SHORT COMMUNICATION

Cytoplasmic expression of E-cadherin and β -Catenin correlated with LOH and hypermethylation of the APC gene in oral squamous cell carcinomas

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BACKGROUND: Inactivation of the adenomatous polyposis coli (APC) gene results in accumulation and translocation of β -Catenin, which are important for malignant development. The aim of the present study is to investigate the possible role of APC/ β -Catenin pathway in oral squamous cell carcinomas.

METHODS: The DNA from 34 patients was examined for loss of heterozygosity (LOH) at two markers surrounding the APC, and for hypermethylation of the APC promoter by using methylation-specific polymerase chain reaction (MS-PCR). Fifteen of 34 samples were stained immunohistochemically to show the expression of E-cadherin and β -Catenin.

RESULTS: We found that cytoplasmic rather than membrane staining of E-cadherin and β -Catenin was a prominent aberrant tumour-related alteration, and that this expression was mainly present in moderately and poorly differentiated tumours. LOH and hypermethylation of the APC promoter was found in four of 31 and five of 34 carcinoma samples, respectively. Four of five cases presenting LOH/hypermethylation showed cytoplasmic expression of E-cadherin and β -Catenin by immunohistochemical (IHC) staining.

CONCLUSION: The present results indicate that LOH at the APC locus or hypermethylation of the APC promoter 1a may lead to free β -Catenin accumulation in cytoplasm of oral carcinoma cells and thereby to oral malignant progression.

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Introduction

β-Catenin, a cytoplasmic protein, is often involved in regulating E-cadherin-mediated cell adhesion and in the WNT-1 (wingless) signalling pathway. β -Catenin is continuously inactivated by phosphorylation when bound to a protein complex including the adenomatous polyposis coli (APC) gene product and it is commonly accepted that the key tumour-suppressor function of the APC gene lies in its ability to destabilize free β -Catenin. Accumulation and translocation of β -Catenin caused by loss of function of APC may thus activate transcription factors (1) and initiate tumorigenesis in colorectal cancer (2). There are reports showing altered expression of β -Catenin, as well as genetic changes of the *APC* gene in oral carcinomas, however, less attention has been paid to epigenetic changes of the APC gene in oral tumorigenesis (3). In the present work, we investigate loss of heterozygosity (LOH) and hypermethylation of the APC gene promoter and expression of E-cadherin and β -Catenin, in order to elucidate the possible role of the APC/ β -Catenin pathway in oral carcinomas.

Materials and methods

Frozen surgical specimens from 34 oral squamous cell carcinomas were obtained for the present study (16 patients came from Odense University Hospital, Denmark, and 18 patients came from Mackay Memorial Hospital, Taipei). A laser microdissection (LCM) system (PALM, Leica, Heidelberg, Germany) was used to separate diseased and normal tissues. DNA was extracted by routine procedures (Qiagen, Qiagen GmbH, Hilden, Germany). A radioactive polymerase chain reaction (PCR) was performed for screening LOH at the APC locus by using two markers at 5q21 including D5S656 and D5S1965 (http://www.gdb.org/). Genomic DNA was treated with sodium bisulphite and methylation-specific PCR (MS-PCR) was performed for detecting methylation of the APC promoter 1a. Methylated samples in MS-PCR were further analysed by fluores-

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cence melting curve analysis (MS-MCA). Fifteen unfixed samples were stained immunohistochemically to show the expression of E-cadherin and β -Catenin by using the monoclonal mouse antibodies (Zymed Laboratories Inc., South San Francisco, CA, USA). Both antibodies were immunoglobulin G (IgG), and one serves as control for the other. Specificity of the antibodies have been thoroughly tested and optimized in previous experiments (4, 5). All above methods were described in our previous study (6) except methylation of the *APC* promoter 1a (7).

Correlation analyses were performed using Fisher's exact probability test.

Results and discussion

A close interaction among E-cadherin, β -Catenin and APC proteins has been demonstrated in tumorigenesis, and indicated that alterations of the *APC* gene can result in accumulation of β -Catenin in colorectal and breast tumour cells (2, 8). The present result showed a similar expression pattern of E-cadherin and β -Catenin: staining was seen at the cell membrane in basal and parabasal layers in normal epithelium and as a mixture of cytoplasmic and membrane staining of the tumour cells in nine of 15 samples investigated by immunohistochemistry of oral carcinomas. Three cases showed loss of β -Catenin expression and three showed membrane

staining as in normal epithelium, total loss of E-cadherin expression was not found in the present study (Fig. 1). Cytoplasmic expression was predominantly present in moderately and poorly differentiated tumours with significant correlation between grade levels and expression patterns of β -Catenin and E-cadherin (P = 0.039) (Table 1). It is therefore suggested that the cytoplasmic expression pattern instead of membrane staining of β -Catenin is a frequent aberrant tumour-related alteration rather than loss or reduction of expression, which has been described previously (9).

Mutation of the *APC* gene has been found in oral carcinomas but there is no information whether the mutation is related to overexpression of β -Catenin (10). We found four of 31 informative carcinoma samples showing LOH at 5q21. Two cases showed LOH at both markers, the remaining two cases only showed LOH at D5S656 (Fig. 2). Two of them were related to cytoplasmic expression of β -Catenin, one was related to loss of β -Catenin expression, the remaining one was not available for IHC staining. Because of the low frequency of LOH and the limited number of patients it was difficult to show differences in patients from Denmark and Taipei, although LOH at *APC* gene locus has been demonstrated more frequently in Western than in Asian patients (11–13).

Hypermethylation of the APC gene promoter 1a was found in five of 34 carcinoma samples (Fig. 3); all



Figure 1 IHC staining of E-cadherin and β -Catenin showing similar expression pattern in both normal and tumour cells except for indication in Table 1 and example (d). (a) β -Catenin expression is seen as membrane staining in normal epithelium; (b) β -Catenin expression is seen as unchanged membrane staining in tumour (no. 27088); (c) arrows indicate accumulated expression of β -Catenin in cytoplasm in part of tumour cells, which showing hypermethylation of the *APC* gene in Fig. 2 (no. 1592); (d) different expression only in a small tumour island in the same case of (c), loss of β -Catenin expression (d1) but retain of E-cadherin expression (d2) (no. 1592).

Number	Number of pathology	Histological grade ^a	LOH at 5q21	Hypermethylation of the APC gene	IHC staining	
					E-cadherin	β -Catenin
1	19395	II	_	_	2	2
2	25941	III	_	+	2	2 ^b
3	15374	Ι	_	_	1	1
4	18034	II	NA	_	1	1
5	31572	Ι	+	_	1 ^c	0
6	33379	II	_	_	2°	2^{c}
7	28753	II	+	_	2 ^b	2 ^b
8	19274	Ι	_	_	2	2
9	2132	Ι	_	_	2 ^c	2 ^b
10	1592	III	_	+	2 ^b	2
11	17093	II	+	_	2	2
12	29627	III	_	_	2	2
13	21394	Ι	_	_	1 ^c	0
14	27088	Ver. Ca.	-	_	1	1
15	33103	I	_	-	1	0

Table 1 Hypermethylation and loss of heterozygosity (LOH) of the *APC* gene, and the correlated expression of E-cadherin and β -Catenin in 15 oral carcinomas

^aHistological grade: I is well differentiated, II is moderately differentiated and III is poorly differentiated. Ver. Ca. indicates vertucous carcinoma. The grade level was correlated with cytoplamic expression of E-cadherin and β -Catenin (P = 0.039), but not with the alteration of the APC gene (P = 0.257, n = 34) (data of Taipei patients were not shown in above table, which including one grade II tumour showing LOH, one grade I and two grade II tumours showing hypermethylation).

IHC staining localization: 1, membrane staining as same as normal epithelium, seen in three cases; 2, membrane staining mixed with cytoplasmic staining, seen in nine cases; 0, completely negative staining, seen in three cases of β -Catenin staining; staining intensity: ^bstronger intensity than normal control, ^cweaker than normal control.



Figure 2 Loss of heterozygosity (LOH) analysis of 5q21 in oral squamous cell carcinomas. T, tumour; N, normal tissue. Arrows indicate LOH at both markers in two cases.



Figure 3 Methylation-specific polymerase chain reaction (MS-PCR) analysis of the *APC* gene promoter in oral squamous cell carcinomas. Genomic DNA was treated with sodium bisulphite and PCR-amplified with primer pairs specific for methylated (M) and unmethylated (U) alleles. SssI-methylated DNA provided positive controls for methylated *APC* alleles. Tum, tumour; Con, connective tissue.

samples showing aberrant methylation using MS-PCR were confirmed by MS-MCA (data not showed). Two of these five cases also showed cytoplasmic expression of

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β-Catenin, the remaining three cases were not available for IHC staining. LOH at 5q21 and hypermethylaion of the *APC* gene promoter 1a were not found concomitantly. No significant correlation was found between histopathological grade of carcinomas and alteration of the *APC* gene (P = 0.257).

A recent study of patients with head and neck cancer showed that the 5 year-survival rate was significantly lower in a group that showed β -Catenin cytoplasmic accumulation than in the non-accumulation group, although only one case showed mutation of the APC gene and no gene mutation in β -Catenin exon 3 was found. This indicates that the APC and β -Catenin mutation are not the cause of β -Catenin accumulation in head and neck cancer. Hypermethylation of the APC gene has been found as a frequent event in colorectal, gastric, bladder, lung, breast cancer and melanoma (1, 2, 7, 8), however, only one report describes methylation of the APC gene promoter in oral carcinomas. Here, it was shown that the aberrant expressions or abnormal localization of E-cadherin and β-Catenin proteins were independent of methylation status or mutations of the APC gene, suggesting that the Wnt pathway-related genes play a very limited role in the development of oral squamous cell carcinomas (3).

Conclusively, our study showed that alteration of the *APC* gene was seen in four of nine cases, which showed cytoplasmic expression of β -Catenin. Such accumulation may be important during the oral malignant progression as it is mainly expressed in moderately or poorly differentiated tumours. However, total loss of β -Catenin expression in three cases indicated that other mechanisms should be involved in oral carcinogenesis, as well.

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