# Loss of CYLD might be associated with development of salivary gland tumors

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Abstract. Molecular studies of cylindromas, which arise from the eccrine or apocrine cells of the skin, have demonstrated frequent alterations at chromosome 16q12-13, recently found to house the cylindromatosis (CYLD) gene. CYLD, a tumor suppressor gene, has deubiquitinating enzyme activity and inhibits the activation of transcription factor NF- $\kappa$ B. Loss of the deubiquitinating activity of CYLD is correlated with tumorigenesis. It has been reported that the expression of CYLD is observed in various organs. We demonstrated previously that human salivary gland tumor (SGT) cell line, HSG spontaneously expresses CYLD and also found that adenoid cystic carcinoma (ACC) arising from the hard palate was distinctly positive for CYLD, immunohistochemically. However, it is unclear whether loss of CYLD is associated with development of SGTs. This study examined CYLD function in SGT cells and attempted to clarify whether CYLD is associated with development of SGTs. The expression of CYLD and NF-KB mRNAs in HSG cells was increased by TNF-α. Translocation of NF-κB protein from the cytoplasm to the nucleus in HSG cells peaked at 30 min after TNF- $\alpha$  stimulation, then decreased at 60 min, whereas that of CYLD protein increased gradually in a timedependent manner. Luciferase reporter assay indicated that TNF-α induced a 5-fold increase of NF-κB-dependent transcription at 4 h, which was further enhanced by knockdown of CYLD using RNA interference. Taken together, these data demonstrated that the levels of both CYLD and NF-KB mRNAs accumulated in HSG cells during 24 h after TNF-a stimulation, although the NF-kB activity in the cells was at least negatively regulated by CYLD. Immunohistochemical examinations revealed that there are several correlations between the expression of CYLD and NF- $\kappa$ B-related factors in 17 cases of ACC tissues. These findings suggest that loss of CYLD is associated with development of SGTs.

# Introduction

Molecular studies of familial and sporadic cylindromas, that arise from the eccrine or apocrine cells of the skin (1,2), have shown frequent alterations at chromosome 16q12-13, recently found to house the cylindromatosis (CYLD) gene (3-6). CYLD, a tumor suppressor gene, has deubiquitinating enzyme activity and inhibits the activation of transcription factor NF- $\kappa$ B (5,7-9), which has key roles in inflammation, immune responses, tumorigenesis and protection against apoptosis (10-12). In most cells, NF- $\kappa$ B is kept inactive in the cytoplasm as a heterodimeric complex composed of p50 and p65 (RelA) subunits bound to the inhibitory protein, inhibitor of KB  $(I\kappa B\alpha)$  (13-15). Insight into the signaling mechanisms that lead to IkBa phosphorylation have identified a high-molecularweight protein complex known collectively as the IkB kinase (IKK) signalosome and including IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ also known as NF-KB essential modulator (NEMO) (16,17). IKKα and IKKβ have been identified as catalytic subunits, whereas IKKy is a regulatory subunit (16,18). Generally, after stimulation by various reagents, IkBa is phosphorylated at serine residues 32 and 36 by IKK $\alpha$  and IKK $\beta$ , together with the scaffold protein NEMO/IKKy (7-9,19). Serine phosphorylation results in polyubiquitination of IkB and its subsequent degradation by the proteasome, allowing NF-kB to translocate to the nucleus and activate its target gene (11,19,20). At this point, CYLD binds to NEMO/IKKy and appears to regulate its activity through deubiquitination of TRAF2 (8,9). Therefore, loss of the deubiquitinating activity of CYLD is correlated with tumorigenesis. It has been reported that expression of CYLD is detectable in brain, testis, skeletal muscle, spleen, liver, heart, lung and leukocytes (5). We have also demonstrated previously (21) that the human salivary gland tumor cell line, HSG, spontaneously expresses CYLD mRNA and protein.

Adenoid cystic carcinoma (ACC) is well known as a typical malignant salivary gland tumor. Facial palsy caused by perineural invasion of ACCs is particularly frequent. ACC

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was previously referred to as cylindroma because of its remarkable histologic resemblance to dermal cylindroma. We further found that ACC arising from the hard palate was distinctly positive for CYLD and NF- $\kappa$ B-related factors, immunohistochemically (21). However, it is unclear whether loss of CYLD is associated with development of salivary gland tumors.

The present study was conducted to examine the function of CYLD in HSG cells. The expression and distribution of CYLD and NF- $\kappa$ B-related factors were also investigated in 17 cases of ACC, to elucidate whether CYLD is associated with development of salivary gland tumors.

#### Materials and methods

*Reagents*. Rabbit anti-human CYLD polyclonal antibody (PAb CYLD), mouse anti-human RelA monoclonal antibody (MAb RelA), rabbit anti-human I $\kappa$ B $\alpha$  polyclonal antibody (PAb I $\kappa$ B $\alpha$ ), mouse anti-human IKK $\alpha$  monoclonal antibody (MAb IKK $\alpha$ ) and goat anti-human IKK $\beta$  polyclonal antibody (PAb IKK $\beta$ ) were purchased from Santa Cruz Biotechnology (CA, USA).

*Cell culture*. The HSG cells established by Shirasuna *et al* (22) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ ml penicillin and 100  $\mu$ g/ml streptomycin, and grown to confluence in 25-cm<sup>2</sup> culture flasks at 37°C in a humidified 5% CO<sub>2</sub> incubator until use.

RNA extraction and RT-PCR. Total-RNA was extracted from the HSG cells using the acid-guanidinium-phenol-chloroform (AGPC) method reported previously (21). Random hexamerprimed single-stranded cDNA was synthesized with an RNA LA PCR<sup>™</sup> Kit (Takara Shuzo, Shiga, Japan) from 1.0 µg of total-RNA in a final volume of 20  $\mu$ l with 0.25 units of avian myeloblastosis virus reverse transcriptase at 42°C for 60 min. An aliquot  $(1.0 \ \mu l)$  of the reaction mixture was diluted with 10  $\mu$ l of PCR buffer containing 4 pmole each of 5' and 3' CYLD, NF-κB and β-actin primer sets. The forward and reverse sequences of the oligonucleotides were as follows: CYLD forward primer, 5'-TCG CTG GAC TGG AAC TGG AAG-3'; reverse primer, 5'-GGA TGA AGG TGG ACT TGA GTG TTG-3'; NF-κB forward primer, 5'-CAC TTA TGG ACA ACT ATG AGG TCT CTG G-3'; reverse primer, 5'-CTG TCT TGT GGA CAA CGC AGT GGA ATT TTA GG-3'; β-actin forward primer, 5'-GTG GGG CGC CCC AGG CAC CA-3'; reverse primer, 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'. The PCR reaction was then performed with 2.5 units of Takara LA Taq<sup>™</sup> (Takara). The samples were subjected to denaturation at 94°C for 2 min. This was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for CYLD mRNA and 65°C for NF-κB mRNA detections for 1 min each other, and extension at 72°C for 1 min. For the last cycle, had the extension period was 10 min. The amplified DNA was electrophoresed on a 2.0% agarose gel, stained with ethidium bromide and visualized on a UV illuminator, then photographed.

*Protein extraction*. To examine RelA translocation to the nucleus and the expression kinetics of CYLD, we used a

subcellular proteome extraction kit (S-PEK) (Calbiochem, Darmstadt, Germany) in accordance with the manufacturer's instructions to extract the cytoplasm, membrane and nucleus fractions of HSG cells. Cells were treated with 10 ng/ml TNF- $\alpha$  for various periods (0, 15, 30 and 60 min), pelleted (5x10<sup>6</sup> cells), washed twice, resuspended in 1 ml of ice-cold Extraction I containing 5  $\mu$ l of protease inhibitor mixture, and then incubated for 10 min at 4°C with gentle agitation. The suspension was centrifuged at 1000 x g at 4°C for 10 min, and the supernatant was used as the cytoplasm fraction; the pellet was resuspended in 1 ml of ice-cold Extraction II containing 5  $\mu$ l of protease inhibitor mixture and incubated for 30 min at 4°C. It was then centrifuged at 6000 x g at 4°C for 10 min, and the supernatant used as the membrane fraction; the pellet was resuspended in 500  $\mu$ l of ice-cold Extraction III containing 5  $\mu$ l of protease inhibitor mixture and 1.5 µl of Benzonase® and incubated for 10 min at 4°C with gentle agitation. It was then centrifuged at 7000 x g at 4°C for 10 min, and the supernatant was used as the nucleus fraction. Protein concentrations were measured by Bio-Rad protein assay. Each supernatant fraction was subjected to immunoblot analysis.

*Immunoblot analysis*. For the detection of ReIA and CYLD protein by gel electrophoresis,  $30-\mu$ g protein samples were mixed with an equal volume of SDS-PAGE sample buffer and boiled for 5 min. The samples were then loaded and separated on polyacrylamide gel of appropriate percentage, and the proteins were electroblotted onto nitrocellulose membranes. Immunoblot analysis was carried out with each antibody according to the method described previously (21).

Transfection of HSG cells with plasmids and luciferase reporter assay. HSG cells (1x10<sup>5</sup> cells/ml) were cultured for 12 h in 24-well culture plates containing RPMI-1640 supplemented with 10% FBS. pTKkB2luc, the thymidine kinase (TK) luciferase construct containing five copies of the kB motif from the CXCL10/IP-10 gene, has been described earlier (23). Cells were transiently transfected with pTKkB2luc and pRL-TK reference Renilla luciferase plasmid (Promega, Madison, WI, USA) by using FuGene transfection reagents (Roche, Nutley, NJ, USA), in accordance with the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with TNF- $\alpha$  for various periods (0, 1, 4, 8 and 24 h). Firefly and Renilla luciferase activities were assayed using reagents provided by Promega, in accordance with the instructions supplied. For standardization of transfection efficiency, the luciferase activity from pTKkB2luc was normalized to the Renilla luciferase activity. The pGL3 control luciferase plasmid was purchased from Promega.

*RNA-mediated interference*. Small interfering RNAs (siRNAs) specific for human CYLD and scrambled (control) were synthesized by Sigma-Aldrich, Inc. (Ishikari, Japan). The sense and antisense strand sequences of the oligonucleotides were as follows: CYLD siRNA sense, CAG AUU GAG CGC UGU AAC UCU; antisense, AGU UAC AGC GCU CAA UCU GAU; control siRNA sense, UUC GAC GUA AUC GGG UCU ACA; antisense, UAG ACC CGA UUA

CGU CGA AUU. FuGene 6 transfection reagent was mixed with 10  $\mu$ M CYLD or 10  $\mu$ M control siRNA (3:3.4  $\mu$ l) in serum-free medium, to a total volume of 100  $\mu$ l and incubated for 30 min at room temperature. For siRNA delivery, HSG cells (1x10<sup>5</sup> cells/ml) were rinsed with serumfree medium and transfected in 24-well plates with an NF- $\kappa$ B-dependent luciferase reporter plasmid and either a CYLD siRNA duplex or a control siRNA using FuGene 6 transfection reagents for 48 h at 37°C. Cells were treated with TNF- $\alpha$  for 4 h, and subjected to luciferase reporter assay and immunoblot analysis.

Primary tumor samples. Formalin-fixed, paraffin-embedded specimens were obtained by surgical biopsy from 17 patients with ACC treated at Meikai University Hospital, Japan. The pathological diagnosis of oral lesions was based on histological examination of hematoxylin and eosin-stained slides and made according to the WHO classification (24). The TNM stage was determined according to the TNM pathological classification of the UICC (International Union Against Cancer) (25). None of the patients had undergone preoperative chemotherapy or radiotherapy. The labeling index was defined as the percentage of tumor cells displaying immunoreactivity, and calculated by counting the number of CYLD and NF-KBrelated factor-positive tumor cells among 1,000 tumor cells in each section. Tissue sections with <5% reactive cells were defined as negative (-), and those with  $\geq 5\%$  positive reactive cells were defined as positive (+).

Immunostaining of adenoid cystic carcinomas. The sections were immersed in absolute methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature to block endogenous peroxidase activity. Immunohistochemistry was carried out with each antibody according to the method reported previously (21).

*Ethical considerations*. The study was approved by the Research Ethics Committee of Meikai University School of Dentistry, Saitama, Japan.

#### Results

Detection of CYLD and RelA mRNAs in HSG cells stimulated with TNF- $\alpha$ . To examine how expression of CYLD and RelA mRNA is regulated in HSG cells upon stimulation with TNF- $\alpha$ , semi-quantitative RT-PCR was carried out. This showed that the expression of both CYLD and RelA mRNA was increased in HSG cells stimulated with TNF- $\alpha$ , in comparison with non-stimulated HSG cells (Fig. 1).

Correlation between expression of CYLD and RelA protein in HSG cells. To examine how the expression of RelA and CYLD protein is regulated in HSG cells upon stimulation with TNF- $\alpha$ , immunoblot analysis was carried out. The immunoblot data were also evaluated by densitometric analysis. Translocation of RelA from the cytoplasm to the nucleus in HSG cells was reached a peak at 30 min, then decreased at 60 min (Fig. 2A and B, left). When the time course of RelA protein expression was analyzed up to 24 h, nuclear translocation of RelA was



Figure 1. Semi-quantitative RT-PCR. The mRNA expressions of both CYLD and RelA were increased in HSG cells stimulated with 10 ng/ml TNF- $\alpha$  for 24 h, in comparison with non-stimulated HSG cells. As an internal control,  $\beta$ -actin mRNA detection revealed that the total-RNA obtained was intact.

further decreased (data not shown). CYLD was localized mainly in the cell membrane, and its intensity in HSG cells after treatment with TNF- $\alpha$  increased gradually with time during 60 min (Fig. 2A and B, right). Thereafter, expression of CYLD protein decreased gradually up to 24 h (data not shown).

*NF*-κ*B* activation in HSG cells stimulated with TNF-α. To investigate the effect of TNF-α on NF-κB-dependent transcriptional activity in HSG cells, luciferase reporter assay was carried out. TNF-α caused strong induction of luciferase activity (Fig. 3). Maximum κB-dependent transcription was observed at 4 h, and this induced a 5-fold increase in luciferase activity compared with cells not exposed to TNF-α. This did not increase further with time. However, constitutive NF-κB activity was not observed in HSG cells. The increase in luciferase activity was completely dependent on the presence of κB sites, since the control plasmid lacking the κB elements did not respond to TNF-α.

CYLD is a negative regulator of NF-κB activation in HSG cells. To determine whether endogenous CYLD indeed functions as a negative regulator of NF-κB activation by TNF- $\alpha$  in HSG cells, we used a siRNA approach to reduce the expression of CYLD and determined the effects on the basal and TNF- $\alpha$ -induced activity of NF- $\kappa$ B. As expected, the CYLD protein was markedly reduced by CYLD siRNA (Fig. 4A). We then assessed the effect of CYLD siRNA on NF- $\kappa$ B-dependent transcriptional activity by TNF- $\alpha$  for 4 h. As shown in Fig. 4B, CYLD knockdown by CYLD siRNA greatly enhanced NF- $\kappa$ B activation by TNF- $\alpha$  in HSG cells, in comparison with transfection using CYLD scrambled siRNA. These data suggested that CYLD functions as a negative regulator of NF- $\kappa$ B activation induced by TNF- $\alpha$  in HSG cells.

Immunohistochemical detection of CYLD and NF- $\kappa$ B-related factors and clinicopathological variables in ACC tissues. The correlations between expression of CYLD and NF- $\kappa$ Brelated factors and clinicopathological variables in ACC tissues



Figure 2. Immunoblot analysis. (A) Measurable translocation of ReIA from the cytoplasm to the nucleus in HSG cells was clearly observed at 30 min, then decreased at 60 min. CYLD was mainly localized in the cell membrane and the intensity in HSG cells after treatment with 10 ng/ml TNF- $\alpha$  gradually increased with time during 60 min. (B) The data were also evaluated by densitometric analysis. Filters were scanned and computer-generated images were analyzed with the National Institutes of Health IMAGE program to obtain densitometric values. For each series of samples (cytoplasm, cell membrane and nucleus), the relative density of each image was calculated and expressed as a percentage of the value (arbitrarily set at 100) indicated by an asterisk.



Figure 3. Luciferase reporter assay. Maximal  $\kappa$ B-dependent transcription was observed with 10 ng/ml TNF- $\alpha$  at 4 h. This induced a 5-fold increase in luciferase activity compared with cells not exposed to TNF- $\alpha$ , which showed no further increase with time. Constitutive NF- $\kappa$ B activity was not observed in HSG cells. The increase in luciferase activity was completely dependent on the presence of  $\kappa$ B sites, since the control plasmid lacking the  $\kappa$ B elements showed no response to TNF- $\alpha$ . The relative luciferase activities are shown as -fold induction compared with the activity of untreated control samples at 24 h. Each column and bar represents the mean  $\pm$  SEM of three independent experiments.

are summarized in Table I. Immunohistochemistry was carried out in a total of 17 cases of ACC at various stages. Histologically, all the cases of ACC analyzed in this study were cribriform. Immunohistochemistry showed that CYLD and NF-kB-related factors were present sporadically and/or in aggregates in the cribriform and duct-like structure of ACC tissues. Positive reactivity with PAb CYLD was clearly observed on the membrane and in the cytoplasm of cancer cells in 10 of 17 cases (58.8%) of ACC (Fig. 5A), whereas positive reactivity with MAb RelA was observed in the cytoplasm in 12 cases (70.6%) (Fig. 5B). Positive reactivity with PAb I $\kappa$ B $\alpha$  was observed in the nucleus in 13 cases (76.5%) (Fig. 5C). There was an inverse relationship between the expression of CYLD and that of RelA. Conversely, there was a positive relationship between RelA and IkBa. Positive reactivity with MAb IKKa was strongly observed in the cytoplasm of cancer cells in 14 cases (82.4%) (Fig. 5D), and positive reactivity with PAb IKKB was weakly observed in the cytoplasm in 4 cases (23.5%) (Fig. 5E). These results indicated that IKKa is expressed more dominantly than IKKB in ACC tissues. However, no significant association was found between expression of CYLD or NF-KB-related factor and clinicopathological variables (age, gender, location, TNM and stage).



Figure 4. CYLD siRNA-mediated interference. (A) Endogenous CYLD protein was markedly reduced by CYLD siRNA. (B) CYLD siRNA greatly enhanced activation of NF- $\kappa$ B by TNF- $\alpha$  in HSG cells, in comparison with CYLD scrambled siRNA transfection. The relative luciferase activities are shown as -fold induction compared with the activity of untreated samples. Each column and bar represents the mean  $\pm$  SEM of three independent experiments.

## Discussion

In the present study, we investigated how the expression of CYLD and NF-KB mRNA is regulated in salivary gland tumor cells upon stimulation with TNF- $\alpha$ . It was found that the levels of CYLD and NF-KB mRNAs expression were clearly increased in HSG cells during 24 h after treatment with TNF- $\alpha$ . We also found that translocation of NF- $\kappa$ B from the cytoplasm to the nucleus in HSG cells peaked as early as 30 min after treatment with TNF- $\alpha$ , then decreased at 60 min, whereas CYLD protein on the HSG cell membrane gradually increased with time during 60 min. Furthermore, luciferase reporter assay indicated that TNF- $\alpha$  induced a 5-fold increase of NF- $\kappa$ B at 4 h compared with cells not exposed to TNF- $\alpha$ , and that thereafter the NF-KB activity decreased gradually, although constitutive NF-kB activity was not observed in HSG cells. Taken together, these data suggest that the levels of both CYLD and NF-KB mRNAs accumulated in HSG cells during 24 h after TNF- $\alpha$  stimulation, although the NF- $\kappa B$ activity in the cells was at least negatively regulated by CYLD. To clarify this possibility, CYLD knockdown analysis was carried out using siRNA, and this showed that NF-KB activation was greatly enhanced at 4 h after TNF-a stimulation in HSG cells. These results were consistent with a previous report (9), and suggested that although CYLD negatively regulates NF- $\kappa$ B activation induced by TNF- $\alpha$  in HSG cells, it has no effect on constitutive NF-KB activity. Constitutive NF-KB activity is present in many tumor cells and is associated with the induction of genes involved in cell survival and also in tumor progression and metastasis (11,26-28). However, more recent studies have also indicated that inhibition of NF-kB activity leads to cell proliferation and development of

Table I. Correlation between the expression of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , CYLD, IKK $\alpha$ , IKK $\beta$  and clinicopathological variables in adenoid cystic carcinomas.

No.	Age	Gender	Location	TNM	Stage	CYLD	NF-κB	ΙκΒα	ΙΚΚα	IKKß
1	58	F	Oral floor	T1N0M0	Ι	+	_	+	+	
2	62	F	Sinus	T2N1M0	III	+	-	-	+	-
3	50	F	Submandibular gland	T2N0M0	Π	+	-	-	+	-
4	31	F	Submandibular gland	T3N1M0	IVA	+	-	-	+	-
5	73	F	Oral floor	T2N0M0	Π	+	-	-	+	-
6	64	М	Oral floor	T2N0M0	Π	+	++	+++	+++	
7	58	F	Oral floor	T3N0M0	III	+	++	+	++	-
8	44	F	Submandibular gland	T3N0M0	III	+	++	+++	+	+
9	57	М	Hard palate	T1N0M0	Ι	+	++	+	++	+
10	46	М	Oral floor	T2N0M0	Π	++	+	+	+	-
11	59	F	Nasal cavity	T2N0M0	Π	-	++	++	++	-
12	27	F	Hard palate	T2N1M0	III	-	+	+	+	+
13	66	F	Oral floor	T4N3M0	IVB	-	+	+	-	-
14	70	F	Oral floor	T2N0M0	Π	-	+	+	-	-
15	58	F	Hard palate	T4N1M0	IVA	-	+	+	-	-
16	52	М	Submandibular gland	T1N0M0	Ι	-	++	+++	+	+
17	81	М	Buccal mucosa	T1N0M0	Ι	-	+	++	+	-



Figure 5. Immunohistochemistry. Immunoreactivity of CYLD and NF- $\kappa$ B-related factors were found sporadically and/or in aggregates in the cribriform and duct-like structure of ACC tissue. (A) A positive reaction with PAb CYLD was clearly observed in the membrane and cytoplasm of cancer cells (x66). (B) A positive reaction with MAb RelA was observed in the cytoplasm of cancer cells (x66). (C) A positive reaction with PAb I $\kappa$ B was observed in the nucleus of cancer cells (x66). (D) A strongly positive reaction with MAb IKK $\alpha$  was observed in the cytoplasm of cancer cells (x66). (E) A weakly positive reaction with PAb IKK $\beta$  was observed in the cytoplasm of cancer cells (x66).

squamous cell carcinoma (29). Furthermore, some types of tumor cells such as colon cancer cells show a decreased level of NF- $\kappa$ B activity (30). These lines of study, together with the results presented herein, suggest that down-regulation of NF- $\kappa$ B activity might be involved in tumorigenesis in certain types of cancer cells via increased cell proliferative activity.

On the other hand, although ACCs are not so aggressive biologically, they can give rise to metastases many years after excision of the primary tumor. Furthermore, the frequency of CYLD expression in ACC is largely unknown. In this study, therefore, the frequency of CYLD expression, together with expression of NF-kB-related factors, was investigated in 17 cases of ACC. No correlations were detected between clinicopathological variables and expression of CYLD or NF-kB-related factors. However, expression of CYLD was negatively correlated with that of RelA, i.e., RelA tended to be absent in cases expressing CYLD. These results imply that NF- $\kappa$ B activation is inhibited in cases with normal CYLD function, whereas inversely it tends to be overexpressed in cases where CYLD is not functional. Hence, these findings suggest that CYLD may be dysfunctional in ACC cases where CYLD is not detectable, or that NF- $\kappa$ B is activated despite the expression of CYLD. Twelve of 17 ACC cases (70.6%) were suspected to have CYLD dysfunction, thus indicating that CYLD might be a useful marker of tumor development and prognosis in

ACC. This observation also suggests that CYLD mutation is significantly associated with ACC tumorigenesis, and that there is a substantial need for analysis of CYLD gene alterations in ACC. Furthermore, there was a positive correlation between the expression of RelA and that of IkBa, i.e., RelApositive cases were mostly positive for IkBa. Whereas RelA that was efficiently expressed in 12 of the 17 ACC cases (70.6%) was observed in the cytoplasm of most tumor cells, IkB $\alpha$  was detected in the nucleus. Although the IkB proteins were first identified as cytoplasmic inhibitors of NF-KB proteins, it is now clear that IkB proteins also act in the nucleus (31). In addition, the finding that IKK $\alpha$  expression was stronger than IKK $\beta$  expression suggests that IKK $\alpha$  may have a dominant role in comparison to IKKB in ACC tissues, although targeted gene disruptions have demonstrated that IKK $\beta$  (but not IKK $\alpha$ ) is largely responsible for cytokineinduced I $\kappa$ B degradation, the NF- $\kappa$ B activation pathway and suppression of apoptosis (32-34). In light of the present findings, these phenomena are quite difficult to understand, because NF-KB is generally activated by IKBa phosphorylation. Conversely, down-regulation of IkBa phosphorylation prevents NF-KB translocation to the nucleus, and the expression of NF-κB-responsive genes is thereby arrested. As a consequence, these phenomena may promote ACC cell apoptosis. However, ACC never regresses as a result of autonomic apoptosis, but instead progresses slowly. This

implies that ACC is not so aggressive, although it does have a potential for high malignancy. Although we cannot account for these results, it has been demonstrated that I $\kappa$ B $\alpha$  regenerates the cytoplasmic pool of NF- $\kappa$ B in preparation for subsequent reactivation (31). It has also been suggested that although IKK $\alpha$  is dispensable for I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation, it may be required in the PI3K/AKT pathway that leads to the phosphorylation of I $\kappa$ B $\alpha$  and activation of NF- $\kappa$ B (34). From these combined data, and the fact that expression of IKK $\beta$  in ACC tissues was hardly detectable, it is speculated that an unknown event in the progression mechanisms of ACC exists. In this regard, determining the signaling pathway occurring in these events might be the key to elucidating the mechanism of development of adenoid cystic carcinomas.

Based on these *in vitro* and *in vivo* observations, one can hypothesize that loss of CYLD function leads to NF- $\kappa$ B activation and subsequently anti-apoptosis, and that as a consequence, it might be associated with tumorigenesis, including growth, development and perineural invasion in human salivary gland tumors, such as ACC.

Further investigation of the role of CYLD, including its interaction with NF- $\kappa$ B-related factors and analysis of CYLD gene alterations in salivary gland tumor cells, will be required to establish a strategy for CYLD-based therapy of salivary gland tumors.

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