Novel PRKD Gene Rearrangements and Variant Fusions in Cribriform Adenocarcinoma of Salivary Gland Origin

Ilan Weinreb, Lei Zhang, Laxmi MS Tirunagari, Yun-Shao Sung, Liang-Chun Chen, Bayardo Perez-Ordenez, Blaise A Clarke, Alena Skalova, Simion I Chiosea, Raja R Seethala, Daryl Wagott, Paul C Boutros, Christine How, Fei-Fei Liu, Jonathan C Irish, David P Goldstein, Ralph Gilbert, Nasir ud Din, Adel Assaad, Jason L Hornick, Lester DR Thompson, and Cristina R Antonescu

1Department of Pathology, University Health Network, Toronto, ON, Canada
2Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada
3Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY
4Department of Pathology, Charles University in Prague, Faculty of Medicine, Plzen, Czech Republic
5Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA
6Informatics and Bio-computing Program, Ontario Institute for Cancer Research, Toronto, ON, Canada
7Ontario Cancer Institute, University Health Network, Toronto, Canada
8Department of Medical Biophysics, University of Toronto, Toronto, Canada
9Department of Radiation Oncology, Princess Margaret Cancer Centre and University of Toronto, Toronto, Ontario
10Department of Otolaryngology-Head and Neck Surgery/Surgical Oncology, Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
11Department of Pathology, Aga Khan University Hospital, Karachi, Pakistan
12Department of Pathology, Virginia Mason Hospital & Seattle Medical Center, WA
13Department of Pathology, Brigham & Women’s Hospital and Harvard Medical School, Boston, MA
14Department of Pathology, Woodland Hills Medical Center, Woodland, CA

Polymorphous low-grade adenocarcinoma (PLGA) and cribriform adenocarcinoma of minor salivary gland (CAMSG) are low-grade carcinomas arising most often in oral cavity and oropharynx, respectively. Controversy exists as to whether these tumors represent separate entities or variants of one spectrum, as they appear to have significant overlap, but also clinicopathologic differences. As many salivary carcinomas harbor recurrent translocations, paired-end RNA sequencing and FusionSeq data analysis was applied for novel fusion discovery on two CAMSGs and two PLGAs. Validated rearrangements were then screened by fluorescence in situ hybridization (FISH) in 60 cases. Histologic classification was performed without knowledge of fusion status and included: 21 CAMSG, 18 classic PLGA, and 21 with “mixed/indeterminate” features. The RNAseq of 2 CAMSGs showed ARID1A-PRKD1 and DDX3X-PRKD1 fusions, respectively, while no fusion candidates were identified in two PLGAs. FISH for PRKD1 rearrangements identified 11 additional cases (22%), two more showing ARID1A-PRKD1 fusions. As PRKD2 and PRKD3 share similar functions with PRKD1 in the diacylglycerol and protein kinase C signal transduction pathway, we expanded the investigation for these genes by FISH. Six additional cases each showed PRKD2 and PRKD3 rearrangements. Of the 26 (43%) fusion-positive tumors, there were 16 (80%) CAMSGs and 9 (45%) indeterminate cases. A PRKD2 rearrangement was detected in one PLGA (6%). We describe novel and recurrent gene rearrangements in PRKD1–3 primarily in CAMSG, suggesting a possible pathogenetic dichotomy from “classic” PLGA. However, the presence of similar genetic findings in half of the indeterminate cases and a single PLGA suggests a possible shared pathogenesis for these tumor types.

INTRODUCTION

Salivary gland cancers are diverse and numerous subtypes have been described with important clinical and biological implications. They show significant morphologic overlap and this distinction can be problematic on small biopsies of head and neck tumors. A particularly enigmatic tumor category is the so-called polymorphous low-grade adenocarcinoma (PLGA), which was first described by Evans and Batsakis (1984). PLGA...
usually arises in the oral cavity, particularly in the palate and can show a wide range of morphologic patterns. It has been described to show “polymorphous architecture with uniform cytology.” The main differential diagnoses for PLGA includes pleomorphic adenoma and adenoid cystic carcinoma, with both of these tumor types also frequently arising in the palate. This morphologic overlap is due to the shared cribriform and tubular growth in all three entities. This distinction is not merely academic as pleomorphic adenoma is benign, while adenoid cystic carcinoma has a high mortality rate. Conversely, PLGA is malignant but rarely causes death due to disease (Perez-Ordonez et al., 1998; Castle et al., 1999). PLGA also has a different clinical behavior with occasional cervical lymph node metastases, but only very rare hematogenous spread (Evans and Batsakis, 1984). Adenoid cystic carcinoma shows the opposite pattern.

Controversy has arisen as to whether PLGA represents a single pathologic entity or whether it should be regarded as a spectrum of tumors with extensive morphologic overlap. The large variety of growth patterns seen in PLGA would suggest a potential “waste basket” diagnosis. Some have argued for exclusion of papillary cases from the PLGA category due to their more aggressive course. Recently, a new entity was described in salivary gland, which most likely was previously regarded as PLGA, under the names “cribriform adenocarcinoma of the tongue (CAT)” (Michal et al., 1999) and presently as “cribriform adenocarcinoma of minor salivary gland (CAMSG).” The latter was based on a follow up paper highlighting the wider spectrum of oral cavity sites of origin (Skalova et al., 2011). This tumor was not originally included in the 2005 World Health Organization classification of head and neck tumors as a separate category, but mentioned as a pattern of PLGA that may or may not represent a unique entity (Luna and Wenig, 2005). CAMSG shows solid sheets and nests with slit-like spaces and nuclei reminiscent of papillary thyroid cancer and does not show the architectural diversity attributed to PLGA (Michal et al., 1999; Skalova et al., 2011).

There have been no recurrent molecular abnormalities reported in either PLGA or CAMSG. Occasional nonrecurrent cytogenetic findings have been found in PLGA with some potential overlap with adenoid cystic carcinoma (Martins et al., 2001). Recently, we and others have identified specific fusion transcripts in other salivary gland cancers, including mucoepidermoid carcinoma (Tonon et al., 2003), adenoid cystic carcinoma (Persson et al., 2009), mammary analogue secretory carcinoma (Skalova et al., 2010), and hyalinizing clear cell carcinoma (Antonescu et al., 2011). It can be speculated that tumors with monomorphic cytomorphology in the PLGA/CAMSG spectrum are likely candidates for similar recurrent fusion events. To investigate this hypothesis and interrogate the pathogenetic relationship of tumors included in this morphologic spectrum, next generation RNA sequencing was used on PLGA and CAMSG for novel fusion discovery.

**MATERIAL AND METHODS**

The institutional pathology files of the authors were searched for the diagnosis of “polymorphous low-grade adenocarcinoma (PLGA)” and “cribriform adenocarcinoma of minor salivary gland (CAMSG)”.

Hematoxylin and eosin (H&E) stained slides were available for review and were reclassified (IW). Tumors were classified as “classic PLGA” \((n = 18)\) when they exhibited a combination of short fascicles, cribriform structures, and a targetoid arrangement of cords of cells around blood vessels and/or nerves. Cases were classified as CAMSG \((n = 21)\) when they showed a combination of solid nests and sheets of cells with slit-like or punched out rounded lumina, papillary structures, and a glomeruloid arrangement of cells. Cytologically, CAMSG displayed nuclei with more open or vesicular chromatin. In addition, a subset of lesions was grouped in the “indeterminate” or “mixed” category \((n = 21)\). These included cases within the PLGA spectrum but showing mixed or unusual morphology (e.g., basoloid nests, apocrine tubules, canalicul-like areas, and so forth). Many of the cases sent in as PLGA by collaborators ended up being reclassified into the “indeterminate” category. Only cases that were largely papillary were reclassified from PLGA to CAMSG and this is based on the current observation (by IW) that papillary structures are more common in CAMSG than previously appreciated. All cases reclassified as other salivary gland carcinomas or as “adenocarcinoma, not otherwise specified” were excluded from the study.

**Fluorescence In Situ Hybridization (FISH)**

FISH was performed on 4-µm-thick sections of formalin-fixed paraffin-embedded tissue on all 60 cases. This was performed using custom bacterial artificial chromosome (BAC) probes, flanking the PRKD1, PRKD2, PRKD3, ARID1A, and DDX3X
genes in separate assays. The BAC clones were obtained from BACPAC sources of Children’s Hospital of Oakland Research Institute (CHORI; Oakland, CA) (http://bapac.chori.org) and were chosen according to the USCS genome browser (http://genome.ucsc.edu; Supporting Information Table 1). DNA from individual BACs was isolated according to the manufacturer’s instructions and labeled with different fluorochromes in a nick translation reaction (Antonescu et al., 2010). They were then denatured, hybridized to pretreated unstained coated slides, incubated, washed, and mounted with DAPI in an antifade solution, as previously described (Antonescu et al., 2010). The genomic location of each BAC set was verified by hybridizing them to normal metaphase chromosomes. Two hundred nonoverlapping nuclei were scored using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Water- town, MA). A case was confirmed as positive for rearrangement when ≥20% of the nuclei examined showed a break-apart signal pattern.

**RNA Sequencing**

Total RNA was prepared for RNA sequencing in accordance with the standard Illumina mRNA sample preparation protocol (Illumina), as previously described (Antonescu et al., 2013). Briefly, mRNA was isolated with oligo(dT) magnetic beads from total RNA (2 μg) extracted from the index cases. The mRNA was fragmented by incubation at 94°C for 2.5 min in fragmentation buffer. To reduce the inclusion of artificial chimeric transcripts due to random priming of transcript fragments into the sequencing library because of inefficient A-tailing reactions that lead to self-ligation of blunt-ended template molecules, an additional gel size-selection step (capturing 350–400 bp) was introduced prior to the adapter ligation step (Quail et al., 2008). The adapter-ligated library was then enriched by PCR for 15 cycles and purified. The library was sized and quantified using DNA1000 kit (Agilent) on an Agilent 2100 Bioanalyzer according to the manufacturer’s instructions. Paired-end RNA sequencing at read lengths of 51 bp was performed with the HiSeq 2000 (Illumina). Across the four samples, a total of about 261 million paired-end reads were generated.

**Analysis of RNA Sequencing Results with FusionSeq**

All reads were independently aligned with STAR alignment software against the human genome refer-

ence sequence (hg19) and a splice junction library, simultaneously (Dobin et al., 2013). The mapped reads were converted into Mapped Read Format (Habegger et al., 2011) and analyzed with FusionSeq (Sboner et al., 2010) to identify potential fusion transcripts. The FusionSeq algorithm is a computational method successfully applied to paired-end RNA-seq data for the identification of chimeric fusion transcripts (Antonescu et al., 2013; Mosquera et al., 2013). Briefly, paired-end reads mapped to different genes are first used to identify candidate chimeric transcripts. A cascade of filters, each taking into account different sources of noise in RNA sequencing results, is then applied to remove spurious fusion transcript candidates. A confident list of fusion candidates is generated and they are statistically ranked to prioritize the experimental validation. In this case, the DASPER score was used (difference between the observed and analytically calculated expected SPER); a higher DASPER score indicates a greater likelihood that the fusion candidate is authentic and did not occur randomly. See Sboner et al. (2010) for further details about FusionSeq.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

An RNA aliquot of the CAMSG index cases (SA2 and SA6) was used for RT-PCR to confirm the novel fusion transcripts. The RNA quality was determined by Eukaryote Total RNA Nano Assay and cDNA quality was tested for PGK housekeeping gene (247 bp amplified product). RT-PCR was performed using the advantage 2 PCR kit (Clontech, Mountain View, CA). For case SA2 RT-PCR was run for 29 cycles at a 64.5°C annealing temperature, using the following primers: ARIXIA exon 1 fwd: 5’-CATGGCCCTCGAAGTGCTG3’ and PRKD1 exon 12 rev: 5’-GCAGACGTTCTGTGTTGG-3’. For case SA6, the RT-PCR was run at 64.5°C annealing temperature for 31 cycles, using the following primers: DDX3X exon 7 fwd: 5’-GTGGAAACATTGAGCTTACTCG-3’ and PRKD1 exon 14rev: 5’-ACTGCGCTTCCATCTCACTGTG-3’. The PCR product was confirmed by Sanger sequencing.

**Functional Studies to Define the Oncogenic Role of Recurrent ARID1A-PRKD1 Fusion, Including Its Expression into Normal Human Salivary Gland Cell Line**

Full length cDNA fusion ARID1A-PRKD1 was amplified from index patient SA2 using forward
ARID1A (5′-GCAACGGTCGCCGACATGGCCGCCGCGAGGTCGCCCCCGC-3′) and reverse PRKD1 (5′-GCAACGGTCGCCGACATGGCCGCCGAGGTCGCCCCCGC-3′) primers. The amplified DNA was digested with RsII and XhoI (New England Biolabs, Ipswich, MA) restriction enzymes and cloned into a modified pCD510B vector with a FLAG tag on the N-terminus (a generous gift from Dr. Arul Chinnaiyan). Lentivirus was produced by cotransfecting either pCD510B-ARID1A-PRKD1 construct or the empty vector along with the helper plasmids psPAX2 and pVSVG (Addgene, Cambridge, MA). Collected and concentrated viral supernatant (40 and 64 hrs after transfection) was used to transduce NIH-3T3 cells or normal salivary gland epithelial cells (HPAM1), purchased from ATCC (Manassas, VA), with a wide age-range at diagnosis of 24–84 years (mean 61 years). The tumors occurred in the palate (n = 27), buccal mucosa (n = 7), lip (n = 6), base of tongue (n = 5), maxilla/mandible (n = 4), nasopharynx (n = 3), parotid (n = 2), and 1 each in the tonsil, floor of mouth, mobile tongue, and nasal cavity. In two cases, the anatomic location was not available, but appeared to be oral mucosa and oropharynx histologically. The tumors ranged from 0.7 to 5.0 cm (mean 2.5 cm). The tumors were reclassified into three groups including those that showed “classic PLGA” morphology (n = 18), “classic CAMSG” (n = 21), and “indeterminate/mixed morphology” (n = 2); Fig. 1). The classic PLGA group generally consisted of short fascicles of tumor and a targetoid arrangement of some of the nests around blood vessels or nerves (Figs. 1A and 1B). The classic CAMSG cases showed sheets and large nests of tumor with slit-like or rounded glandular spaces (Fig. 1C). These lacked the hyalinized material of the cribriform areas of classic PLGA, but instead were either empty or had pink homogenous secretions, mucoid material, or extravasated red blood cells. The tumors often had pseudopapillary and glomeruloid formations (Fig. 1D). The tumor nuclei were generally optically clear, although this was not striking in every case. The final category of “indeterminate/mixed” was a heterogeneous group that fit the description of the PLGA spectrum but had mixed features, or focal unusual morphology such as canalicular ribbons. All tumors had minimal mitotic activity (<3 MF/10 HPFs) and were low grade.

Follow up and treatment was not available on many cases due to: (1) the number of consult cases, (2) biopsy only cases, and (3) loss to follow up in this cohort. Among the CAMSG group there

**RESULTS**

**Histological and Clinical Findings**

A total of 60 cases of “polymorphous low-grade adenocarcinoma (PLGA)” and “cribriform adenocarcinoma of minor salivary gland (CAMSG)” were selected. The patients with available clinical information included 43 females and 17 males, with a wide age-range at diagnosis of 24–84 years (mean 61 years). The tumors occurred in the palate (n = 27), buccal mucosa (n = 7), lip (n = 6), base of tongue (n = 5), maxilla/mandible (n = 4), nasopharynx (n = 3), parotid (n = 2), and 1 each in the tonsil, floor of mouth, mobile tongue, and nasal cavity. In two cases, the anatomic location was not available, but appeared to be oral mucosa and oropharynx histologically. The tumors ranged from 0.7 to 5.0 cm (mean 2.5 cm). The tumors were reclassified into three groups including those that showed “classic PLGA” morphology (n = 18), “classic CAMSG” (n = 21), and “indeterminate/mixed morphology” (n = 2); Fig. 1). The classic PLGA group generally consisted of short fascicles of tumor and a targetoid arrangement of some of the nests around blood vessels or nerves (Figs. 1A and 1B). The classic CAMSG cases showed sheets and large nests of tumor with slit-like or rounded glandular spaces (Fig. 1C). These lacked the hyalinized material of the cribriform areas of classic PLGA, but instead were either empty or had pink homogenous secretions, mucoid material, or extravasated red blood cells. The tumors often had pseudopapillary and glomeruloid formations (Fig. 1D). The tumor nuclei were generally optically clear, although this was not striking in every case. The final category of “indeterminate/mixed” was a heterogeneous group that fit the description of the PLGA spectrum but had mixed features, or focal unusual morphology such as canalicular ribbons. All tumors had minimal mitotic activity (<3 MF/10 HPFs) and were low grade.

Follow up and treatment was not available on many cases due to: (1) the number of consult cases, (2) biopsy only cases, and (3) loss to follow up in this cohort. Among the CAMSG group there

**Gene Expression Analysis by Quantitative PCR and Immunoblotting of ARID1A-PRKD1 Oncoprotein in fusion-expressing HPAM1 Salivary Gland Cells**

Total RNA was isolated from tissue culture cells using RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using the TaqMan reverse transcription kit (Life Technologies). Predesigned TaqMan Gene Expression Assays and TaqMan Universal PCR master mix (Life Technologies) were used on a ViiATM 7 Real-Time PCR machine (Life Technologies). Fifteen nanograms of cDNA was used for each sample, and each sample was run in triplicates. GAPDH was used as an endogenous control and the relative expression of genes was calculated using ΔΔCt method (Life Technologies).

Protein lysates were extracted from one ARID1A-PRKD1 positive SA2 sample as well as from the HPAM1 cell line with and without expression of ARID1A-PRKD1 fusion. Electrophoresis and Immunoblotting were done using 30 µg of the lysate following standard protocols. Rabbit polyclonal antibodies for PRKD1 (Cell Signaling, Danvers, MA, catalog # 2052; 1:2,000 dilution) and B-actin (cell signaling, 1:1,000 dilution) were used for detection.

**RESULTS**

**Histological and Clinical Findings**

A total of 60 cases of “polymorphous low-grade adenocarcinoma (PLGA)” and “cribriform adenocarcinoma of minor salivary gland (CAMSG)” were selected. The patients with available clinical information included 43 females and 17 males, with a wide age-range at diagnosis of 24–84 years (mean 61 years). The tumors occurred in the palate (n = 27), buccal mucosa (n = 7), lip (n = 6), base of tongue (n = 5), maxilla/mandible (n = 4), nasopharynx (n = 3), parotid (n = 2), and 1 each in the tonsil, floor of mouth, mobile tongue, and nasal cavity. In two cases, the anatomic location was not available, but appeared to be oral mucosa and oropharynx histologically. The tumors ranged from 0.7 to 5.0 cm (mean 2.5 cm). The tumors were reclassified into three groups including those that showed “classic PLGA” morphology (n = 18), “classic CAMSG” (n = 21), and “indeterminate/mixed morphology” (n = 2); Fig. 1). The classic PLGA group generally consisted of short fascicles of tumor and a targetoid arrangement of some of the nests around blood vessels or nerves (Figs. 1A and 1B). The classic CAMSG cases showed sheets and large nests of tumor with slit-like or rounded glandular spaces (Fig. 1C). These lacked the hyalinized material of the cribriform areas of classic PLGA, but instead were either empty or had pink homogenous secretions, mucoid material, or extravasated red blood cells. The tumors often had pseudopapillary and glomeruloid formations (Fig. 1D). The tumor nuclei were generally optically clear, although this was not striking in every case. The final category of “indeterminate/mixed” was a heterogeneous group that fit the description of the PLGA spectrum but had mixed features, or focal unusual morphology such as canalicular ribbons. All tumors had minimal mitotic activity (<3 MF/10 HPFs) and were low grade.

Follow up and treatment was not available on many cases due to: (1) the number of consult cases, (2) biopsy only cases, and (3) loss to follow up in this cohort. Among the CAMSG group there
were eight cases known to have a neck dissection, of which seven showed lymph node metastases, often multiple and large. Most of the classic PLGAs did not have a neck dissection, however, the information was incomplete and this may represent a selection/referral bias where smaller biopsies and excisions represented consults. Management details for these patients were often unavailable.

**Novel ARID1A-PRKD1 and DDX3X-PRKD1 Fusions Identified by RNA-seq and FusionSeq**

The samples with frozen material (SA2 and SA6) were subjected to RNA sequencing to identify potential fusion candidates (Figs. 2 and 3). An ARID1A-PRKD1 fusion transcript was selected by FusionSeq as the top candidate with a DASPER score of 1.56 in SA2 and a DDX3X-PRKD1 fusion transcript was selected for SA6 with a DASPER score of 1.34. Read alignments suggested a fusion of ARID1A exon 1 on chromosome 1 with PRKD1 exon 12 on chromosome 14 (Fig. 2A) for SA2 and fusion of DDX3X exon 7 on chromosome X to PRKD1 exon 11 in SA6 (Fig. 3A). RT-PCR confirmed the presence of a fusion transcript of ARID1A exon 1 to exon 12 of PRKD1 in SA2 and DDX3X exon 7 to exon 11 of PRKD1 in SA6. No equivalent t(1;14)(p36.11;q12) or t(X;14)(p11.4;q12) has been described by conventional cytogenetics in PLGA or any other salivary gland tumor to our knowledge, and these chromosomal breakpoints are not listed in the Mitelman database for cytogenetics findings (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

**Fluorescence In Situ Hybridization (FISH)**

All 60 cases were then investigated for both ARID1A and PRKD1. Three cases, including the index case SA2, were confirmed to have gene rearrangements in both ARID1A and PRKD1. All three showed classic CAMSG morphology (Fig. 2B). An additional CAMSG case showed ARID1A break-apart alone without PRKD1. The index case SA6 with the DDX3X-PRKD1 fusion predicted by sequencing and RT-PCR was further confirmed to have FISH rearrangements in both DDX3X and PRKD1 and showed classic CAMSG morphology. However, this case also had interesting trabecular areas with a “tall cell” appearance to the cells (Fig. 3B). Nine additional cases with...
PRKD1 rearrangement were identified, four showing classic CAMSG morphology, and five being in the indeterminate/mixed features category (Fig. 4). These could not be classified more specifically. Most of the positive cases showed classic break-apart signals; however, two of the PRKD1 rearranged tumors showed a signal pattern consistent with an intrachromosomal rearrangement and one showed a signal pattern consistent with an unbalanced translocation with loss of 5' PRKD1 material. None of the cases in the “classic PLGA” category showed abnormalities of these genes. A total of 14 cases showed evidence of a rearrangement in one or both of these genes (14/58; 24%); with all except one case showing PRKD1 gene rearrangement (22%). Two cases failed FISH testing.

As the PRKD1 gene shares biologic function with PRKD2 and PRKD3, these genes were further investigated by FISH as well. PRKD2 testing showed six additional positive cases (6/45; 13%). These cases included three classic CAMSG cases, two indeterminate, and one PLGA. In hindsight, this last case showed unusual canalicular ribbons and hemorrhage-filled microcystic spaces not typically seen in PLGA in addition to the more traditional fascicular and targetoid areas. PRKD3 rearrangement was found in six additional cases (6/38; 16%), representing four classic CAMSG and two indeterminate cases (Fig. 5).

Of the fusion-positive cases (Table 1), the tumors arose in a slightly younger group of patients as compared to the total with a range of 24–84 years (mean 59.0 years); approximately 4 years younger than the fusion-negative group ranging from 27 to 83 (mean 63 years). The fusion-positive cases occurred over a wide site
distribution, including nasopharynx and parotid. Of the seven cases with known lymph node metastases, all were classified as CAMSG, six showed rearrangement of one of the PRKD genes and five were located in the base of tongue/pharynx. In fact all 10 cases occurring in pharynx (seven oropharynx and three nasopharynx) were positive for a PRKD rearrangement.

**ARID1A-PRKD1** Induces Similar Transcriptional Abnormalities in a Normal Salivary Gland Cell Line (HPAM1) as Seen in fusion-positive CAMSG Tumors

Based on RNA sequencing data, we first selected 462 upregulated transcripts in the SA samples with the RPKM (Habegger et al., 2011) values above 100, compared to a wide range of other tumor types. We then applied information content for constructing sequence logos as an additional ranking parameter (Schneider and Stephens 1990), which identifies genes that have null expression in most samples except for the subset of interest (i.e., SA tumors). Log fold change between PRKD1-positive SA and all other tumors was the last ranking parameter. Based on these criteria, three top-ranked transcripts were identified: CLDN10, CRISPLD1 and MUC7 (Supporting Information Fig. 1). The mRNA expression of these genes was then investigated by real-time PCR in the normal salivary gland cells (HPAM1) as well as in the ARID1A-PRKD1 transduced HPAM1. Two of the three genes analyzed, CLDN10 and MUC7, showed increased expression exclusively in HPAM1 transduced with ARID1A-PRKD1 (Supporting Information Fig. 1). Furthermore, real-time PCR performed on the HPAM1 expressing the ARID1A-PRKD1 fusion transcript and corresponding controls showed significant upregulation of PRKD1 mRNA (Supporting Information Fig. 2).

![Figure 3. DDX3X-PRKD1 gene fusion in a parotid cribriform adenocarcinoma of salivary gland (SA6).](Image)

(A) Schematic representation of the DDX3X-PRKD1 fusion indicating the loci that are joined together; DDX3X exon 7 being fused to PRKD1 exon 11 (top image). Experimental validation of the fusion by RT-PCR shows the junction sequence between exon 7 of DDX3X and exon 11 of PRKD1 (bottom image); (B) The index case SA6 showed some typical CAMSG morphology but also showed trabecular architecture with a columnar or "tall cell" appearance; (C) FISH for DDX3X shows break apart signals with one normal fused yellow signal and separate 5' green and 3' red signals indicating rearrangement of the gene (arrows). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Figure 4. Variant morphologies in PRKD1 rearranged tumors without DDX3X or ARID1A. (A, B) An indeterminate case showing tubular (A) and glomeruloid (B) features. One PRKD1 rearranged tumor in the nasopharynx showed abundant canalicular like growth (C). Another tumor showed an unusual pattern of cribriform and papillary-cystic architecture (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. Most cases with PRKD2 and PRKD3 showed classic CAMSG morphology or indeterminate features (A, B). A lymph node metastasis of a PRKD2 rearranged tumor is shown here with typical glomeruloid morphology (A). Another PRKD3 rearranged CAMSG case with papillary features is shown here (B). PRKD2 (C) and PRKD3 (D) FISH for these cases showed classic break-apart signals. A single PRKD2 rearranged case in the parotid (E) showed a classic PLGA targetoid/fascicular growth pattern (right) with foci of canalicular growth (left). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Expression of Truncated PRKD1 Protein in ARID1A-PRKD1-Transduced Cells and a PRKD1-Rearranged CAMSG Tumor

Western blotting was performed on the ARID1A-PRKD1 transduced NIH3-3T3 and corresponding controls, including NIH3-3T3 cells transduced with the empty vector, as well as the index case SA2, positive for the ARID1A-PRKD1 fusion. The fusion-positive samples showed a 74 kDa size band, in keeping with truncated PRKD1 protein, in contrast to the 115 kDa wild type PRKD1 protein (Supporting Information Fig. 3). Additional samples, such as one angiosarcoma, one PEC-oma and three GISTs were included displaying only the 115 kDa wild type band (data not shown).

DISCUSSION

PLGA is a salivary gland cancer, which was first described by Evans and Batsakis (1984). PLGA is known for its heterogeneous architecture but uniform cytology (Evans and Batsakis, 1984). The tumors occur in women more often than men and most arise in the palate (Luna and Wenig, 2005). PLGAs are generally small and easily treated surgically and unlike the principal differential diagnosis, namely adenoid cystic carcinoma, they have an excellent prognosis with a small incidence of recurrence and cervical lymph node metastases overall (Castle et al., 1999; Seethala et al., 2010). Extrapalatal site has been linked to a more aggressive course (Seethala et al., 2010). Even when recurrent and metastatic, many PLGAs can still be cured and hematogenous spread and mortality are rare. Evans and Luna (2000) revisited the entity in a series of 40 cases with long term follow up in 2000 and found that papillary growth was one feature that could be associated with more cervical lymph node metastases, and although “low grade” there have been reports of adverse outcomes in PLGA (Perez-Ordonez et al., 1998; Castle et al., 1999) and occasional cases of “high grade transformation/dedifferentiation” (Simpson et al., 2002).
Despite its “polymorphous” pattern it was still considered one entity until recently. Michal et al. (1999) first described “cribriform adenocarcinoma of the tongue (CAT)”. This tumor was felt to be unique in that it usually arose in the base of tongue, had cervical lymph node metastases at presentation, and had characteristically optically clear nuclei, reminiscent of papillary thyroid cancer. Despite this behavior, the prognosis was still excellent. However, the CAT entity was not universally accepted as its own entity and was considered a variant pattern of PLGA by the World Health Organization “blue book” classification of head and neck tumors (Luna and Wenig, 2005). A follow up paper from the same authors described a larger series of new cases in 2011, now under the appellation “cribriform adenocarcinoma of minor salivary gland origin (CAMSG)” (Skalova et al., 2011). This was considered its own entity, not just because of clinical presentation, but also based on the immunohistochemical and ultrastructural evidence of “myosecretory differentiation,” which is absent in PLGA. Whether CAMSG will be accepted as unique in future classifications remains to be seen.

We have observed that many putative cases of CAMSG have papillary and glomeruloid structures, sometimes diffusely, which were seen only focally in the original descriptions of the entity (Michal et al., 1999; Skalova et al., 2011). We have hypothesized that these cases may in fact show the same papillary pattern that was first thought by Evans and Luna to represent a potential prognosticator for greater metastatic spread to lymph nodes in PLGA (Evans and Luna, 2000). To explore this controversial spectrum of tumors, further, we used next generation RNA sequencing on two candidate CAMSG cases and two classic PLGAs and analyzed the data using FusionSeq, which is a bioinformatics tool that has been proven to predict fusions using paired-end RNA sequencing data (Sboner et al., 2010; Antonescu et al., 2013; Mosquera et al., 2013). No candidate fusion was found in the two sequenced classic PLGAs. The index CAMSG case SA2 was predicted to have an ARID1A-PRKD1 chimeric transcript and this was validated using RT-PCR and further confirmed by FISH. In addition, the index CAMSG case SA6 was found to have a DDX3X-PRKD1 fusion, which was also confirmed by RT-PCR and FISH.

The FISH probes for ARID1A and PRKD1 genes were then applied to a series of 60 cases that spanned the spectrum from typical CAMSG, classic PLGAs, and indeterminate/mixed cases. A total of 14 cases (24%) had rearrangement of ARID1A and/or PRKD1. Three cases showed both, suggesting an ARID1A-PRKD1 fusion. These cases had identical morphology and were considered typical CAMSG cases. They occurred in base of tongue (n = 2) and buccal mucosa (n = 1). The CAMSG case with DDX3X-PRKD1 occurred in the parotid and showed some unusual features, including areas of trabecular architecture with a “tall cell” appearance. An additional case of CAMSG showed only ARID1A rearrangement, while nine cases showed break-apart in PRKD1, without associated ARID1A or DDX3X rearrangements, four of which were typical CAMSG cases. In screening the remaining cases for PRKD2 and PRKD3 gene abnormalities by FISH, an additional six cases each showed PRKD2 and PRKD3 rearrangements. Of these, seven showed classic CAMSG morphology, four were indeterminate, and one was a possible PLGA.

A total of 16/20 (80%) typical CAMSG cases showed gene rearrangements in one of these genes, with 15/20 (75%) showing abnormalities in one of the PRKD genes. The indeterminate/mixed category showed 9/20 (45%) cases with rearrangements in one of these genes. On retrospective review of the fusion-positive cases, one new feature not seen in fusion-negative cases was canicular architecture. This appearance has not been previously thought to be part of the CAMSG spectrum and requires further investigation. Otherwise no specific findings in the indeterminate group were predictive of fusion status. Remarkably, only one PLGA case had abnormalities in one of these genes, a parotid tumor with PRKD2 rearrangement (1/18; 6%). Although the association of CAMSG histology with the presence of PRKD gene rearrangements is striking compared to classic PLGA, several limitations in our study preclude a definitive conclusion on CAMSG being a unique and distinctive entity.

The most apparent reason to use caution is due to the difficulty in the histologic classification encountered in up to one-third of cases. This diagnostic challenge was in keeping with the significant morphologic overlap shared by these two entities and the potential inclusion of these entities as part of a spectrum. In addition, a large percentage of the indeterminate category was positive for PRKD rearrangements and one potential parotid PLGA was positive for PRKD2 as well. Although this latter case was an isolated example, it highlights the need for a larger number of cases.
in all categories to explore the potential for these genes to be involved in classic PLGA as well. ARID1A, DDX3X, and PRKD genes have not been implicated in a translocation/fusion previously and very little is known of the function of PRKD genes or their potential role in oncogenesis. ARID1A is a gene well known to be involved in chromatin remodeling and has been implicated in gynecological cancers previously (Wiegand et al., 2010). The loss of ARID1A function leads to aberrations in DNA repair. Interestingly, DDX3X is also a gene involved in DNA repair (Sun et al., 2013) and has been implicated in a number of cancers including medulloblastoma (Jones et al., 2012) and breast cancer (Botlagunta et al., 2008). This may provide an as yet unknown link between DDX3X and ARID1A, at least in terms of how promoter swapping may activate PRKD1 in these tumors. The PRKD1 gene encodes a kinase that has previously been implicated in colorectal, breast, esophageal, laryngeal, and other cancers, although deletion and/or gene silencing appear to be the mechanisms associated with colorectal and breast cancers (Borges et al., 2013; Brim et al., 2014), while activation is associated with esophageal and laryngeal cancers (Fountzilas et al., 2014; Xie et al., 2014). Along with the related PRKD2 and PRKD3 genes, it is associated with signal transduction in the diacylglycerol (DAG) and protein kinase C (PKC) pathway. When activated PRKD family of kinases have a role in signal transduction, trafficking, migration, differentiation and proliferation (Xie et al., 2014) and potentially may serve as targets for future drug therapies. Further studies are required to elucidate the mechanism of PRKD gene activation in these tumors and how this leads to tumor proliferation.

In summary, recurrent PRKD1, PRKD2, and PRKD3 rearrangements, including ARID1A-PRKD1 and DDX3X-PRKD1 fusions, are associated with the “polymorphous low-grade adenocarcinoma (PLGA) / cribriform adenocarcinoma (CAMSG)” spectrum of salivary gland cancer. The fusion-positive group appears to cluster mainly in CAMSG and indeterminate cases rather than in “classic PLGA,” however, a large percentage of cases in this spectrum remain uncharacterized genetically. The presence of PRKD gene abnormalities in indeterminate cases precludes using them as diagnostic markers or confirming the uniqueness of CAMSG at this time, although it appears to be the first evidence of a difference between CAMSG and “classic PLGA.” Further sequencing studies of fusion-negative cases are required to identify additional genetic markers that may fill in this gap and larger annotated cohorts that include patient outcome are also required to address the potential role of PRKD with the alleged greater tendency for CAMSG to metastasize to cervical lymph nodes (Evans and Luna, 2000; Skalova et al., 2011).

REFERENCES


