Human Pathology (2019) **93**, 37–47

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Human PATHOLOGY

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Original contribution

# Biomarker immunoprofile and molecular characteristics in salivary duct carcinoma: clinicopathological and prognostic implications☆,☆☆

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Received 10 June 2019; revised 1 August 2019; accepted 7 August 2019

**Keywords:**
Salivary gland neoplasm;
Salivary duct carcinoma;
Immunoprofile;
Prognosis;
NGS

**Summary** Salivary duct carcinoma (SDC) is one of the most aggressive salivary gland tumors, and prognosis remains poor for most patients. The aim of this study was to investigate the prognostic implications of biomarker immunoprofile in a cohort of SDC and to identify molecular characteristics through next-generation sequencing (NGS) in a subset of cases. Clinicopathological and follow-up information of 25 cases diagnosed as SDC was collected. Immunoexpression of AR, HER-2/*neu,* GATA3, CK5/6, and MIB1 was analyzed, and *ERBB2* (*HER-2/Neu*) gene amplification was investigated by fluorescence in situ hybridization. Cases were classified under the "SDC revised classification system." Eight SDC cases were analyzed by targeted NGS for detection of gene fusions and variants. Overall survival and disease-free survival were analyzed with Kaplan-Meier curves and Cox regression. Most cases expressed AR (100%), GATA3 (73%), and CK5/6 (76.5%), and 42% expressed *HER-2/neu. ERBB2* gene amplification was proven by fluorescence in situ hybridization in 7 of 15 (46%) cases. *Apocrine HER2* (AR+/HER2+) subtype was significantly associated with lower overall survival ($P$ = .05). NGS analysis revealed 9 pathogenic mutations in 7 SDC cases, and the most frequently mutated gene was *HRAS* (4/9) followed by *PIK3CA* (2/9) and *TP53* (2/9). One case (1/9) presented homozygous deletion of locus 9p21 (*CDKN2A*), and another case (1/9) showed *MDM2* amplification. In conclusion, we demonstrated that *Apocrine HER2* (AR+/HER2+) is a potential biomarker of poor outcome in SDC. Furthermore, NGS analysis revealed recurrent mutations in SDC.


☆ Competing interests: The authors have no conflicts of interest to disclose.
☆☆ Funding/Support: This study was partly supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, São Paulo - SP, Brazil (Finance Code 001).
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https://doi.org/10.1016/j.humpath.2019.08.009
0046-8177/

## 1. Introduction

Salivary duct carcinoma (SDC) is as an aggressive malignancy microscopically resembling high-grade mammary ductal carcinoma [1]. This tumor accounts for up to 2% of all salivary gland neoplasms and usually occurs in major salivary glands (especially in the parotid gland), being most often diagnosed in male patients more than 50 years old [2]. SDC can arise de novo or as the malignant component of carcinoma ex pleomorphic adenoma [1].

SDC most likely derives from cells of the excretory ducts and histologically is characterized by pleomorphic polygonal and eosinophilic cells arranged in solid nests, ductal structures, cribriform arrangements, or small cords, usually embedded in a desmoplastic stroma and presenting high mitotic frequency and areas of comedonecrosis [2,3]. At immunohistochemical analysis, this tumor usually presents broad expression of low–molecular weight cytokeratins and epithelial membrane antigen, and it is typically negative for S-100 and p63 [2]. As in breast carcinoma, SDC often presents overexpression of HER-2/*neu,* androgen receptor (AR), and epidermal growth factor receptor (EGFR) [4,5]. On the other hand, SDC rarely shows expression of estrogen receptor or progesterone receptor, which is observed in more than 75% of breast carcinoma cases [2,3,6].

Currently, treatment choice for SDC includes complete surgical resection of the primary site and neck dissection followed by adjuvant chemo/radiotherapy [7]. Notwithstanding, recurrence and distant metastasis are fairly common in SDC, and prognosis remains poor, with 5-year overall survival ranging from 12% to 55% [7]. Case series have reported partial response or stable disease in patients treated with androgen deprivation therapy or with *ErbB2/HER-2* inhibitor (trastuzumab); however, it is still not possible to predict which patients are likely to benefit from these therapies [8].

Recent studies using targeted and whole-exome sequencing have identified genomic alterations common to SDC, many of which may become actionable targets for this tumor. Along with, SDCs harbor an average mutational burden of 1.7 mutations per megabase, which is higher than observed in other salivary carcinomas [9]. The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway is possibly the most frequently altered pathway in this type of tumor, with recent studies demonstrating mutations and copy number alterations in *PIK3CA*, *PTEN*, *RICTOR*, *AKT1*, *AKT3*, and/or *PIK3R1* [6,10-12]. Mutations in TP53, HRAS/NRAS, cyclin D1/CDK pathways are also found in SDC, as well as mutations in ERBB2, EGFR and BRAF genes [6,9,12].

Understanding the molecular pathogenesis of SDC and other salivary gland tumors is essential to the discovery of new biomarkers for molecular diagnosis and the eventual development of new individualized therapeutic strategies. Therefore, the purpose of this study was to investigate the biomarker immunoprofile in a cohort of SDC cases to investigate its relationship with clinicopathological data and patient survival and also to identify molecular characteristics through next-generation sequencing (NGS) in a subset of cases.

## 2. Materials and methods

### 2.1. Samples

This research was approved by the local ethics committee.

Twenty-eight cases originally diagnosed as SDC were retrieved from the consultation files of the Salivary Gland Tumor Registry at the Department of Pathology, Faculty of Medicine in Plzen, and Biopticka Laboratory Ltd, Plzen, Czech Republic. For conventional microscopy, the excised tissues were fixed in formalin, routinely processed, embedded in paraffin (FFPE), cut, and stained with hematoxylin and eosin. The histopathologic features of all tumors and the immunohistochemical stains, when available, were reviewed by 2 pathologists. A diagnosis of SDC was confirmed in cases that displayed histologic features consistent with original description in conjunction with the appropriate immunohistochemical profile.

After histopathological and immunohistochemical analysis, 1 case was reclassified as carcinoma ex pleomorphic adenoma not otherwise specified, and 1 case as a high-grade anaplastic carcinoma. Following fusion detection analysis, we observed 1 case harboring an *ETV6-NTRK3* fusion;

**Table 1** Antibodies used for immunohistochemical study

| Antibody specificity | Clone | Dilution | Antigen retrieval/time (min) | Source |
|---|---|---|---|---|
| CK7 | OV-TL 12/30 | 1:200 | CC1/36 | DakoCytomation |
| S100 protein | Polyclonal | RTU | CC1/20 | Ventana |
| GATA-3 | L50-823 | 1:200 | CC1/52 | BioCareMedical |
| MIB1 | 30-9 | RTU | CC1/64 | Ventana |
| Androgen receptor | SP107 | RTU | CC1/64 | Cell Marque |
| p63 | 4A4 | RTU | CC1/64 | Ventana |
| HER2/neu | 4B5 | RTU | CC1/36 | Ventana |
| CK5/6 | D5/16 B4 | 1:50 | CC1/36 | Dako |

NOTE. CC1—EDTA buffer, pH 8.6.
Abbreviation: RTU, ready to use.

therefore, it was reclassified as a high-grade secretory carcinoma (Supplementary Fig. 1) and not further tested. The histologic and immunohistochemistry results for these cases are summarized in Supplementary Table 1.

Therefore, 25 cases with confirmed diagnosis of SDC were included in this study.

When available, clinical follow-up information was obtained from the patients, from their physicians, or from referring pathologists.

## 2.2. Immunohistochemistry

Immunohistochemistry for CK7, CK5/6, p63, S100, MIB1, AR, *HER-2/neu*, and GATA3 was performed as described previously [13]. Antibodies, clones, and antigen retrieval methods are described in Table 1. Appropriate positive and negative controls were used.

For immunohistochemistry analysis, cases were divided into 2 groups: positive and negative. *HER2* positivity was defined according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for breast cancer [14]; cases were considered positive when >10% of tumor cells presented a circumferential membrane staining (*HER2* 3+). For other antibodies, cases were also considered positive when >10% neoplastic cells were stained. Only nuclear staining was considered for AR and GATA3; for CK5/6 and CK7, moderate to strong cytoplasmic staining in neoplastic cells was considered positive; and for S100, strong nuclear and cytoplasmic staining was considered positive. For MIB1 (Ki-67), nuclear staining was considered positive, and a semiquantitative analysis was performed and staining was expressed as a percentage (proliferation index); furthermore, cases presenting a proliferation index of <40% were considered MIB1-low and cases with ≥40% were categorized as MIB1-high according to Takase et al [15].

## 2.3. Revised classification based on biomarker immunoprofiling

After analysis of immunohistochemical results, 24 cases were classified according to the SDC revised classification into 5 subtypes, as previously described [15]. The categories for SDCs were:

a. Apocrine A (AR+/HER2−/MIB1-low);
b. Apocrine B (AR+/HER2−/MIB1-high);
c. Apocrine HER2 (AR+/HER2+);
d. HER2-enriched (AR-/HER2+);
e. Double negative (AR−/HER2−).

## 2.4. Fluorescence in situ hybridization analysis

*ERBB2* and *MDM2* amplification and *ETV6* break apart were investigated through fluorescence in situ hybridization (FISH).

Four-micrometer-thick FFPE sections were placed onto positively charged slides, routinely deparaffinized, incubated in 1× Target Retrieval Solution Citrate at pH 6 (Dako, Glostrup, Denmark) at 95 °C for 40 minutes, and subsequently cooled for 20 minutes at room temperature in the same solution. Slides were washed in deionized water for 5 minutes and digested in protease solution with pepsin (0.5 mg/mL, Sigma Aldrich, St Louis, MO) in 0.01 mol/L HCl at 37°C for 45 to 75 minutes according the sample conditions. Slides were then placed into deionized water for 5 minutes, dehydrated in a series of ethanol solution (70%, 85%, and 96% for 2 minutes each), and air dried.

For *ERBB2, CDKN2A*, and *MDM2* aberration detection, PathVysion HER-2 DNA Probe Kit (Vysis/Abbott Molecular - Abbott Park, Illinois, U.S.A), ZytoLight SPEC MDM2/CEN 12 Dual Color Probe (ZytoVision Gmbh - Bremerhaven Germany), and ZytoLight SPEC CDKN2A/CEN 9 Dual Color Probe were used.

ZytoVision probes were factory premixed. Vysis ETV6 Break Apart probe was mixed with deionized water and LSI Buffer (Vysis/Abbott Molecular) in 1:2:7 ratio.

Probes were applied onto the specimens, covered with a glass coverslip, and sealed with rubber cement. Slides were incubated in the ThermoBrite instrument (StatSpin/Iris Sample Processing, Westwood, MA) with codenaturation at 85°C for 8 minutes and hybridization at 37°C for 16 hours. Rubbercemented coverslip was then removed, and the slide was placed in posthybridization wash solution (2× SSC + 0.3% NP-40) at 72°C for 2 minutes. The slides were air dried in the dark, counterstained with 4′,6′-diamidino-2-phenylindole (Vysis/Abbott Molecular), coverslipped, and immediately examined. Appropriate positive and negative controls for each FISH probe were used.

## 2.5. FISH interpretation

The sections were examined with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) using a ×100 objective and filter sets triple-band pass (4′,6′-diamidino-2-phenylindole/SpectrumGreen/SpectrumOrange), dual-band pass (SpectrumGreen/SpectrumOrange), and single-band pass (SpectrumGreen or SpectrumOrange). For *ETV6* break-apart probe, a minimum of 100 randomly selected, nonoverlapping tumor cell nuclei were evaluated for the presence of yellow, orange, and green fluorescent signals. For break-apart probe, yellow signals were considered negative, whereas separate orange and green signals were considered positive. Cutoff values were set to more than 10% of the nuclei for breakapart probe showing split signals (mean + 3 standard deviation in normal non-neoplastic control tissues). Nuclei presenting only 1 color were excluded from the analysis. Amplification of *MDM2* was evaluated according the manufacturer's instructions. Amplification of *ERBB2* was evaluated according the 2013 ASCO/CAP guidelines [14] and manufacturer's instructions.

### 2.6. Targeted NGS

Tumor-rich regions were macrodissected from FFPE blocks. Total nucleic acid for fusion detection was purified using Agencourt FormaPure Kit (Beckman Coulter, Brea, CA) using manufacturer's protocol modified with an overnight digest and an additional 1-hour 80°C incubation as recommended by ArcherDX (ArcherDX Inc, Boulder, CO). DNA and RNA acids were quantified using the Qubit Broad Range DNA and RNA Assay Kits, respectively (Thermo Fisher Scientific - Waltham, MA USA). The FusionPlex Archer Solid Tumor Kit (fusions in 53 genes) and Comprehensive Thyroid and Lung kit (fusions in 36 genes and hotspots in 18 genes) were used for fusion detection, and Variant Plex Solid Tumor kit (hotspots in 67 genes) was used for tumor mutation analysis (ArcherDX Inc). Lists of analyzed genes can be found in the supplements. Both libraries were prepared per manufacturer's protocols and sequenced on a NextSeq sequencer (Illumina). Fusion detection was performed using the Archer Analysis software (v5; ArcherDX Inc). Fusion parameters were set to a minimum of 5 valid fusion reads with a minimum of 3 unique start sites within the valid fusion reads. Detected variants were filtered for variant allele frequency >0.05, variant population frequency <0.001, and synonymous or noncoding variants and manually checked for the clinical interpretation.

### 2.7. Statistical analysis

Associations between clinical variables and immunohistochemistry results were analyzed using Fisher exact test. Overall survival (OS) and disease-free survival (DFS) rates were evaluated by the Kaplan-Meier method and by univariate and multivariate Cox proportional hazard models adjusted for age groups, sex, primary tumor site, and tumor stage. The hazard ratio and 95% confidence interval were used to evaluate associations. Statistical analyses were performed using the software R version 3.5.1 and RStudio Desktop 1.1.456. $P < .05$ was considered statistically significant. Cases reclassified as high-grade secretory carcinoma or high-grade not otherwise specified carcinoma and high-grade anaplastic carcinoma were excluded from statistical analysis.

## 3. Results

### 3.1. Clinical features

The patient group comprised 22 men and 3 women aged between 24 and 95 years with a mean age at diagnosis of 66.3 years and median of 64 years. Most tumors (61.5%) occurred in the parotid gland followed by the submandibular gland (38.5%). Most patients were diagnosed at stages III or IV. Three patients (11.5%) had recurrent disease, and 7 patients (26.9%) presented distant metastasis. Mean follow-up time was 34 months, and 12 patients (46.2%) died of disease. Clinical and demographic data are summarized in Table 2.

Almost all patients (n = 22) were treated by radical surgical excision of the tumor and involved gland followed by radio/chemotherapy or both. Among the other 3 patients, 1 died of disease 2 months after diagnosis, 1 was treated with radiotherapy only and is alive with disease for 36 months, and the last 1 was treated with radiotherapy and chemotherapy and died of other causes (with disease) after 60 months.

### 3.2. Microscopic and immunohistochemical findings

Thirteen cases were classified as invasive SDC arising de novo and were characterized by neoplastic cells often arranged in small nests, tubules, or aggregates resembling distended ducts, mostly showing apocrine differentiation and exhibiting varying degrees of pleomorphism, hyperchromasia, and frequent mitoses. Tumor stroma was frequently desmoplastic, and comedonecrosis was a common finding (Fig. 1).

The other 12 cases were characterized as SDC arising as the malignant component of a carcinoma ex pleomorphic adenoma. In those cases, evidence of pleomorphic adenoma was confirmed, presenting often as a separate sclerotic nodule. One case was classified as a carcinoma ex pleomorphic adenoma with an in situ component of SDC.

In general, most studied cases were positive for CK7, CK5/6, AR, and GATA3, and 42% were positive for *HER-2/neu* (Table 2) Thermo Fisher Scientific - Waltham, MA USA. Representative images of positive immunostaining are shown in Fig. 2.

### 3.3. FISH results for *ERBB2* (*HER-2/Neu*) gene amplification

Fifteen cases were investigated for *ERBB2* (*HER-2/neu*) gene amplification by FISH. Among those cases, 7 (46%) were proved to have amplification of *ERBB2* (*HER-2/neu*). All cases presenting *ERBB2* (*HER-2/neu*) amplification were immunohistochemically classified as HER2 3+. Four cases that presented HER2 2+ did not exhibit amplification of this gene.

### 3.4. Results of SDC revised classification

After analyzing biomarker immunoprofile, 24 cases were categorized according to the SDC revised classification. Fifteen cases (62.5%) were classified as *Apocrine HER2* (AR+/HER2+) subtype, 5 cases (20.8%) were *Apocrine B* (AR+/HER2−/MIB1-high), and 4 cases (16.7%) were *Apocrine A* (AR+/HER2−/MIB1-low).

### 3.5. Molecular genetic findings

Nine cases were analyzed by NGS using the FusionPlex Solid Tumor and Comprehensive Thyroid and Lung kits, and a VariantPlex Solid Tumor kits (ArcherDX). Results of molecular findings are summarized in Table 3.

**Table 2** Detailed clinicopathological, immunohistochemical, and molecular information of the 25 studied cases

| Case no. | Sex | Age | Diagnosis | Site | AR | MIB1 | HER2 | HER2/neu amplification (FISH) | GATA3 | CK5/6 | TNM | Disease status | Distant metastasis | Recurrence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 60 | SDC | Submandibular gland | + | 60% | 2+ | – | + | + | T2N2bM0 | NED | Yes | No |
| 2 | M | 79 | SDC | Submandibular gland | + | 40% | 2+ | – | + | + | T3N1M0 | DOD | Yes | No |
| 3 | M | 77 | SDC | Parotid | + | 60% | 3+ | + | – | ND | T4N2Mx | DOD | No | Yes |
| 4 | M | 63 | SDC ex PA | Parotid | + | 70% | 1+ | – | + | + | T2N0M0 | DOD | Yes | No |
| 5 | F | 49 | SDC ex PA | Submandibular gland | + | 80% | 2+ | – | – | + | T1N0M0 | DOD | No | No |
| 6 | M | 59 | SDC ex PA | Parotid | + | 50% | – | NA | + | – | T2N0M0 | DOD | Yes | No |
| 7 | M | 83 | SDC ex PA | Parotid | + | 40% | 3+ | + | ND | ND | T3N0M0 | DOC | No | No |
| 8 | F | 55 | SDC ex PA | Parotid | + | 40% | 2+ | ND | – | + | T3N0M0 | NED | No | No |
| 9 | M | 52 | SDC | Submandibular gland | + | 40% | 3+ | + | + | – | T3N0M1 | DOD | Yes | No |
| 10 | M | 69 | SDC ex PA | Parotid | + | 40% | – | NA | – | + | TxN2M0 | DOC | No | No |
| 11 | M | 86 | SDC | Parotid | + | 40% | 3+ | + | + | ND | T4N0Mx | DOC | No | No |
| 12 | M | 60 | SDC | Submandibular gland | + | 80% | – | – | + | – | T2N0Mx | DOD | No | Yes |
| 13 | F | 54 | SDC | Submandibular gland | + | 40% | 3+ | + | + | + | T1N2Bm1 | DOD | Yes | Yes |
| 14 | M | 53 | SDC ex PA | Parotid | + | 30% | 3+ | ND | + | ND | T4N2aM0 | DOD | No | No |
| 15 | M | 66 | SDC | Parotid | + | 40% | – | ND | + | + | T3N0Mx | AWD | No | No |
| 16 | M | 60 | SDC ex PA | Parotid | + | ND | 3+ | ND | + | ND | T3N0Mx | NtA | No | No |
| 17 | M | 95 | SDC | Submandibular gland | + | 25% | 2+ | - | + | ND | T4aN2bM1 | DOD | No | No |
| 18 | M | 24 | SDC | Submandibular gland | + | 55% | 3+ | ND | + | ND | T3N1M0 | AWD | Yes | No |
| 19 | M | 61 | SDC ex PA | Submandibular gland | + | 45% | – | - | – | + | T4aN2bM1 | NED | No | No |
| 20 | M | 64 | SDC ex PA | Parotid | + | 15% | - | ND | + | ND | T1N0M0 | NED | No | No |
| 21 | M | 89 | SDC ex PA | Parotid | + | 40% | ND | ND | + | ND | T4aN1M0 | AWD | No | No |
| 22 | M | 63 | SDC | Parotid | + | 30% | – | - | – | + | T3N2bM1 | + | No | No |
| 23 | M | 65 | SDC | Parotid | + | 50% | 3+ | + | + | + | T2N2bM1 | AWD | No | No |
| 24 | M | 73 | SDC | Parotid | + | 40% | 3+ | + | + | ND | T2N2bM1 | DOD | No | No |
| 25 | M | 70 | SDC ex PA | Submandibular gland | + | 60% | 3+ | ND | + | – | T1N0M0 | AWD | Yes | No |

Abbreviations: AWD, alive with disease; DOC, died of other causes; DOD, died of disease; NA, not analyzable; ND, not done; NED, no evidence of disease; NtA, not available; +, not available.

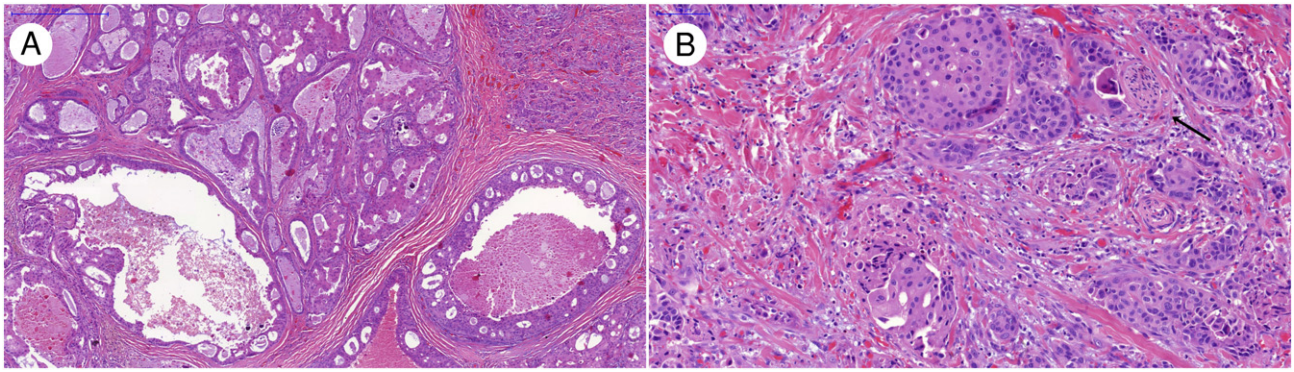


**Fig. 1** Representative histologic features of salivary duct carcinoma. A, SDC showing dilated ductal structures embedded in densely hyalinized stroma, with areas of comedonecrosis. B, SDC cells exhibiting large pleomorphic nuclei and abudant eosinophilic cytoplasms. Area of perineural invasion is also observed (arrow).

Fusion detection analysis revealed 1 case harboring an *ETV6-NTRK3* fusion; therefore, it was reclassified as a high-grade secretory carcinoma and not further tested (Supplementary Table 1 and Supplementary Fig. 1). No other gene fusions were detected in the remaining 8 cases, tested at least with 1 of the fusion detection kits. Nine pathogenic/likely pathogenic mutations were detected in 7 cases. All of detected variants that were in the regions of interest of the Comprehensive Thyroid and Lung panel were also detected from RNA (HRAS mutations in cases 26 and 27). The following transcripts were used for variant annotation: *HRAS* - NM_001130442.1, *TP53* - NM_000546.5, *PIK3CA* - NM_006218.2, *PTEN* - NM_000314.4, *TERT* - NM_198253.2, *AKT1* - NM_005163.2, *PTEN* - NM_000314.6. Three cases were positive for amplification of *ERBB2* gene using FISH probes (example in Fig. 3), and corresponding overexpression using NGS was detected. Homozygous deletion of locus 9p21 (*CDKN2A*) was detected in case 26. Case 17 showed amplification of *MDM2*. The other cases were negative for *CDKN2A* deletion or *MDM2* amplification.

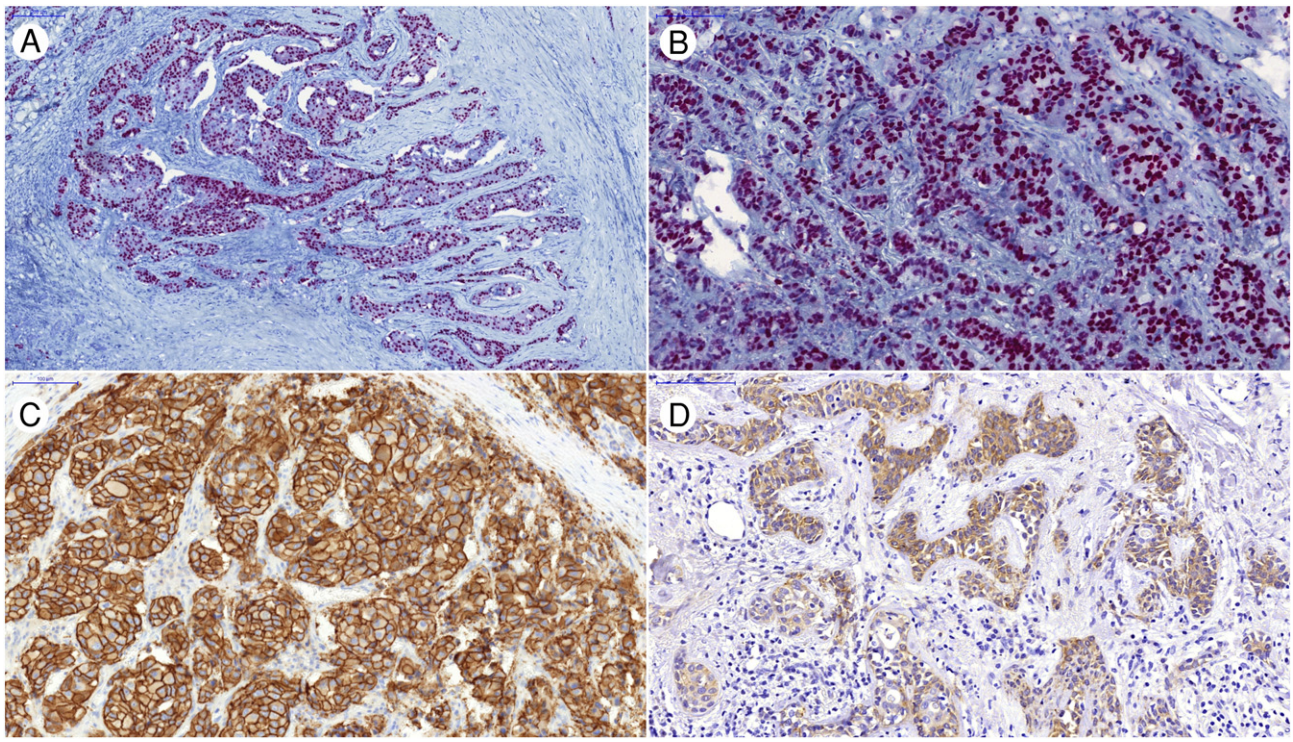


**Fig. 2** Representative images of positive immunohistochemical reactions. A, Androgen receptor diffuse nuclear immunostaining. B, GATA3 diffuse nuclear immunostaining. C, Diffuse and strong membranous immunostaining of HER2/neu. D, Moderate cytoplasmic expression of CK5/6.

**Table 3** Results of NGS and FISH analysis in 8 cases of SDC

| Case | AST (fusions) [a] | CTL (fusions and variants) [b] | VST (variants) [c] | *HER2/Neu* [d] | *CDKN2A* [e] | *MDM2* [f] |
|---|---|---|---|---|---|---|
| 1 | Negative | NA | NA | Negative | Negative | Negative |
| 2 | Negative | HRAS c.37G > C p.Gly13Arg, freq:25% | NA | Negative | NA | Negative |
| 15 | Negative | Negative | TERT c.-146C > T (C250T), AF:50% | Negative | Negative | Negative |
| 16 | Negative | Negative | PTEN c.1003C > T p.Arg335Ter, AF:90% | Positive | Negative | Positive |
| 22 | Negative | AKT1 c.49G > A p.Glu17Lys, freq: 60% | NA | Negative | Negative | Negative |
| 23 | Negative | HRAS c.182A > G p.Gln61Arg, freq: 55% | HRAS c.182A > G p.Gln61Arg, AF:42%<br>PIK3CA c.3140A > G p.His1047Arg, AF:17% | Negative | Deletion | Negative |
| 24 | Negative | HRAS c.37G > C p.Gly13Arg, freq: 62% | HRAS c.37G > C p.Gly13Arg, AF:39%<br>PIK3CA c.3140A > G p.His1047Arg, AF:8%<br>TP53 c.817C > T p.Arg273Cys, AF:10% | Positive | Negative | Negative |
| 25 | Negative | Negative | HRAS c.187G > A p.Glu63Lys, AF:10%<br>TP53 c.830G > T p.Cys277Phe, AF:58% | Positive | Negative | Negative |

Abbreviations: AF, alelle frequency; AST, FusionPlex Solid Tumor kit for fusions detection; *CDKN2A*, FISH enumeration of *CDKN2A*; CTL, Comprehensive Thyroid and Lung kit for detecting fusions and variants; freq, frequency of mutated reads (RNA); *HER2/Neu*, FISH enumeration of *ERBB1 (HER2/Neu)*; *MDM2*, FISH enumeration of *MDM2*; VST, VariantPlex Solid Tumor kit for detecting variants.

[a] Eight of 8 tested cases.
[b] Seven of 8 tested cases.
[c] Five of 8 tested cases.
[d] Eight of 8 tested cases.
[e] Seven of 8 tested cases.
[f] Eight of 8 tested cases.

### 3.6. Association of biomarker immunoprofile and *HER-2/neu* gene amplification with clinicopathological factors

Results of comparisons made between immunohistochemistry and FISH results with clinicopathological factors are summarized in Table 4. We observed that CK5/6-positive cases were more often diagnosed at tumor stage III/IV ($P$ = .05). Other variables were not significantly associated to biomarker immunoprofile or *ERBB2* gene amplification.

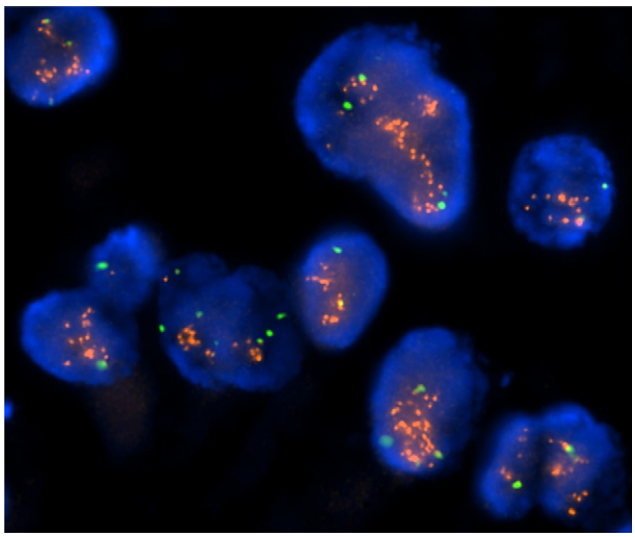

**Fig. 3** SDC exhibiting *ERBB2* (*HER2/neu*) amplification (FISH).

### 3.7. Results of survival analysis

Median survival time for *Apocrine A* (AR+/HER2−/MIB1-low), *Apocrine B* (AR+/HER2−/MIB1-high), and *Apocrine HER2* (AR+/HER2+) cases were, respectively, 48, 60.13, and 30.56 months. We observed that *Apocrine HER2* subtype was significantly associated with lower OS (hazard ratio = 0.15 [confidence interval: 0.014-1.74], $P$ = .05). Those results are summarized in Supplementary Table 2, and Kaplan-Meier curves are visualized in Supplementary Fig. 2.

Other variables were not associated with survival. Results of further survival analysis are summarized in Table 5.

## 4. Discussion

Although SDC is a relatively uncommon entity, many studies over the past years have identified potential biomarkers for this malignancy, and the introduction of HER2 and AR targeted therapies is already a reality for some patients [8]. Nevertheless, the precise correlation of immunoprofile and patients' clinicopathological characteristics and disease prognosis has not been fully explored for this tumor [15].

SDC reportedly presents *HER2/neu* positivity varying from 15% to 44% [2-4,16,17] in studies that used the scoring system recommended by the 2007 ASCO/CAP guideline for breast cancer [18]. In this study, we used the updated 2013 ASCO/CAP guideline to evaluate *HER2/neu* immunoexpression [14]. In this guideline, the cutoff immunohistochemical level

**Table 4** Association of biomarker immunoprofile with clinicopathological factors in studied cases of SDC

| Variable | AR [a] | | *P* [g] | HER2/*neu* [b] | | *P* [g] | HER2/*neu* [c] amplification | | *P* [g] | GATA3 [d] | | *P* [g] | CK5/6 [e] | | *P* [g] | MIB1 [f] index | | *P* [g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Neg | Pos | | Neg | Pos | | Neg | Pos | | Neg | Pos | | Neg | Pos | | Low | High | |
| Age, y | | | | | | | | | | | | | | | | | | |
| ≤60 | 0 | 10 | .99 | 5 | 5 | .68 | 3 | 2 | 1.00 | 2 | 8 | .97 | 3 | 4 | .29 | 1 | 8 | .61 |
| >60 | 0 | 15 | | 5 | 9 | | 5 | 5 | | 3 | 11 | | 1 | 7 | | 4 | 10 | |
| Sex | | | | | | | | | | | | | | | | | | |
| Male | 0 | 22 | .99 | 12 | 9 | .26 | 7 | 6 | 1.00 | 3 | 17 | .28 | 4 | 7 | .51 | 5 | 14 | .56 |
| Female | 0 | 3 | | 2 | 1 | | 1 | 1 | | 2 | 2 | | 0 | 4 | | 0 | 4 | |
| T classification | | | | | | | | | | | | | | | | | | |
| 1-2 | 0 | 9 | .99 | 7 | 3 | .66 | 4 | 3 | .22 | 1 | 8 | .37 | 3 | 4 | .58 | 1 | 8 | .48 |
| 3-4-x | 0 | 16 | | 7 | 7 | | 4 | 4 | | 4 | 11 | | 1 | 7 | | 4 | 14 | |
| N classification | | | | | | | | | | | | | | | | | | |
| 0 | 0 | 13 | .99 | 7 | 5 | .68 | 3 | 3 | .77 | 4 | 8 | .66 | 4 | 4 | .10 | 1 | 9 | .32 |
| 1-2 | 0 | 12 | | 7 | 5 | | 5 | 4 | | 3 | 11 | | 0 | 7 | | 4 | 9 | |
| Tumor stage | | | | | | | | | | | | | | | | | | |
| I/II | 0 | 6 | .99 | 5 | 1 | .62 | 3 | 0 | .30 | 1 | 4 | 1.00 | 3 | 2 | **.05** [h] | 1 | 5 | .55 |
| III/IV | 0 | 19 | | 9 | 9 | | 5 | 7 | | 4 | 15 | | 1 | 9 | | 4 | 13 | |
| Primary site | | | | | | | | | | | | | | | | | | |
| Parotid gland | 0 | 15 | .99 | 8 | 6 | .39 | 6 | 2 | .24 | 3 | 12 | .69 | 1 | 6 | .29 | 4 | 9 | .99 |
| Submandibular gland | 0 | 10 | | 6 | 4 | | 2 | 5 | | 2 | 7 | | 3 | 5 | | 1 | 9 | |
| Histologic origin | | | | | | | | | | | | | | | | | | |
| De novo | 0 | 13 | .99 | 7 | 6 | 0.68 | 5 | 6 | .15 | 2 | 11 | .63 | 2 | 5 | 1.00 | 2 | 11 | .16 |
| Ex pleomorphic adenoma | 0 | 12 | | 7 | 4 | | 3 | 1 | | 3 | 8 | | 2 | 6 | | 3 | 7 | |

[a] Twenty-five cases tested.
[b] Twenty-five cases tested.
[c] Fifteen cases tested.
[d] Twenty-four cases tested.
[e] Fifteen cases tested.
[f] Twenty-three cases tested.
[g] Fisher exact test.
[h] Statistically significant.

**Table 5** Results of univariate and multivariate survival analysis for clinicopathological and immunoprofile characteristics of salivary duct carcinoma

| Factor | No. of cases | Univariate analysis | | | | Multivariate analysis [b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | OS | | DFS | | OS | | DFS | |
| | | HR (95% CI) | *P* | HR (95% CI) | *P* | HR (95% CI) | *P* | HR (95% CI) | *P* |
| Sex | | | .99 | | .83 | 0.99 (0.21-4.57) | .99 | 0.66 (0.13-3.23) | .61 |
| Female | 5 | 1.01 (0.28-3.56) | | 0.88 (0.24-3.23) | | | | | |
| Male [a] | 22 | | | | | | | | |
| Age groups | | 1.23 (0.48-3.12) | .66 | | .72 | 1.08 (0.27-4.30) | .90 | 1.83 (0.47-7.10) | .37 |
| ≤60 y | 10 | | | 0.85 (0.32-2.23) | | | | | |
| >60 y [a] | 15 | | | | | | | | |
| T | | | | | .39 | 2.11 (0.41-10.88) | .37 | 1.03 (0.22-4.79) | .96 |
| T1/T2 | 10 | 1.85 (0.73-4.65) | .18 | 1.51 (0.57-3.92) | | | | | |
| T3/T4/Tx [a] | 15 | | | | | | | | |
| N | | | .74 | | .39 | 1.48 (0.38-5.72) | .56 | 2.60 (0.57-11.81) | .21 |
| N0 | 12 | 1.16 (0.46-2.94) | | 1.47 (0.57-3.75) | | | | | |
| N1/N2 [a] | 13 | | | | | | | | |
| Tumor staging | | | .57 | | .40 | 0.78 (0.24-2.51) | .68 | 0.54 (0.14-1.99) | .35 |
| I/II | 6 | | | 1.56 (0.57-4.24) | | | | | |
| III/IV [a] | 19 | 1.35 (0.48-3.80) | | | | | | | |
| Distant metastasis | | | .26 | 0.81 (0.32-2.06) | .64 | 0.33 (0.06-1.69) | .18 | 1.01 (0.22-4.63) | .98 |
| Absent | 15 | 0.60 (0.24-1.52) | | | | | | | |
| Present [a] | 8 | | | | | | | | |
| Primary tumor site | | | .50 | | .77 | 0.84 (0.16-4.21) | .83 | 1.08 (0.22-5.31) | .91 |
| Parotid gland | 15 | 0.70 (0.24-1.99) | | 0.85 (0.30-2.44) | | | | | |
| Submandibular gland [a] | 10 | | | | | | | | |
| HER-2 | | | | | .56 | 0.46 (0.13-1.60) | .22 | 0.55 (0.14-2.05) | .37 |
| Positive | 11 | 0.67 (0.23-1.69) | | 0.73 (0.25-2.09) | | | | | |
| Negative [a] | 13 | | | | | | | | |
| GATA3 | | | .24 | | .26 | 1.40 (0.45-4.29) | .55 | 1.05 (0.31-3.54) | .93 |
| Positive | 17 | 1.72 (0.69-4.28) | | 1.70 (0.66-4.39) | | | | | |
| Negative [a] | 5 | | | | | | | | |
| HER2/*neu* amplification | | | .20 | | .14 | 1.13 (0.43-2.96) | .79 | 1.38 (0.48-4.00) | .54 |
| Positive | 7 | 0.60 (0.27-1.32) | | 0.55 (0.25-1.22) | | | | | |
| Negative [a] | 8 | | | | | | | | |

NOTE. Log-rank (Mantel-Cox) test.

[a] Comparison factor.

[b] Adjusted for sex, age groups, tumor staging, and site.

for *HER2* positivity was reduced from 30% to 10%. We found *HER2* overexpression in 42% of cases. On the other hand, Takase et al [15] used the same scoring guidelines in their study and observed a high expression of *HER2* in 46% of SDC cases. They also observed a high concordance between *HER2* overexpression and gene amplification. In the current study, we observed *HER2* amplification in 7 of 15 SDC cases, and all 7 cases were also HER2 3+ immunopositive.

*HER2* overexpression has been associated with poor prognosis in patients with breast cancer [19]. On the contrary, we did not observe any associations between *HER2* status and clinicopathological/prognostic parameters in SDC cases. Other recent studies also demonstrated that *HER2* is not a prognostic factor for SDC [4,15-17].

AR is usually expressed in SDC [15,17,20-22], and in this study, we observed AR immunoreactivity in 25 of 25 SDC cases, which was a similar percentage to that observed in other recent series [15,23,24]. Absence of AR expression has been associated with poor outcome in SDC patients [4,15,20] as well as in prostate and breast cancers [25,26]. Yet, the role of AR in the molecular pathogenesis of SDC has not been fully understood [23].

A less studied possible biomarker of SDC is the GATA protein biding 3 (GATA3). GATA3 is a member of the GATA family of zinc finger transcription factors, and binding of GATA members, in general, is thought to promote differentiation, development, and/or cell proliferation of different tissues and cell types, including breast, kidney, nerves, skin, and T-lymphocytes [27,28]. Few studies have showed that GATA3 is overexpressed in about 100% of SDCs [17,27,29,30]. However, in the present study, we observed GATA3 expression in 19 of 26 (73.08%) SDC cases. Although no statistical associations were identified between GATA3 expression and patients' outcome, studies with larger

cohorts would be helpful to unveil a possible relation between GATA3 expression and SDC.

The expression of basal cell markers is usually absent or low in SDC; however, some cases present positive expression of CK5/6 [2]. In our study, we observed positive CK5/6 expression in 13 of 15 SDC cases. This number is higher than previously reported [3,15], although it can possibly be explained by the small sample investigated. We also observed that CK5/6-positive cases were significantly associated to higher tumor stage. In a larger sample of 149 SDCs, Takase et al [15] observed that CK5/6 overexpression was an independent prognostic factor for this tumor.

Biomarker immunoprofile has been largely used to classify breast cancer into molecular groups, and these groups were proven to have distinct clinical behavior and response to chemotherapy [31]. Because of the similarities between breast ductal carcinoma and SDC, Di Palma et al [3] introduced a classification for SDC where it is classified into 4 main groups: luminal AR positive (AR+/HER2−), HER2 (AR any/HER+), basal-like (AR−/HER2−/EGFR and/or CK5/6+), and intermediate (others). In 2015, Masubuchi et al [4] proposed a new classification system similar to the one used for breast cancer based on AR, HER2, and Ki-67 staining. Recently, this revised classification was evaluated in a large SDC cohort and proven to be a good prognosis predictor [15].

In the present study, most cases (15/26) were classified as *Apocrine HER2* (AR+/HER2+), and we observed that this subtype showed lower OS. These results are in accordance with the ones described by Takase et al [15], who observed that *Apocrine B* (AR+/HER2−/MIB1-high), *Apocrine HER2* (AR+/HER2+), *HER2 enriched* (AR−/HER2+), and *Double negative* (AR−/HER2−) SDC subtypes had worst prognosis when compared to *Apocrine A* (AR+/HER2−/MIB1-low) subtype. Although we had few cases in our sample, in each subtype, we were able to demonstrate that the revised classification system might be a useful predictor of prognosis for some subtypes of SDC; however, further studies are needed to prove this relation.

Besides investigating immunoprofile, we performed NGS analysis in 10 cases of our sample. As said before, this analysis revealed 1 case harboring an *ETV6-NTRK3* translocation, which is well known to be exclusively found in secretory carcinoma; therefore, this case was reclassified as a high-grade secretory carcinoma. One case was not analyzable and was excluded. Furthermore, the other 8 cases (8/8 studied cases) did not present any detectable translocations.

Nine pathogenic mutations were detected in 7 of 7 SDC cases; the most mutated gene was *HRAS* followed by *PIK3CA*, *TP53*, and other genes (*AKT1, PTEN, TERT*). All of these mutations were described as pathogenic in the database of clinical relevance of mutations ClinVar [32].

Recent studies investigating the molecular pathology of SDC have demonstrated mutations in diverse genes in this tumor [6,9,10,12,17,30,33]. Mutations in gene *TP53* have been demonstrated to be one of the most frequent in SDC, varying from 37.5% to 68% of studied cases [12,33], and we observed 2 *TP53* mutations in our cohort, c.817C > T p.Arg273Cys in case 27 and c.830G > T p.Cys277Phe in case 28.

In this study, *HRAS* mutations were the most frequent, affecting 4 of 8 studied patients. Other studies have also demonstrated *HRAS* mutations to be common in SDC, with a frequency varying from 13% to 56.2% [6,12,17,33]. This is a highly relevant finding in the biological and therapeutic point of view because *HRAS* mutations are known to reduce sensitivity to HER2 inhibition [12,30]. We observed 1 case with *HRAS* mutation that presented *HER2* gene amplification, and this was also observed in the studies by Khoo et al [30] and Chiosea et al [6].

We also identified mutations in genes *PTEN* (c.1003C > T, p.Arg335Ter) and *AKT1* (c.49G > A p.Glu17Lys). *PTEN* is a frequently disrupted tumor suppressor in cancer, and its lipid phosphatase activity antagonizes the (PI3K/AKT/mTOR pathway to repress tumor cell growth and survival [9,12,30]. Mutations in *PTEN* and *AKT1* in SDC have also been identified in other studies [6,9,10,12,17,30]. Furthermore, *PTEN* loss has been reported to be a frequent finding in SDC [6,11,30,33], and in a large cohort of 151 SDCs, Shimura et al [33] demonstrated that loss of *PTEN* was significantly associated to higher T stage and to lower expression of AR. These findings imply an important role of *PI3K/AKT/mTOR* pathway signaling in the tumorigenesis of SDC and could implicate new therapeutic targets for individualized treatment.

Furthermore, 1 case (of 7) harbored a mutation in the human telomerase reverse transcriptase (*TERT*) gene. This seems to be a rare event in SDC, as reported by Dalin et al [9], who found no mutations in this gene in 31 SDC cases.

Additionally, we observed that 1 SDC case harboring a *PTEN* mutation also presented amplification of the gene *MDM2*. This seems to be a rare event, as described by Grünewald et al [34], who identified *MDM2* amplification in 3 cases of 51 SDCs, and by Chiosea et al [6], who observed 1 SDC case (of 37) with amplified *MDM2*. Interestingly, those studies observed that *MDM2* was amplified simultaneously with p53 mutation in 3 cases.

In conclusion, we observed that SDC *Apocrine HER2* (AR+/HER2+) subtype is related to decreased survival, which indicates that the revised classification system might be a useful predictor of prognosis for some subtypes of SDC, and validation of this system is highly recommended for future researches. Furthermore, NGS analysis in a small subset of SDC cases revealed mutations in *HRAS*, *AKT1, PTEN, TP53, CDKN2A,* and *TERT* genes in 7 of 7 cases. Those preliminary results support the use of targeted NGS for unrevealing the molecular pathogenesis underlying SDC and identifying possible individualized therapeutic targets.

## Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.08.009.

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