

Detection of Novel Actionable Genetic Changes in Salivary Duct Carcinoma Helps Direct Patient Treatment

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Abstract

Purpose: Salivary duct carcinomas (SDC) are a rare and aggressive subtype of salivary gland cancers for which cytotoxic chemotherapy has limited efficacy. We investigated whether genotyping analysis could detect novel tumor-specific mutations that would help direct SDC patient treatment using targeted agents.

Experimental Design: We genotyped 27 SDC archival specimens from patients followed at Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary (Boston, MA) between 2000 and 2011. These included the tumors of 8 patients who were tested prospectively. Targeted mutational analysis of 13 clinically relevant cancer genes was conducted using SNaPshot multiplexed genotyping. FISH was conducted to detect *HER2* gene amplification. Patient medical records and tumor histopathologic features were retrospectively reviewed.

Results: Mutually exclusive genetic aberrations were detected in 15 of 27 (56%) tumors, including 2 (7%) mutations in *BRAF*, 5 (19%) mutations in *PIK3CA*, and 8 (30%) cases of *HER2* gene amplification. To our knowledge, this is the first time that *BRAF* and *PIK3CA* mutations have been reported in this tumor type. Prospective clinical testing of 8 patients with SDC identified actionable genetic alterations in 6 tumors and influenced therapeutic decisions for all 6 patients.

Conclusion: SNaPshot molecular profiling identified novel genetic changes in SDCs, expanded the therapeutic options for patients with this rare tumor, and is changing SDC management at our institution. These findings highlight the importance of using broad-based genetic profiling to expedite the identification of effective-targeted therapies for patients with rare malignancies. *Clin Cancer Res*; 19(2); 480–90. ©2012 AACR.

Introduction

Salivary gland cancers are rare, accounting for less than 5% of all cancers of the head and neck, and are among the most heterogeneous primary tumors that arise in the body, with very different natural histories according to the specific histologic subtypes (1). Salivary duct carcinomas (SDC) account for approximately 6% of all salivary gland cancers and are among the most aggressive salivary malignancies (2).

First described in 1968, SDC histopathology is characterized by solid and cribriform cell nests with central necrosis resembling ductal carcinoma of the breast (3). SDC can occur *de novo* or as carcinoma ex-pleomorphic adenomas and primarily involve the major salivary glands (96%), particularly the parotid. The average age at diagnosis is 50 years and they tend to occur in men (approximately 75%). Patients typically present with an enlarging mass in the parotid accompanied by facial nerve dysfunction, and often with locally advanced disease. Despite aggressive clinical management (complete surgical resection and chemoradiation) about 65% of patients with SDC die from progressive disease by 48 months (4).

In addition to the histologic resemblance between SDC and breast ductal carcinoma, overexpression of the *HER2/neu* is also a feature shared by both tumor types (5–8). Review of the literature indicates that approximately 37% of SDC (64 of 173 cases) exhibit strong (3+ score) *HER2* overexpression, as assessed by immunohistochemistry (IHC), and 72% of these (46 of 64 cases) are positive for *HER2* gene amplification, as determined by FISH or chromogenic *in situ* hybridization (CISH; refs. 9–17). In contrast to breast carcinoma, SDC are consistently negative for estrogen- α and progesterone receptors, whereas the

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Translational Relevance

Broad-based tumor genotyping is expediting the clinical development of effective-targeted therapies, which is particularly helpful for patients with rare malignancies of poorly understood pathogenesis, who have limited therapeutic options. We applied a multiplexed tumor genotyping assay focused on clinically actionable targets, SNaPshot, and *HER2* FISH to study salivary duct carcinoma (SDC), a rare and aggressive salivary gland tumor for which current chemotherapeutic regimens are largely ineffective. We identified mutually exclusive genetic aberrations in 15 of 27 (56%) tumors and detected, for the first time in SDC, activating mutations in the *PIK3CA* and *BRAF* genes while confirming the presence of *HER2* gene amplification in a subset of cases. These findings were rapidly translated to clinical care, expanding the therapeutic options for patients with SDC. Indeed, prospective clinical testing of 8 patients with SDC identified actionable genetic alterations in 6 tumors, prompting treatment of all 6 patients with targeted therapies.

expression of androgen receptor (AR) is observed in the majority of cases (18).

Given the rarity of the disease, establishing effective systemic therapy for such patients has been a challenge. Clinical trials are primarily limited to small phase II studies for patients with salivary gland cancers of multiple histologies, and have yielded modest response rates (19, 20). The need for better therapies has prompted a number of efforts to characterize the biologic underpinnings of the various salivary gland histologies. With the rapidly expanding new generation of targeted therapies, we now have the ability to explore new treatments based on tumor-specific genetic markers. These approaches are especially critical in patients for whom no proven conventional treatment options are known.

We developed a multiplexed tumor genotyping clinical assay (SNaPshot) that has been adopted at our institution for over 3 years, to help guide patient treatment of various malignancies (21). Clinical SNaPshot testing of 1 patient with SDC detected a novel targetable mutation in the *BRAF* oncogene (V600E). On the basis of this finding, we extended our genotyping efforts to a retrospective cohort of 19 patients with SDC, and conducted prospective clinical testing of 7 additional patients. We identified actionable genetic alterations in 15 (56%) cases and our data impacted therapeutic decisions for 6 patients.

Patients and Methods

Patient selection

SDC cases diagnosed between 2000 and 2010 were identified by searching the Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary (Boston, MA) records. Excess archival human material, in the form of

formalin-fixed paraffin-embedded (FFPE) blocks, was retrieved for 19 patients and used under a research protocol approved by the Institutional Review Board for human research (reference: 2008-P-002165; MGH). Between 2009 and 2011, 8 patients were tested prospectively as part of their standard-of-care. SNaPshot tumor genotyping has been available at our institution as a clinical test since March 2009, and is offered to any patient with cancer at the discretion of the treating physician (21). Prospective clinical genotyping is carried out on FFPE surgical material and requires the patient's written informed consent. Patient medical records were reviewed for demographics, treatment, and outcomes. Stage at diagnosis was determined according to the American Joint Committee on Cancer (AJCC) guidelines (22).

Genetic analysis

Archival surgical specimens were reviewed by a pathologist (V. Nardi) to select the most appropriate tumor-enriched area for analysis. Total nucleic acid was extracted from FFPE tumor tissue obtained by manual macro-dissection, or by coring the paraffin block with a 1.5 mm dermal punch, followed by extraction using a modified FormaPure System (Agencourt Bioscience Corporation; ref. 21). Multiplexed mutational analysis of a panel of 13 cancer genes, which included *BRAF*, *EGFR*, *KRAS*, *NRAS*, *PIK3CA*, and *TP53* (Supplementary Table S1), was conducted using a previously reported tumor genotyping protocol that was developed by our group, and is based on Applied Biosystems' SNaPshot platform (21).

The mutational changes identified by SNaPshot genotyping were confirmed by Sanger sequencing, using previously described conditions (21). For *PIK3CA* E545K and H1047R mutations, we conducted direct sequencing of M13-tagged PCR products generated using the following primer pairs: *PIK3CA* exon 9, 5'-TGTAACGACGGCCAGTCTGTAATCCAGAGGGAAA-3' (forward) and 5'-CAGGAAACAGCTATGACCCATGCTGAGATCAGCCAAAT-3' (reverse); and *PIK3CA* exon 20, 5'-TGTAACGACGGCCAGTCATTTGCTCCAACTGACCA-3' (forward) and 5'-CAGGAAACAGCTATGACCTGTGGAATCCAGAGTGAGCTT-3' (reverse). Thermocycling was conducted at 95°C for 8 minutes, followed by 45 cycles of 95°C for 20 seconds, annealing at 58°C (exon 20) or at 68°C (exon 9) for 30 seconds and 72°C for 1 minute, and 1 last cycle of 72°C for 3 minutes.

Confirmation of *BRAF* V600E mutations was conducted using direct sequencing of PCR products generated using the following primer pair: *BRAF* exon 15, 5'-TCATAATGCTTGCTCTGATAGGA-3' (forward) and 5'-GGCCAAATTTAATCAGTGGA-3' (reverse). Thermocycling was conducted at 94°C for 2 minutes, followed by 38 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds and 72°C for 45 seconds, and 1 last cycle of 72°C for 10 minutes.

FISH was conducted on 5- μ m sections of FFPE tumor tissue, to evaluate copy number changes in the *HER2* gene. Dual-color FISH was carried out using the PathVysion assay (Abbott Molecular), which includes 1 probe specific to the chromosome 17q *HER2* locus and a copy number control

probe recognizing centromere 17 (CEP17). *HER2* gene amplification was determined following previously established guidelines, which define amplification as a *HER2*: centromere 17 (CEP17) signal ratio greater than 2.2 scored in 50 tumor nuclei (23).

Immunohistochemistry

Five-micrometer thick tissue sections were cut from selected paraffin blocks, mounted onto charged glass slides, and air-dried overnight in a 58°C convection oven. Immunohistochemical staining was conducted on a fully automated Leica Bond Platform using the Leica Refine Detection Kit, including antigen retrieval (EDTA 9.0 for 20 minutes) and deparaffinization steps. We used the AR antibody from Dako (1:50 dilution). A positive control (section of known AR-positive prostate cancer) was run simultaneously. Immunostaining for AR was identified by a dark brown stain confined exclusively to the nucleus. Cases were considered positive if staining was observed in more than 50% of tumor cell nuclei, negative when seen in fewer than 5%, and weak or moderate if staining was detected in 5% to 20% or 21% to 50% of tumor cell nuclei, respectively (24). All staining results were evaluated by a pathologist (P.M. Sadow).

Statistical methods

Hypothesis tests for the association between clinicopathologic features and the *PIK3CA*, *BRAF*, and *HER2* status were conducted with Fisher exact tests. Survival curves were estimated using the Kaplan–Meier estimator; HRs were estimated using the Cox proportional hazards model, and *P* values for comparing survival distributions, were calculated using log-rank tests.

Results

Clinical characteristics of the cohort

Patient demographics show a median age at presentation of 64 years (range, 24–88), a high male to female ratio (8:1), and advanced disease at presentation, with 19 of 27 (70%) patients presenting with lymph node metastasis and 4 of 27 (15%) with distant metastasis (Tables 1 and 2). The majority (82%) of patients presented with stage IV disease (Table 1), and the overall prognosis was poor with 44% of initially resectable patients experiencing disease recurrence at a median of 10.03 months (range, 1.1–97.12; Supplementary Table S2).

Representative images of SDC histology are illustrated in Supplementary Fig. S1A–S1D showing infiltrative ductal elements, cellular pleomorphism, high mitotic activity, and an exquisite resemblance to ductal carcinoma of the breast. Characteristic positive staining for *HER2* and for AR are also depicted (Supplementary Fig. S1E and S1F, respectively). Additional histopathologic features identified in our cohort are outlined in Table 2. Perineural and lymphovascular invasion were observed in 20 (74%) and 15 (56%) cases, extraglandular extension in 16 (59%) cases, and the presence of positive margins was detected in 14 (52%) tumors. Tumor cell necrosis was detected in 12 (44%) specimens. Lymph node involvement and extranodal extension were identified in 19 (70%) and 9 (33%) cases, respectively.

Molecular profiling of SDC identifies *HER2* gene amplification and novel activating mutations in *PIK3CA* and *BRAF*

SNaPshot mutational profiling identified mutually exclusive point mutations in 7 of 27 (26%) SDC specimens, of which 24 were primary tumors and 3 were metastases

Table 1. Baseline characteristics of SDC patients

	All patients <i>n</i> = 27 No. (%)	<i>PIK3CA</i> mutational status		<i>BRAF</i> mutational status		<i>HER2</i> amplification status	
		Positive <i>n</i> = 5 No. (%)	Negative <i>n</i> = 22 No. (%)	Positive <i>n</i> = 2 No. (%)	Negative <i>n</i> = 25 No. (%)	Positive <i>n</i> = 8 No. (%)	Negative <i>n</i> = 19 No. (%)
Age, y							
Median	64	54	64	57	65	69	62
Range	24–88	39–76	24–88	52–62	24–88	55–84	24–88
Gender							
Male	24 (89)	4 (80)	20 (91)	2 (100)	22 (88)	6 (75)	18 (95)
Female	3 (11)	1 (20)	2 (9)	0	3 (12)	2 (25)	1 (5)
Stage							
I	1 (4)	0	1 (4.5)	0	1 (4)	0	1 (5)
II	1 (4)	0	1 (4.5)	0	1 (4)	1 (12)	0
III	2 (7)	0	2 (9)	0	2 (8)	0	2 (11)
IVA	17 (63)	4 (80)	13 (59)	2 (100)	15 (60)	5 (63)	12 (63)
IVB	1 (4)	0	0	0	1 (4)	0	1 (5)
IVC	4 (15)	1 (20)	4 (18)	0	4 (16)	2 (25)	2 (11)
Unknown	1 (4)	0	1 (4.5)	0	1 (4)	0	1 (5)

Table 2. SDC tumor characteristics

	All patients <i>n</i> = 27 No. (%)	<i>PIK3CA</i> mutational status		<i>BRAF</i> mutational status		<i>HER2</i> amplification status	
		Positive <i>n</i> = 5 No. (%)	Negative <i>n</i> = 22 No. (%)	Positive <i>n</i> = 2 No. (%)	Negative <i>n</i> = 25 No. (%)	Positive <i>n</i> = 8 No. (%)	Negative <i>n</i> = 19 No. (%)
Necrosis							
Absent	7 (26)	0	7 (32)	1 (50)	6 (24)	2 (25)	5 (26)
Present	12 (44)	2 (40)	10 (45)	1 (50)	11 (44)	5 (63)	7 (37)
Unknown	8 (30)	3 (60)	5 (23)	0	8 (32)	1 (12)	7 (37)
Margins ^a							
Absent	9 (33)	2 (40)	7 (32)	0	9 (36)	2 (25)	7 (37)
Present	14 (52)	3 (60)	11 (50)	2 (100)	12 (48)	5 (63)	9 (47)
Unknown	4 (15)	0	4 (18)	0	4 (16)	1 (12)	3 (16)
Perineural invasion							
Absent	6 (22)	0	6 (27)	0	6 (24)	2 (25)	4 (21)
Present	20 (74)	5 (100)	15 (68)	1 (50)	19 (76)	6 (75)	14 (74)
Unknown	1 (4)	0	1 (5)	1 (50)	0	0	1 (5)
Lymphovascular invasion							
Absent	9 (33)	1 (20)	8 (36)	0	9 (36)	1 (12)	8 (42)
Present	15 (56)	3 (60)	12 (55)	2 (100)	13 (52)	5 (63)	10 (53)
Unknown	3 (11)	1 (20)	2 (9)	0	3 (12)	2 (25)	1 (5)
Extraglandular extension ^a							
Absent	8 (30)	0	8 (36)	1 (50)	7 (28)	2 (25)	6 (32)
Present	16 (59)	4 (80)	12 (55)	1 (50)	15 (60)	5 (63)	11 (58)
Unknown	3 (11)	1 (20)	2 (9)	0	3 (12)	1 (12)	2 (10)
Lymph node involvement ^a							
Absent	7 (26)	0	7 (32)	1 (50)	6 (24)	1 (12)	6 (32)
Present	19 (70)	5 (100)	14 (64)	1 (50)	18 (72)	7 (88)	12 (63)
Unknown	1 (4)	0	1 (5)	0	1 (4)	0	1 (5)
Extranodal extension ^a							
Absent	7 (26)	0	7 (32)	0	7 (28)	3 (38)	4 (21)
Present	9 (33)	3 (60)	6 (27)	1 (50)	8 (32)	4 (50)	5 (26)
Unknown	11 (41)	2 (20)	9 (41)	1 (50)	10 (40)	1 (12)	10 (53)

^aThese features are relevant for adjuvant therapy.

(Supplementary Table S3). All mutations were confirmed by Sanger sequencing. Five (19%) tumors harbored activating mutations in the p110- α subunit of the phosphatidylinositol 3-kinase (*PIK3CA*) gene. Three of these substitutions were located within the kinase domain of *PIK3CA* (H1047R; Fig. 1A), and the other 2 mapped to the helical domain (E545K; Fig. 1B). In our cohort, patients with *PIK3CA*-mutant SDC presented with stage IV disease (5/5), perineural invasion (5/5), extraglandular extension (4/4), positive lymph nodes (5/5), and extranodal tumor extension in the 3 cases with available information (3/3; Tables 1 and 2). While these features could be suggestive of a more aggressive tumor behavior, prognosis is universally poor in patients with this disease. In addition, we were limited by the size of our cohort, and found no statistically significant association between the presence of a *PIK3CA* mutation and any clinicopathologic features, time to recurrence, or overall survival.

SNaPshot testing detected the *BRAF* V600E kinase activating mutation in 2 (7%) additional SDC cases (Fig. 1C). Both *BRAF*-mutant tumors had positive margins, exhibited lymphovascular invasion, and were identified in male patients who presented with stage IVA disease (Tables 1 and 2).

The *HER2* gene encodes for a receptor tyrosine kinase often amplified and overexpressed in SDC (5–17). Using FISH, we detected *HER2* gene amplification in 8 of 27 cases (30%). Representative images of SDC cases exhibiting normal *HER2* copy number and *HER2* gene amplification are illustrated in Fig. 1D and E, respectively. Review of the pathology reports for all SDC cases revealed that *HER2* IHC had been requested at the time of diagnosis for 12 of 27 tumors. We found complete correlation between *HER2* gene amplification by FISH and strong *HER2* overexpression (3+ score) by IHC, for all SDC cases with available IHC data. None of the *HER2*-amplified tumors had concurrent

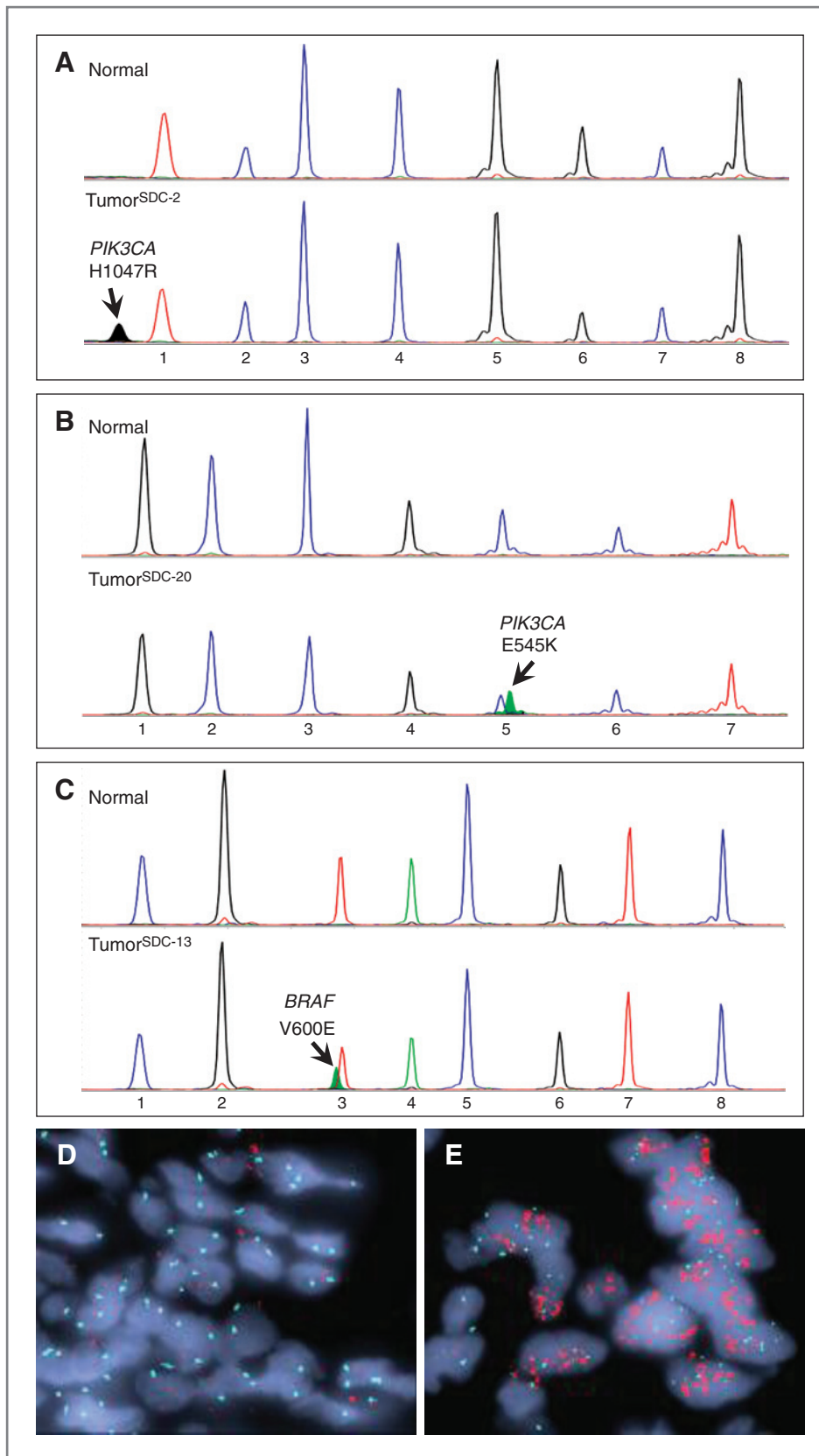


Figure 1. Detection of *PIK3CA* and *BRAF* activating mutations and *HER2* amplification in SDC. A–C, SNaPshot mutational profiling was conducted on nucleic acid extracted from SDC archival specimens run in parallel with a normal genomic DNA control. Examples of the 3 different mutated codons are shown with the black arrows pointing to the mutant alleles: *PIK3CA* H1047R (c.3140 A>G) in SDC-2 (A), *PIK3CA* E545K (c.1633G>A) in SDC-20 (B), and *BRAF* V600E (c.1799T>A) in SDC-13 (C). Assayed loci for each panel were as follows: (A) 1. *PIK3CA* 3140, 2. *CTNNB1* 101, 3. *JAK2* 1849, 4. *BRAF* 1798, 5. *NRAS* 37, 6. *PIK3CA* 1636, 7. *APC* 4348, and 8. *APC* 3340; (B) 1. *KRAS* 34, 2. *EGFR* 2235_49del F, 3. *EGFR* 2369, 4. *NRAS* 181, 5. *PIK3CA* 1633, 6. *CTNNB1* 94, and 7. *CTNNB1* 121; and (C) 1. *EGFR* 2235_49del R, 2. *NRAS* 38, 3. *BRAF* 1799, 4. *NRAS* 182, 5. *PIK3CA* 263, 6. *TP53* 742, 7. *CTNNB1* 95, and 8. *CTNNB1* 122. D, *HER2* FISH-negative SDC display, on average, 2 red signals (*HER2* probe Spectrum Orange: RP11–94L15) and 2 blue signals (Spectrum Aqua probe for chromosome 17 centromere) per nucleus [4',6-diamidino-2-phenylindole (DAPI) counterstaining]. E, *HER2* FISH-amplified SDC, showing clusters of red signals (*HER2* probe) and 2 blue signals (chromosome 17 centromere probe) per nucleus (DAPI counterstaining).

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point mutations in *PIK3CA* or *BRAF*. Six of 8 (75%) patients with *HER2*-amplified tumors were male, and 7 (88%) presented with advanced disease (stage IV; Table 1). Of the 8 *HER2*-amplified tumors, 7 (88%) had lymph node involvement, 6 (75%) showed perineural invasion, and 5 (62%) presented with at least one of the following characteristics: positive resection margins, lymphovascular invasion, extraglandular extension, or necrosis (Table 2). We found no statistically significant associations between the examined clinicopathologic features and SDC *HER2* status.

IHC analysis revealed that AR was strongly expressed by the majority of SDC in our cohort (24/27) confirming previous findings in the literature (Supplementary Fig. S1F; ref. 18). Of the 3 SDC cases that were negative for AR expression, 2 exhibited *HER2* gene amplification, as detected by FISH, and none of them harbored activating mutations in oncogenes detected by SNaPshot genotyping (Supplementary Table S3). The limited number of cases precluded any significant association between the AR status of SDC tumors and other genetic changes or clinicopathologic features.

Stratification of the overall patient population according to the mutational status of their tumors, revealed no statistically significant differences in outcome, either when each genetic alteration was analyzed separately or when the 3 types of changes were grouped together (Fig. 2 and Supplementary Table S2). However, patients with SDC whose tumors harbored a genetic abnormality (*PIK3CA* mutation, *BRAF* mutation, or *HER2* amplification) experienced a trend toward a reduced event-free survival ($P = 0.1102$) when compared with patients whose tumors had no detectable oncogenic change (Fig. 2B).

Genotype-driven targeted therapies for SDC

SNaPshot genotyping of 1 SDC case was first requested as a clinical test for patient SDC-13 (Table 3). The finding of a *BRAF* V600E mutation in this patient's tumor prompted the current study and his clinical case is presented later.

Patient SDC-13 was a 66-year-old male who presented with a large right parotid mass (6.2 cm in largest dimension) in June 2007, for which he underwent right total parotidectomy and modified radical neck dissection. Histology revealed stage IVA (22) SDC with positive surgical margins and 7 of 49 involved lymph nodes. Adjuvant radiotherapy to the parotid bed and ipsilateral neck irradiation (6,400 cGy in 200 cGy fractions), with weekly concurrent carboplatin (area under the curve 1.5) and paclitaxel (30 mg/m²) administration, lasted 7 weeks and was completed in December 2007.

In August 2008, routine FDG-PET-CT surveillance showed a solitary hypermetabolic L1 bone lesion that was treated with hemilaminotomy and facetectomy, followed by radiotherapy. Subsequent imaging in February 2009 showed new hypermetabolic bone lesions and mediastinal adenopathy. Monthly zoledronic acid infusions and cyclophosphamide plus doxorubicin were initiated, but chemotherapy was discontinued in October 2009 due to disease progression.

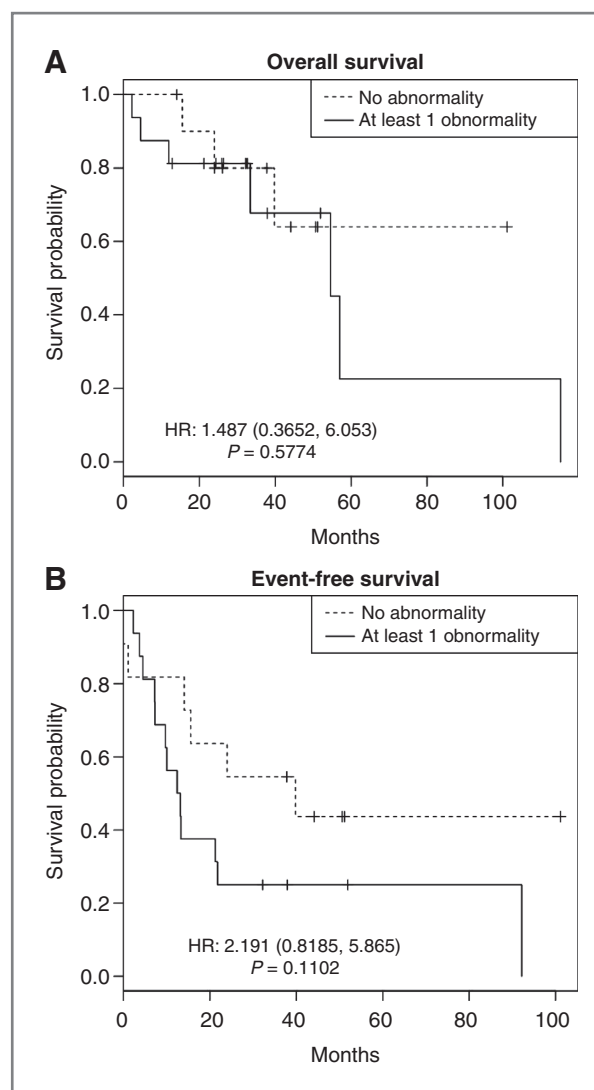


Figure 2. Observed survival of 27 patients with SDC according to molecular status. Overall survival (A) and event-free survival (B) depicted according to the presence or absence of genetic abnormalities (*HER2* gene amplification, or mutations in *PIK3CA* or *BRAF*). Statistical analysis revealed no significant difference but showed a trend toward better survival for patients without a detected oncogenic abnormality

At that point, SNaPshot analysis of the tumor was conducted. Identification of a *BRAF* V600E mutation led to the patient's enrollment in a phase II clinical trial investigating a mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor in patients with *BRAF*-mutant tumors. Treatment started in November 2009 and by March 2010 the disease seemed stable (22% reduction in mediastinal adenopathy), according to the Response Evaluation Criteria in Solid Tumors (RECIST). Eight months after initiation of therapy with the experimental MEK inhibitor (July 2010), a restaging computed tomography (CT) showed an increase in mediastinal adenopathy (Fig. 3A) and treatment was discontinued.

Table 3. Summary of therapeutic decisions

UPN	Genetic alteration	Treatment based on tumor genotype (TTP) ^a
SDC-13	<i>BRAF</i> V600E (c.1799T>A)	Phase I clinical trial MEK inhibitor (8 mo) Phase I clinical trial Combination of <i>BRAF</i> inhibitor + MEK inhibitor (12 mo) Phase I clinical trial Combination of PI3K/mTOR inhibitor + MEK inhibitor (NA)
SDC-20	<i>PIK3CA</i> E545K (c.1633G>A)	Phase I clinical trial PI3K inhibitor (3.7 mo) Phase I clinical trial Combination of PI3K inhibitor + MEK inhibitor (NA) Phase I clinical trial TORC1/2 inhibitor (5 mo)
SDC-21	<i>PIK3CA</i> H1047R (c.3140A>G)	Phase I clinical trial PI3K/mTOR inhibitor (8 wks)
SDC-27	<i>PIK3CA</i> H1047R (c.3140A>G)	Phase I clinical trial PI3K inhibitor (ND)
SDC-24	<i>HER2</i> amplification	Off label treatment Concurrent RT + chemotherapy, followed by adjuvant trastuzumab for solitary metastasis (12 mo)
SDC-26	<i>HER2</i> amplification	Off label treatment Concurrent RT + chemotherapy + trastuzumab, followed by adjuvant trastuzumab for initial postoperative therapy (6 mo) Off label treatment Whole brain RT, followed by combination of trastuzumab + lapatinib for brain metastases (ND)

NOTE: Prospective tumor genotyping helped guide treatment decisions for patients with SDC whose tumors harbored oncogenic mutations.

Abbreviations: NA, not applicable, patient experienced disease progression under treatment; ND, not determined, treatment ongoing; TTP, time to disease progression.

^aAll treatment of metastatic disease except where noted.

In August 2010, the patient enrolled in a phase I study of combined BRAF and MEK inhibitors GSK2118436 and GSK1123212. By September 2010, a restaging CT scan of the chest, abdomen, and pelvis showed a partial response with a 52% decrease in mediastinal adenopathy and stable bone lesions (Fig. 3B). The patient remained on this study for 12 months, until August 2011 when, after 14 cycles, imaging showed progressive disease.

In October 2011, the patient enrolled on a phase I clinical trial investigating combined PI3K/mTOR and MEK inhibitors. Shortly after starting the third cycle, head CT conducted after a fall showed multiple new brain metastases. The patient came off study, elected to forgo whole brain radiotherapy, and passed away in December 2011. In summary, 16 month after being diagnosed with advanced stage SDC and after undergoing 2 surgeries and several rounds of

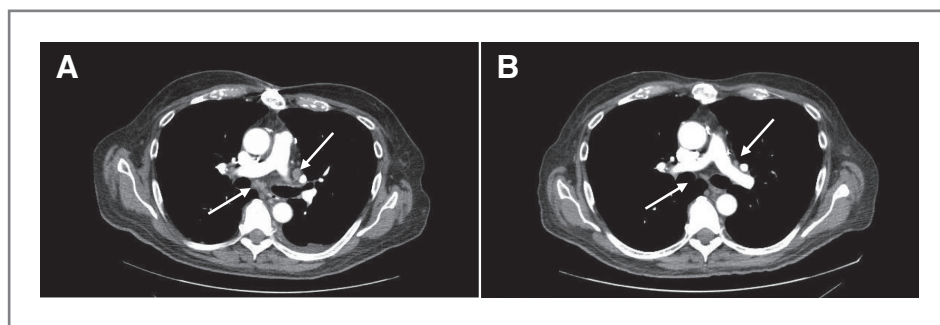


Figure 3. Effect of combination therapy with a BRAF and a MEK inhibitor in patient SDC-13, who carried a *BRAF* V600E-mutant tumor. A, pretreatment chest CT scan showing enlarged mediastinal lymph nodes (arrows). B, posttreatment/restaging CT scan showing 52% shrinkage of mediastinal lymphadenopathy (arrows).

radiotherapy and chemotherapy, patient SDC-13 presented with new metastases and progressive disease. Discovery of a *BRAF* V600E mutation in his tumor uncovered new therapeutic options and led to his enrollment in 3 consecutive clinical trials. He had partial responses to 2 of these protocols and his disease remained progression-free for a total of 20 months. His targeted treatments are summarized in Table 3 (patient SDC-13).

Three additional patients in our cohort entered clinical trials based on prospective SNaPshot genotyping data showing activating mutations in *PIK3CA* (Table 3; SDC-20, SDC-21, and SDC-27). For patient SDC-20 with a *PIK3CA* E545K mutant tumor, benefit from targeted agents was not negligible. He showed partial response to 2 experimental drugs and his combined progression-free survival lasted 8.7 months (Table 3). Patient SDC-21 harbored a *PIK3CA* H1047R mutant tumor and her response to treatment was less remarkable, with progressive disease being detected just 8 weeks after enrollment in a study using a dual PI3K/mTOR inhibitor. Patient SDC-27 initiated treatment with a phosphoinositide 3-kinase (PI3K) inhibitor at the time of writing, thus follow-up information is still unavailable.

Finally, 2 of 8 SDC patients with *HER2*-amplified tumors were tested prospectively and received off-label adjuvant treatment with the anti-*HER2* monoclonal antibody trastuzumab (Table 3; SDC-24 and SDC-26). There was a period of disease stabilization in both cases, with 1 patient experiencing disease progression after 6 months and the other one after 1 year of treatment.

Discussion

Targeted cancer therapies may represent the most promising strategy to fight rare aggressive malignancies that do not respond well to cytotoxic chemotherapy, such as salivary gland cancers. So far, targeted treatments used in salivary gland cancers have not always been adjusted to match particular histologic classes or specific genetic changes (6, 19, 25). In all, few objective responses have been reported, and no agent has emerged as a standard-of-care for the treatment of loco-regionally recurrent and/or distant metastatic disease. In this study, we attempted to close the gap for one of the most aggressive subtypes of salivary gland cancers, that is, SDC.

Our approach uncovered novel and mutually exclusive mutations in *PIK3CA* (19%) and *BRAF* (7%). The discovery of *PIK3CA* variants in SDC provides a new genetic link between this malignancy and ductal carcinoma of the breast, further highlighting the similarities between these 2 tumor types. In addition, driver mutations in *PIK3CA* and *BRAF* are molecular targets for therapeutic agents under clinical development and could potentially represent new hope for patients suffering from this aggressive disease.

In agreement with previous reports, our patient population consisted primarily of males (89%) older than 50 years of age (median of 64 years), who presented with advanced disease (82% with stage IV; refs. 4, 26, 27).

In addition to the histologic resemblance between SDC and breast ductal carcinoma, another feature common to both tumor types is the strong overexpression of *HER2*, which is detected in a subset of cases and highly correlates with amplification of the *HER2* locus. We detected *HER2* gene amplification in 8 of 27 (30%) SDC cases, consistent with the overall frequency reported in the literature (47 in 173 cases, 27%) (9–17).

For patients with *HER2*-positive breast cancer, treatment with anti-*HER2* agents, such as trastuzumab, can significantly improve disease-free and overall survival (28–30). These findings provide a rationale for exploring the effects of trastuzumab in the treatment of *HER2*-positive SDC. So far, only few patients with SDC have been enrolled in treatment regimens that included *HER2* antagonists, and some case reports have shown encouraging results with an occasional complete response (13, 25, 31, 32).

In our study, 2 patients with *HER2*-amplified SDC were treated with adjuvant trastuzumab therapy (Table 3). They both experienced a period of stable disease after initiation of trastuzumab treatment, which lasted 6 months (SDC-26) and 1 year (SDC-24). The 2 patients are still alive at the time of writing, 13 and 33 months, respectively, since the date of diagnosis. Their survival is within the range previously described for reported cases of patients with SDC treated with trastuzumab (13, 31, 32).

Consistent with previous studies, the majority of SDC in our cohort (24/27) were AR-positive by IHC, including the 5 patients who were treated prospectively. Being AR so distinctively and strongly expressed in SDC, androgen deprivation therapy is emerging as a possible targeted approach for patients with this malignancy. In small series, androgen deprivation therapy has shown clinical benefit in a discrete number of patients with AR-positive SDC (33, 34).

We identified activating mutations in *PIK3CA* (19%) and in *BRAF* (7%) in a subset of SDC that were AR-positive and -negative for *HER2* gene amplification. This is an interesting finding, as *BRAF* and *PIK3CA* encode for downstream targets of oncogenic signaling pathways triggered by the EGF family of receptor tyrosine kinases, which include *HER2* and EGF receptor (EGFR; ref. 35). Of note, a recent report described the presence of *EGFR* mutations in 9% of SDC (15). Furthermore, while our article was in preparation, another group reported *PTEN* loss in 1 patient with SDC (36). *PTEN* is a negative regulator of PI3K signaling, thus loss of function of the *PTEN* tumor suppressor results in activation of the PI3K/AKT/mTOR pathway (37).

The oncogenic roles of *BRAF* and *PIK3CA* have been extensively studied and supported by functional analyses in cell line and animal tumor models for other malignancies (38, 39), but this type of mechanistic data are still lacking for SDC. Of note, targeted inhibitors of *BRAF* and PI3K/mTOR signaling have shown promising results in a variety of cancers (40–43). While functional studies will be necessary to definitely assess SDC tumor dependency on these oncogenes, taken together with previous studies, our observations suggest that abnormal activation of intracellular pathways (such as PI3K/Akt/mTOR and RAS/RAF/MEK/ERK)

downstream of EGFR/HER2 signaling, may contribute to SDC tumorigenesis. The current data also raise the possibility that SDC malignancy could be triggered by distinct genetic drivers, including mutations in *PIK3CA*, *BRAF*, and *EGFR*, loss of *PTEN* and overexpression of *HER2*.

PIK3CA is mutated in a variety of epithelial tumors, including head and neck squamous cell carcinomas and breast cancer (44, 45). Potent PI3K and PI3K/mTOR inhibitors are currently undergoing testing in genotype-driven clinical trials for patients with tumors carrying the hyperactive kinase. To our knowledge, this is the first time that activating mutations in *PIK3CA* have been systematically studied and reported in SDC. In our cohort, 5 of 27 (19%) specimens carried activating mutations in the kinase domain or in the helical domain of *PIK3CA*, and had tumor features suggestive of more aggressive disease. On the basis of prospective genotyping, 3 patients (SDC-20, SDC-21, and SDC-27) enrolled in clinical trials that included PI3K, mTOR, and MEK inhibitors (Table 3). In a recent report, 2 patients with SDC with extensive cutaneous metastases were treated with the mTOR inhibitor temsirolimus, in combination with the antiangiogenic agent bevacizumab. Both patients responded rapidly and dramatically to therapy but the follow-up was short (<4 months), as their treatment was discontinued for reasons unrelated to the study drugs. *PTEN* loss was identified in the tumor of one of the patients, and none of them harbored mutations in *PIK3CA* (36). In our study, 1 patient (SDC-21) presented with progressive disease shortly (8 weeks) after initiation of treatment with an experimental PI3K/mTOR inhibitor, but patient SDC-20 had lasting partial responses to 2 targeted agents, maintaining progression-free disease for a total of 8.7 months (Table 3).

BRAF is a signaling component of the mitogen-activated protein kinase (RAS/RAF/MEK/ERK) pathway that promotes cell proliferation downstream of receptor tyrosine kinase signaling. The *BRAF* gene is mutated in a wide variety of cancers and the V600E mutation accounts for more than 95% of all *BRAF* somatic variants described in human tumors (46). Second generation *BRAF* inhibitors have shown impressive results in patients with *BRAF* V600E-mutated malignant melanomas, with tumor regression being observed in the majority of cases (47, 48). A strategy to overcome reactivation of the RAS/RAF/MEK/ERK pathway, which can occur in the context of acquired resistance when targeting *BRAF*, consists in inhibiting downstream targets of the pathway, such as the MEK1/2 kinases. Multiple clinical trials are now testing MEK inhibitors in *BRAF*-mutant cancers. Therefore, it was interesting for us to detect *BRAF* V600E mutations in 2 of our patients (7%) and to use this information to guide treatment for one of them, who was tested as part of routine clinical management (SDC-13). This patient had partial response to 2 experimental treatments that used *BRAF* and MEK inhibitors, and his disease remained free from progression for a total of 20 months while on targeted therapy regimens (Table 3). Of note, in 2 recent clinical trials, treatment of *BRAF*-mutant advanced melanoma patients with vemurafenib resulted in median

progression-free survivals of 5.3 and 6.8 months (47, 49). In all, patient SDC-13 survived 54 months after diagnosis, which compares favorably with previously reported survival of SDC (4).

In conclusion, we identified novel mutations in SDC, and by prospectively testing 8 patients, we detected actionable genetic alterations in 6 of them, which helped guide their treatment with molecularly targeted therapies. While these are encouraging developments, it is hard to delineate clinical benefits in few patients with SDC who were treated with targeted agents based on somatic molecular findings (*HER2* gene amplification or the presence of *BRAF* or *PIK3CA* mutations). Because SDC is so rare and typically presents as a high-grade salivary cancer that requires nodal resection, the prognostic and therapeutic implications of our findings are difficult to access. At this point, there is no good estimate on what the expected time to disease progression should be, and it is unclear whether a few months of disease-free survival will translate into increased overall survival. Ultimately, larger studies with more homogeneous patient cohorts will be necessary to properly evaluate the clinical benefits of using targeted treatment strategies in SDC.

Our findings reiterate that broad base genetic profiling is a valuable approach in the identification of actionable mutations in rare tumors. We also show that, when applied to all tumor types in a clinical setting, large-scale tumor genotyping strategies can enable the rapid translation of novel findings to patient care, which, if successful, could have a significant impact in rare and highly aggressive malignancies, such as SDC.

Disclosure of Potential Conflicts of Interest

D.R. Borger is a consultant/advisory board member of BioReference Laboratories. A.J. Iafrate has ownership interest (including patents) in Snapshot patent. L.J. Wirth is a consultant/advisory board member of Novartis. D. Dias-Santagata is a consultant/advisory board member of BioReference Laboratories, Inc. No potential conflicts of interest were disclosed by the other authors.

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