the youngest patient reported to date [26]. In another case involving a young patient, MASC in the parotid gland presented as a secondary malignancy in a 14-year old male survivor of atypical teratoid rhabdoid tumour [29]. Salivary gland tumours are rare in children and our findings suggest that MASC should be considered in the list of salivary neoplasms encountered in children.

Interestingly, six MASCs showed ECE and three showed PNI including one case with increased *ETV6* copies. Histological features of aggressive behaviour such as ECE and PNI are relatively uncommon in MASC, however; they have been reported in some cases [30].

Eight of the 11 MASCs including the two '*in situ*' cases were completely negative for DOG-1, and three cases showed only weak focal positivity. This is similar to the findings of Chênevert et al. who showed restricted DOG-1 expression in MASC with focal positivity in some cases whereas strong and diffuse DOG-1 staining was seen in all ACCs compared to other entities [9]. Further characterisation of DOG-1 and mammaglobin expression in other salivary tumours would be beneficial for understanding distribution of these markers.

MASCs have been shown to exhibit a range of clinical behaviours from indolent to highly aggressive [2]. Skalova et al., showed that 12/15 patients were disease-free after 22-120 months follow-up, with local recurrence seen in 3/15 and lymph node metastasis in 2/15 [1]. Chiosea et al. showed a mean disease-free survival of 92 months (95% CI, range 71–115) in

28 MASC patients and 121 months (95% CI, range 92–149) in 38 patients with ACC suggesting a more aggressive disease course for MASC [27]. However, there was no significant difference between the two groups. Another study reported local recurrence in 3/9 cases after a median time of 44 months (range, 10–101) [31]. Out of the 172 MASC cases reported in the literature until early 2015, only seven patients died from disease. In two patients death followed distant metastases, one had multiple locoregional recurrences, and one followed unspecified recurrence [1,4,27]. The remaining three involved the parotid gland showing high-grade transformation with an aggressive clinical behaviour [37]. These high grade lesions showed strong membrane staining for EGFR and β-catenin, and nuclear staining for cyclin-D1 in addition to diffuse staining for S-100. *ETV6* gene rearrangement was seen in all cases. There was no evidence of mutations for *TP53* and *CTNNB1* genes or copy number aberration of *EGFR* and *CCND1* genes. Patients with high-grade MASC died of disseminated disease within two to six years of diagnosis.

A recent study analysed adipophilin (a lipid marker) expression in MASC showing larger lipid droplets in MASC compared to other salivary tumours [23]. Similarly, Carbonic anhydrase VI has recently been reported as an acinar marker and was shown to differentiate MASC from ACC with a sensitivity and specificity similar to DOG1 [38]. These findings suggest potential use of these markers as immunohistochemical tools [23]. Furthermore, two cases of non-*NTRK* gene fusion with *ETV6 (ETV6-X fusion*) have also been reported which appears to correlate with more aggressive histological features such as PNI and LVI [39].

In conclusion, analysis of 31 ACC and 11 MASCs show a distinctive staining profile for MASC. All MASCs show absence of PAS-D positive acinar granules, but show a characteristic strong globular PAS staining in microcysts and intercellular spaces. An immunoprofile of strong positive staining for S100 and mammoglobin with an absence of DOG-1 positivity may also be specific in addition to Stat5a and GCDFP-15 being useful

markers. The same PAS-D and immunoprofile in cases with increased *ETV6* copy number is a novel finding suggesting that this molecular change may also be a feature of MASC.

FIGURE LEGENDS

Figure 1. Representative images showing FISH with the Vysis *ETV6* probe. A) Nuclei demonstrating a non-rearranged *ETV6* signal. B) Nuclei demonstrating a rearranged *ETV6* signal pattern (n=4/37). Green/orange overlapping signals represent intact *ETV6*, green and orange separated signals represent *ETV6* rearrangement (n=4). C) Nuclei demonstrating increased copies of *ETV6* fusion signal (n=2/42) (original magnification x100).

Figure 2. Photomicrographs showing H&E staining of representative cases. ***A and B***. MASC- Microcystic pattern. ***C***. MASC- Papillary-cystic pattern. ***D.*** ACC with clear cell change. ***E***. ACC- Microcystic pattern. ***F.*** ACC- Solid patternt. ***G***. ACC- Follicular pattern. ***H***. ACC- Papillary-cystic pattern.

Figure 3. Photomicrographs showing PAS-D staining. ***A and B***. MASCs showing lack of PAS-D resistant zymogen granules and globular intraluminal PAS-D positive secretions. ***C and D***. ACCs showing abundant PAS-D positive cytoplasmic granules.

Figure 4. Representative photomicrographs showing immunohistochemical staining in MASCs. ***A and B***. MASC- Cam5.2. ***C and D.*** The same case with increased *ETV6* copy numbers showing expression of CK14 (C) and αSMA (D).

Figure 5. Photomicrographs showing S100 staining in MASCs. ***A-B***. MASC- Micro-cystic variant. ***C***. MASC- Papillary-cystic variant. ***D***. No staining was seen in one case with rearranged *ETV6*.

Figure 6. Representative photomicrographs showing immunohistochemical staining in MASCs. ***A***. DOG-1 in normal salivary tissue. ***B-C***. Absence of DOG-1 staining ***D***. Focal luminal DOG-1 staining in one MASC. ***E-F***. Mammaglobin. ***G.*** Absence of mammaglobin staining in one MASC. ***H***. Stat5a.

Figure 7. Representative photomicrographs showing immunohistochemical staining in ACCs. ***A***. S100. ***B***. Stat5a. ***C-D***. DOG-1. ***E***. GCDFP15. ***F.*** Mammaglobin. ***G***. MUC1. ***H***. MUC4.

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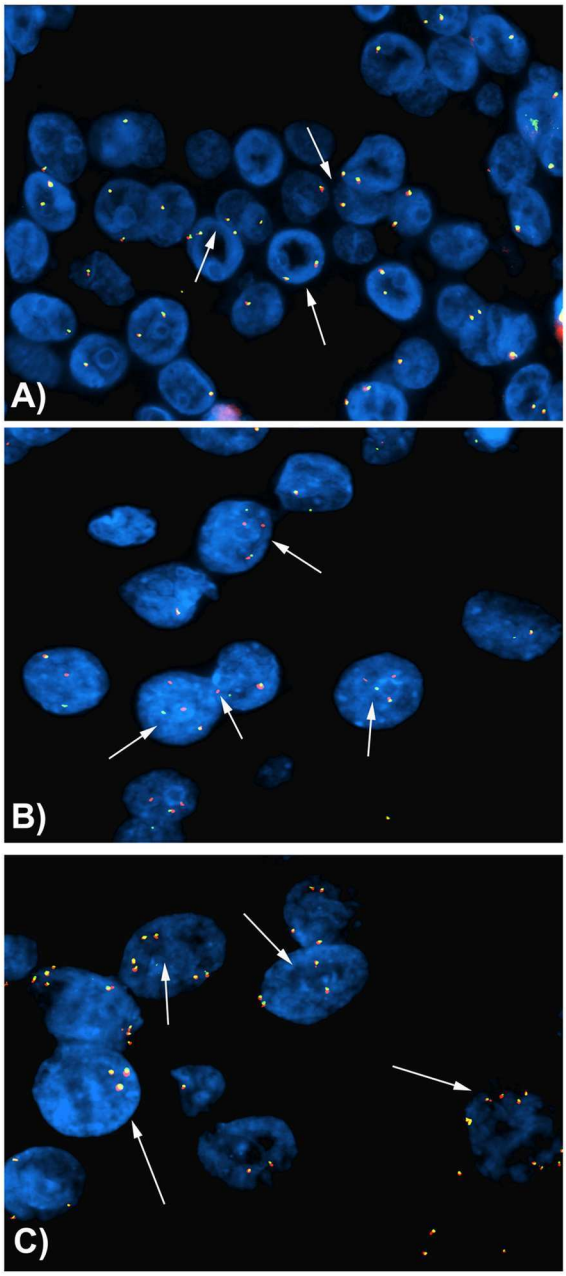
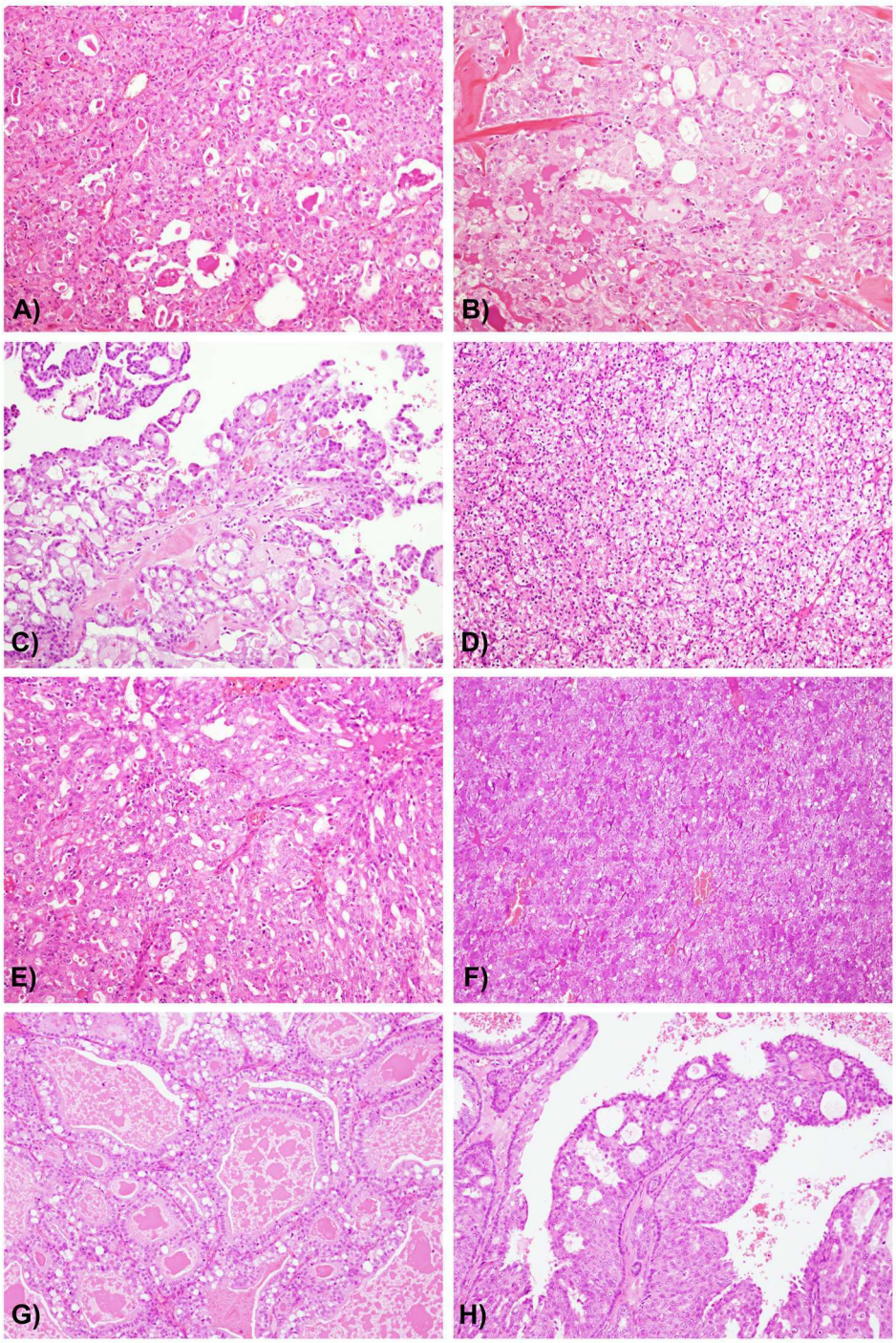
Antibody	Dilution	Pre-treatment	Manufacturer	Cat. No.	No of cases*
CK7	1:50	EDTA	Dako	M7018	42
CK8	1:200	Citrate	Abcam	AB2531	37
Cam5.2	1:100	Citrate	BD Bioscience	345779	42
CK14	1:100	Citrate	Abcam	AB7800	42
SMA	1:100	Citrate	Dako	M0851	42
P63	1:50	EDTA	Dako	M7247	37
S100	1:2000	Citrate	Dako	Z0311	42
Amylase	1:200	Citrate	Sigma	WH0000276M4	37
MUC1	1:50	Citrate	Abcam	AB15481	37
MUC4	1:200	Citrate	Abcam	AB60720	37
STAT5a	1:200	Citrate	Abcam	AB32364	37
GCDFP-15	1:200	Citrate	Abcam	AB1319	37
Mammaglobin	1:200	Citrate	Dako	M3625	37
DOG-1	1:100	Retrieval Solution pH 6.0 RE7113, Leica	Leica Microsystems	NCL-L-DOG-1	42

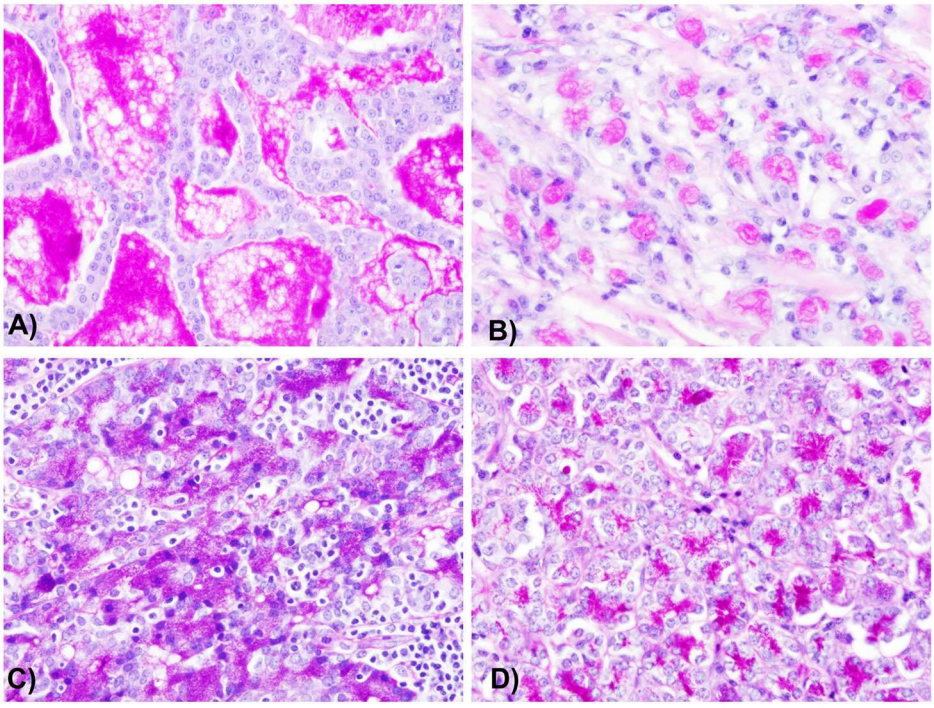
Table 1. Primary antibodies used in this study with their respective dilutions, antigen retrieval methods and manufacturer specifics. * 37 cases represent the retrospective cohort analysed in TMAs. 42 cases include the five additional MASC cases diagnosed between 2013 and 2015.

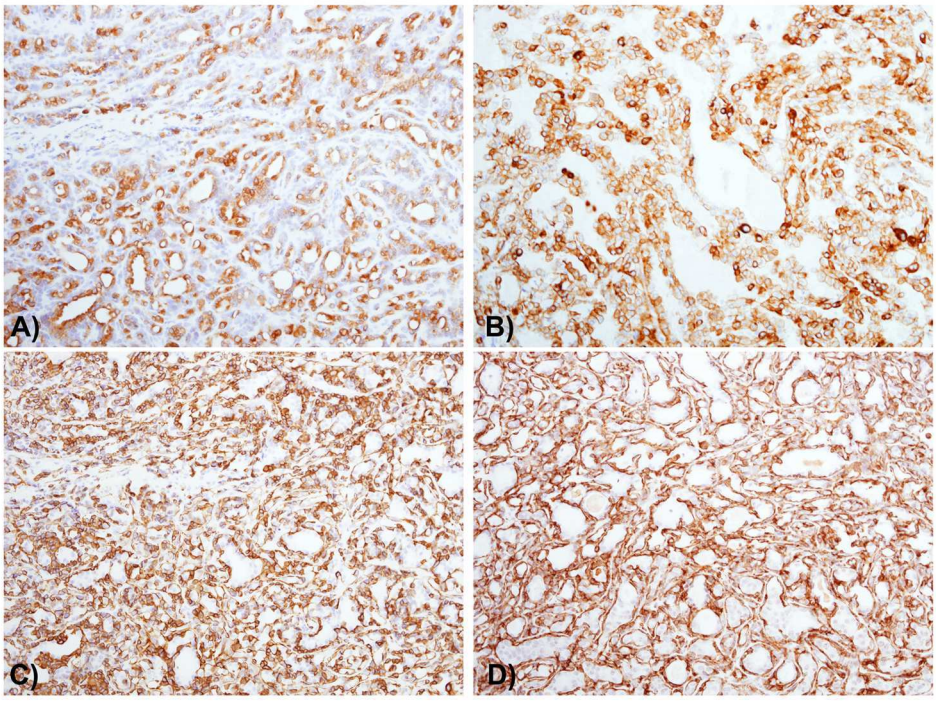
Table 2. Details of FISH analysis with percentage of abnormal cells showing the *ETV6* rearrangement or increased copy number. At least 50 nuclei were analysed for each case.

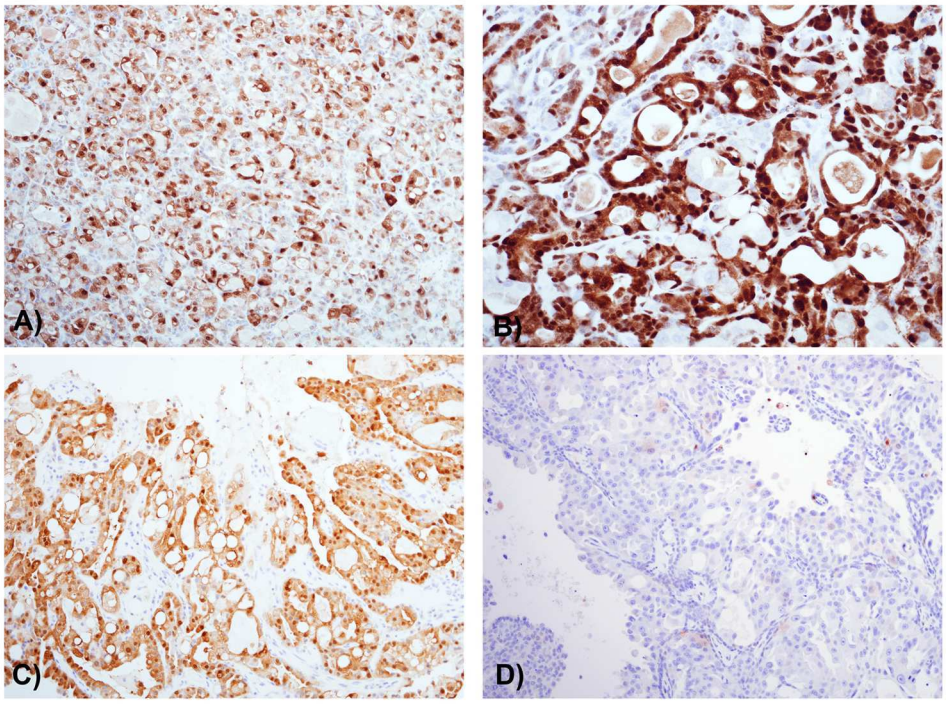
Case number	Abnormal cells
10	90%
14	96%
19	88%
23	88%
38	74%
39	80%
40	92%
41	96%
42	35%
28	56% increased *ETV6* copy number
20	94% increased *ETV6* copy number

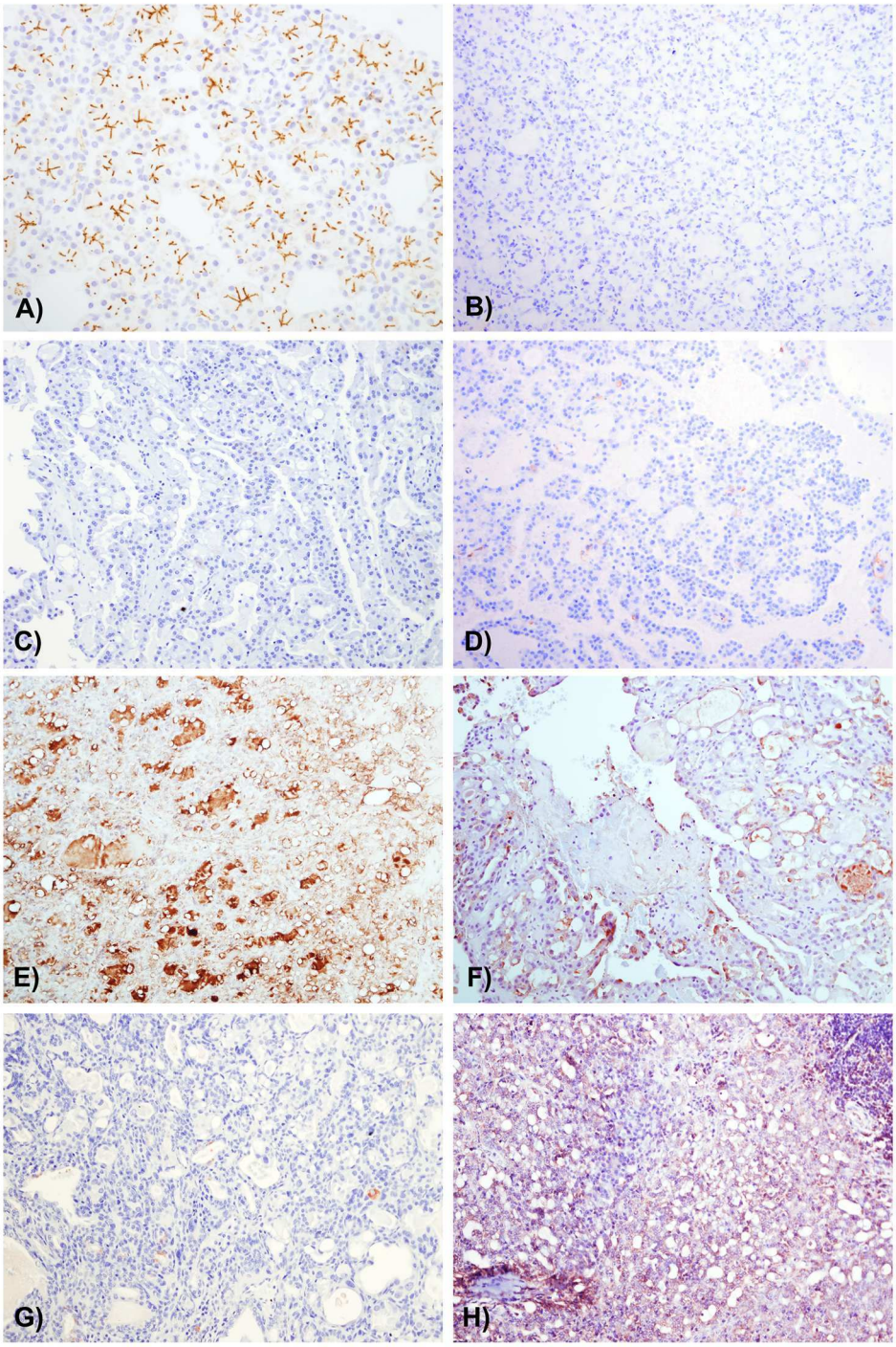


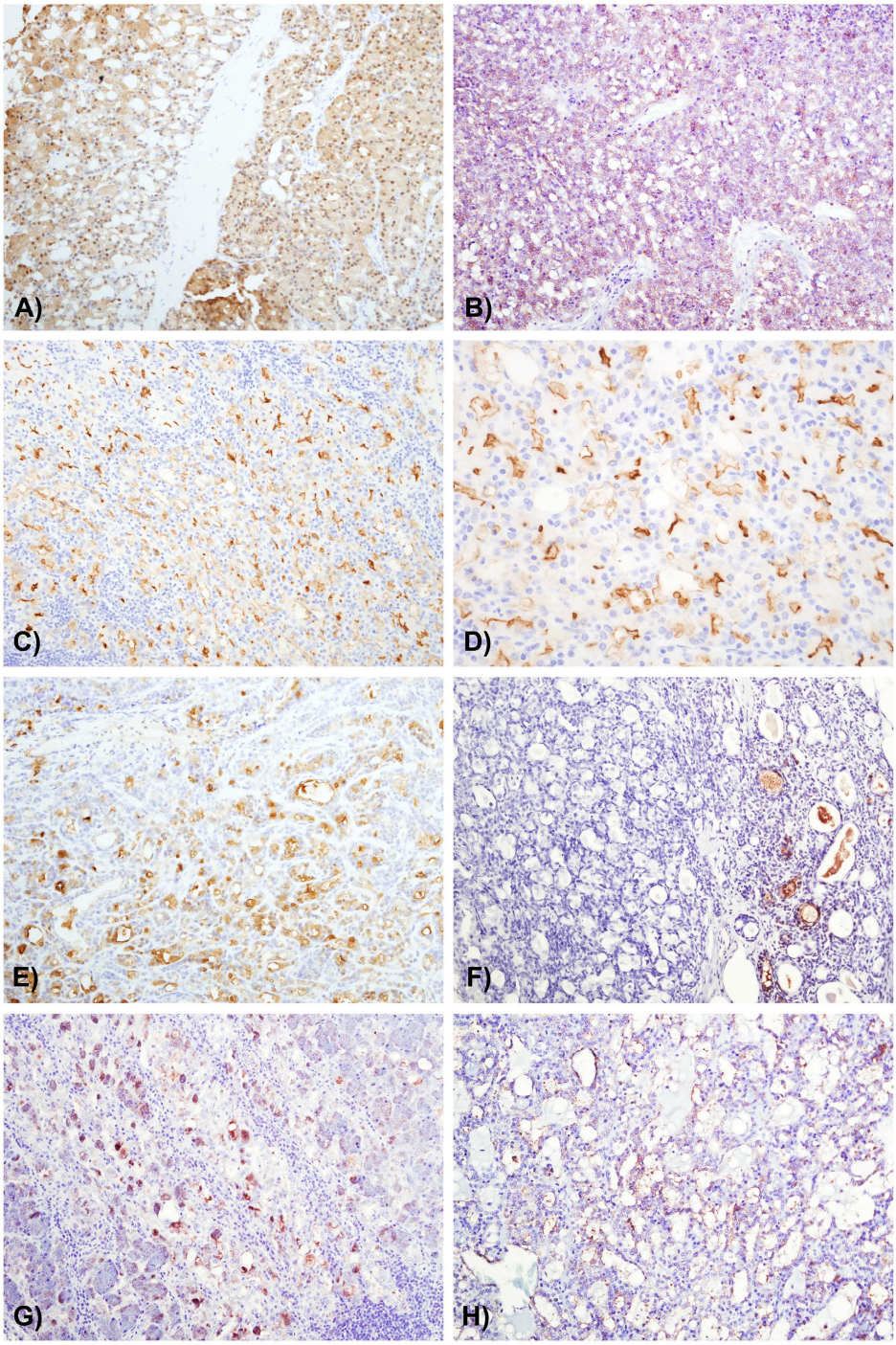












Statement of Clinical Relevance

MASC of salivary glands harbours an *ETV6* translocation with a suggested aggressive clinical course. PAS-D and DOG1 staining can differentiate it from Acinic cell carcinoma. Tumours with increased *ETV6* copies show a similar immunoprofile and should be considered as MASCs.