Loss of heterozygosity at 9q33 and hypermethylation of the DBCCR1 gene in oral squamous cell carcinoma

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Oral cancer comprises about 3% of all newly diagnosed cancer cases in the Western countries. Despite advances in therapy, the 5-year survival rate after diagnosis is still poor and remains ~50% (Landis et al., 1999; Silverman, 2001). Clinically, oral carcinomas often develop in a two-step process. The first step is characterised by the appearance of potentially malignant lesions such as leukoplakias and erythroplakias, and the second step is characterised by the development of carcinomas. However, clinical and histopathological features are insufficient measures for predicting the prognosis of potentially malignant lesions (Warnakulasuriya, 2000, 2001). Furthermore, a recent study indicated that clinically and histologically normal mucosa adjacent to tumours may harbour patches of genetically altered cells (Braakhuis et al., 2003). It is, therefore, important to find molecular markers for identifying the minor fraction of oral lesions that will develop into carcinoma.

Loss of heterozygosity (LOH) at multiple chromosome regions and genetic and epigenetic alterations of several proto-oncogenes and tumour suppressor genes have been demonstrated in oral carcinomas, including alterations of the TP53, p16, p15, MGMT, E-cadherin genes and RAS (Califano et al., 1996; Partridge et al., 1999; Williams, 2000; Ogi et al., 2002; Viswanathan et al., 2003). In addition, our previous study showed that hypermethylation of the ABO gene promoter was associated with loss of expression of A/B antigen in approximately one-third of oral squamous cell carcinomas (Gao et al., 2004). LOH at 9q34, in which the ABO gene is located, was also a frequent event in these tumours. However, a number of tumours from AO and BO heterozygotes showed deletion of the O allele, which does not encode a functional glycosyltransferase, suggesting the existence of an additional tumour suppressor gene on chromosome 9q. The DBCCR1 (deleted in bladder cancer chromosomal region candidate 1) gene at chromosome 9q33 was identified as a putative tumour suppressor gene that is frequently targeted by hypermethylation in transitional cell carcinomas of the bladder (Habuchi et al., 1997, 1998, 2001; Nishiyama et al., 1999). There are, at present, no reports of DBCCR1 alterations in other cancers. The aim of this study was to examine for LOH at the 9q33 region and determine the methylation status of DBCCR1 in oral squamous cell carcinomas and potentially malignant oral lesions.

MATERIALS AND METHODS

Sample preparation

Surgical specimens of oral lesions were obtained from School of Dentistry, National Yang-Ming University, Taipei, and Odense University Hospital, Denmark. The median age of the patients was 60 years (range 35–89 years); there were six women and 32 men. The materials included unfixed frozen tissues from 34 patients with oral squamous cell carcinoma and four patients with potentially malignant lesions (leukoplakia with epithelial dysplasia). A
laser microdissection system (PALM) was used to separate tumour cells or leukoplakia epithelium from normal connective tissue. In seven cases, tumour-adjacent epithelium was isolated as well. DNA was extracted by routine procedures using the DNeasy Kit (Qiagen). Informed consent and approval by the Ethics Committee were obtained according to Danish legislation.

LOH analysis

DNA from tumour or leukoplakia lesions and matched normal tissues was screened for LOH at the 9q33 region using the three microsatellite markers, D9S195, D9S1872 (http://www.gdb.org) and 9-11407. The latter marker was designed by one of us (HE) and is located at 300 kb upstream of exon 1 of the DBCCR1 gene, according to GenBank accession no. AF027734). The primers for the unmethylated reaction were 5'-TTATGTTGTAATTGTGTTGTT-3' and 5'-CAACTCA-CATCCAAAAACACACAC-3', which amplify a 269-bp product (positions 15–283), and the primers for the methylated reaction were 5'-TTTGAATTGTGTTGTTGTT-3' and 5'-TCCGAACAC-GAGCGAAA-3', which amplify a 253-bp product (positions 22–274). PCR was carried out using the HotStarTaq Kit (Qiagen); the annealing temperatures for the unmethylated and methylated reactions were 60 and 62°C, respectively. Primer sequences and reaction conditions for MS-PCR analysis of the ABO gene promoter were as described (Kominato et al., 1999; Gao et al., 2004). The PCR products were resolved on 2% agarose gels. DNA treated with SssI methyltransferase (New England Biolabs) served as the methylated control.

For methylation-specific melting-curve analysis (MS-MCA) of DBCCR1, the primers were 5'-GGGAGGTAGGGAGGTAGG-3' and 5'-AAAATCCCTAACTCCTAAACACACAC-3', which amplify a 117-bp product (positions 127–243). PCR and subsequent MCA were carried out as previously described (Worm et al., 2001) using the LightCycler (Roche) and the FastStart DNA Master SYBR Green I Kit (Roche). Reactions were started by initial denaturation at 95°C for 10 min, followed by cycling at 95°C for 10 s, a transition from 72 to 66°C at 0.5°C cycle–1 for 10 s and 72°C for 20 s. Melting-curve analysis was performed immediately after amplification by measuring the fluorescence of SYBR Green I during a linear temperature transition from 70 to 95°C at 0.05°C s–1. Fluorescence data were converted into melting peaks by the LightCycler software.

Table 1  Hypermethylation of DBCCR1 and LOH at 9q33 in oral squamous cell carcinomas and leukoplakias with dysplasia

| Case # | Sex | Age | DBCCR1 | ABO | D9S1872 | D9S195 | 9-11407 |
|--------|-----|-----|--------|-----|---------|--------|---------|
| Carcinomas | | | | | | |
| 1 | CT5 | M | 54 | + | – | – | – | + |
| 2 | CT6 | M | 56 | – | – | – | – | – |
| 3 | CT7 | M | 60 | + | – | – | – | – |
| 4 | CT8 | M | 62 | – | – | – | – | – |
| 5 | CT10 | M | 57 | – | + | NA | NA | NA |
| 6 | CT11 | M | 57 | – | – | – | – | – |
| 7 | CT12 | M | 50 | – | – | – | – | – |
| 8 | CT14 | M | 50 | – | – | – | – | – |
| 9 | CT15 | M | 53 | – | – | – | – | – |
| 10 | CT16 | M | 37 | – | – | – | – | – |
| 11 | CT17 | M | 57 | – | – | – | – | – |
| 12 | CT18 | M | 65 | + | + | – | – | – |
| 13 | CT19 | M | 58 | – | – | – | – | – |
| 14 | CT20 | M | 54 | – | – | – | – | – |
| 15 | CT21 | M | 71 | – | – | – | – | – |
| 16 | CT22 | M | 65 | – | – | – | – | – |
| 17 | CT23 | M | 35 | +* | +* | – | – | – |
| 18 | CTGx | F | 57 | + | + | + | + | + |
| 19 | 30365 | M | 66 | + | – | NA | NA | NA |
| 20 | 19395 | M | 61 | + | – | – | – | + |
| 21 | 25941 | M | 65 | + | – | – | – | + |
| 22 | 15374 | M | 69 | + | – | – | – | + |
| 23 | 18034 | M | 76 | – | – | NA | NA | NA |
| 24 | 31572T1 | F | 55 | + | + | – | – | + |
| 25 | 31572T2 | | | + | + | + | + | + |
| 26 | 33379 | M | 84 | – | – | – | – | – |
| 27 | 28753 | F | 52 | – | – | – | – | + |
| 28 | 19274 | M | 61 | – | – | + | + | + |
| 29 | 2132 | M | 58 | +* | + | – | – | – |
| 30 | 1592 | F | 71 | – | – | – | – | – |
| 31 | 17093 | M | 60 | – | – | + | + | + |
| 32 | 29627 | M | 61 | – | – | – | – | – |
| 33 | 21394 | M | 69 | + | – | – | – | + |
| 34 | 33103 | M | 69 | – | – | – | – | – |

Leukoplakias with dysplasia

| Case # | Sex | Age | DBCCR1 | ABO | D9S1872 | D9S195 |
|--------|-----|-----|--------|-----|---------|--------|
| 35 | 6042 | F | 60 | – | – | – | – |
| 36 | 24710 | M | 54 | – | – | – | – |
| 37 | 24722 | M | 53 | + | – | – | – |
| 38 | 16050 | M | 51 | – | – | – | – |

NA = no available information; T1 = well-differentiated tumour cells adjacent to normal epithelium; T2 = poorly-differentiated tumour cells far away from normal epithelium.

Table 1  Hypermethylation of DBCCR1 and LOH at 9q33 in oral squamous cell carcinomas and leukoplakias with dysplasia

Methylation analysis

Genomic DNA was treated with sodium bisulphite as described previously (Clark et al., 1994). For methylation-specific PCR (MS-PCR) analysis of the DBCCR1 promoter (GenBank accession no. AF027734), the primers for the unmethylated reaction were 5'-TTATGTTGTAATTGTGTTGTT-3' and 5'-CAACTCA-CATCCAAAAACACACAC-3', which amplify a 269-bp product (positions 15–283), and the primers for the methylated reaction were 5'-TTTGAATTGTGTTGTTGTT-3' and 5'-TCCGAACAC-GAGCGAAA-3', which amplify a 253-bp product (positions 22–274). PCR was carried out using the HotStarTaq Kit (Qiagen); the annealing temperatures for the unmethylated and methylated reactions were 60 and 62°C, respectively. Primer sequences and reaction conditions for MS-PCR analysis of the ABO gene promoter were as described (Kominato et al., 1999; Gao et al., 2004). The PCR products were resolved on 2% agarose gels. DNA treated with SssI methyltransferase (New England Biolabs) served as the methylated control.

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Figure 1  LOH analysis of 9q33 in oral squamous cell carcinomas. T, tumour; N, normal tissue; T1, well-differentiated tumour cells adjacent to normal epithelium; T2, poorly-differentiated tumour cells far away from normal epithelium. Arrows indicate LOH.
(Ver.3.39) by plotting the negative derivative of fluorescence over temperature vs. temperature (−dF/dT vs. T).

**Statistical analysis**

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

**RESULTS**

**LOH analysis of chromosome 9q33**

LOH analysis of the 9q33 region using three microsatellite markers showed allelic loss in 10 of 31 (32%) informative cases of oral squamous cell carcinoma (Table 1; see Figure 1 for examples). Among these, four showed LOH at D9S1872, six at D9S195, and seven at 9-11407. Notably, three cases showed LOH at 9-11407 located ~300 kb upstream of *DBCCR1*, but retention of D9S195 located in intron 1 of *DBCCR1*. In one case, in which DNA was isolated from both well- and poor-differentiated tumour cells from the same tumour, LOH at D9S195 was found in both populations, but only the poor-differentiated tumour cells showed LOH at 9-11407 and D9S1872 (Figure 1). LOH at D9S1872 was also found in one of four leukoplakias with dysplasia. No LOH was found in epithelial tissues adjacent to the tumours.

**Methylation analysis**

Hypermethylation of the *DBCCR1* gene promoter was present in 15 out of 34 (44%) oral squamous cell carcinomas, as determined by MS-PCR analysis (Table 1; see Figure 2 for examples). In three out of seven cases, *DBCCR1* hypermethylation was also found in tumour-adjacent tissues, including two hyperplastic and one histologically normal epithelium. To further characterise *DBCCR1* methylation patterns in oral carcinomas and to exclude possible false-positive MS-PCR results, all samples showing a positive signal for methylated *DBCCR1* alleles using MS-PCR were also examined using MS-MCA (Figure 2). Aberrant methylation was confirmed in all cases. However, in one case (#31572), well- and poor-differentiated tumour cells isolated from the same lesion showed different methylation patterns (Figure 2). Hypermethylation of the *DBCCR1* gene was also found in two of four leukoplakias with dysplasia, none of which showed LOH at 9q33 (Table 1).

Concomitant LOH at 9q33 and hypermethylation of the *DBCCR1* gene were found in seven carcinomas (*P* = 0.057); however, this...
and neck carcinomas have demonstrated LOH involving the 9q32-
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as a candidate tumour suppressor (Habuchi

DBCCR1

Methylation analysis of the

DBCCR1

frequently involved microsatellite marker D9S195, which is located

suppressor gene involved in oral carcinogenesis. Notably, LOH

suppressor genes known to be targeted by promoter hypermethy-

different techniques showed aberrant hypermethylation in 44% of

DISCUSSION

Substantial evidence suggests that aberrant hypermethylation of

promoter CpG islands may constitute an alternative mechanism to

intragenic mutations and deletions for inactivation of tumour

suppressor genes (Worm and Guldberg, 2002; Nephew and Huang,

2003). Hypermethylation of the DBCCR1 gene as well as LOH and

homozgyous deletions at the DBCCR1 locus have been shown to be

frequent events in bladder cancer (Fujiwara et al, 2004), but there was no correlation between the

DBCCR1 and ABO hypermethylation events (P = 0.11; Tables 1 and 2).

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Table 2 Correlation analysis of LOH at 9q33 and DBCCR1 and ABO

hypermethylation

| Methylation of DBCCR1 | No | M (%) | U (%) | P-value |
|-----------------------|----|-------|-------|---------|
| Methylation of ABO    | M  | 11    | 7 (63.6) | 4 (36.4) | 0.11 |
|                       | U  | 23    | 8 (34.8) | 15 (65.2) |       |
| LOH at 9q33           | +  | 10    | 7 (70.0) | 3 (30.0) | 0.057 |
|                       | −  | 24    | 8 (33.3) | 16 (67.7) |       |
| at 9-11047            | +  | 7     | 6 (85.7) | 1 (14.3) |       |
|                       | −  | 27    | 9 (33.3) | 18 (66.7) | 0.018 |
| at D9S1872            | +  | 4     | 2 (50.0) | 2 (50.0) |       |
|                       | −  | 30    | 13 (43.3) | 17 (56.7) | 0.60  |
| at D9S195             | +  | 6     | 4 (66.7) | 2 (33.3) |       |
|                       | −  | 28    | 11 (39.3) | 17 (60.7) | 0.22  |

M = hypermethylation; U = no methylation.

correlation was only significant for microsatellite marker 9-11407,

which is located ~ 300 kb upstream of exon 1 of the DBCCR1 gene

(Table 2). Hypermethylation of ABO was found in 11 out of 34

(32%) tumour samples and in three adjacent epithelia (Table 1)

(Gao et al, 2004), but there was no correlation between the

DBCCR1 and ABO hypermethylation events (P = 0.11; Tables 1 and 2).

Genetic and epigenetic alterations of the DBCCR1 gene were not

restricted to oral carcinomas. LOH at 9q33 was also demonstrated

in one of four patients with severe epithelial dysplasia, and

DBCCR1 hypermethylation was present in another two of these

cases. Abrupt hypermethylation levels were found even in tumour-

adjacent epithelia with no histopathological evidence of

malignancy, suggesting that it may represent an early event in oral

malignant development. In bladder cancer, field carcinisation has

been attributed to age-related methylation of

DBCCR1

epithelium (Habuchi et al, 2001). The presence of DBCCR1

hypermethylation in oral tumour-adjacent epithelium is of great

interest and should be further investigated in order to elucidate

whether local recurrence or field carcinisation in oral cancer

patients can be explained, at least in some cases, by the existence

of a DBCCR1-hypermethylated field in histologically normal

epithelium.

In the present work, we were not able to detect any divergence

between the two groups of patients, which were of different ethic

origin and exposed to different environmental factors (betel/

tobacco and alcohol/tobacco). However, the material is too limited

to make any firm conclusions. In a new prospective study, we are

investigating whether the methylation and LOH status have a

clinical significance.

There is still little information about the possible function of the

DBCCR1 gene in carcinogenesis. Unresolved issues include the

apparent lack of DBCCR1 expression in most normal tissues and the

unclear correlation between hypermethylation and transcriptional

silencing of this gene (Habuchi et al, 1998), questioning the role

of DBCCR1 as a tumour suppressor in the homeostasis of

normal cells. Previous cell cycle studies suggested that DBCCR1

growsuppressing and antiproliferative activities mediated via

modulation of the G1 checkpoint. Overexpression of DBCCR1

caused a slower G1 transition rather than G1 arrest and did not

affect apoptosis (Nishiyama et al, 2001). Although these functional

studies and the high rate of DBCCR1 hypermethylation in oral

squamous cell carcinomas support the candidacy of DBCCR1 as a

tumour suppressor at 9q33, additional studies are required to

unravel its possible role in oral malignant development.
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