PLAG1 and *CYLD* do not play a role in the tumorigenesis of adenoid cystic carcinoma

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Abstract. The pleiomorphic adenoma gene 1 (*PLAG1*) gene is activated in a subset of pleomorphic adenomas of the salivary gland by gene fusion. Germ-line mutation in cylindromatosis (*CYLD*), a tumor suppressor gene, causes familial cylindromatosis and Brook-Spiegler syndrome. In the present study, aberrations in *PLAG1* and *CYLD* were investigated in adenoid cystic carcinoma (ACC) of the salivary gland. Reverse-transcription PCR and PCR direct sequencing were performed to detect gene fusion of *PLAG1* and mutation of *CYLD* in 34 ACC tissues. No *PLAG1* fusion was detected in ACC. However, silent mutation of *CYLD* was detected in 2 cases of ACC, but no missense mutation was detected in ACC. These results suggest that *PLAG1* and *CYLD* do not play a role in ACC tumorigenesis.

Introduction

Adenoid cystic carcinoma (ACC), a relatively rare tumor occurring mainly in the salivary glands, is a slow growing but highly malignant tumor. In recent years, cancer treatment has shifted to molecular-targeted therapy based on molecular aberrations in specific neoplasms. The molecular pathology of ACC, however, has not been fully elucidated.

Pleiomorphic adenoma gene 1 (*PLAG1*) and cylindromatosis (*CYLD*) are genes known to affect tumorigenesis. *PLAG1* is commonly rearranged in a subset of pleomorphic adenoma (PA) of the salivary gland by chromosomal aberrations, resulting in gene fusion. Several fusion partners of *PLAG1*, including *CTNNB1*, *CHCHD7*, *LLIR* and *LIFR*, have been identified (1-7). PLAG1 protein is a zinc finger protein that functions as a DNA-binding transcription factor. Deregulated transcription of various genes by abnormally expressed PLAG1 is hypothesized to play a major role in the development of PA. PA is the most common neoplasm of the salivary gland and shares specific morphological characteristics with ACC. ACC and PA tumors are composed of epithelial and myoepithelial cells. Ultrastructural analysis indicates that these tumors have a similar histogenetic basis (8). However, the role of *PLAG1* in the development of ACC remains unknown. Matsuyama *et al* (9) analyzed two cases of ACC and identified no fusion genes involving *PLAG1*.

CYLD is a tumor suppressor gene, the germ-line mutation of which causes familial cylindromatosis and Brook-Spiegler syndrome (10). The gene encodes a cytoplasmic protein that functions as a deubiquitinating enzyme. CYLD protein plays a role in cell proliferation and survival by negatively regulating nuclear factor-κB (11). There are morphological similarities between cutaneous cylindroma and ACC, and ACC was previously considered to be a cylindroma (12).

The present study was designed to determine the role of the *CYLD* gene in ACC of the salivary gland.

Materials and methods

Materials. A total of 34 paraffin-embedded blocks of ACC of the major and minor salivary glands were retrieved from the archival specimens maintained at the Pathology Center of Oita University Hospital (Oita, Japan). The study was approved by the ethic committee of Oita University, Faculty of Medicine.

Reverse-transcription (RT)-PCR. RT-PCR analysis for the detection of *PLAG1* gene fusion was performed using the method described by Matsuyama *et al* (9) with minor modifications. RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue using the Qiagen RNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, 10-μm FFPE tumor sections of each sample were digested with proteinase K in lysis buffer. Total RNA adsorbed to the column provided in the kit was collected in the elution buffer. The extracted total RNA was reverse transcribed to cDNA using the First-Strand cDNA Synthesis kit (GE Healthcare, Tokyo, Japan).

In the present study, *PLAG1*-associated fusion transcripts with catenin β 1 (*CTNNB1*), coiled-coil-helix-coiled-coil-helix domain containing 7 (*CHCHD7*), leukemia inhibitory factor receptor α (*LIFR*) and transcription elongation factor A (SII), 1

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Table I. Primers for RT-PCR.

Primer designation	Sequence (5'-3')	Size of PCR product (bp)
Reverse		
PLAG1-exon 2R	gaccgtcacagaatgaagca	
PLAG1-exon 3R	gccatgcccattgactcttc	
Forward		
CHCHD7-exon 1F	gtgagccattgacgtgtttg	124 (with exon 2R), 123 and 228 (with exon 3R)
CTNNB1-exon 1F	gaggaaggtctgaggagcag	159 (with exon 2R), 158 and 263 (with exon 3R)
LIFR-exon 1F	agctcagaaagggagcctct	105 (with exon 2R), 104 and 209 (with exon 3R)
TCEA1-exon 1F	gctttgccaagaagatggac	106 (with exon 2R), 105 and 210 (with exon 3R)

PLAG1, pleiomorphic adenoma gene 1; *CHCHD7*, coiled-coil-helix-coiled-coil-helix domain containing 7; *CTNNB1*, catenin β 1; *LIFR*, leukemia inhibitory factor receptor α; *TCEA1*, transcription elongation factor A (SII), 1.

Table II. Primers used in PCR.

Target	Forward	Reverse	Size of PCR product (bp)
Exon 4-1	tcttttgcggttttatgacaa	cggtactttaaggagcttttgtg	199
Exon 4-2	tcaagaatgcagcgttacaga	agaactgcatgaggttgctct	171
Exon 4-3	gtggggcattcaaggattc	aggctgaacctctcctcaca	173
Exon 4-4	gcaacctcatgcagttctctt	tttcttccccagatctcagc	194
Exon 4-5	aatagacgtgggctgtcctg	cagacacacatgaacacaaacaa	187
Exon 5-1	ccccttttcctatggatcgt	ctttccaatgcagtgtcatca	198
Exon 5-2	agattgtggcgtgtttgttg	tcctggcaaaacatcacaga	199
Exon 5-3	tcgaacttcctcctttggaa	gatatttaatccaaaattttcttacca	159
Exon 6-1	tttggaggattctttatggaaaa	aacacacgcaaaactacaaagc	151
Exon 6-2	gggatggaagatttgatgga	aaccaaacaccacctgttcc	188
Exon 7	ctcaaatccactgtgggtga	accttaaagcccagcaatga	190
Exon 8	tttctcttctataagaatttgccttt	ggcattatgcaaattactaaaggtt	198
Exon 9-1	tttttaaatgaaacttttcttgttcc	tggattgtggttgtgagtcaa	118
Exon 9-2	ggatctacctcagaccctgga	tctgatgagttagaaagaaaggatca	173
Exon 10-1	gagtcaatatccttgaatacatttctg	attgggcatcttggtgagac	194
Exon 10-2	accgttcttcaccaccactc	caagggtggactctcttgga	194
Exon 10-3	attggccacagtccactttc	attcagtcctggtggctgac	198
Exon 10-4	cctgggaactcacatggtct	gcgaaatctgcacaaaacct	191
Exon 11-1	ggcacggtataatgcatattga	gctgcaatgatgcaaaccta	168
Exon 11-2	gcgctgtttgtgaaactgaa	aaaacactgtcaccatcacctaa	186
Exon 12-1	ttttgcatcaaaatacaaaaacatt	ctccaagccttctttttcca	184
Exon 12-2	ttttcagcatttggaggcta	cctgcctcatggcactatct	197
Exon 13	gaaaattatccttttttcttttgcag	aggcaaaatagcaatttgttttc	178
Exon 14-1	tccagcctgagtgatagagtga	gatgcagcctccacctttt	195
Exon 14-2	tgtgtgccacaaaaattatgaa	cccccaactacacagacaca	174
Exon 15	tgatttaaaaattttgcctgtga	catgtctgttgaataatggcagt	194
Exon 16-1	ttaacattttgatttaagcatttga	cctctgcaaatttcaggttactg	199
Exon 16-2	ttcccacaattcagcagttg	aagactcccacagactttcaca	112
Exon 17-1	tgttttgtttgacagccatga	tctgttatatttaattccagagaagga	187
Exon 17-2	attcagatgcctcgatttgg	tgccttgggaaatactgtgtc	199
Exon 18	cccttccccttctcacattt	tccattaagtgaagggaagctc	166
Exon 19-1	ttgaactcctgacctcgtga	gcagagaacagcaaataactcca	195
Exon 19-2	cccaaagacttacccgactg	gcagaagaaaggcgttttca	190
Exon 20-1	tcactggcaaaagggtttaga	gcatcacaaagcagtcttcg	200
Exon 20-2	tctggaagacctgcattcct	acagaactgccagctcgaat	191

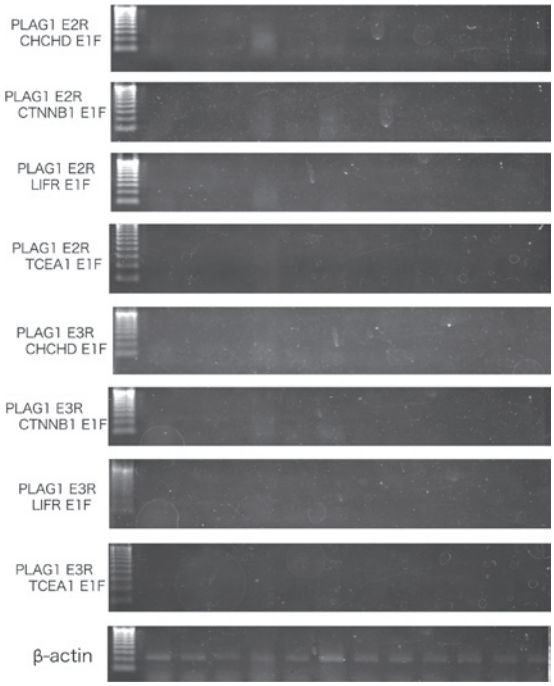

Figure 1. Representative results of RT-PCR. Eight primer pairs were tested for the presence of fusion transcripts involving *PLAG1*. No product was identified in each primer pair. In the bottom panel, note the products for β-actin, indicative of successful RNA extraction and reverse transcription. RT-PCR, reverse-transcription PCR; *PLAG1*, pleiomorphic adenoma gene 1; *CHCHD*, coiled-coil-helix-coiled-coil-helix domain; *CTNNB1*, catenin β 1; *LIFR*, leukemia inhibitory factor receptor α; *TCEA1*, transcription elongation factor A (SII), 1.

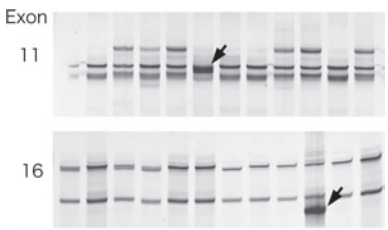

Figure 2. Results of SSCP analysis for exons 11 and 16 of *CYLD*. The aberrant band is indicated by arrows. These products were subjected to DNA sequencing. SSCP, single strand conformational polymorphism; *CYLD*, cylindromatosis.

(*TCEA1*) were analyzed. The sequence data of the primers is presented in Table I. The primer sequences were those reported by Matsuyama *et al* (9). PCR was performed using 2.5 units Taq DNA polymerase (AmpliTaq Gold; Perkin Elmer, Norwalk, CT, USA), 1.5 mmol/l $MgCl_2$, PCR buffer (Perkin Elmer), 200 μmol/l each DNP (Perkin Elmer) and 0.2 μmol forward and reverse primers. The 35-cycle PCR amplification consisted of 35 cycles of denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec and elongation at 72˚C for 30 sec, followed by a final extension at 72˚C for 10 min.

PCR-single strand conformational polymorphism (SSCP) direct sequencing. Tumor cells were purified from the specimen by laser micro-dissection (LMD) using the Leica LMD system (Leica Microsystems, Wetzlar, Germany). In brief, FFPE specimens were sectioned at 10-μm thickness and placed on

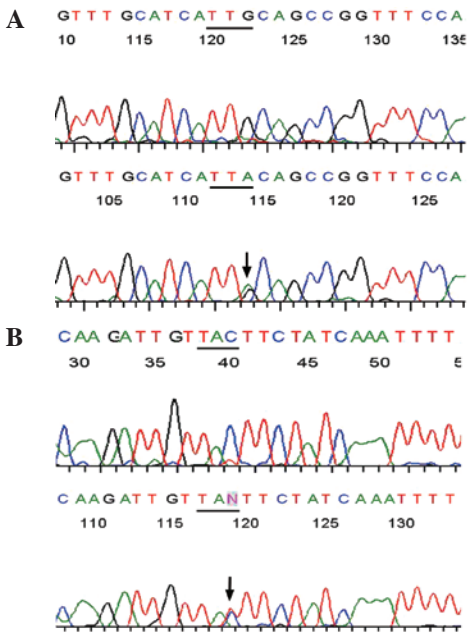

Figure 3. Results of sequence analysis of exons (A) 11 and (B) 16 of *CYLD* in the samples presented in Fig. 2. (A) Guanine, the 3rd nucleotide of codon 548, is substituted with adenine. (B) Cytosine, the 3rd nucleotide of codon 713, is substituted with thymine. These point mutations are not associated with amino acid substitutions. The exon and codon numbers were obtained from the Reference Sequence (reference nos. NG_012061.1 and NM_015247.2). *CYLD*, cylindromatosis.

membrane slides (Leica Microsystems). Following staining with toluidine blue, tumor cells were selected and dissected out using a laser beam under a microscope. Care was paid to avoid contamination by normal tissue surrounding the ACC cells. The dissected tumor cells were digested with proteinase K and DNA was purified using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. The primers for amplification of the *CYLD* gene coding exons were designed using Primer 3 (http://primer3.sourceforge.net/webif.php). Table II lists the sequence data of the primers. PCR was performed in 25-μl sample volumes as follows: 5 min at 95˚C followed by 35 cycles of 30 sec at 95˚C, 30 sec at 64˚C and 30 sec at 72˚C. For SSCP analysis, the PCR products were denatured by heating in a solution of 50% formamide and 10 mM ethylenediaminetetraacetic acid and then separated on a 12.5% polyacrylamide gel using the Genephore system (Amersham Pharmacia, Uppsala, Sweden). Following denaturation, single-stranded DNA underwent 3-dimensional folding and assumed a unique conformational state based on the base sequence. The majority of single base changes are detected as mobility shifts (12). The gels were silver stained using a kit (Amersham Pharmacia) to detect the mobility shifts. Mutational analysis was performed for cases demonstrating gene aberration as determined by SSCP. Purified PCR products from ACC and normal tissue adjacent to the tumor were directly sequenced using the BigDye Terminator Cycle sequencing Ready Reaction mix and ABI310 genetic analyzer (both Applied Biosystems, Foster City, CA, USA).

Results

Representative RT-PCR results are presented in Fig. 1. The β-actin product was detected in each case. RT-PCR products

for fusion genes, involving *PLAG1*, were not obtained at the expected sizes.

Since 35 primer pairs were prepared, a total of 1,190 PCR analyses were performed to examine the coding region of *CYLD* in the 34 cases of ACC. PCR products were obtained in ~75% of the PCR analyses. The results of PCR-SSCP analysis are presented in Fig. 2 and aberrant bands are indicated by arrows. These PCR products were subjected to direct sequencing.

Fig. 3 presents results of direct sequencing. The sample with an aberration in exon 11, identified by SSCP analysis, (Fig. 2A) was found to exhibit a silent mutation at codon 548. The sample with an aberration in exon 16 (Fig. 2B) was also identified to have a silent mutation, located at codon 713.

Discussion

It is well known that c-KIT, a proto-oncogene and therapeutic target, is recurrently expressed in ACC (14,15). A previous study reported that the chromosomal translocation t(6;9), which is associated with overexpression of MYB, is frequently found in ACC (16). Thus, knowledge of the molecular pathology of ACC is increasing, however, the molecular features of ACC remain to be elucidated. In the current study, gene-fusion involving *PLAG1* and the mutational status of *CYLD* were investigated.

PLAG1, encoding a zinc finger protein, is consistently rearranged in PAs of the salivary glands. Through chromosomal translocation, abnormal expression of *PLAG1* is driven by a constitutionally active promoter. Overexpression of PLAG1, acting as a transcription factor, causes deregulation of a variety of PLAG1 target genes. The aberrant expression of these target genes is hypothesized to be the cause of PA (17). Aberrations in *PLAG1* have been detected in neoplasms other than PA. Chromosomal rearrangement involving *PLAG1* are present in the majority of lipoblastomas (18,19). Although the fusion partner for *PLAG1* varies, *PLAG1* with a strong promoter following chromosomal rearrangement has been identified in lipoblastoma as well as PA (19). Thus, aberrant expression of PLAG1 occurs in these neoplasms, acting as an oncogene. In the present study, the gene fusions of *PLAG1* and several fusion partners, specifically, *CTNNB1*, *CHCHD7*, *LIFR* and *TCEA1,* were analyzed. These gene fusions have been detected in PA (9). Based on the results of RT-PCR, no gene fusion involving *PLAG1* was detected in ACC. These results are consistent with observations reported by Matsuyama *et al* (9). ACC and PA have similar histogenetic properties (8), however, the karyotypical aberrations differ from each other (20,21). In this study, chromosomal abnormalities of ACC were not tested, however, gene fusion, including *PLAG1,* was investigated in a relatively large number of cases. Results indicate that the mechanism involved in the tumorigenesis of ACC is different from that of PA.

Since cylindroma is a cutaneous neoplasm, cylindroma and ACC do not share histogenetic characteristics, however, myoepithelial cells participate in tumor formation in both types of neoplasms (22). Thus, cylindroma and ACC share morphological characteristics. *CYLD*, encoding a deubiquitinating enzyme, is associated with cylindromatosis, multiple familial trichoepithelioma and Brooke-Spiegler syndrome (10). In addition to these tumors, loss of CYLD expression is observed in various types of skin cancer, including basal cell and squamous cell carcinoma (23). Choi *et al* (24) identified loss of heterozygosity at the *CYLD* locus in basal cell adenoma of the salivary gland. Thus, *CYLD* may play a role in tumorigenesis in various neoplasms.

In the present study, the mutational status of *CYLD* was investigated in ACC. A silent mutation was detected in only two cases, indicating that *CYLD* does not play a role in ACC tumorigenesis comparable to that in Brooke-Spiegler syndrome.

In the present study, no gene fusions of *PLAG1* or mutations of *CYLD* were identified, indicating that these genes are not involved in ACC tumorigenesis.

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