Next-Generation Sequencing in Salivary Gland Basal Cell Adenocarcinoma and Basal Cell Adenoma

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Abstract Basal cell adenoma and basal cell adenocarcinoma represent basaloid salivary gland neoplasms that show cyto-morphologic similarity but differ at the histologic level by their invasive qualities, as adenocarcinomas show invasion beyond their capsule, a finding not seen in the adenomas. Due to the low incidence of these tumors, the molecular mechanism underlying their pathogenesis is poorly understood. We sought to further delineate these neoplasms through mutation profiling by targeted next-generation sequencing (NGS). Twenty cases (basal cell adenocarcinoma = 10; basal cell adenoma = 10) were retrospectively selected from a previous analysis. NGS was performed using the Ion AmpliSeq^{TM} Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA). The data was analyzed using the Ion Torrent Suite Software (Life Technologies) followed by a laboratory-developed pipeline. One of eight cases of basal cell adenocarcinoma had a mutation, which was an activating mutation in *PIK3CA* (*c.3140A>G*, p.H1047R). No mutations were detected in the remaining basal cell adenocarcinomas. In the basal cell adenomas, the *CTNNT1* p.I35T mutation was identified in three of nine (3/9) cases. A missense mutation in the *ATM* gene (*c.2572T>C*, p.F858L) was seen in a basal cell adenoma with an allele frequency of 53 %, raising the possibility of a germline mutation. The overall findings suggest that although there is cytomorphologic similarity, differences exist between these two tumors at the histologic and genetic level. Although the numbers of cases are limited, the aberrations in genes affecting different signaling

pathways in the basal cell adenocarcinoma versus the basal cell adenomas suggest that basal cell adenocarcinoma likely arises de novo and not from basal cell adenoma.

Keywords Basal cell adenoma · Basal cell adenocarcinoma · Salivary gland neoplasms · Next generation sequencing (NGS)

Introduction

Basal cell adenoma and basal cell adenocarcinoma both represent uncommon salivary gland neoplasms with basaloid cell proliferation. Basal cell adenoma represents approximately 1–3 % of salivary gland neoplasms [1, 2]. Basal cell adenoma is a benign neoplasm with local recurrence in approximately 7 % of cases, with the majority of recurrences showing membranous architecture [3]. Basal cell adenocarcinoma is less common, representing approximately 1–2 % of salivary gland neoplasms and is characterized by an indolent clinical course with local recurrence in approximately 17–50 % of cases. It rarely has distant metastasis or even causes death [2–6]. There have been reports of basal cell adenocarcinoma arising from an underlying basal cell adenoma and some have proposed that basal cell adenocarcinoma represents malignant transformation of basal cell adenoma, however others consider that these two neoplasms are separate entities with different tumorigenesis pathways [7–9].

Several studies have compared the morphologic and immunohistochemical features of basal cell adenoma with basal cell adenocarcinoma to aid in distinguishing these neoplasms from each other [3, 6, 10, 11]. However, malignancy is typically established on the basis of

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identifying invasion into the surrounding benign tissues, including perineural invasion [3, 5]. Recent studies have focused on beta-catenin expression by immunohistochemistry for separation of the two with mixed results [11, 12]. Beta-catenin is a major player in one of the Wnt pathways. Wnt increases the stability of beta-catenin. This stabilization results in an increase of cytoplasmic beta-catenin and ultimately, increased nuclear beta-catenin which in turn stimulates transcriptional activity and increased expression of several genes, including the genes for cyclin D1 and *c-Myc*. In this capacity, beta-catenin functions as if it were an oncogene [13].

The aim of this study was to further study the molecular profiles of these rare neoplasms and to identify common oncogenic mutations in basal cell adenoma and basal cell adenocarcinoma by next generation sequencing.

Materials and Methods

Patient Selection

The patients were selected from a prior study of basal cell adenomas and adenocarcinomas [3]. Both neoplasm types exhibit a basaloid population of cells arranged in solid, trabecular or tubular patterns with one tumor showing a membranous pattern. The separation of basal cell adenocarcinoma from basal cell adenoma was defined as invasion of the surrounding gland in the former. All of the tumors studied were the original tumor (first occurrence) and no patients had an associated syndrome. The study was approved by the Institutional Review Board.

Extraction of Genomic DNA

Ten cases of basal cell adenocarcinoma and ten cases of basal cell adenoma of the major and minor salivary glands were identified and corresponding formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from the University of Iowa archives [3]. One H&E-stained slide and 10 unstained sections (6 microns in thickness) were cut. Areas of interest were circled on the H&E slide and corresponding areas from the unstained slides were manually microdissected using a razor blade. The paraffin flakes were placed in a 1.5 ml microcentrifuge tube, deparaffinized with 1200 μ L of xylene, vortexed, and centrifuged at 16,000g $\times$ 5 min. The tissue pellet was washed with 95 % ethanol twice before proceeding with the extraction. Genomic DNA was isolated from the microdissected FFPE sections using the QIAamp DNA FFPE Tissue kit (Qiagen, Valencia, CA).

Next Generation Sequencing (NGS)

NGS was performed using the Ion AmpliSeq^{TM} Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA) which contains 50 oncogenes and tumor suppressors (for a listing of the 50 genes targeted on this panel, see <https://tools.thermofisher.com/content/sfs/brochures/Ion-AmpliSeq-Cancer-Hotspot-Panel-Flyer.pdf>). The test was performed on Ion Torrent PGM according to manufacturer's instruction. Briefly, 10 ng of gDNA was used for preparation of the amplicon libraries using the Ion AmpliSeq^{TM}2.0 technology. The libraries were bar-coded, clonally amplified by emulsion PCR in vitro, and sequenced on an Ion 318 Chip. The data was analyzed using the Ion Torrent Suite Software followed by a laboratory-developed pipeline. The assay was validated for clinical use by our molecular pathology laboratory. The sensitivity of this test is 5 % which was obtained by serial dilution of mutation-positive DNA into mutation-negative patient DNA.

Immunohistochemistry

An immunochemical study was carried out for beta-catenin expression were carried out as follows. Four micron sections were cut from the FFPE tissue, deparaffinized and then brought through xylenes and alcohols to distilled water. Heat epitope-induced retrieval (Biocare Decloaking Chamber, Concord, CA) was carried out with antibody to beta-catenin (Beta-catenin (BD Biosciences, San Jose, CA) at 1:6000 dilution for 60 min at room temperature. Negative controls were performed with non-specific antibody. After exposure to antibody, the slides were washed in buffer and then Dako Mouse Envision System HRP (Dako) was applied for 30 min at room temperature. After washing with buffer, Dako DAB Plus (Dako) was applied followed by Dako DAB enhancer (Dako). After washing in buffer, the slides were counterstained with hematoxylin. Staining was assessed for nuclear expression and graded as 0, 1(weak), 2(moderate), and 3(strong) staining intensity.

Results

The results of NGS studies are shown in Table 1. Only the mutations detected with the Ion AmpliSeq Cancer Hotspot Panel v2 that were present are listed. All other genes on the panel were negative for mutations.

Mutations

Of the 10 basal cell adenocarcinomas, the sequencing testing failed in 2 cases due to suboptimal DNA quality. Of

Table 1 Clinicopathologic and NGS finding summary of basal cell adenocarcinomas and basal cell adenomas

Type	Morphology	Site	Gene mutation, nucleotide	Predicted effect	Followup status/time (months)/recurrence
BCAC	Solid	Sublingual gland	<i>PIK3CA</i> c.3140A>G, p.H1047R	Activating mutation	Alive/91 months/no recurrence
BCAC	Solid/tubular	Parotid gland	None		Alive/170 months/no recurrence
BCAC	Trabecular	Parotid gland	None		Alive/10 months/no recurrence
BCAC	Tubular	Parotid gland	None		Alive/34 months/no recurrence
BCAC	Solid	Parotid gland	None		Dead/34 months with recurrence
BCAC	Trabecular	Parotid gland	None		Alive/19 months/no recurrence
BCAC	Solid	Parotid gland	None		Alive/10 months/no recurrence
BCAC	Tubular	Parotid gland	None		Alive/15 months/no recurrence
BCA	Trabecular	Parotid gland	<i>CTNNB1</i> , c.104T>C, p.I35T	Affects degradation of beta-catenin	Alive/39 months/no recurrence
BCA	Solid/tubular	Parotid gland	<i>CTNNB1</i> , c.104T>C, p.I35T	Affects degradation of beta-catenin	Alive/12 months/no recurrence
BCA	Trabecular	Parotid gland	<i>CTNNB1</i> , c.104T>C, p.I35T	Affects degradation of beta-catenin	Alive/39 months/no recurrence
BCA	Membranous	Parotid gland	<i>ATM</i> , c.2572T>C, p.F858L	Inactivating mutation of tumor suppressor	Alive/55 months/no recurrence
BCA	Solid/tubular	Parotid gland	None		Alive/15 months/no recurrence
BCA	Trabecular	Parotid gland	None		Alive/79 months/no recurrence
BCA	Solid	Parotid gland	None		Alive/33 months/no recurrence
BCA	Trabecular	Buccal mucosa	None		Alive/10 months/no recurrence
BCA	Tubular/Sol	Parotid gland	None		Alive/66/no recurrence

BCAC basal cell adenocarcinoma, BCA basal cell adenoma

Fig. 1 Basal cell adenocarcinoma with invasion of cells into the surrounding periglandular tissue. This tumor exhibited a *PIK3CA* mutation

the 8 cases in which the testing was successful, an activating mutation in the *PIK3CA* gene was identified in one, c.3140A>G, which is predicted to cause an amino acid

change from histidine to arginine at position 1047, p.H1047R (Figs. 1, 2).

Of the 10 basal cell adenomas, the sequencing testing failed in one case. Of the 9 cases in which the testing was successful, mutations were identified in four of nine basal cell adenomas (44 %). Of these 4 cases, we found 3 cases with a substitution mutation of the *CTNNB1* gene, c.104T>C, which is predicted to cause a replacement of isoleucine to threonine at codon 35 (p.I35T) (Figs. 3, 4). One basal cell adenoma had a missense mutation in the *ATM* gene, c.2572T>C, which is predicted to result in a substitution of phenylalanine by leucine at amino acid position 858 (p.F858L) (Figs. 5, 6).

Immunohistochemistry

Beta-catenin staining was found in one of the basal cell adenocarcinomas in this series, but was not associated with a *CTNNB1* mutation. Beta-catenin staining was found in 5 of the basal cell adenomas. Of these, expression was found in all three associated with the *CTNNB1* mutated cases but also in two cases that had no mutation. All cases that stained had strong (3+) nuclear staining.

Fig. 2 Integrative genomic viewer of the sequencing of the tumor seen in Fig. 1. An activating mutation was found in the *PIK3CA* gene (c.3140A>G, p.H1047R) as indicated between the dotted lines

Discussion

Basal cell adenoma and basal cell adenocarcinoma represent relatively uncommon basaloid neoplasms of the salivary glands. Differentiating between these two neoplasms can be challenging due to extensive morphologic and immunohistochemical overlap between these neoplasms. Malignancy is typically established on the basis of identifying invasion into the surrounding benign tissues, including perineural invasion and capillary-lymphatic invasion [3, 5]. While it has been postulated by some that basal cell adenocarcinoma arises out of basal cell adenoma [7–9], the neoplasms with mutations in our series showed differing mutations, suggesting that basal cell adenoma and basal cell adenocarcinoma, while similar in cytologic

Fig. 3 Basal cell adenoma. This neoplasm exhibited a *CTNNB1* mutation, as did two other basal cell adenomas

appearance, are likely unrelated in direct molecular etiology.

One of the basal adenocarcinomas and four of the basal cell adenomas had mutations detected, with two cases of basal cell adenocarcinoma and one basal cell adenoma having failed NGS results due to suboptimal DNA quality. Of interest, the *PI3K* gene family was found to be implicated either directly or indirectly in a number of both basal cell adenocarcinomas and basal cell adenomas.

An activating mutation was found in the *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)* gene in a basal cell adenocarcinoma with a solid pattern. The *PI3K* family consists of a group of eight catalytic subunits that is split into three classes, I, II, and III. *PIK3CA* is a gene on chromosome 3q26 which encodes the p110 alpha subunit of class IA phosphatidylinositol 3-kinase. This subunit is critical for the control of protein synthesis, angiogenesis, and cellular proliferation through the PI3K/Akt pathway. *PIK3CA* is an oncogene and mutations in the gene have been found in many tumor types [13].

Activating mutations in *CTNNB1* were found in 3 of the basal cell adenomas. Of interest, this genetic mutation was seen in only the basal cell adenomas in our study. While our study sample is small, this unusual distribution of *CTNNB1* mutations is intriguing. The *CTNNB1* (catenin beta-1) gene is situated on chromosome 3p21 and codes for beta-catenin. Beta-catenin has roles in cell–cell attachment but also in cell proliferation. Gain of function mutations in the *CTNNB1* gene can inhibit the degradation of beta-catenin which then accumulates within the cell. The Wnt signaling pathway, which is involved in cell proliferation and differentiation, requires beta-catenin as a controlling protein. The Wnt signaling pathway is also reported to be

Fig. 4 Integrative genomic viewer of the sequencing of the tumor seen in Fig. 3. An activating mutation in the *CTNNB1* gene is present (*c.104T>C*, *p.I35T*) as indicated between the *dotted lines*

important in the biology of adenoid cystic carcinoma [14]. While our basal cell adenomas showed beta-catenin expression in 5 of 10 cases, Kawahara's study of 22 patients with basal cell adenoma, nuclear beta catenin staining was found in 21 of 22 cases [12]. However, in that study, the authors found *CTTNB1* mutations in only 11 of the 22 tumors tested. In contrast to our paper, Jung et al. [15] found that nearly all basal cell adenomas and basal cell adenocarcinomas express nuclear beta-catenin. In that study, none of the adenoid cystic carcinomas expressed beta-catenin. In a study of a mixture of salivary gland adenocarcinomas of varying types, Lill et al. [16] found that one of two basal cell adenocarcinomas expressed beta-catenin, although the authors considered both cytoplasmic as well as nuclear staining in their overall evaluation.

One of the basal cell adenomas had an inactivating mutation (p.F858L) in the ataxia-telangiectasia, mutated (*ATM*) gene, a member of the *PI3K* superfamily. This variant has been reported in hematopoietic and lymphoid neoplasm (<http://cancer.sanger.ac.uk/cosmic/mutation/overview?id=21826>) and a case of lung carcinoma [17]. The mutant allele frequency of 53 % also raised the possibility of a germline mutation. Of interest, this mutation occurred in a basal cell adenoma that had a membranous pattern, a pattern that is associated with a high incidence of recurrence [18]. The *ATM* gene encodes a protein that has an increasingly recognized role in responding to DNA double stranded breaks. Maintenance of genome stability is a required cellular activity as DNA damage that occurs must be repaired and/or the cell cycle arrested in order that abnormal DNA does not get replicated. Mechanisms of

Fig. 5 Basal cell adenoma, membranous pattern. This neoplasm had an inactivating mutation of the tumor suppressor gene, *ATM*

action of *ATM* involve phosphorylation of molecules that are critical for responding to DNA damage. *ATM* gene mutations have been best known to be associated with ataxia-telangiectasia, a disease characterized by neuromotor dysfunction, immunodeficiency, and propensity for development of malignancies. However, more recently, investigators have found that patients without ataxia-telangiectasia have tumors with *ATM* mutations, most of these neoplasms represented by carcinomas of pancreas and breast [19].

One gene that was not examined was the *CCND1* gene as it is not present on the commercially available panel we utilized. *CCND1* codes for cyclin D1, a regulator of cyclin-dependent kinases important in cell cycle regulation. Cyclin D1 has been reported to be expressed in a variety of salivary gland tumors and likely plays a role in tumorigenesis [15, 16, 20].

Although this is an admittedly small sample size, the data suggest that activating mutations in oncogenes may be more likely a mechanism for the development of basal cell adenocarcinoma, while the Wnt signaling pathway may play more of a role in the pathogenesis of basal cell adenoma. In addition, as there were no identical activating mutations in the adenocarcinomas versus the adenoma, this may give evidence that basal cell adenocarcinoma likely arises de novo and not from basal cell adenoma. The overall findings may also suggest that although there is cyto-morphologic similarity, differences exist between the two tumors at the observable microscopic and genetic level. It would be of interest to further define whether or not the molecular findings are related to the invasive capabilities of these two cyto-histologically similar tumors.

Fig. 6 Integrative genomic viewer of the sequencing of the tumor seen in Fig. 5. An inactivating mutation in the *ATM* gene was found (c.2572T>C, p.F858L) as indicated between the dotted lines

Further molecular studies to explore additional genetic abnormalities may improve classification of basal cell adenoma and basal cell adenocarcinoma as well as to explore additional potential targeted therapies for basal cell adenocarcinoma.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to report.

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