LOH at chromosome 9q34.3 and the Notch1 gene methylation are less involved in oral squamous cell carcinomas

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BACKGROUND: Previous studies of oral carcinomas have shown that both loss of heterozygosity (LOH) and hypermethylation at chromosome 9q33 to 9q34.2 are frequent. The present study investigates the frequency of Notch1 gene methylation and LOH at 9q34.3 region.

METHODS: Gene promoter hypermethylation of the Notch1 gene was analysed by methylation-specific PCR and LOH was analysed using microsatellite markers.

RESULTS: We found LOH at 9q34.3 in three patients and methylation of the Notch1 gene only in two patients with oral carcinoma.

CONCLUSION: Comparing with the alterations at 9q33 to 34.2 regions, LOH at 9q34.3 and methylation of the Notch1 gene was less involved in oral squamous cell carcinomas.


Keywords: loss of heterozygosity; methylation; Notch1; oral carcinoma

Introduction

Loss or deletion of chromosome regions is a frequent event in oral carcinoma, but only a few studies have shown changes at chromosome 9q (1), although this is frequently found in other tumours, especially in bladder cancers (2, 3). We have previously shown loss of heterozygosity (LOH) at 9q33 and 9q34.1-2 regions in oral carcinoma, related to the DBCCR1 and ABO genes (4, 5). The frequent LOH of chromosome 9q can be essential for the tumour development as it may involve in several tumour suppressor genes, but it has been argued that it is not an important event during tumour progression as loss of chromosome 9q sequences occurs in a completely random fashion (6). As it has been hypothesized that hot-spots for LOH in cancers are also frequent targets for aberrant hypermethylation (7), inactivation of tumour suppressor genes may be caused by combination of LOH and hypermethylation, as shown for the DBCCR1 and ABO genes in oral carcinomas (4, 5). In the present study, we investigate the methylation status of Notch1 (located on 9q34.3) gene promoters and LOH at 9q34.3 to the telomeric region in oral carcinomas.

Patients and methods

Samples and DNA preparation

The materials included unfixed frozen tissues from 34 patients with oral squamous cell carcinoma, which have previously been investigated for LOH and gene promoter hypermethylation of ABO and DBCCR1 genes (4, 5).

Cell lines

SCC4, SCC9 and SCC25 cell lines derived from squamous cell carcinomas of the human tongue (ATCC, Rockville, MD, USA), were cultured as recommended by ATCC. DNA and cytoplasmic RNA from three cell lines were obtained, using DNeasy Kit and RNeasy Mini Kit (Qiagen, Albertslund, Denmark).

LOH analysis

DNA from tumour and corresponding normal tissue was screened for LOH using three microsatellite markers at 9q34.3 to telomeric region, D9S158, D9S905 and D9S2168, comparing with two markers at the region between 9q33.3 and 9q34.1, D9S1818 and D9S159 (http://www.gdb.org). LOH and microsatellite instability (MSI) was identified as described elsewhere (4, 5).

Methylation analysis

Genomic DNA was treated with sodium bisulphite as described previously (4, 5). Methylation-specific PCR (MSP) was performed for methylation analysis of the Notch1 promoter, the primers were within a CpG island
region, for the unmethylated reaction were, 5'-GTTTTG TTTTTTTATGTGTTTGG-3' and 5'-CAAACACC TAAAACACTTCTCTATT-3', which amplify a 168-bp product (positions -630 to -463), and the primers for the methylated reaction were 5'-GTTTCGTTTTTTTTATT TCGTTTC-3' and 5'-GAACGCGCTAAAACACTTCTCGTTT-3' which amplify a 167-bp product (positions -630 to -464). The annealing temperatures for the unmethylated and methylated reactions were 57°C and 55°C, respectively. DNA treated with SsIl methyltransferase (New England Biolabs, Beverly, MA, USA) served as the methylated control. To confirm the methylation results, methylated PCR product was purified by Gel Purification Kit (Qiagen), then purified DNA was subcloned into vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) and eight positive clones were sequenced (8).

RT-PCR
Reverse transcription reaction was performed with 2 µg DNA-free RNA using First-strand cDNA Synthesis Kit (Amersham Biosciences, Little Chalfont, UK) and RT-PCR was performed (8). The primers for the Notch1 gene were Notch1-F, 5'-CAGGCAATCGAGGACT ATG-3', Notch1-R, 5'-GCAGTGCCGTTTTGTTGT TCTCACAG-3' (430 bp product, 1069–1498 bp). The GAPDH gene was amplified as an internal control. The annealing temperature was 55°C for Notch1 and 62°C for GAPDH.

Results and discussion
In the present study, hypermethylation of the Notch1 gene promoter was demonstrated in two carcinoma samples but not in corresponding normal tissues. The pattern was confirmed by PCR product direct sequencing. One showed full methylation at all 15 CpGs by collecting sequencing data from eight clones, the other one showed partly methylation at 6/15 of CpGs located at both upstream/downstream regions (for example, see Fig. 1). LOH was found in three of 31 (10%) informative cases of oral carcinomas at 9q 34.3 region, where the Notch1 gene located, using three microsatellite markers (Table 1). In contrast, LOH at 9q33.3 to 34.1 region using two microsatellite markers was found in nine of 31 (29%) (Table 1), and shown similar frequency to adjacent regions at 9q33 and 9q34.1-2, which hypermethylation of the promoters of DBCCR1 and ABO genes also often occurred (4, 5).

Notch1 is a gene located at the region of 9q34. The Notch gene family codes for proteins that are involved in multiple pathways that control both cell proliferation and apoptosis and in this way is involved in malignant progression (9). It has further been demonstrated that the Notch1 gene may function as a tumour suppressor gene in mouse skin (10), and a decreased expression level for Notch1, 2 and 3 and Hes1, a known target for the Notch signal, was noticed (11).

In order to investigate whether such a deregulation of Notch signalling in oral cancer results from a methylation mechanism, we analysed the gene promoter methylation and mRNA expression of Notch1 gene in three oral carcinoma cell lines. The results from SCC4 and SCC25 showed an inverse correlation between methylation status and expression level, suggesting an epigenetic mechanism in the regulation of Notch1 gene in oral carcinoma; but such correlation was not found in SCC9 cells indicating mechanisms other than methylation may also involve in the regulation (Fig. 2).

The present results combined with previous studies revealed that when we have detected hypermethylation of one of the sites investigated, there is a 50% chance of the same site showing LOH (4, 5). Similar findings have been demonstrated in both studies of gastric cancer and oesophageal cancer, which suggests that LOH and hypermethylation are active in conjunction with carcinogenesis (12, 13). We also could demonstrate that both LOH and hypermethylation were found with a decreased tendency from 9q33 to the telomeric region, pairwise matched genes of DBCCR1, ABO and Notch1. No hypermethylation of the TSC1 (the tuberous sclerosis) gene promoter was found in the patients with oral carcinomas and normal tissues, although it locates...
Table 1  LOH at chromosome 9q33.1 to 9q34.3 in oral carcinomas

<table>
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<th>Markers</th>
<th>Position (cM)*</th>
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<th>Distance</th>
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<th>CTGx</th>
<th>17093</th>
<th>CT8</th>
<th>CT15</th>
<th>19274</th>
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*Three markers started from 129.74 cM (9q33.1) have shown LOH from 15.4% to 26.9% in a previous study (6).

Seven markers from 144.67 to 150.92 cM (9q34.1–2) have shown LOH from 4% to 28% in a previous study (7).

References


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