AKT3 drives adenoid cystic carcinoma development in salivary glands

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Introduction
The serine/threonine kinase AKT is a critical effector downstream of the phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway, and it is also a vulnerable node to be targeted once hyperactivated in tumorigenesis. Its expression and activation controls cellular processes such as cell growth, proliferation, cell survival, and neo-vascularization and was shown to mediate cancer progression [1, 2]. The AKT family consists of three different but highly homologous
gene products AKT1, AKT2, and AKT3, which are considered to be attractive targets for the design of small molecule-based anticancer therapies [3, 4]. However, more recent studies demonstrate isoform-specific, opposing functions of individual isoforms in cancer [5–10], and a better understanding of isoform-specific functions is a prerequisite for AKT targeting therapies. Being maybe the least studied AKT isoform, AKT3 was found to be upregulated in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines [11, 12]. Moreover, knockdown of AKT isoforms has been reported to abrogate invasive growth of salivary gland cancer (SGC) cell lines [13].

Salivary gland cancers are a rare group of malignancies accounting for <0.5% of all cancers and around 3-5% of all head and neck cancers. The World Health Organization (WHO) distinguishes between 24 subtypes of SGC, and all of them exhibit different morphological and pathological features. SGC predominantly arise in one of the three major salivary glands (submandibular, sublingual, and parotid gland), with adenoid cystic carcinomas (ACC), mucoepidermoid carcinomas, and polymorphous low-grade adenocarcinomas being the most abundant SGC subtypes [14, 15]. Generally, salivary gland tumors are surgically resected. However, nonresectable, recurrent, and metastatic high-grade SGC respond only weakly to cytotoxic chemotherapy, and targeted therapies are not available in most SGC subtypes, culminating in poor prognosis [16–18]. In order to develop targeted therapies in this field, a better understanding of salivary gland tumorigenesis is required, and more recent studies addressing the mutational landscape of SGC may facilitate the identification of novel oncogenic drivers in SGC [19, 20]. Intriguingly, these studies revealed mutations in genes implicated in the PI3K-AKT-mTOR signaling cascade, suggesting a prominent role of this pathway in salivary gland tumorigenesis. Indeed, recurrent mutations resulting in activation of the PI3K-AKT-mTOR pathway were found in 30% of ACC[21], and activated (phosphorylated) AKT isoforms and downstream mTOR is enhanced in ACC as compared to healthy adjacent tissue [22, 23].

Being a central signal mediator in the PI3K-AKT-mTOR signaling pathway, we aimed to investigate a possible implication of AKT3 signaling in breast and salivary gland tumorigenesis. Hence, we generated transgenic mice conditionally expressing Akt3 under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter, which directs expression to the mammary and salivary glands [24, 25].

Material and Methods

Animals

For the establishment of TetO-Akt3 transgenic mice, a cDNA encoding the murine Akt3 containing an N-terminal myristoylation signal and a C-terminal HA tag was cloned in an expression vector containing the tetracycline-responsive Tet-op promoter and an IRES-luciferase [25]. Plasmid DNA was linearized and microinjected into the pronucleus of FVB/N oocytes. TetO-Akt3 founders were identified by PCR and a TetO-Akt3 transgenic line was established in the FVB/N genetic background. For the generation of MMTV-tTA/TetO-Akt3 double transgenic animals TetO-Akt3 transgenic were crossed with MMTV-tTA mice [25]. In all described experiments, littermates were used as controls. All mice were kept and bred under standardized conditions according to an ethical animal license protocol complying with the current Austrian Law. Genotyping of the mice was performed using the primer pairs (P1/P2) for detection of the Akt3-transgene and the primer pairs (P3/P4) for detection of the MMTV promoter (see Table S1).

RNA and real-time quantitative PCR

RNA was isolated with TRIzol Reagent (Life Technologies, Rockford, IL, USA) according to the manufacturer’s instructions. RNA was treated with DNaseI (Thermo Fisher Scientific, Waltham, MA, USA) prior to reverse transcription by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using random hexamer primers. cDNA derived from 50 ng total RNA was used per reaction. qRT-PCR was performed using SYBR Green. Mouse Actb and 28S as housekeeping controls were detected using the primer pairs P5/P6 and P7/P8, respectively. Akt3 transgene detection was performed using the primer pairs P9/P10 (see Table S1).

Western blot analysis

Salivary gland tissue homogenates were prepared from snap-frozen salivary gland tissues in RIPA buffer (Cell signaling technology, Danvers, MA, USA Cat.No- 9806). Tissue homogenates were cleared by centrifugation at 4°C for 15 min at 15.0000 g. Protein concentration was determined using the Bradford protein assay method (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. RNA was isolated with TRIzol Reagent (Life Technologies, Rockford, IL, USA) according to the current Austrian Law. Genotyping of the mice was performed using the primer pairs (P1/P2) for detection of the Akt3-transgene and the primer pairs (P3/P4) for detection of the MMTV promoter (see Table S1).

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**Histology**

Mouse salivary gland tissues were fixed in 4% formaldehyde solution and embedded into paraffin. Five-micrometer thick tissue sections were deparaffinized and rehydrated. Sections were either stained with hematoxylin and eosin or subjected to immunohistochemical staining. Stained slides were scanned with TissueFaxs software (TissueGnostics Gmbh, Vienna, Austria). Quantification of the tumor area, tumor burden, and staining intensities was done with HistoQuest software (TissueGnostics Gmbh). For immunohistochemical stainings, antigen retrieval was performed using Target Retrieval Solution, pH 6.0 (Dako, Santa Clara, CA, USA). Endogenous peroxidase activity was diminished by incubating sections with 3% hydrogen peroxide for 10 min. Sections were blocked with M.O.M blocking solution (Vector Laboratories, Burlingame, CA, USA) for 1 h prior to primary antibody incubation. Used primary antibodies were anti-HA Tag (1:1000, Rabbit mAb, Cell signaling technology, Cat-No-2367), anti-Ki-67 (1:400, Rabbit mAb, Cell signaling technology, Cat-No-9027), anti-α-SMA (1:200, Mouse mAb, MS-113-P0), and anti-CC3 (1:300, Rabbit Ab, Cell signaling technology, Cat-No-9961). Signal detection was performed using IDetect™ Universal Mouse Kit-HRP (Empire genomics) and 3,3′-Diaminobenzidin (DAB) as chromogenic substrate.

**Dox treatment of mice**

Dt mice with established salivary gland tumors were treated with Dox (Sigma Aldrich, Cat-No-D9891) for three consecutive weeks. Dox was applied at a final concentration of 1 mg/mL. Dox in drinking water containing 1% sucrose (Sigma Aldrich, Cat-No- S0389). The prepared solution was changed twice a week. Measurement of the tumor volume was performed every second day using a caliper. Tumor volume was calculated using the following equation: (width*width*length)/2.

**Human data**

We used the cBioPortal for Cancer Genomics browser [26, 27] to analyze the TCGA dataset for head and neck cancer patients [28]. To identify patients with increased AKT3 mRNA expression, we applied a z-score of ±1.5 RNASeq V2 RSEM. We also used the publically available GEO dataset GSE10300 for analysis. Probe set for AKT3 was 212607_at.

**Statistics**

All values are given as means ± SD. Comparison between two groups was made by Student’s t-test. For Kaplan–Meier analysis, a log-rank test was performed.

**Results**

**Akt3 overexpression triggers salivary gland tumor formation**

We generated TetO-Akt3 transgenic mice by injecting linearized plasmid DNA encoding the mouse Akt3 cDNA containing an N-terminal myristoylation signal and a C-terminal Human influenza hemagglutinin (HA)-tag under control of the tetracycline-responsive Tet-op promoter and an IRES-luciferase into the pronucleus of FVB/N oocytes. To study the role of Akt3 in salivary and mammary gland tumorigenesis, we then took advantage of the Tet-Off system and crossed MMTV-tTA transgenic mice [25] with TetO-Akt3 transgenic mice. Breeding these strains lead to the generation of MMTV-tTA/TetO-Akt3 double transgenic animals (hereafter: Dt). These mice show sustained expression of HA-tagged Akt3 in mammary and salivary glands which can be switched off by Doxycycline (Dox) treatment (Fig. 1A).

To validate the model, we performed real-time quantitative PCR analysis (qRT-PCR) using primers specific for the transgene and confirmed transgenic mRNA expression in the Dt mouse group at 8 weeks of age (Fig. 1B). On the protein level, we confirmed expression of the HA-tagged Akt3 which resulted into increased levels of total Akt protein, verified by an antibody against pan-Akt. Notably, we detected massive activation of Akt in Dt mice, which was absent in wild-type ctrl mice (Fig. 1C). Intriguingly, Dt mice suffered from the formation of salivary gland cancer with 100% penetrance and with a median tumor-free survival of 134 days (Fig. 1D). Tumors isolated from Dt mice stained positive for HA-tagged Akt3 by immunohistochemistry (IHC) (Fig. 1E). At the time of sacrifice due to tumor burden and according to the animal law, mammary glands of Dt mice did not exhibit any malignant phenotype and Ctrl mice were tumor-free (data not shown). These data suggest an essential function of Akt3 in the rapid and aggressive development of salivary gland tumors. Hence, these results did not only validate the transgenic mouse model used in this study, but also confirmed an oncogenic role for high Akt3 expression as driver for salivary gland tumor progression.

**Akt3-driven salivary gland tumors exhibit adenoid cystic carcinoma characteristics**

To characterize the tumors, we dissected the major salivary glands and consecutive hematoxylin-eosin (H&E)-stained tissue sections were examined by a board-certified pathologist (HP). All tumors analyzed displayed similar immunopathologic features and were classified as adenoid cystic carcinomas (ACC). ACC were found in all major salivary
glands, that is, the sublingual, the submandibular, and parotid glands, and ACC formation led to complete disruption of the normal histological architecture of the salivary gland (Fig. 2A). Indeed, the histopathology of the glands of Dt mice indicates nodular proliferation of uniform basaloid cells, consistent with ACC. Typical large cystic spaces can be seen including pseudoglandular spaces covered by cuboidal cells as well as areas with microglandular patterns (Fig. 2B). Furthermore, in the pseudolumina, a mucus-like material is present, which is composed of proteinaceous fluid, containing mainly fibronectin and collagen type IV (Fig. 2B). Infiltration of the carcinoma into a newly formed lymph node confirms the malignant potential of this tumor (Fig. 2C). However, perineural invasion and metastasis, for example, in the lung, as often observed in human patients suffering from ACC [29–31], were not observed when we sacrificed tumor-bearing mice. Moreover, we found tumor areas positive for α-smooth muscle actin (α-SMA), a feature of ACC pathology (Fig. 2D) [14]. Next, we checked proliferation in salivary gland tissue of Dt mice compared to wild-type Ctrl mice by IHC staining for Ki-67. We detected significant higher proliferation rate in tumor-bearing glands in Dt mice, both in 8 week-old mice and in mice older than 12 weeks,

Figure 1. Akt3 overexpression provokes salivary gland tumorigenesis: (A) Scheme depicting breeding strategy of MMTV-driven expression of HA-tagged Akt3 via tTA. Dox administration represses transgenic Akt3 expression. (B) Expression of the Akt3 transgene in salivary gland tissues of Dt animals was verified by qRT-PCR (n = 5) analysis and (C) by western blot analysis. (D) Kaplan–Meier plot depicting time until tumors were clearly visible in Ctrl and Dt mice (n > 10 mice per group). (E) IHC of salivary glands of Ctrl andDt mice probed for HA expression. Pictures on the right show a higher magnification of the same sections.

Figure 2. Tumors show an adenoid cystic carcinoma pathology. (A) Representative H&E stainings of sublingual, submandibular, and parotid glands in Ctrl versus Dt mice. (B) H&E staining of adenoid cystic carcinoma of the salivary gland depicting the typical large cystic spaces and small pseudoglandular spaces covered by cuboidal cells (left) and microglandular pattern with solid areas. The proteinaceous material is stained in violet. (C) H&E staining of a newly formed lymph node. White arrows indicate infiltrating carcinomas. (D) ACC in Dt mice show negative and positive areas for α-SMA staining (E) Representative pictures of immunohistochemistry for the proliferation marker Ki67 of salivary gland sections of Ctrl and tumor-bearing Dt mice at 8 weeks and >12 weeks of age. Ki67+ were quantified using TissueQnostic software. Data are presented as mean ± sd and were analyzed by Student’s t-test (n = 3, *P < 0.05).
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further confirming the contribution of Akt3 to ACC pro-
geression (Fig. 2E). Notably, we did not notice tumor forma-
tion in the minor salivary gland, presumably because of
insufficient transgene expression.

**Abrogation of Akt3 overexpression mediates tumor regression**

Next, we tested whether rising ACC become addictive to
Akt3 overexpression by taking advantage of our Dt mouse
model. Hence, we treated salivary gland tumor-harboring
mice with doxycycline (Dox), thereby abrogating transgenic
Akt3 overexpression. Administration of Dox immediately
triggered regression of tumors in all mice tested (n = 3, 
Fig. 3A). Indeed, we observed reduction in tumor burden
and tumor number by analysis of H&E-stained salivary
gland sections and normalization of the salivary gland
structure following Dox treatment (Fig. 3B–D). Regression
of tumors correlated with decreased expression of the
Akt3 transgene in the salivary glands as verified by IHC
staining of the HA tag and by qPCR (Fig. 4A and D)
and was not only reflected by decreased proliferation of
tumors in Dox-treated mice as tested by Ki-67 staining
(Fig. 4B and E), but also by increased apoptosis. Abrogation
of Akt3 overexpression triggered activation of the apoptotic
cascade, as evidenced by positive IHC staining for cleaved
caspase 3, which was completely absent in non-Dox-treated
Dt mice (Fig. 4C and E). Altogether, these data demon-
strate the dependence of ACC tumorigenesis on Akt3
expression and further validates our mouse model as a
potent tool to study Akt3-driven salivary gland
tumorigenesis.

**Discussion**

The MMTV promoter has been widely used to condition-
ally manipulate gene expression in secretory glands and
serves as a valuable tool to study disease in salivary and
mammary glands [25, 32–35]. We took advantage of this
model by crossing the MMTV-tTA mouse [25] into mice
expressing Akt3 under TetO control, which were generated
in our laboratory for this study. Dt mice developed sali-
vary gland ACC within a few weeks of age, which became
clearly visible within 4–5 months.

The clinical management of SGC remains still chal-
lenging. Major obstacles for a better outcome in the treat-
ment of SGC patients are high recurrence rates and the
high metastatic potential of these tumors, as well as their
poor response to chemotherapy [36]. Recent exon and
whole genome sequences suggest alternative treatment
strategies targeting abundant MYB-NFIB fusion oncogenes,
or genes involved in NOTCH1 or FGF receptor signaling,
as well as targeting the PI3K/AKT/mTOR pathway[21, 37,
38]. However, drugs for targeted therapies have not over-
come the hurdles into clinical application for SGC yet.
Indeed, seeking for novel treatment options using small
molecule inhibitors is difficult for rare diseases such as
SGC in general and salivary ACC in particular, since
adequate preclinical models are barely available. This work
demonstrates the pro-oncogenic potential of Akt3 in sali-
vary glands to drive ACC in a novel genetically modified
mouse model. These data are in line with a previous
report showing that siRNA-mediated AKT3 knockdown
limits invasive growth of human salivary gland cell lines
[13]. Also, highly phosphorylated AKT levels in ACC

![Figure 3. Dox treatment leads to regression of established tumors. (A). Relative tumor volume upon doxycycline (Dox) treatment of mice measured using a caliper. Treatment was started when tumors reached a volume of 500–750 mm³ (B & C). Representative H&E staining of ACCs in salivary glands of Dt mice w/o treatment and with Dox treatment. (D) Quantitation of tumor area and tumor number using TissueQnostic software. Graphs represent mean ± SD, data were analyzed by Student’s t-test. (n = 3, **P < 0.01).](image-url)
tissue were associated with an increased risk for tumor relapse [23]. In contrast, a recent publication showed that activation of AKT was also associated with better prognosis of salivary gland ACC patients [22]. This discrepancy might arise because of distinct functions of the different AKT isoforms, suggesting the need for AKT isoform-specific inhibitors in the treatment of salivary gland ACC patients [39].

Indeed, despite the high sequence similarity of the family members, different functions for the AKT isoforms were reported with respect to cancer. For example, AKT1 was revealed as an oncogene in mammary cancer, and AKT2 primarily acts on metastatic dissemination[5, 40, 41]. In contrast, AKT3, but not AKT1 and AKT2, was required for the growth of triple-negative breast cancer cell lines [12]. However, the genetic landscape in breast ACC compared to salivary gland ACC is different, and so are morphologic and clinical features [42, 43]. In our model, the dominant phenotype in salivary glands precluded us to investigate the role of Akt3 in mammary gland tumorigenesis. We acknowledge that it is unclear whether our model assures adequate Akt3 expression in mammary glands during the whole life span of our mice, and whether expression levels achieved would be sufficient...
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to drive mammary gland tumorigenesis. Therefore, we want to emphasize that our work by no means rules out an oncogenic role of Akt3 in development of cancers of the mammary gland.

Altogether, our data demonstrate the potent oncogenic role of AKT3 for ACC pathogenesis in vivo. Furthermore, our novel mouse model has the potential to serve as a valuable tool to study salivary gland ACC and develop new therapeutic strategies.

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Conflict of Interest

The authors declare no potential conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Sequences of primers used for genotyping and SYBR green-based qRT-PCR.