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Loss of the Adenomatous Polyposis Coli Gene and Human Papillomavirus Infection in Oral Carcinogenesis

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Recent evidence suggests that loss of heterozygosity (LOH) of the adenomatous polyposis coli (APC) gene plays a role in colorectal tumorigenesis and other cancers. However, little is known as to whether the APC gene contributes to the pathogenesis of oral squamous cell carcinoma. To assess involvement of both the APC gene and the human papillomavirus (HPV) in the development of oral pre-malignant and malignant lesions, we analysed DNA from 14 paired oral normal and pre-malignant or malignant paraffin-embedded biopsy specimens, and DNA from cultured normal and HPV 16-immortalised oral epithelial cells for the presence of LOH of APC and for HPV infection, using PCR based techniques. LOH of APC occurred in 80% of cases of oral epithelial dysplasia, 67% of carcinoma *in situ*, 50% of invasive squamous cell carcinoma cases, and in the HPV 16-immortalised oral epithelial cells. HPV was detected in half of the biopsy specimens, with HPV 16 as the dominant type. More than half of the carcinoma cases were found to contain both LOH of APC and HPV infection. These results suggest that LOH of APC is an early event during oral tumorigenesis. Our findings also suggest a strong correlation between HPV infection, particularly HPV 16, and LOH of the APC gene in oral squamous cell carcinomas. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

As in other cancers, oral squamous cell carcinoma is thought to result from accumulation of genetic alterations that affect the normal cell growth and result in transformation of the normal cell to a malignant cell. Although cancer of the oral cavity (together with cancers of the lip and pharynx) is globally the sixth most common cancer in both sexes, the molecular genetic mechanisms of the development of these tumours still remain unclear. In the past few years, some genetic alterations of oncogenes and tumour suppressor genes associated with oral cancer have been discovered, including mutations of the *p53* tumour suppressor gene [1], *c-myc*, *ras*, *int-2*, *hst-1*, *bcl-1*, *prad-1*, EGFR and E-cadherin [2, 3]. Human papillomavirus (HPV) infection, particularly HPV type 16, has also been considered as one of the aetiological agents in the development of oral cancer [4]. Therefore, it is likely that abnormalities at multiple genetic loci and viral infection are associated with the pathogenesis of these tumours.

The gene for adenomatous polyposis coli (APC) was first discovered as the gene involved in familial APC, an inherited disease predisposing to colorectal carcinomas [5]. It has been localised by linkage analysis to 5q21-22. APC gene mutation is thought to play an important role in the development of colon cancer [5]. However, a high incidence of loss of heterozygosity (LOH) of APC has also been demonstrated in cancers of the upper aerodigestive organs, such as the oesophagus [6] and lung [7]. Although these neoplasms are associated with predisposing factors similar to oral squamous cell carcinoma, e.g. smoking and/or excessive alcohol use, little is known as to whether LOH of the APC gene is involved in the pathogenesis of oral pre-malignant and malignant lesions. In the present study we analysed allelic loss of the APC gene in formalin-fixed paraffin-embedded biopsy specimens and also in a HPV-immortalised oral epithelial cultured cell line, using polymorphic DNA markers to determine the incidence of LOH of APC in the development of oral cancer. Prevalence of HPV infection in the biopsy specimens was also determined using the PCR technique. Furthermore, the correlation between LOH of APC and HPV infection was analysed.

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MATERIALS AND METHODS

Biopsy tissue samples

Tissue from oral pre-malignant and malignant epithelial lesions were obtained from 14 unrelated patients from the Division of Oral Pathology, University of Washington Med-

ical Center, Seattle. They include five epithelial dysplasias, three carcinomas *in situ*, and six invasive squamous cell carcinomas. All samples were formalin fixed and paraffin-embedded. Two 5 μm sections from each specimen were used for DNA extraction.

Monolayer cultures of oral epithelial cells

One cell line was also used in this study—a line of normal human oral epithelial cells which had been immortalised by the HPV E6/E7 genes (Dr D. Oda, University of Washington) and had now reached passage 350. The technique for obtaining and culturing normal oral epithelial cells has been described previously in detail [8]. Briefly, specimens obtained from healthy patients undergoing surgery for impacted third molar removal were washed immediately in cold, sterile phosphate-buffered saline (PBS). After removing excess and damaged epithelium and connective tissue, the healthy specimens were cut into small pieces and incubated overnight in Dispase II (Boehringer Mannheim, Mannheim, Germany) at 4°C. Surface epithelium was mechanically separated and trypsinised to dissociate the cells into a single cell suspension. The cells were centrifuged and resuspended in KGM medium (Clonetics, San Diego, California, U.S.A.), then seeded on plastic dishes (Falcon, Lincoln Park, New Jersey, U.S.A.) and fed every 48 h. Upon confluence, the cells were trypsinised with 0.05% trypsin in 0.53 mM EDTA (Gibco, Grand Island, New York, U.S.A.) and re-seeded.

Immortalisation with HPV type 16, E6/E7 open reading frame

Normal epithelial cells at 50% confluence were transfected with HPV 16 E6/E7 open reading frames (ORFs) using a recombinant retroviral system previously described [9]. HPV E6/E7 ORFs were cloned into the murine-based retroviral vector LXSN to create the vector pLXSN 16E6/E7. The constructs were transfected into a packaging cell line PA317 and recombinant retroviruses were collected in the supernatant and used for infection of early passage oral epithelial cells. Cells were selected with G418 at 120 μg/ml in KGM medium and passaged in culture. Normal control cells and normal cells with pLXSN vector were also treated with G418. Over 95% of the pLXSN 16E6/E7 infected cells were healthy after the G418 treatment. They were trypsinised and passaged 1:5 twice while maintained in G418, after which they were passaged in non-selective keratinocyte SFM (Gibco) as described [9]. The pLXSN vector control and normal cells survived for seven to nine passages, whereas those cells that were infected with pLXSN 16E6/E7 became immortal. In this study, pLXSN 16E6/E7 immortalised cells were used at passage 305.

Microdissection

Microdissection of selected populations of normal oral epithelial cells, mild to moderately dysplastic cells, carcinoma *in situ* cells, and invasive squamous cell carcinoma cells from formalin-fixed, paraffin-embedded specimens was performed under direct light microscope visualisation using a surgical scalpel blade. Two 5 μm sections were used for each specimen.

DNA preparation

Cellular DNA from the biopsy sections was prepared as described by Wright and Manos [10]. DNA from the pellets of normal and immortalised cells was extracted by the method of Kawasaki [11]. Digestion with proteinase K and ethanol precipitation was used to prepare specimens for DNA and for PCR amplification.

Detection of LOH of APC genes

Polymorphic DNA marker. LOH was detected on the basis of PCR amplification of polymorphisms near or within the APC genes. Two sets of primers for PCR amplification of markers were selected and synthesised based on previous literature: DP1—deleted in polyposis 1 [12] and exon 11 [6]. The sequence from the DP1 gene, which is located 30–70 KB downstream from the APC gene, has been considered as a microsatellite marker which is extremely useful for clinical diagnosis in families segregating an allele for APC [13]. An APC dinucleotide microsatellite polymorphism was analysed by the method of Spirio *et al.* [12] with modifications for formalin-fixed tissues. Briefly, the PCR reaction mixture was of 25 μl total volume containing 0.25 μg genomic DNA, 10 mM Tris–HCl, pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 100 mM dNTPs (10 mM dCTP), 75 ng of each primer, 5 μCi of [α-^{32}P] dCTP (3000 Ci/mmol), and 1 U of *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.). The reaction mixtures were initially denatured in an automated thermal cycler (Perkin-Elmer Cetus DNA Thermal Cycler) at 94°C for 3 min. This was followed by 35 cycles of 94°C for 1 min (denaturation), 58°C for 1 min (annealing), and 72°C for 1 min (extension), followed by cooling to 4°C. The radioactively amplified PCR products were then separated on a formatted 8% denaturing acrylamide gel electrophoresis system and analysed by autoradiography.

PCR-RFLP analysis. LOH of APC gene exon 11 amplified a 133-base pair target sequence containing a polymorphic *Rsa* I restriction site with restriction fragments of 87 and 46 base pairs. The LOH of APC exon 11 was examined using a similar PCR mix and cycling conditions as for the DP1 gene, except that unlabelled dCTP was substituted for the 5 μCi of [α-^{32}P] dCTP. Eight microlitres of the 133 base pair PCR product were digested with 2 U of *Rsa* I (Amersham International, Amersham, U.K.) at 37°C for 2 h and electrophoresed on 8% non-denaturing polyacrylamide gels. The DNA was visualised in the gels under UV light after staining with ethidium bromide.

Detection of HPV infection

All tissue specimens were analysed for the presence and type of HPV DNA by polymerase chain reaction (PCR) amplification of total cellular DNA preparations. For these analyses, two separate PCR based tests were used: with HPV type consensus primers [MY11 and MY09 [14]) and with type-specific primers [15]. Amplification reactions were performed using standard techniques previously described by our group [16] and others [14].

RESULTS

LOH in oral epithelial cells

A total of 28 samples of pre-malignant and malignant formalin-fixed oral epithelial lesions and the corresponding normal tissues from 14 patients, one HPV 16-immortalised oral epithelial cell line and cultured normal oral epithelial cells were studied for LOH of the APC gene. All the samples and

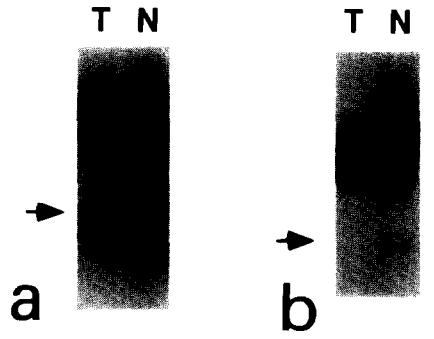

Fig. 1. Results of LOH in oral epithelial dysplasia (a) and squamous cell carcinoma (b) using a dinucleotide microsatellite located DP1 of the APC gene. Lanes: T, tumour tissue; N, corresponding normal tissue. Allele losses are indicated by arrowheads.

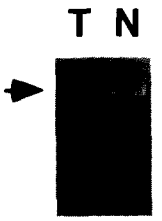

Fig. 2. Results of LOH at exon 11 of the APC gene detected by PCR-LOH assay. Each tumour DNA (T) lane is immediately followed by its corresponding normal DNA (N). LOH of APC was detected when the DNA of the oral lesions showed a loss of the single uncut band—133 bp. Allele loss is indicated by arrowhead.

Table 1

Histology	Number of cases	LOH of APC (DP1/exon 11)	HPV+
Dysplasia	5	4/5 (80%)	2/5 (40%)
Carcinoma *in situ*	3	2/3 (67%)	3/3 (100%)
Invasive carcinoma	6	3/6 (50%)	2/6 (33%)
		$P>0.05$	$P>0.05$
Total	14	9/14 (64%)	7/14 (50%)

the immortalised cell line were informative for LOH analysis at the APC loci. A representative example of LOH of APC DP1 is illustrated in Fig. 1. The typical LOH at exon 11 of APC is either the 133-bp allele lacking the *Rsa* I restriction site (Fig. 2) or the allele containing the site, represented by bands at 85 bp and 48 bp. In 4/5 cases (80%), LOH was observed in epithelial dysplasias, 2/3 cases (67%) of the carcinomas *in situ*, and 3/6 cases (50%) of the invasive carcinomas. Statistically, there is no significant difference among the three groups (Table 1). The HPV 16-immortalised cell line was also found to have allelic loss of APC at the exon 11 region.

HPV infection in oral lesions

Using PCR with both specific and consensus primers, HPV DNA was detected in 7/14 (50%) of the oral lesions. Of these, two were epithelial dysplasias, three were carcinomas *in situ* and two were invasive carcinomas (Table 1). The majority of positive cases (6/7, or 86%) were HPV type 16, while the other was type 6.

DISCUSSION

In spite of the fact that the use of tobacco and alcohol has been well documented as the major risk factor for oral cancer, the molecular mechanisms of the pathogenesis of oral squamous cell carcinoma are poorly understood. Recently, frequent LOH on chromosomes 3p, 5q, 11q, 13q, 17p in head and neck cancers, including oral cancers, has been reported [17–19]. These findings suggest that these genetic alterations may play a role in the development and progression of these tumours. In addition to these chromosomal changes, *p53* mutation has been found at increased incidence in squamous cell carcinoma of the head and neck [1].

HPV infection has been considered to be one of the aetiological factors in oral cancer. While there is a strong correlation of specific HPV types with development of both oral and anogenital cancer, it is generally accepted that the presence of HPV alone does not necessarily lead to cancer [20]. Other co-factors, particularly tobacco and alcohol, appear to be necessary and synergistic for full oncogenic expression in the oral cavity [21].

The candidate tumour suppressor gene, APC, is thought to play a key role at an early stage in colon carcinogenesis [22] and recent evidence shows LOH of the APC gene is also associated with other cancers, i.e. oesophageal [6], lung [7], breast [23] and gastric [24]. In squamous cell carcinomas of the head and neck (SCCHN), a high incidence of LOH of the APC gene has recently been reported [17, 25], Ah-See *et al.* [17] found 43% of SCCHN to contain LOH/imbalance at the 5q region, at or near the APC locus. They found this to be the most common region for loss of 50 markers in SCCHN. Uzawa and colleagues [25] also claimed that LOH of the APC gene (exon 11) was as high as 72.7% in the informative cases. These findings indicate that LOH of the APC gene may play a role in the development of SCCHN.

Our results of specific LOH are clearly distinguished from random genetic losses/imbalances due to generalised chromosomal instability, which normally occur in less than 20% of specimens [17]. In the present study, we found a high frequency of allelic loss of the APC gene, not only in the cases with oral invasive squamous cell carcinoma, but also in epithelial dysplasia. The latter is considered as a premalignant lesion at high risk of progressing to invasive carcinoma [26]. Histologically, epithelial dysplasias range from mild to moderate or severe. Epithelial dysplasias (80%) were just as likely as carcinomas *in situ* (67%) and invasive carcinomas (50%) to show LOH of APC. This strongly suggests that LOH of APC occurs as an early event during oral tumorigenesis and this allelic loss remains with progression from benign to invasive carcinoma. This finding is consistent with a previous report that APC mutations play a major role in the early development of colorectal carcinomas and can be detected in the earliest tumour that could be analysed [22].

Unlike previous publications in which investigators used microsatellites for detecting LOH of APC from fresh tissues or cultured cells [6, 7, 17–19, 25], we examined LOH of APC using degraded DNA extracted from formalin-fixed, paraffin-embedded tissues. 9 out of 14 cases (64%) were shown to contain LOH in this study, which is not statistically different

from the results of previous investigations of LOH of APC in fresh cells, which range from 43 to 72.7% [17, 22, 25, 27, 28]. Our findings also are supported by a previous report [29] that the PCR-based technique is a rapid, specific and efficient method of screening for LOH from formalin-fixed tumour tissues. Thus, it could be used for large retrospective and epidemiological investigations.

Using PCR techniques, HPV DNA has been detected in 50% of oral lesions and HPV 16 is the dominant type in this study. More than half of the HPV-positive cases with oral carcinoma (5/9, or 56%) demonstrated both HPV DNA and LOH of the APC gene. A similar result was found in our line of normal oral epithelial cells immortalised by a viral oncogene, HPV 16 E6/E7 (Dr D. Oda, University of Washington). This cell line has exhibited significant progression of chromosomal abnormalities by cytogenetic analysis (Dr D. Oda, University of Washington). One of the common chromosomal abnormalities was in the 5q region, at or near the APC gene. This change was also confirmed by our RFLP study, i.e. LOH at exon 11 of the APC gene was detected. These findings suggest a strong correlation between HPV infection, particularly HPV 16, and LOH of the APC gene in oral squamous cell carcinomas. While it seems probable that the co-operation of another carcinogenic factor such as tobacco or alcohol is usually necessary for full tumorigenic transformation, HPV probably does not initiate or cause changes but allows them to occur spontaneously with passaging or from carcinogenic insult. The APC gene may provide a useful predictive marker for the development of oral SCC, since loss of heterozygosity is seen at the early stages of benign oral epithelial changes and early dysplasia.

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