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Alterations of Notch pathway in patients with adenoid cystic carcinoma of the trachea and its impact on survival

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ABSTRACT

Introduction: Adenoid cystic carcinoma (ACC) of the trachea lacks of well-characterized molecular markers. There is currently no specific treatment for metastatic ACC of the trachea. This study aimed to identify genomic mutations of Notch pathway and investigate the efficacy of *NOTCH* inhibitor in ACC of the trachea.

Methods: **73** Patients with ACC of the trachea at four institutions from 2008 to 2016 were identified. Analysis of hotspot mutations in cancer-related genes of Notch pathway was performed using next generation sequencing. Gene-expression and functional analyses were performed to study the mechanism of activation through mutation. Univariable and multivariable Cox regression models were used to predict overall survival (OS). Patient-derived xenograft (PDX) models were established and treated with *NOTCH* inhibitor Brontictuzumab.

Results: Gain-of-function mutations of the *NOTCH1* gene occurred in 12 (16.4%) tumors, leading to stabilization of the intracellular cleaved form of *NOTCH1* (ICN1). *NOTCH1* mutation was associated with increased *NOTCH1* activation and its target gene *HES1*. Mutations in *NOTCH2* (3/73), *NOTCH4* (2/73), *JAG1* (1/73) and *FBXW7* (2/73) were also identified in 8 (11.0%) patients. A strong inverse correlation of expression was observed between *FBXW7* and *HES1*. *NOTCH1* mutation was associated with solid subtype ($P = 0.02$), younger age at diagnosis ($P = 0.041$) and shorter overall survival (OS) ($P = 0.017$). *NOTCH1* mutation was not an independent prognostic factor in the presence of histologic subtype and resection margin. Brontictuzumab significantly reduced tumor growth in *NOTCH1*-mutated PDX.

Conclusion: *NOTCH1* mutation is associated with activation of Notch pathway in ACC of the trachea. *NOTCH1* is a potential target for therapeutic intervention in patients with ACC of the trachea.

1. Introduction

Adenoid cystic carcinoma (ACC) of the trachea has a predilection for perineural invasion and a tendency for local recurrence and late metastasis. The tumors were histologically classified into tubular, cribriform, and solid subtypes [1,2]. Surgical resection is the main treatment for ACC, but resectability mainly depends on the size, location and extension of tumor [3]. Because of mucosal or submucosal spread in the airway, complete resection is not always possible and R0 resections are achieved in approximately 50% of patients [4]. Radiotherapy is usually recommended after incomplete resections [5]. Currently, there is no specific treatment for metastatic ACC of the trachea. ACC harbors activating mutations in *NOTCH1* [6]. Notch signaling is active in luminal epithelial components of low-grade ACC. Its further activation by somatic Notch pathway mutation likely underlies the switch to solid histology and the aggressive clinical course [7].

The Notch pathway is involved in cancer-relevant functions, including maintenance of stem cells, cell fate specification, proliferation, and angiogenesis [8].

In humans, there are four Notch receptors (*NOTCH* 1-4) that, upon engagement by ligands of the Delta/Serrate/Lag2 (DSL) family, are proteolytically cleaved to release the intracellular domain of Notch (NICD), which translocates into the nucleus to modulate gene expression such as *HES1*. The generation and stability of NICD is regulated by the ubiquitin ligase complexes containing *FBXW7* [9]. Ferrarotto et al. found that 14 of 102 patients with ACC (13.7%) were found to harbor *NOTCH1* mutations. However, this study population consisted of only 11 cases of ACC of the trachea and most (82/102) originated from

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salivary glands [10]. A diagnostic feature of ACC is a t (6;9) rearrangement that translocates *MYB* to the *NFIB* locus and results in high *MYB* expression [11–14]. *MYB* coordinates with distinct regulatory programs in alternate cell lineages in ACC cooperating with the Notch pathway [11]. The molecular underpinning of ACC of the trachea other than the *MYB-NFIB* fusion gene remains largely unexplored. The contribution of somatic *NOTCH1* mutation to ACC of the trachea has yet to be determined. The aim of this study is to identify *NOTCH1* mutations in ACC of the trachea and to assess the antitumor activity of Notch inhibitor in ACC of the trachea.

2. Materials and methods

2.1. Patient selection

Patients with ACC of the trachea from four institutions in China were enrolled in this study from June 2008 to July 2016. Hematoxylin and eosin–stained slides from all selected study patients were reviewed independently by two pathologists (Dr. Fu xinge and Dr. Gu Yingying, the First Affiliated Hospital of Guangzhou Medical University) who were blinded to clinical information and immunohistochemical and cytogenetic results. ACCs of the trachea were classified according to the predominant histologic pattern (cribriform, solid, and tubular). Disagreements were resolved by consensus. Tumor samples were genotyped by target-sequencing platform. Ethical approval was obtained from each institutional review board. Written informed consent for tumor sample collection was obtained from all patients.

2.2. Genomic analysis

Total genomic DNA was extracted from paraffin-embedded sections using the DNA Extraction Kit (Qiagen, California, USA). The DNA was directly used as template for PCR reactions. Target exome sequencing or analysis of hotspot mutations in *NOTCH*-related genes was performed using next generation sequencing. Details are available in the Data Supplement.

2.3. Expression vectors for NOTCH1 mutants

Expression vectors for the different FLAG-*NOTCH1* mutants were engineered into a C-terminal FLAG-tagged version of full-length *NOTCH1* (pCDNA3 FL-*NOTCH1*) by site-directed mutagenesis. Details are available in the Data Supplement.

2.4. Immunohistochemistry assay (IHC)

The following primary antibodies were used in this study: cleaved *NOTCH1* (Val1744) antibody from Cell Signaling (Danvers, MA) and *FBXW7* antibody from Abcam (Ab109617, Cambridge, MA). Details are available in the Data Supplement.

2.5. Immunofluorescence assay (IF)

Immunofluorescence assay was performed as previously described [15]. Antibodies were anti-FLAG (rabbit) and anti–activated-*NOTCH1* (Ab8925, Abcam). Fluorochrome-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. DAPI (4′,6-diamidino-2-phenylindole) was purchased from Sigma (CA, USA).

2.6. Fluorescent in-situ hybridization (FISH)

MYB/NFIB rearrangements were detected by FISH assay. In nuclei containing the *MYB-NFIB* fusion, green and red signals from the *MYB* and *NFIB* genes overlap in a red/green (yellow) signal. Details are available in the Data Supplement.

2.7. Western blotting assay

Antibodies of activated anti-*NOTCH1*-Valine 1744 (Cell Signaling Technology), anti-FLAG (Sigma Co., CA, USA) and anti-Vinculin (ab73412, Abcam Co., CA, USA) were used as primary antibodies. The blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). Proteins were visualized with electrochemical luminescence detection system (GE Healthcare, CA) [15].

2.8. Luciferase assay

Luciferase activity in the cell lysates was determined using the Dual-Luciferase Reporter Assay Kit (Promega) as previously described [16]. HEK293 cells were cotransfected with the *NOTCH1* mutant plasmid containing luciferase reporter gene and the Renilla luciferase reporter plasmid, and tested 48 h after transfection. Cells were treated with *NOTCH1* inhibitor Brontictuzumab (OMP 52M51) immediately after transfection. Luciferase activity was normalized to the Renilla transfection control and to *NOTCH1* expression levels. The assays were repeated at least three times in duplicate.

2.9. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol plus kit (TaKaRa, Japan). First-strand cDNA synthesis was performed using Promega kit. Synthesized cDNA was used for qRT-PCR analysis using Lightcycler (Roche, Switzerland). All samples were amplified in duplicates. Relative changes in the amount of transcripts in each sample were determined with β-actin normalization of mRNA levels.

2.10. Patient-derived xenograft (PDX) models

PDX models of ACC of the trachea (ACCX2 (*NOTCH1* S2467* mutation), ACCX3 (*NOTCH1* S2276 fs mutation), and ACCX7 (*NOTCH1* WT)) were established under International Animal Care and Use Committee-approved protocols as described previously [17]. Briefly, fresh resected tumor tissues of ACC of the trachea were mechanically sectioned into 1–2 mm fragments and placed in RPMI supplemented with 10% FBS. One to four tumor fragments were then moved into the cutaneous incisions on the bilateral flank regions per nude mice (nu/nu). The tumor were allowed to grow to a maximum diameter of 1.5 cm, and were then surgically harvested. Three xenografts were created per patient tumor. Xenograft tumors were minced and passaged into new nude mice as described above. The identity of these PDX tumors was determined by short tandem repeat (STR) profiling that confirmed the match with the primary human tumors used to generate this PDX model (data not shown). The passage number for the xenograft models was as follows: ACCX2—passage 2; ACCX3—passage 3; ACCX7—passage 1. Nude mice of 6–8 weeks old were implanted with tumor fragments (1–2 mm) from established PDX ACC models. PDX tumors were allowed to grow to an average volume of approximately 200 mm^3 before mice were randomized into either treatment group (Notch1 inhibitor Brontictuzumab) or control group (Poly-ethylene glycol/Kolliphor® EL in PBS) (7–9 mice per group; ACCX2 and ACCX3, eight treated versus seven controls; ACCX7, nine treated versus seven controls). Brontictuzumab was intraperitoneally injected at a dose of 10 mg/kg every two weeks for two total doses. Tumor volume was calculated using the following formula: $(\text{short diameter})^2 \times (\text{long diameter})/2$. Tumor volume was assessed every three days for four weeks. Tumor volume and weight of the mice were monitored three times a week. Tumor growth inhibition were evaluated for each group using tumor volume before and after treatment. Statistical significance was calculated using two-way student *t*-test. On day 30, the mice were euthanized, and the xenograft tumors were removed. Tumor volume and weight of the xenograft tumors between treated and control group were