

# Alteration of *SMAD4* does not participate in tumorigenesis of adenoid cystic carcinoma of the salivary gland

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**Abstract.** *SMAD4*, also known as *DPC4* (deleted in pancreatic carcinoma 4) was initially identified as a tumor-suppressor gene. It is located on 18q21, a region frequently deleted in pancreatic carcinoma. Functionally inactivating mutation of the gene also occurs in many cases of pancreatic cancer. Functional loss of *SMAD4* is frequently detected not only in pancreatic carcinoma but also in colorectal carcinoma. However, in other human cancers, *SMAD4* aberrations are seen only occasionally. The purpose of this study was to elucidate the role of *SMAD4* in adenoid cystic carcinoma of the salivary glands. We examined 34 cases of adenoid cystic carcinoma for loss of heterozygosity (LOH) of the *SMAD4* locus and for the existence of mutations of the gene. LOH was detected in 2/14 informative cases. No mutations were detected in any of the 34 cases. In conclusion, the infrequent LOH of the *SMAD4* gene locus and lack of *SMAD4* mutations indicate that *SMAD4* does not play a role in the tumorigenesis of adenoid cystic carcinoma of the salivary gland.

## Introduction

Nearly 90% of human pancreatic carcinomas show allelic loss at chromosome 18q (1). *SMAD4*, also known as *DPC4*, was originally isolated from human chromosome 18q21.1 as a candidate tumor suppressor gene in this region (1,2) and was found to be mutated in pancreatic carcinomas (3). As a result of this loss of normal function in the vast majority of pancreatic carcinomas, *SMAD4* is thought to play a major role in pancreatic carcinogenesis. Previous studies have indicated that the *SMAD4* protein mediates the tumor growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway, which negatively regulates the growth of epithelial cells (4). *SMAD4* is also thought to be involved in familial juvenile polyposis, which is characterized

by autosomal dominant inheritance and a predisposition to hamartomatous polyps and gastrointestinal cancer (5). The somatic alteration of *SMAD4* found in pancreatic carcinomas is also frequently detected in colorectal carcinomas (6), but in other human cancers mutation of this gene is seen only occasionally (6).

Adenoid cystic carcinoma (ACC) is a slow-growing neoplasm arising predominantly in the major and minor salivary gland. It is highly malignant and shows a remarkable rate of recurrence (7,8). Mutations of the tumor suppressor gene *TP53* are sometimes detected in ACC (9,10). In addition, the c-erbB2 and c-Kit proteins, which harbor tyrosine kinase activity and act as oncoproteins, are frequently expressed in ACC (11,12). However, little-to-nothing is known about the development of ACC.

The present retrospective study was designed to characterize genetic alterations in *SMAD4* in cases of ACC by detecting loss of heterozygosity (LOH) at 18q21.1 and by screening the entire *SMAD4* gene for mutations. DNA sequencing in 34 cases of ACC was used.

## Materials and methods

**Laser microdissection and DNA extraction.** 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. The 34 ACC cases included cribriform type (n=17), tubular type (n=13) and solid type (n=4). Prior to DNA extraction, laser microdissection was performed using the Leica Microdissection System (Leica, Wetzlar, Germany) to dissect tumor cells only for analysis. Normal salivary gland tissue was also dissected in particular cases that contained abundant normal salivary gland tissue adjacent to the ACC for the preparation of normal genomic DNA for LOH analysis. The dissected specimens were digested in 10 mM Tris-HCl pH 7.5 containing 1% sodium dodecyl sulfate at 55°C for 16 h. The lysate containing genomic DNA was cleaned using a MiniElute Reaction Cleanup Kit (Qiagen, Hilden, Germany) according to the instructions provided by manufacturer.

**18q21.1 LOH analysis.** The microsatellite marker D18S1110 was used for LOH analysis. The sequences of the primers were based on the Genome Database (www.gdb.org. Accession ID: GDB608763). Genomic DNA obtained from tumors and adja-

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Table I. Sequence data of the primers.

Primer	Sequence	Size of product (bp)
SMAD4 E1-1 F	ttccttgcaacgtagctg	249
SMAD4 E1-1 R	tctccttcagcttcttaccaaa	
SMAD4 E1-2 F	tgccatagacaagggtggaga	250
SMAD4 E1-2 R	gcttgaaaggaaacgtagcaa	
SMAD4 E2 F	tgacacatgaataaatggtcggt	241
SMAD4 E2 R	tgtttcttaggatgaaagcaaagtc	
SMAD4 E3 F	tgtttctattgttttcccttt	154
SMAD4 E3 R	ctgccgctcacacaactaa	
SMAD4 E4-1 F	ttttctgggaatagaagcttataaaaa	198
SMAD4 E4-1 R	agctgggggtgctgtatgtct	
SMAD4 E4-2 F	tcaattcaaacatccagca	213
SMAD4 E4-2 R	cccccaagtgactacataaaa	
SMAD4 E5 F	ccatgttaatgtcttctgttctctc	230
SMAD4 E5 R	cccacatgggttaatttgct	
SMAD4 E6 F	taaccatgtgggccttaat	207
SMAD4 E6 R	cagaaacaaagccctaccaa	
SMAD4 E7 F	tcttagacattgcataagctgtttt	249
SMAD4 E7 R	tgtgctttcaatcaccact	
SMAD4 E8-1 F	tgtggagtgaagtgaagc	181
SMAD4 E8-1 R	gggtccacgtatccatcaac	
SMAD4 E8-2 F	tccttcaagctgccctattg	199
SMAD4 E8-2 R	catgggaaacataaccttgaa	
SMAD4 E9-1 F	gctatctttggtttatgtgatcttt	248
SMAD4 E9-1R	acgccagcttctctgtcta	
SMAD4 E9-2 F	gggtgcacataggcaaggt	250
SMAD4 E9-2 R	ttcctccaccagatttca	
SMAD4 E10 F	ggcattggttttaattgtatgga	292
SMAD4 E10 R	tgctcaagaactaatcaactgag	
SMAD4 E11-1 F	atcacctgtccctctgatg	246
SMAD4 E11-1 R	aagggttggtgctgcaatc	
SMAD4 E11-2 F	tggtgtgatgacctcgctc	208
SMAD4 E11-2 R	ccccaacggtaaaagacct	

E, exon; F, forward; R, reverse; bp, base pairs.

cent normal salivary gland tissues was amplified using these primers, and the products were electrophoresed and visualized using the GenePhor DNA Separation System (Pharmacia, Uppsala, Sweden).

**PCR-direct sequencing.** To check for mutational aberrations, all the coding exons of *SMAD4* were amplified using polymerase chain reaction (PCR). The sequences of the primers are listed in Table I. A total of 16 pairs of primers were designed.

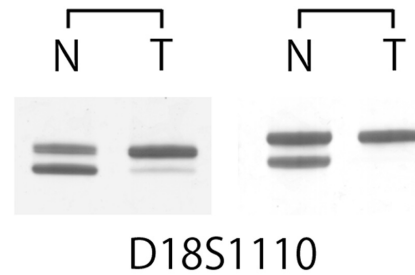


Figure 1. Loss of heterozygosity analysis using microsatellite marker D18S1110. Both cases show loss of heterozygosity at the locus of the *SMAD4* gene. Two heterozygote PCR products were obtained from the normal salivary gland tissue (N). PCR product from one allele is markedly decreased or absent in the corresponding adenoid cystic carcinoma (T).

To avoid low productivity, some exons were amplified separately. PCR products were sequenced using the BigDye cycle sequencing kit and ABI310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

## Results

**LOH analysis.** A total of 34 cases were tested for LOH analysis, and 14 informative cases were identified as having heterozygosity at the D18S1110 locus. Two of the 14 cases showed LOH (Fig. 1).

**Mutational analysis.** We tested all the coding exons of *SMAD4* and found no mutations in any of the 34 cases of ACC.

## Discussion

The TGF- $\beta$  signaling pathway is very complex, has multiple levels of regulation, and is involved in various processes including development, wound healing, fibrosis, carcinogenesis, angiogenesis and immunity (13). TGF- $\beta$  binds to a heteromeric complex of transmembrane serine/threonine kinases, type I and II receptors, and the activated receptor phosphorylates SMAD2 and SMAD3, the so called 'receptor specific SMADs'. Upon phosphorylation, these receptor-specific SMADs form a complex with second class SMADs - 'common-partner SMADs' - such as SMAD4. The complex formed with phosphorylated SMAD2 or SMAD3 and SMAD4 translocates to the nucleus, where it activates transcription of the target genes involved in cell proliferation. TGF- $\beta$  signaling prevents progression through the cell cycle by inducing the expression of various cyclin-dependent kinase inhibitors, which arrest the cell cycle during the transition from the G1 to S phase. Thus, TGF- $\beta$  signaling acts as a tumor suppressor (4,14).

The TGF- $\beta$  signaling pathway can be disrupted by aberration of its signal transducers, such as the SMADs. *SMAD4*, also known as DPC4 (deleted in pancreatic carcinoma 4), was initially identified as a tumor suppressor gene (2). *SMAD4* is located on 18q21, a region deleted in 90% of pancreatic carcinomas (1). The human *SMAD4* gene contains 11 coding exons with a predicted 552-amino acid coding sequence containing two functional regions, termed mad homology 1 and 2 (MH1 and MH2) (2). The MH2 region participates in homo-

and hetero-oligomerization with SMAD family proteins (15). The MH2 domain of SMAD4 is also involved in transcriptional activation and nuclear localization (16). The majority of *SMAD4* gene mutations in human cancer occur at the MH2 domain (17). In pancreatic cancer, somatic mutational events occur with high frequency (2,18,19). In total, approximately 55% of pancreatic cancers show inactivation of the *SMAD4* gene (17). In colorectal cancer, the frequency of mutational events in the *SMAD4* gene increases with the progression of clinical stages (6). Mutations of the *SMAD4* gene were detected in approximately 30% of advanced-stage colorectal cancers (6). Alterations of the *SMAD4* gene are prevalent in pancreatic and colorectal cancer, while the mutation is less frequent in other types of malignancy, such as acute myeloid leukemia, biliary tract carcinoma, ovarian cancer, small intestinal carcinoma, gastric carcinoma, head/neck squamous cell carcinoma, hepatocellular carcinoma or lung carcinoma (17). No *SMAD4* mutations were detected in breast cancer (20) or prostatic cancer (21).

In the present study, we tested the locus of the *SMAD4* gene for LOH as well as the entire coding region of the gene in ACC for mutations. The results revealed infrequent LOH and no mutation of this gene in ACC. This indicates that the *SMAD4* gene remains normally conserved in the majority of ACCs, and that production of the SMAD4 protein is not disturbed in ACC tumor cells.

In conclusion, although *SMAD4* gene aberration plays an important role in the carcinogenesis of pancreatic and colorectal tumors, is not involved in the development of ACC.

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