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Cooperation Between Pten and Smad4 in Murine Salivary Gland Tumor Formation and Progression

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Abstract

Salivary gland tumor (SGT) is a rare tumor type, which exhibits broad-spectrum phenotypic, biological, and clinical heterogeneity. Currently, the molecular mechanisms that cause SGT pathogenesis remain poorly understood. A lack of animal models that faithfully recapitulate the naturally occurring process of human SGTs has hampered research progress on this field. In this report, we developed an inducible keratin 5-driven conditional knockout mouse model to delete gene(s) of interest in murine salivary gland upon local RU486 delivery. We have deleted two major tumor suppressors, Pten, a negative regulator of the PI3K pathway, and Smad4, the central signaling mediator of TGFβ pathway, in the murine salivary gland. Our results have shown that deletion of either Pten or Smad4 in murine salivary gland resulted in pleomorphic adenomas, the most common tumor in human SGT patients. Deletion of both Pten and Smad4 in murine salivary gland developed several malignancies, with salivary adenoid cystic carcinoma (SACC) being the most frequently seen. Molecular characterization showed that SACC exhibited mTOR activation and TGFβ1 overexpression. Examination of human SGT clinical samples revealed that loss of Pten and Smad4 is common in human SACC samples, particularly in the most aggressive solid form, and is

Abbreviations: CxPA, carcinoma ex PA; GEMM, genetically engineered mouse model; HNSCC, head and neck squamous cell carcinomas; IF, immunofluorescence; K5, keratin 5; PI3K, phosphatidylinositol 3-kinase; SACC, salivary adenoid cystic carcinoma; SDC, salivary ductal carcinoma; SG, salivary gland; SGT, salivary gland tumor; SPA, salivary pleomorphic adenoma; SMA, smooth muscle actin; TGFβ, transforming growth factor β; YFP, yellow fluorescence protein

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correlated with survival of SACC patients, highlighting the human relevance of the murine models. In summary, our results offer significant insight into synergistic role of Pten and Smad4 in SGT, providing a rationale for targeting mTOR and/or TGFβ signaling to control SGT formation and progression.

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Introduction

Salivary gland tumors (SGTs), although uncommon, account for approximately 5% of all head and neck tumors and are composed of more than 20 histopathological subtypes with widely varied clinical outcomes [1, 2]. Both rarity of incidence and heterogeneity of pathology pose challenges for SGT studies, resulting in SGT being one of the least studied tumor types [3]. The most common subtype of SGT is salivary pleomorphic adenoma (SPA). Although benign, about 20% of SPAs will eventually progress to malignancy such as carcinoma ex PA (CxPA) or salivary ductal carcinoma (SDC) [4]. Salivary adenoid cystic carcinoma (SACC) is among the most common malignant SGTs; it exhibits slow but persistent tumor progression and frequent recurrence and metastasis [5]. Current therapeutic options for human SGT are limited. Depending on their location, some SGTs are difficult to remove completely. Radiation therapy is less effective for clinical treatment. The effects of current chemotherapies have been disappointing [6]. Thus, there is a need to acquire understanding of the molecular mechanisms of SGT pathogenesis that can be used toward the development of novel therapeutic approaches and that will allow clinicians to improve survival and quality of life for patients with SGT. Roadblocks impeding progress toward the realization of clinical diagnostics and therapeutics in this field are a lack of characterization of SGTs at the molecular level and a lack of research tools such as mouse models that mimic human SGTs.

Molecular characterization of human SGTs has been made recently. Of note, the application of exome sequencing on samples from the SGT biorepository has shown that, in addition to commonly seen chromosome translocations, molecular alterations of Pten and/or Smad4 occur in human SGTs [7–10]. Pten, best known as a lipid phosphatase, negatively regulates the phosphatidylinositol 3-kinase (PI3K) pathway, which is activated in multiple human cancers [11]. Alteration of Pten has been reported in several subtypes of human SGTs. For example, we and others have recently shown that loss of Pten expression is common in human SACC patients, with the solid form most frequent [12, 13]. Loss of heterozygosity and a germline mutation of Pten have been reported in human epithelial myoepithelial carcinomas of salivary gland [14] and a case of acinic cell carcinoma patients [15], respectively. Moreover, recent studies showed that loss of Pten is fairly common in multiple human SGTs and is associated with a worse prognosis, relapse, and metastasis [10, 16–19]. Smad4, the central signaling mediator for the transforming growth factor β (TGFβ) pathway, is a tumor suppressor for multiple human cancers [20], including its mutations in human SGTs [8]. In addition, TGFβ signaling has been known to regulate development and differentiation of salivary gland [21, 22]. And overexpression of TGFβ1 ligand has been reported in human CxPAs [23] and promotes migration and invasion of SACCs [24].

We have seen murine SGTs development in our Pten and/or Smad4 conditional deletion mouse models for head and neck squamous cell carcinoma driven by the Keratin 5 (K5) promoter [25]. We further modified RU486 delivery specifically into murine salivary glands and noticed that deletion of either Pten or Smad4 in murine salivary gland leads to the development of SPAs and that combined deletion of both Pten and Smad4 results in malignant progression of murine SGT, with SACC the most common. Examination of human SGT samples showed that reduced expression of Pten and Smad4 is common in human SACC clinical samples, particularly in the most aggressive solid form, and are correlated with survival of SACC patients, validating the clinical relevance of the murine SGT models. Furthermore, the murine SGTs exhibit activation of mTOR and elevated TGFβ1 ligand. Thus, our study will not only reveal molecular mechanisms of Pten and Smad4 in tumor formation and malignant progression of SGT but also identify novel targets for SGT therapy.

Material and Methods

Generation and Characterization of the Inducible Head and Neck–Specific Pten and/or Smad4 Genetically Engineered Mouse Model

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Cancer and Use Committees of the University of Colorado Anschutz Medical Campus. All mouse experiments were performed in a C57BL/6 background. The inducible *K5CrePR1* mouse was reported previously [26]. The *Pten*-floxed (*Ptenflf*) mouse has been originally generated by Dr. Hong Wu, from University of California at Los Angeles [27], and was purchased from Jackson Laboratory (Bar Harbor, ME, USA). The *Smad4*-floxed mice (*Smad4flf*) have been provided by Dr. Chuxia Deng at the National Institutes of Health [28]. The *K5CrePR1* mice were mated with homozygous *Ptenflf* mice or *Smad4flf* mice. The offspring, *K5CrePR1.Ptenfl/wt* or *K5CrePR1.Smad4fl/wt* hemizygous mice, were cross-bred to *Ptenflf* or *Smad4flf* mice, respectively, to generate *K5CrePR1.Ptenflf, K5CrePR1.Smad4flf* mice or *K5CrePR1.Ptenflf.Smad4flf* mice. PCR genotyping was performed at 3 weeks of age using genomic DNA isolated from tail biopsies. The primers used to detect the *K5CrePR1* transgene were forward CGGTCGATGCAACGAGTGAT and reverse CCACCGT CAGTACGTGAGAT [26], primers to detect *Pten*-floxed gene were forward ACTCAAGGCAGGGATGAGC and reverse AATCTAGGG CCTCTTGTGCC [27], and primers to detect *Smad4*-floxed gene were forward GGGCAGCGTAGCATATAAGA and reverse GACCCAAA CGTCACCTTCAC [25].

To delete *Pten* and/or *Smad4* in murine head and neck epithelia, 100 μl of RU486, dissolved in sesame oil (0.2 μg/μl), was applied in the oral cavity of mouse once a day for 5 days as described previously [29]. The resulting mouse lines *K5CrePR1.Ptenflf, K5CrePR1.Smad4flf*, and *K5CrePR1.Ptenflf.Smad4flf* were selected by using genotyping primers specific for *K5CrePR1*, *Pten*-floxed, and *Smad4*-floxed mice as described above.