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Alterations of $p16^{INK4a}$ tumour suppressor gene in mucoepidermoid carcinoma of the salivary glands

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Abstract. Mucoepidermoid carcinoma (MEC) is common in the salivary glands, but alterations of the $p16^{INK4a}$ tumour suppressor gene are largely unknown. The aim of this study was to analyse $p16^{INK4a}$ gene alterations in MEC, and evaluate their significance for carcinogenesis. Thirty-eight salivary glands with MEC and six normal salivary glands were studied for $p16^{INK4a}$ alterations. In the MEC-affected group, there were 23.7% (9/38) and 13.2% (5/38) cases of homozygous deletion, and 5.3% (2/38) and 2.6% (1/38) cases of point mutation in $p16^{INK4a}$ exon 1 and exon 2, respectively. Hypermethylation of the $p16^{INK4a}$ gene promoter was found in 13 cases (13/38, 34.2%). Alterations of the $p16^{INK4a}$ gene were not found in the normal salivary glands. These findings suggest that the main mechanisms of inactivation of the $p16^{INK4a}$ gene in MEC of the salivary glands are promoter hypermethylation and homozygous deletion.

Keywords: salivary gland; mucoepidermoid carcinoma; $p16^{INK4a}$; DNA methylation; mutation; deletion.

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Mucoepidermoid carcinoma (MEC) is a common malignant tumour in salivary glands. In the WHO classification, MEC is graded as low-grade type (or well differentiated) or high-grade type (poorly differentiated). Between these two well defined types there may exist a third group of intermediate-grade type (moderately differentiated), which have mixed features of high and low grade. The histopathological degree of malignancy and prognosis appear to correlate with subtype. As a whole, MEC is found in major and minor salivary glands without predominance, and there is no distinct gender predominance even when divided into low- and high-grade carcinomas[13]. Most MECs occur in persons of 40–60-year-old, but even children are affected.

Tumour cell proliferation has been correlated with the progression and prognosis of a variety of malignant tumours. Recent advances have elucidated the precise mechanisms of cell-cycle regulatory systems, and have shown that deregulated cell proliferation is a common feature of many cancers[4]. Cell-cycle progression is regulated by many cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. The $p16^{INK4a}$ tumour suppressor gene is one very important kind of CDK inhibitor that can directly combine CDK4 and CDK6 or cyclin–CDKs complexes to regulate the cell cycle.

Alterations of the $p16^{INK4a}$ tumour suppressor gene are often observed in a variety of human cancers, and are considered to play a critical role in the

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transition to malignant growth. Very few investigations have so far been reported of genetic and epigenetic alterations in salivary gland carcinomas[12,19], including MEC[8,12]. The aim of the present study was to detect alterations of the p16^{INK4a} gene in MEC and evaluate their significance for carcinogenesis.

Materials and methods

Tissue specimens

Normal and MEC salivary glands were collected from the Stomatology Hospital and Qilu Hospital, Shandong University from January 2002 to June 2005, and the study was carried out from January 2005 to January 2006. The samples were taken in the operating room. Half of each were snap-frozen in liquid nitrogen and stored at −80 °C for analysis, while the rest were sent to the pathology department.

The samples for DNA extraction were obtained through microdissection after diagnosis. There were six cases of normal salivary glands (four cases in major salivary glands, two cases in minor salivary glands; mean age 41.5 years, range 19–57 years), and 38 cases of MEC classified as low grade (21), intermediate grade (7) and high grade (10) (Table 1). There were 18 men and 20 women in the MEC group, and the mean age at diagnosis was 48.8 years (range 7–79 years). MECs arising in major salivary glands were staged according to the sixth and newest edition of the American Joint Committee on Cancer (AJCC) staging system[6]. MECs arising in minor salivary glands were staged like squamous cell carcinomas according to their sites of origin[13].

Sections from each sample were fixed in 10% neutral buffered formalin, paraffin embedded, and cut at 5 μm for hematoxylin and eosin staining. DNA was extracted from frozen tissue samples using the proteinase K digestion method.

Detection of homozygous deletion and mutation in p16^{INK4a}

Exon 1 and exon 2 of the p16^{INK4a} gene were amplified by PCR and screened for mutation by single-strand conformation polymorphism (SSCP) analysis. PCR products were separated by electrophoresis on 2% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Polyacrylamide gel electrophoresis was used for PCR-SSCP, which was run on a 6% polyacrylamide gel and then silver stained.

Table 1. Characteristics of patients with mucoepidermoid carcinoma

Patient characteristics	Total	Low grade	Intermediate grade	High grade
Number of patients	38	21	7	10
Male	18	11	3	4
Female	20	10	4	6
Median age (years)	50	55	49	40
Range (years)	7–79	7–79	9–76	17–68
Tumour site				
Major salivary gland	21	10	4	7
Minor salivary gland	17	11	3	3
Tumour stage				
I	24	16	5	3
II	6	2	1	3
III	3	2	0	1
IV	5	1	1	3

Methylation-specific PCR

Aberrant methylation was detected using the CpG WIZ Amplification kit (Promega, Madison, WI, USA) following the protocol provided. DNA methylation status in the promoter CpG island of the p16^{INK4a} gene was determined by chemical modification of unmethylated, but not methylated, cytosine to uracil using sodium bisulphate and subsequent PCR using 'unmethylated-specific' and 'methylated-specific' primers. Detailed experimental procedures were previously reported[9]. Unmethylated-specific primers were 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and 5′-CAACCCCAAACCACAACCATAA-3′, and methylated-specific primers were 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ and 5′-GACCCCGAACCGCGACCGTAA-3′.

Statistical analysis

Fisher's exact text was used to assess the significance of differences in frequencies of various parameters tested. A probability value of <0.05 was considered statistically significant.

Results

Homozygous deletion and mutational analysis of p16^{INK4a} gene

PCR amplication was performed to generate a 340-bp fragment of exon 1 and 427-bp fragment of exon 2. The β-actin fragment (600 bp) was also amplified at the same time. The PCR results are shown in (Fig. 1) and PCR-SSCP results in (Fig. 2). Table 2 shows these alteration profiles of the p16^{INK4a} gene in MECs of differing tumour stage and histological grade.

There were 23.7% (9/38) and 13.2% (5/38) homozygous deletions, and 5.3% (2/38) and 2.6% (1/38) point mutations of p16^{INK4a} exon 1 and exon 2 in the MEC group, respectively. There was no statistical difference between different tumour stages or histological grades ($P > 0.05$). There was a significant difference in p16^{INK4a} homozygous deletion rate between low-grade/intermediate-grade MECs (7/28, 25%) and high-grade MECs (6/10, 60%) ($P = 0.02$).

Methylation status of p16^{INK4a} gene promoter

As p16^{INK4a} gene mutations were rare in the MECs, the methylation status of the p16^{INK4a} gene promoter was next examined using methylation-specific PCR. As shown in (Fig. 3), some MEC samples (MEC17,18) were found to be partially methylated, and MEC19 was heavily (or fully) methylated. In the normal salivary glands and some other MEC samples no methylation was observed. Of the 38 MEC samples, methylation of p16^{INK4a} was found in 13 (34.2%, 13/38). All cases of matched normal tissues were shown to be unmethylated. There was no statistical difference according to tumour stage or histological grade ($P > 0.05$).

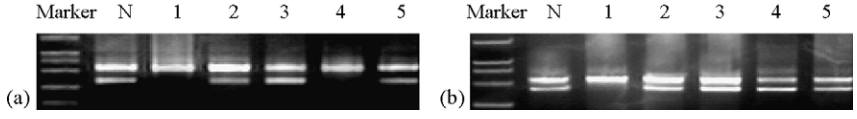

Fig. 1. PCR products of exon 1, 340 bp (a) and exon 2, 427 bp (b). Marker (bp): 2000, 1000, 750, 500, 250. N: normal salivary gland. The upper band: β-actin (600 bp). MEC1: homozygous deletion (HD) in exon 1 and exon 2; MEC4: HD in exon 1; MEC2 and MEC3: no HD in exon 1 or exon 2.