Salivary duct carcinoma (SDC) is an aggressive malignant tumor with a high mortality, which resembles high-grade breast ductal carcinoma in morphology [1]. Most of SDCs are located at the parotid gland, and males are diagnosed approximately three times more frequently than females with SDC [2]. SDC is a rare tumor which only accounts for less than 5% of all head and neck cancer and 1–3% of all salivary gland tumors [3, 4]. The survival of patients with SDC is poor, with most dying within three years [5, 6]. Conventional treatments for SDC patients such as surgery with or without radiotherapy usually lead to high recurrence rate [7]. Therefore, novel treatment methods including targeting chemotherapy in combination with postoperative radiotherapy would be desirable [8]. However, little is known about the molecular profile of this rare disease.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is involved in many critical cellular processes, such as cell proliferation and survival [9]. Genetic alterations in the key components of the PI3K pathway have been identified in diverse human tumors, including the PIK3CA gene [10, 11]. PIK3CA, which is the catalytic subunit p110α of PI3-kinase, has been demonstrated to play an oncogenic role in some human cancers in vivo and in vitro [12]. Somatic mutations of the PIK3CA gene have been also reported frequently in numerous cancer types including head and neck cancers [13–17]. Most of these reported mutations are clustering in the exons 9 and 20 of the PIK3CA gene, where three hotspot mutations (E542K, E545K, and H1047R) reside. All those three PIK3CA hotspot mutations have been proven to be oncogenic and are associated with poor clinical outcomes [18, 19].
We have previously reported high incidence of PIK3CA somatic mutations in head and neck squamous cell carcinoma [13, 14], particularly in pharyngeal cancers [15]. However, the PIK3CA mutation status in patients with SDC was not included in that study. Furthermore, SDC shares many similarities with breast ductal carcinoma both in histology and biology, in which frequent PIK3CA mutation has been identified in human breast cancer [20]. Based on these observations, we investigated the PIK3CA protein expression and genetic mutation in six SDC patients by immunohistochemistry (IHC) and direct genomic DNA sequencing. The results showed that PIK3CA expression was elevated in all six salivary duct carcinomas; 2 of them were identified with PIK3CA hotspot mutations.

2. Materials and Methods

2.1. Patient Samples. Acquisition of tissue specimens was approved by the Columbia University Medical Center (CUMC) Institutional Review Board and performed in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations. A total of six cases of SDC were identified from the archival tissues banked between 1997 and 2012 at the CUMC. The cases were reviewed by two pathologists with scales from 0 to 2+. Samples found to have a genetic alteration in the target exon were subsequently sequenced in the reverse direction to confirm the mutation using the reverse PCR primers. The mutation was then further verified by sequencing of a second independently amplified PCR product from the original genomic DNA template. All sequencings were performed with ABI's 3100 capillary automated sequencers at the DNA facility of Columbia University Medical Center in New York [14].

2.2. Immunohistochemistry. Unstained 5 micron sections were cut from the paraffin blocks of SDC cases and deparaffinized by routine techniques. Tissue sections were treated with 0.01 M tri-sodium citrate buffer and boiled in a microwave for 15 minutes. Slides were then cooled for 10 minutes in tap water before blocking with Dako peroxidase blocking reagent (Catalogue no. S2001, Dako, CA). Primary antibody anti-P13 kinase p110 α (Cell Signaling, MA) was diluted at 1:10 and incubated at room temperature for one hour. For HER2 staining (Epitomics), the condition was 1:250 dilution of the primary antibody and incubation at room temperature overnight. Then, Dako LSAB + System-HRP kit (Catalogue no. K0690, Dako, CA) was used by adding biotinylated link universal and streptavidin-HRP each for 15 minutes at room temperature. Sections were counterstained with hematoxylin. IHC results were scored by the two pathologists with scales from 0 to 2+.

2.3. DNA Extraction from Tissue Specimens. Tumor and adjacent normal tissue were separately microdissected from 10 micron sections cut from each patient's paraffin block. Genomic DNAs were extracted using DNeasy tissue kit (QIA-GEN Inc., CA). The procedures were performed according to the manufacturer's instructions.

2.4. Genomic DNA Sequencing. Exons 9 and 20 of the PIK3CA gene were analyzed by PCR amplification of genomic DNA and direct sequencing of the PCR products [14]. Specific primers for the PIK3CA gene exons 9 and 20 (PIK-E9F: CCAGAGGGAAAAATATGACA; PIK-E9R: CATTTTAGCCTTACCTGTGAC; PIK-E20F: CATTGCCAATGCTCCAGCA; PIK-E20R: GGTCTTTGCCGCTGCTGAGGT) were designed for efficient PCR amplification from paraffin-embedded specimens. PCR products were purified using the GeneClean Turbo Nucleic Acid Purification Kit (QIAGEN, CA). Finally, purified DNA fragments were directly sequenced using the corresponding forward PCR primers.

2.5. Quantitative PCR for Measurement of PIK3CA Amplification. The genomic copy numbers of the PIK3CA allele were analyzed by quantitative real-time PCR measurement of each tumor and corresponding normal tissues. Specific primers (PIK-qF: TGCAAGAATCAGAACTCC; PIK-qR: CAGGAGGCATTCTCCCAAAGTCG) were designed for this genomic real-time PCR of PIK3CA encoding allele from paraffin-embedded specimens. Beta-actin was chosen as the reference gene for this assay (ACTB-qF: TAGAACCTCAGCATGAGCCC; ACT-qR: GTACTGGCATGCAACACAG). The SYBR Green reagents applied for the real-time PCR were purchased from life technologies.

3. Results

3.1. Clinical and Pathologic Findings. The SDC patients in this study show a median age of 62 years (range, 50–71) and a high male to female ratio (5:1). All the SDC cases occurred in the common parotid gland and the tumor sizes ranged from 1.6 cm to 2.3 cm. Histologically, the tumors showed both intraductal and invasive components. The intraductal component had a predominantly cribriform architecture and prominent comedo-type necrosis. The invasive components consisted of irregular infiltrative nests of tumor cells surrounded by prominent fibrosis and sclerosis. The tumor cells had abundant eosinophilic, somewhat granular cytoplasm, and enlarged, mildly pleomorphic nuclei with single prominent nucleoli. Mitotic activity and necrosis were variably present. Perineural invasion was present in all invasive cases (100%) and vascular invasion in four (67%). Evidence of a preexisting pleomorphic adenoma (carcinoma ex-pleomorphic adenoma) was seen in 3 cases (50%), one (SDC5) of which consisted of in situ salivary duct carcinoma where the malignant cells were confined within the capsule of the preexisting pleomorphic adenoma. Metastases to lymph nodes were present in 4 out of 6 cases (67%), where lymph nodes were removed. The clinical and pathologic findings are summarized in Table 1.

3.2. PIK3CA Expression Was Elevated in Salivary Duct Carcinoma. Six specimens of SDC lesions were immunolabeled...
with anti-PI3 Kinase p110α (PIK3CA) antibody to assess the expression level of PIK3CA proteins. IHC results showed focal cytoplasmic positivity in tumor cells compared to normal ductal epithelial cells. Although elevated PIK3CA expression was also observed in some normal ductal epithelial cells of salivary glands immediately surrounding the tumor masses, immunolabeling of the neoplastic cells was of much greater overall intensity (1-2+) relative to the background expression of the nonneoplastic cells inside or adjacent to the neoplastic lesions (0-1+) (Figure 1). Significantly higher expression was observed in patients 2, 3, 4, and 5 than in patients 1 and 6. No apparent difference was observed between the primary tumors and corresponding lymph node metastases.

3.3. Two Hotspot Mutations H1047R and E545K of the PIK3CA Gene Were Identified in Six SDC Patients. To investigate whether genetic mutation is one of the molecular mechanisms that contributed to the elevated PIK3CA expression in SDC, we performed DNA sequencing analyses by microdissecting genomic DNAs from the neoplastic lesions and their adjacent normal tissues in each case. No more than 2-fold difference between the tumor and the corresponding normal components was detected in the four cases (see Supplementary Figure S1 available online at http://dx.doi.org/10.1155/2014/810487). This data suggest that the PIK3CA gene amplification was not a contributing molecular mechanism for the upregulation of the PIK3CA protein expression in SDC tumor cells.

Frequent overexpression of HER2 protein has been reported in the human salivary ductal carcinoma [22]. To investigate if the PIK3CA mutations coexist with HER2 overexpression, we examined HER2 expression in the 5 of those 6 SDC cases by IHC. Three of five patients showed overexpression of HER2 from + to ++ + (Figure 3). No coexistence of HER2 overexpression and PIK3CA mutation was observed among these five cases (Table 1).

4. Discussion

SDC is one of the most aggressive subtypes of salivary gland cancers [1]. Recently, elevated expressions of p53 and HER2/neu have been detected in some SDC patients, which were shown to be associated with recurrence, poor prognosis, and distant metastasis [7, 23]. In this study, we found that oncogenic PIK3CA protein was significantly elevated in the salivary duct carcinoma of all six patients (Figure 1). Furthermore, we detected high incidence of PIK3CA hotspot mutations in SDC patients (33%) (4%, 78%; 95% confidence limits). The two PIK3CA hotspot mutations (E545K and H1047R) are located at the helical domain and the kinase domain of the PIK3CA protein, respectively (Figure 2). These data suggest that oncogenic PIK3CA may play a critical role in the carcinogenesis of this rare disease, possibly from an early stage. These data are consistent with recent studies that reported PIK3CA mutations in patients with SDC [24, 25]. Moreover, Suzuki et al. have also reported positive staining of phosphorylated mTOR (a downstream gene of the PI3K signaling) in 10/12 SDCs examined [26]. Combined with our previous studies that high incidence of PIK3CA gene mutations was identified in the other tumor types of human head and neck squamous cell carcinomas such as pharyngeal cancer [14, 15], the current study further confirmed the importance of oncogenic PIK3CA in the carcinogenesis of salivary duct epithelial cells and highlighted

### Table 1: Clinical and pathologic features of salivary duct carcinoma cases.

| Case | Gender | Age | Location | Tumorsize | Perineural invasion | Margin | Lymph node | Pleomorphic adenoma | HER2 expression | PIK3CA mutation |
|------|--------|-----|----------|-----------|--------------------|--------|------------|-------------------|----------------|-----------------|
| SDC1 | M      | 60  | Parotid  | 2.3 cm    | +                  | −      | 1/2        | −                 | −              | −               |
| SDC2 | M      | 65  | Parotid  | 2.2 cm    | +                  | +      | 15/29      | −                 | −              | +               |
| SDC3 | F      | 50  | Parotid  | 1.7 cm    | +                  | −      | 8/44       | +                 | N/A            | E545K           |
| SDC4 | M      | 58  | Parotid  | 2.0 cm    | +                  | −      | (close)    | 14/45             | ++             | −               |
| SDC5 | M      | 67  | Parotid  | N/A       | N/A                | +      | 0/12       | +                 | −              | −               |
| SDC6 | M      | 71  | Parotid  | 1.6 cm    | +                  | −      | (close)    | N/A               | −              | +++             |

Abbreviations: N/A: not available or not applicable; close: less than 0.1 cm.
Figure 1: Immunohistochemical analysis of salivary duct carcinoma with anti-PIK3CA antibody. Representative PIK3CA IHC results of adjacent normal (a) and (c) and tumor tissues ((b) and (d) to (h)) from SDC patients. Staining of neoplastic cells was of greater overall intensity ((b) and (d) to (h)) relative to staining of nonneoplastic cells (a) and (c). Magnification: 200x.
a critical oncogenic PI3K signaling pathway in human head and neck cancers. It is conceivable that PI3K pathway is frequently dysregulated in SDC and the activated PIK3CA function may have led to the reported increased p-mTOR expression.

Clinical data indicates that SDC2 with hotspot mutation H1047R in PIK3CA kinase domain was a 65-year-old male patient. SDC3 patient with hotspot mutation E545K was the only female patient and was also diagnosed at 50 years-old, which is the youngest among this SDC cohort. Both patients with hotspot PIK3CA mutations were featured with perineural invasion and many lymph node metastases. Interestingly, SDC2 did not exhibit overexpression of HER2, while three of the four cases with wild-type PIK3CA displayed
upregulated HER2 expression. Unfortunately no sufficient tissue remained from the SDC3 case for the HER2 IHC.

Oncogenic PIK3CA is mainly activated through gene amplification and “gain of function” single-nucleotide substitution in human cancers [27, 28]. In this study, all six SDC patients showed upregulated expressions of PIK3CA protein in tumor lesions, but only two of them harbored PIK3CA genetic mutations. Since gene amplification is a logical alternative molecular mechanism for PIK3CA activation [10, 27–29], we examined copy number alteration of PIK3CA by quantitative PCR. No amplification was detected in our samples; therefore, the source of activation is likely upstream of PIK3CA. Our data would recommend that when investigating activation of the PI3 K signaling pathway, other approaches such as IHC should be included to complement genomic DNA sequencing. Indeed, IHC for PIK3CA expression in cervical intraepithelial neoplasia has been shown possessing diagnostic significance for cervical cancer [30]. Thus, the PIK3CA and p-mTOR immunostaining results should be further examined for their high translational potentials as biomarkers for diagnosis/prognosis of human SDC or guiding target therapeutics for patients with SDC.

Despite aggressive surgical resection and postoperative adjuvant radiotherapy, the overall survival rate for SDC patients remains dismal. Clearly, more targets for chemotherapy or adjuvant therapy following surgery or in combination with radiotherapy are very desirable. Here we report high PIK3CA mutation rates in this rare disease with high mortality. Our data also suggests that nongenomic alteration is involved in the dysregulation of the PI3 K signaling pathway. In combination with other reports [24, 25], current data strongly support the notion that the PI3 K signaling pathway plays a critical oncogenic role in the development of human SDC and the prevalence of its dysregulation advocates its potential as a feasible therapeutic target.

Abbreviations
SDC: Salivary duct carcinoma
PCR: Polymerase chain reaction
PI3 K: Phosphatidylinositol 3-kinase
PIP3: Phosphatidylinositol-3, 4, 5-triphosphate.

Conflict of Interests
The authors declare that there is no conflict of interests related to this work.

Acknowledgments
This work was supported by the Florence and Herbert Irving Clinical Research Career Award and the NCI R01 CA109525.
