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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.

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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 hypermethyaltion, 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.

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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 TTTTTTTTATTTTGTTTTG-3′ and 5′-CAAACACC TAAAACTACTTCTCATT-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′-GAACGCCTAAAACTACTTC TCGTT-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 *Sss*I 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′-CAGGCAATCCGAGGACT ATG-3′, Notch1-R, 5′-GCAGTCAGGCGTGTTGT 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 locate 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 TSC_1 (the tuberous sclerosis) gene promoter was found in the patients with oral carcinomas and normal tissues, although it locates

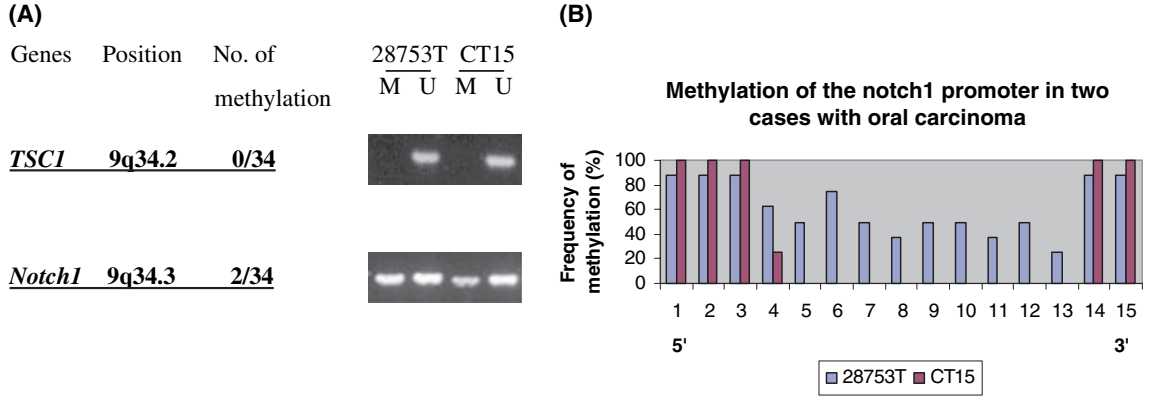

Figure 1 Methylation of the *Notch1* gene was found in two cases of oral carcinomas, but no methylation was found for the *TSC1* gene. (A) Genomic DNA was treated with sodium bisulphite and PCR-amplified with primer pairs specific for methylated (M) and unmethylated (U) alleles. The present data showed that no methylation was found in the *TSC1* gene (the primer details can be provided as requested), and two tumours were found methylation in the *Notch1* gene, but not matched normal tissues (not shown). (B) Methylated DNA product of the *Notch1* gene from two tumour samples was directly sequenced to confirm the methylation status, one showed full methylation at all 15 CpGs (28753T), another one showed partly methylation at 6/15 of CpGs locating at both upstream/downstream regions (CT15).

Table 1 LOH at chromosome 9q33.1 to 9q34.3 in oral carcinomas

Markers	*Position*	*Distance (cM)**	*Case number* CT5	CTGx	17093	CT8	CT15	19274	CT18	19395	31572	28753	27088	CT22	*LOH INF.*	*Percentage (%)*
a																
D9S1881	9q33.3	135.85													4/23	17.4
D9S159	9q34.11	142.51													8/24	33.3
b																
D9S158	9q34.3	161.71													2/23	8.69
D9S905	9q34.3	163.84													1/15	6.66
D9S2168	9q34.3	168.98													1/21	4.76

LOH, loss of heterozygosity – ■; MSI, microsatellite instability – ▨; NI, non-informative – ▬; ROH, retention of heterozygosity – □; LOH INF., case numbers with LOH or MSI/Total informative case numbers.
[a]Three markers started from 129.74 cM (9q33.1) have shown LOH from 15.4% to 26.9% in a previous study (6).
[b]Seven markers from 144.67 to 150.92 cM (9q34.1–2) have shown LOH from 4% to 28% in a previous study (7).

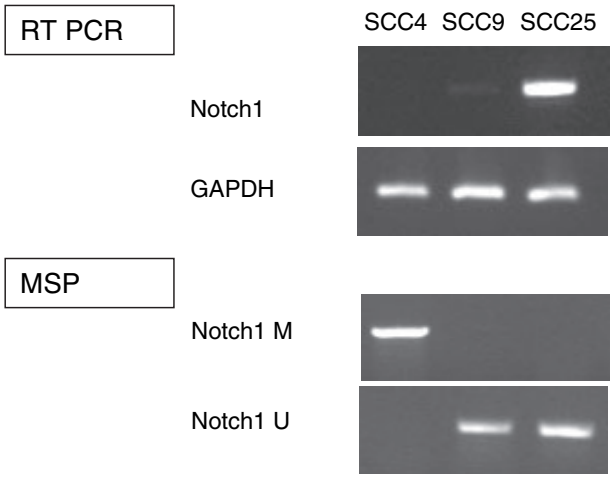

Figure 2 Correlation between *Notch1* methylation and mRNA expression level in three oral squamous cell carcinoma cell lines. In order to see whether methylation of the *Notch1* gene promoter correlated with its expression, RT-PCR was performed for detecting the Notch mRNA level, GAPDH level was as internal control. By comparing the results of MSP (bottom panel) and RT-PCR (top panel), it showed that methylation of the *Notch1* gene promoter was inversely correlated with *Notch1* mRNA level. Full methylation was found in SCC4 but no detectable level of mRNA was shown, no methylation in SCC25 which showed higher *Notch1* mRNA expression. Such correlation was not seen in SCC9 cells.

closed to the *ABO* gene (Fig. 1). We found that LOH appeared at a lower frequency in tongue carcinoma compared with other sites of the oral mucosa, 4/10 vs. 21/24 ($P < 0.01$), suggesting the site-specific mechanism for oral tumour cell development, which has been previously described as a theory of site-specific prevalence of genetic alterations in the studies of head and neck squamous cell carcinomas (14, 15).

In conclusion, as the frequency of LOH at 9q34.3 region and hypermethylation of the *Notch1* gene are much lower than 9q33 to 9q34.2 region, it is unlikely that alteration of *Notch1* gene plays a major role in oral carcinogenesis *in vivo*.

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