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ARTICLE

Frequent and differential mutations of the *CYLD* gene in basal cell salivary neoplasms: linkage to tumor development and progression

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Abstract

Basal cell salivary neoplasms display similar cyto-morphologic features and are classified into adenoma and adenocarcinoma based on the presence or absence of tumor invasion at diagnosis. These neoplasms also share considerable phenotypic resemblance and co-exist with certain dermal adnexal tumors harboring the *CYLD* gene mutations inferring common genetic association. We sequenced the *CYLD* gene in both basal cell adenomas and adenocarcinomas and correlated the findings with CYLD, NF-κB, and β-catenin expression levels and clinicopathologic factors. Twenty mutations were identified and comprised of 3 synonymous and 17 non-synonymous (missense) types involving the coding exons of the *CYLD* gene. Mutations in exons 9–11 were identified in both adenomas and adenocarcinomas, while mutations in exons 12–20, encoding the USP domain, were exclusively found in carcinomas. Although no significant correlation between *CYLD* mutations and expression levels of CYLD, NF-κB, and β-catenin or clinicopathologic parameters was found, basal cell adenocarcinomas with multiple mutations showed reduction in CYLD protein expression and pursued aggressive clinical behavior. Our study revealed high incidence and sequential *CYLD* mutations in both basal cell adenoma and adenocarcinoma supporting a single neoplastic continuum for their evolution and provides evidence for potential diagnostic and therapeutic utility.

Introduction

Basal cell salivary neoplasms encompass benign and malignant forms that display uniform basal cell composition in variable morphologic organizations. Given these overlapping features, they are classified into adenoma and adenocarcinoma based on the state of invasion at diagnosis [1–4]. These characteristics could lead to pre-surgical and/or post-surgical diagnostic and management challenges. We hypothesize that the morphologic equivalence and co-occurrence of adenoma and adenocarcinoma in some patients sustain the concept of a single neoplastic continuum for their development and progression [5–8]. Understanding the development of these tumors, however, is hampered by the paucity of molecular genetic information and the lack of surrogate models [9, 10]. Interestingly, basal cell salivary tumors share considerable morphologic and biological characteristics with certain dermal adnexal tumors with *CYLD* gene mutations [11–18]. In an earlier study, we reported frequent loss of heterozygosity (LOH) at chromosome 16q region and postulated the involvement of *CYLD* gene in concurrent salivary and dermal basal cell neoplasms [19]. This contention has gained further credence with the finding of *CYLD* alteration in a patient with dermal-like basal cell salivary tumors [20].

The *CYLD* gene, mapped to chromosome 16q 12–13 region, spans 56 kb and encodes for protein with

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deubiquiting enzyme function [14, 21, 22]. The CYLD protein consists of a cytoskeletal-associated proline-glycine, a conserved proline rich, and a catalytic of ubiquitin carboxyl-terminal hydrolase domains. The carboxyl-terminal (USP) domain specifically removes the K-63-linked polyubiquitin chain resulting in the deubiquitination of key cell survival regulatory proteins. Consequently, alteration of the CYLD can lead to sustained activation and deregulation of cell survival proteins associated with tumorigenesis [21, 23–28]. Although mutation is the most common *CYLD* alteration, evidence for its down-regulation and activation by genomic and non-genomic modifications have been reported [27–32]. Interestingly, no mutation has been reported to involve exons 1–5 of the N-terminal domain of the *CYLD* gene, suggesting a critical role for this region in the stability of the gene in both wild and mutated states [21, 22, 33]. Currently, the majority of *CYLD* mutations reported in epithelial malignancies have been confined to exons 9–20 and limited to frameshift and nonsense types, implicating disruption of the deubiquitination domain in tumorigenesis [28, 30, 33].

We posit that *CYLD* gene alterations underlie the development and progression of salivary basal cell tumors and that these alterations may have an important diagnostic and clinical implications. To test this hypothesis, *CYLD* gene was sequenced in a series of salivary basal cell adenomas and adenocarcinomas and correlated the findings with the expression of selected downstream effector and the clinicopathologic pathology factors.

Materials and methods

Forty five basal cell salivary neoplasms, 21 (12 adenomas and 9 adenocarcinomas) from Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa and 24 (2 adenomas and 22 adenocarcinomas) from MD Anderson Cancer Center, were assessed in this study. Four tumors from the two siblings were also tested [19]. Eight normal parotid gland specimens comprised the materials for the study. The study was approved by the Institutional Board of the respective institutions. Hematoxylin and eosin-stained sections of tumors from both institutions were independently re-reviewed at MD Anderson by a Head and Neck pathologist. Clinicopathologic characteristics were extracted from the patients' charts, pathologic reports, and review of the available slides.

DNA extraction

DNA extracted from both formalin-fixed paraffin-embedded and available fresh frozen tissues were processed using the Gentra Puregene Tissue Kit (QIAGEN) according to the manufacturer's protocol. DNA was quantified using thee Nanodrop spectrophotometer (Nanodrop). DNA integrity was analyzed using agarose gel electrophoresis.

CYLD gene sequencing

Mutation analysis for exons 9–20 of the *CYLD* gene was performed using Sanger sequencing. For PCR primers, see

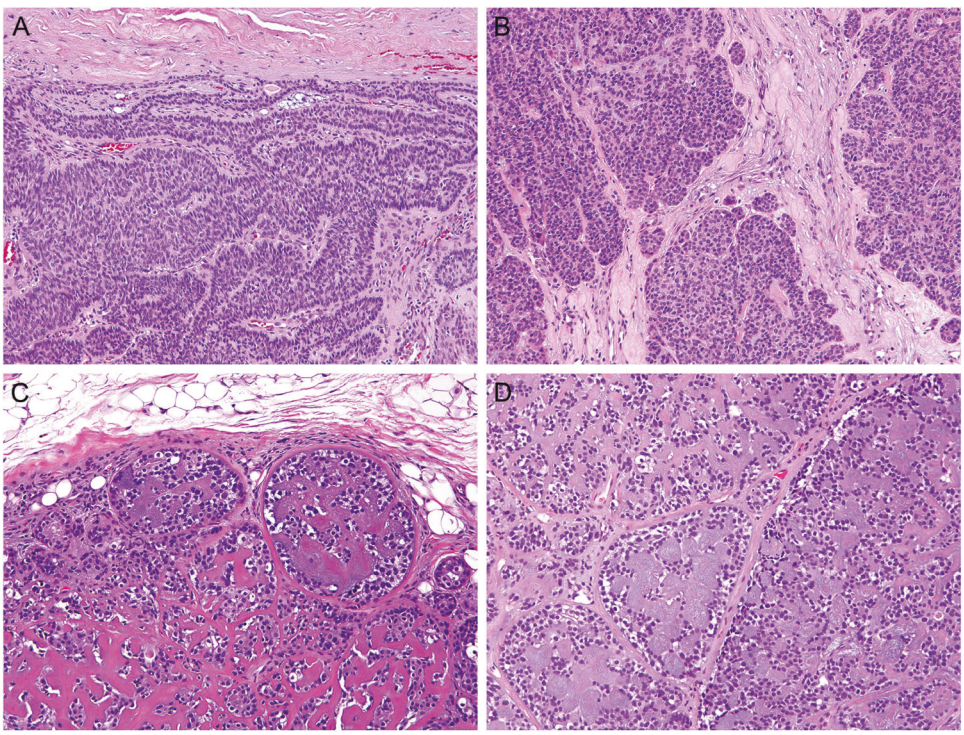

Fig. 1 Cellular resemblance of both basal cell adenoma and adenocarcinoma of salivary gland. **(A, B)** A photomicrograph of basal cell salivary adenoma, note the well-defined tumor and host tissue boundaries. **(C, D)** A contrasting micrograph of basal cell salivary adenocarcinoma with invasive tumor nests into surrounding soft tissue

Supplementary table S1. PCR fragments were purified by ExoSAP-IT (Affimetrix) and analyzed using Applied Biosystems 3730X1 DNA analyzer at GENEWIZ, Inc. (New Jersey, USA) and Sequencing and Microarray Facility at MD Anderson Cancer Center.

Mutation classification

All sequence data were manually checked by ABI viewing program. The following definitions were applied to mutation classification: (1) non-synonymous mutation: missense mutations that alter amino acid sequence, (2) synonymous mutations: mutations with no amino acid substitution. Common variants (single-nucleotide polymorphisms) were filtered (dbSNP databases, version 148) in mutational calling.

Immunohistochemistry (IHC)

IHC was performed on 4 µm thin sections of selected paraffin blocks using Autostainer Link 48 (Dako) according to the manufacturer's instructions. Anti-CYLD (Abcam, ab137524), anti-nuclear factor-κB (anti-NF-κB; Cell signaling, D14E12), and anti-β-Catenin (Dako, Clone: β-Catenin-1) antibodies were used with appropriate antigen retrieval and dilutions. CYLD, NF-κB, and β-Catenin protein expression levels were evaluated for nuclear and/or cytoplasmic staining based on the following criteria: 3+, strong staining in >10% tumor cells; 2+, moderate staining in >10%; 1+, weak staining in >10%; 0, negative or <10% staining. For categorical classification, expression was scored as high (strong and moderate) and low (weak and negative) staining.

Results

Histopathology

Both adenomas and adenocarcinomas were composed of uniform basal cells forming variable morphologic structures with and without dense eosinophilic basement membrane materials (Fig. 1). Both the inner ductal and outer base cells displayed uniform round-to-oval nuclei with nuclear-to-cytoplasmic ratio. Occasionally, slight cellular pleomorphism and rare mitosis were formed in few adenocarcinomas.

Table 1 presents the clinicopathologic parameters of patients with basal cell tumors. Histopathologically, tumors were classified into 11 adenomas and 34 adenocarcinomas. Patients included 22 males and 23 females who ranged in age from 18 to 85 years with a median of 53 years. All tumors were located in the parotid gland. Tumor sizes ranged from 1.5 to 6.0 cm (mean 2.0 cm).

Table 1 Clinicopathologic correlations and *CYLD* alteration in patients with salivary basal cell adenomas and adenocarcinomas

Parameter	BCAd ($n = 11$)	BCAC ($n = 34$)	p-Value[b]
Age			
<60	3	10	
≥60	8	24	=1.00
Sex			
Male	6	16	
Female	5	18	=0.74
Size[a]			
<4 cm	10	20	
≥4 cm	0	9	=0.079
PNI			
Yes	0	12	
No	11	22	=0.044
Metastasis			
Yes	0	11	
No	11	23	=0.042
CYLD mutation			
Yes	4	10	
No	7	24	=0.72
CYLD IHC			
High	11	31	
Low	0	2	=1.00
NF-κB IHC			
High	6	22	
Low	4	9	=0.70
β-Catenin IHC			
High	8	22	
Low	1	5	=0.68

Non-synonymous mutations (missense only in this study) were counted

IHC results of CYLD, NF-κB, and β-catenin were available for 11 BCAds and 33 BCACs, 10 BCAds and 31 BCACs, and 9 BCAds and 27 BCACs, respectively

BCAd basal cell adenoma, *BCAC* basal cell adenocarcinoma, *PNI* perineural invasion, *IHC* Immunohistochemistry

[a]Size is available for 10 BCAds and 29 BCACs

[b]p-Value was calculated by Fisher exact test

CYLD mutation

Figure 2 illustrates *CYLD* mutated locations in basal cell salivary neoplasms, and supplementary tables S2 and S3 present the results of mutation analysis in this study. A total of 23 *CYLD* nucleotide substitutions were identified in 23 of the 45 tumors (51%). Three alterations were single-nucleotide polymorphisms (dbSNP database) identical to those found in corresponding in eight matching normal tissue. The remaining 20 mutations comprised of 17

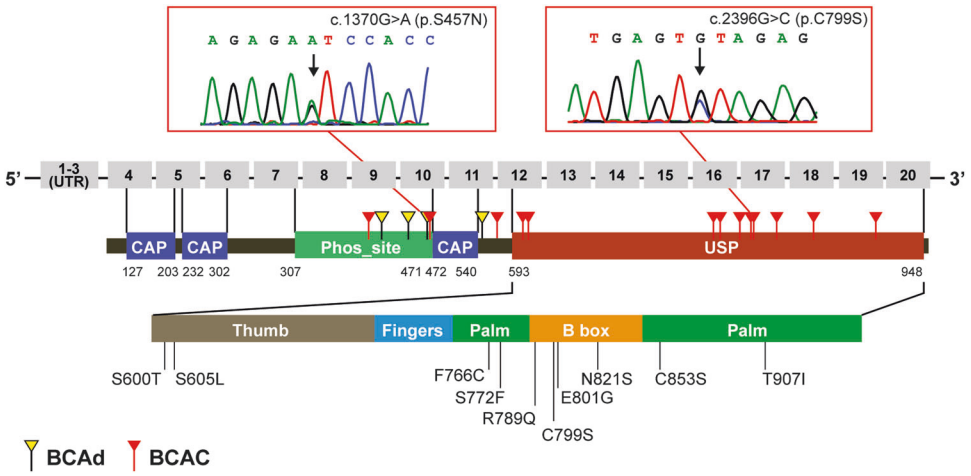

Fig. 2 *CYLD* mutation in salivary basal cell tumors. CYLD protein domain and mutated locations in basal cell adenoma (BCAd, yellow flag) and basal cell adenocarcinoma (BCAC, red flag). Non-synonymous mutations (missense only in this study) were analyzed. Two examples of missense mutations (p.S457N and p.C799S) are represented. Most of the mutations in basal cell adenocarcinoma occurred in USP domain. CAP cytoskeleton-associated protein glycine-rich (CAP-GLY) domain. Phos_site phosphorylation region of CYLD, USP ubiquitin (Ub)-specific protease domain. Adjacent table indicates the result that *CYLD* mutation occurred frequently in USP domain of basal cell adenocarcinoma ($p = 0.087$, Table 2)

Table 2 *CYLD* mutations in salivary basal cell adenomas and adenocarcinomas

Category	USP domain			Non-USP		
	Positive	Negative	*p*-Value	Positive	Negative	*p*-Value
BCAd	0	11	=0.087	4	7	=0.025
BCAC	9[a]	25		2[a]	32	

Non-synonymous mutations (missense only in this study) were analyzed

BCAd basal cell adenoma, *BCAC* basal cell adenocarcinoma

[a]Concurrent two mutations of USP in POBC03 and of non-USP in POBC10 were detected. Two mutations in each USP and non-USP were identified in MDBC10. See also supplementary table S2

nonsynonymous (missense) and 3 synonymous mutations (Supplementary tables S2 and S3). Both single-nucleotide polymorphisms and synonymous mutations were excluded from the analysis. Seventeen missense mutations were identified in 4 of the 11 adenomas (36%, Table 1) and 10 of the 34 adenocarcinomas (29%), where 3 adenocarcinomas had 2 concurrent *CYLD* mutations (Supplementary table S2). Only one mutation was identified in each of the four adenomas. The majority of *CYLD* mutations in adenocarcinomas occurred within the ubiquitin protease domain (exons 12–20, USP domain), while mutations in adenomas were located in exons 9–11 proximal to the USP domain (Fig. 2 and Table 2, $p = 0.087$). The most common sites of mutations within the USP domain were found in exon 18.

CYLD mutation in familial cases

Supplementary table S4 presents the *CYLD* gene mutation in previously published tumors from the two sisters [19]. Interestingly, exact same mutation from the two sisters were found in basal cell adenocarcinoma and were located in the USP domain [c.2839T>C (p.Y947H)]. Also, both trichoepithelioma and basal cell adenocarcinoma in sister #1 retained the exact single-nucleotide polymorphism in exon 18 (p.D804D, rs2066852).

CYLD alterations and expression of selected targets

Tables 3 and 4 present the correlation between *CYLD* gene alteration and expression of target genes. Both nuclear and/or cytoplasmic CYLD protein expression (Fig. 3) was noted in 42 tumors with and without *CYLD* mutations, and both basal cell adenomas and adenocarcinomas showed variably high CYLD protein expression. Two basal cell adenocarcinomas showed decreased expression of CYLD with this gene mutation. Although not significant, trend association between *CYLD* mutations and its protein expression was only observed in basal cell adenocarcinomas ($p = 0.085$, Table 3). A significant association between *CYLD* mutation status (multiple and/or USP domain) and its low

Table 3 Correlation of *CYLD* mutations and its target expression levels in salivary basal cell adenomas and adenocarcinomas

CYLD mutation	IHC								
	CYLD			NF-κB			β-Catenin		
	High	Low	*p*-Value	High	Low	*p*-Value	High	Low	*p*-Value
BCAd									
Yes	4	0		1	3		3	1	
No	7	0	=1.00	5	1	=0.19	5	0	=0.44
BCAC									
Yes	8	2		8	2		7	2	
No	23	0	=0.085	14	7	=0.68	15	3	=1.00

p-Value was calculated by Fisher exact test

BCAd basal cell adenoma, *BCAC* basal cell adenocarcinoma, *IHC* immunohistochemistry

Table 4 Correlation of *CYLD* mutation locations and its target expression levels in salivary basal cell tumors

CYLD mutation	IHC								
	CYLD			NF-κB			β-Catenin		
	High	Low	*p*-Value	High	Low	*p*-Value	High	Low	*p*-Value
Any site									
Yes	12	2		9	5		10	3	
No	30	0	=0.096	19	8	=0.73	20	3	=0.65
USP domain/multiple									
Yes	8	2		8	2		7	2	
No	34	0	=0.048	20	11	=0.46	23	4	=1.00

p-Value was calculated by Fisher exact test

IHC immunohistochemistry

CYLD expression ($p = 0.048$, Table 4) was noted. Both basal cell adenomas and adenocarcinomas expressed both NF-κB and β-Catenin mainly in the cytoplasm and occasionally in the nuclei (Fig. 3). No significant association between *CYLD* mutation and the expression levels of NF-κB and β-catenin in both basal cell adenomas and adenocarcinomas was found (Tables 3 and 4).

Clinicopathologic correlation

Tables 1 and 5 present the clinicopathologic characteristics of the entire and adnocarcinoma cohort, respectively. Compared with adenomas and adenocarcinomas, tumor size showed a trend association with malignant diagnosis ($p = 0.079$, Table 1). Only significant correlation between perineural invasion and metastasis and malignant diagnosis was found ($p = 0.044$ and 0.042, respectively, Table 1). No significant correlation between the expression levels of either NF-κB or β-Catenin and any of the clinicopathologic parameter was found (Tables 1 and 5). In patients with basal cell adenocarcinomas, a trend correlation between *CYLD* mutations and perineural invasion was found ($p = 0.061$, Table 5). Although only three patients with adenocarcinoma had multiple *CYLD* mutations, those showed poor outcome (Table 5).

Discussion

Basal cell salivary neoplasms encompass benign and malignant forms and share common cellular and architectural manifestations. These pathmorphologic resemblances along with their coincident occurrence in some patients infer sequential neoplastic evolution for their development [1–8]. We postulate that basal cell salivary neoplasms evolve as a single neoplastic continuum with an initial adenoma stage and a subsequent malignant state with temporal progression. Identifying driver genetic events underlying basal cell salivary tumor development therefore is fundamental to early diagnosis and predicting progressive disease. We report, for the first time, frequent and differential domain mutations of the *CYLD* gene in benign and malignant basal cell salivary gland tumors. The distribution of the mutations were confined to two distinct clusters: one

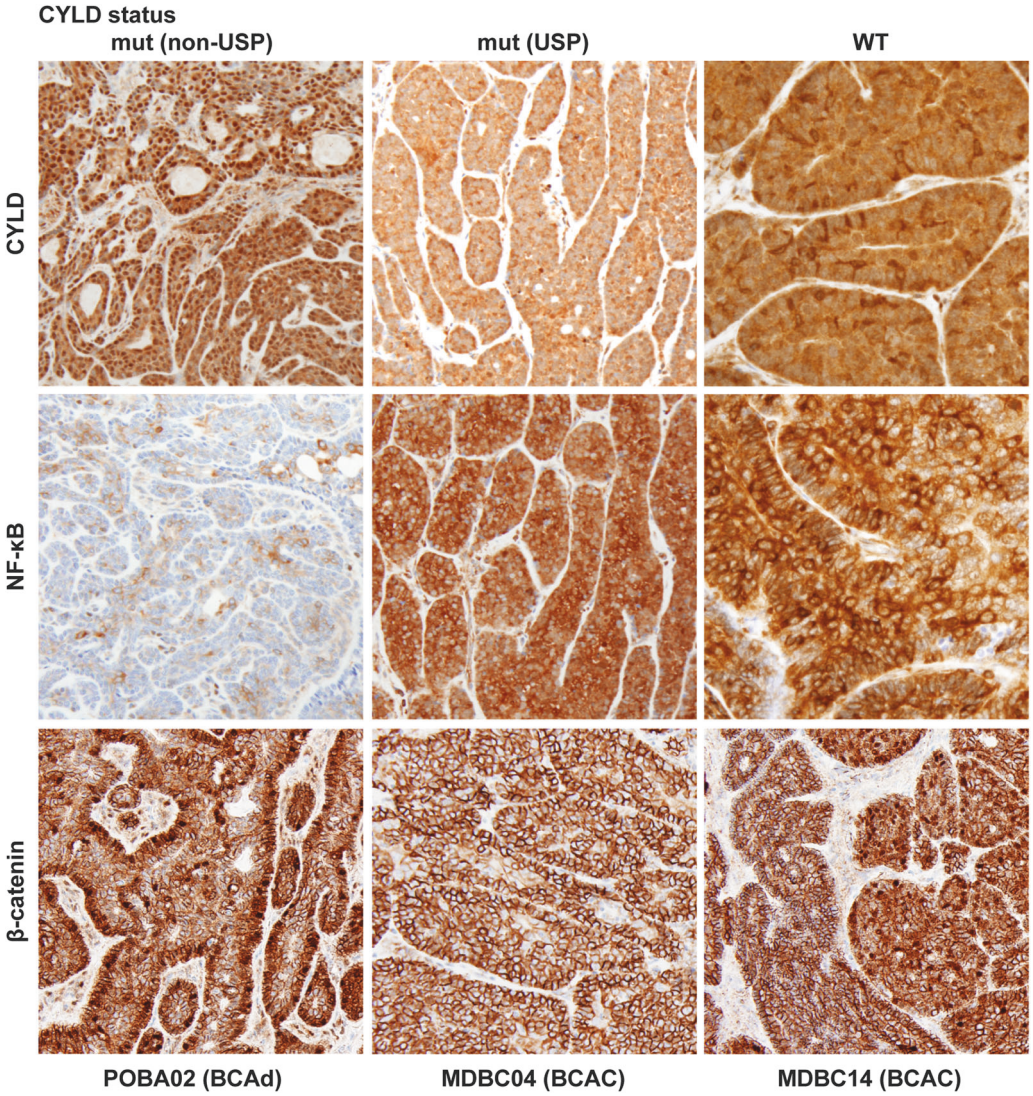

Fig. 3 Expression of CYLD, NF-κB, and β-catenin in salivary basal cell tumors. CYLD expression in an adenoma (POBA02) and adenocarcinoma (MDBC04 and MDBC14) with/without *CYLD* mutation. MDBC04 (*CYLD* mutation in USP) showed weak CYLD expression. Note that strong cytoplasmic expression with occasional nuclear staining of CYLD was found. NF-κB and β-catenin expression level status was not affected by CYLD expression status (see also Table 3). Nuclear positive of β-catenin expression was found in both adenoma and adenocarcinoma. BCAd basal cell adenoma, BCAC basal cell adenocarcinoma

at exons 9–11 that were found in both adenomas and some carcinomas, and the other at exons 12–20 that were exclusively found in carcinomas, suggesting a sequential acquisition with tumor progression [27, 30–32].

A notable hallmark in our study is the restricted *CYLD* gene mutations to the missense type in both benign and malignant forms of basal cell salivary neoplasms. This finding is at variance in its frequency and extent from those reported in dermal adnexal tumors, suggesting tumor context specificity [33]. Interestingly, however, an association between *CYLD* missense mutations and specific dermal adnexal phenotype has been described [33–35]. Together, these findings suggest that *CYLD* missense mutation may play a role in the genotype–phenotype determination of same forms of these tumors. Our results also advance a potential diagnostic and biological implications for the *CYLD* mutations where mutational screening by non-invasive presurgical sampling of primary or recurrent tumors could be a better management of certain patients [33]. In this cohort, although no significant correlation between *CYLD* mutations and patients' outcome was found, we observed that carcinomas with multiple mutations pursued more aggressive clinical course. Large cohort with long follow-up duration of patients with basal cell salivary neoplasms is needed to validate our findings.

Our results indicate the retention of one allele of the *CYLD* gene in basal cell salivary neoplasms as evidenced by the persistent CYLD expression in tumors with

Table 5 Clinicopathologic correlations and *CYLD* alteration in patients with salivary basal cell adenocarcinomas

Parameter	*CYLD* mutation		
	Positive	Negative	*p*-Value[b]
Age, years			
<60	4	6	
≥60	6	18	=0.43
Sex			
Male	6	10	
Female	4	14	=0.46
Size[a]			
<4 cm	7	13	
≥4 cm	2	7	=0.67
PNI			
Yes	1	11	
No	9	13	=0.061
Rec/Met[c]			
Yes	3	8	
No	7	16	=1.00
CYLD IHC			
High	8	23	
Low	2	0	=0.085
NF-κB IHC			
High	8	14	
Low	2	7	=0.68
β-Catenin IHC			
High	7	15	
Low	2	3	=1.00

Non-synonymous mutations (missense only in this study) were counted

IHC results of CYLD, NF-κB, and β-catenin were available for 33, 31, and 27 basal cell adenocarcinomas, respectively

PNI perineural invasion, *IHC* immunohistochemistry, *Rec/Met* Recurrence/Metastasis

[a]Size is available for 29 basal cell adenocarcinomas

[b]*p*-Value was calculated by the Fisher exact test

[c]Two of the three patients with adenocarcinoma harboring multiple *CYLD* mutations had metastasis tumor

mutations and argue against the requirement for inactivation of both alleles of *CYLD* as a tumor-suppressor gene in these tumors [12–14, 28]. Similar findings have been reported in familial dermal tumors where no LOH was found in tumors with mutation of the *CYLD* gene [12, 34–36]. Together, these findings suggest that haploinsufficiency and/or dominant-negative isoform effect [37–40] result in dysregulation of downstream effectors of cellular homeostasis and contribute to basal cell salivary and dermal adnexal tumorigenesis. Accordingly, total loss of the *CYLD* gene would be incompatible with basal cell tumor development and that either LOH or mutations of the *CYLD* underlie the development of majority of basal cell salivary tumors.

In our study, *CYLD* mutations were found in 50% of tumors, suggesting that epigenetic modifications, LOH at chromosome 16q13 region, and/or other alterations including different chromosomal sites could also be associated with basal cell salivary neoplasm development. Notably, alterations of chromosomes 1q22 and 9p21 regions have been reported in dermal adnexal tumors lacking chromosome 16q12-q13 abnormalities, supporting intertumoral and genetic variability [41, 42]. Similarly, alterations in chromosomes 1q and 9p regions have also been identified in multiple basal cell salivary tumors underscoring the kinship of these tumors [43–45]. These findings along with evidence for the involvement of *PTCH1* gene alterations in these tumors suggest that alternative events, exclusive of *CYLD* gene alterations, could play a role in basal cell salivary tumorigenesis [46]. Other non-genetic modifications leading to downregulation of the CYLD expression or through the Wnt and NOTCH signaling pathways could also contribute to tumorigenesis [47–52].

Importantly, our findings highlight a potential diagnostic and therapeutic implications for *CYLD* mutational analysis in patients with these basal cell salivary tumors. Diagnostically, mutational screening of *CYLD*, similar to dermal syndromes, could lead to early classification and stratification of patient for targeted therapy. Recent evidence for an association between *CYLD* somatic mutations and impairment of the tropomyosin kinase signaling provides an attractive therapeutic target for patients with dermal and basal cell salivary tumors [53, 54]. This is further sustained by new data demonstrating singnifcant inhibitory effect of Lestaurtinib on colony formation and tumor cell proliferation in vitro [53]. The availability of these small molecule agents may be employed in future trials of patients with advanced basal cell salivary adenocarcinomas. Further investigations of surrogate models of these tumors may provide a proof of principle for the application of these agents in patients with recurrent and/or metastatic basal cell salivary adenocarcinoma.

In conclusion, our findings support our hypothesis that basal cell salivary neoplasms represent a sequential neoplastic continuum and that mutation of the *CYLD* may allow for early classification and the development of future targeted therapeutic modalities. We also maintain that alterations affecting cell death and senescence pathways underlie the indolent nature of salivary basal cell tumorigenesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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