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Genetic and epigenetic alteration profiles for multiple genes in salivary gland carcinomas

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Summary As combinations of genetic and/or epigenetic alterations occurring during salivary gland carcinogenesis are largely unknown, we here analyzed 36 salivary gland carcinomas (SGCs) for changes in *INK4a/ARF*, *RB1*, *p21*, *p27*, *PTEN*, *p53*, *MDM2* and *O^6-MGMT* genes using methylation specific PCR (MSP), loss of heterozygosity (LOH) assays and mutational analysis with immunohistochemistry (IHC), as well as histone H3 and H4 acetylation status. The *RB1* gene was found to be the most frequently methylated (41.7% of cases), while methylation of *$p27^{Kip1}$* and *O^6-MGMT* were less frequent 8.3% and 5.6%, respectively). Two other genes, *$p21^{Waf1}$* and *PTEN*, were unmethylated in the SGCs examined. *RB1* methylation significantly correlated with loss of expression as determined by IHC ($P = 0.03$), and also a poor prognosis ($P = 0.02$). *p53* mutations were found in 8 cases (22.2%), coupled with *$p14^{ARF}$* hypermethylation in two cases. LOH in *INK4a/ARF* and the *RB1* locus was observed in 33.3% and 28.6% of the lesions, respectively. There was no correlation between 9p21 LOH and methylation of the *INK4a/ARF* gene. Promoter hypermethylation of *RB1* coupled with LOH was evident in three samples immuno-negative for *RB1*. Acetylation of histone H3 and H4 was detected in 6 and 5 cases, respectively. These findings indicate that epigenetic silencing of tumour suppressor genes via promoter hypermethylation might be crucial for salivary gland carcinogenesis, particularly in the *RB1* gene. Thus epigenetic events including methylation and acetylation as well as genetic alterations may have important contributions.

Introduction

Salivary gland carcinomas (SGCs) are relatively uncommon malignancies with widely variable

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histopathologic and biologic characteristics.[1] This makes it difficult to determine the prognosis and select the optimal therapeutic modality.[2] However, it is generally accepted that the outcome for patients with SGCs depends on the site, histology, the extent of disease and completeness of surgery and/or irradiation therapy.[3,4]

Recent studies have clearly shown that development and progression of human malignancies is associated with accumulation of alterations in proto-oncogenes and tumour suppressor genes (TSGs), and this appears also to be the case for SGCs.[5–7] The pathogenesis of human cancers is a heterogenous process involving several pathways, and it has been proposed that the tumour genotype may affect the clinical behavior and the prognosis.[8,9]

Epigenetic alterations, such as DNA methylation and histone acetylation, are also considered to be important for human carcinogenesis. DNA hypermethylation is now known to be involved in the tumour development and progression and methylation profiling of TSGs is a powerful diagnostic tool for early detection and prognosis with various types of cancers.[10] Hypermethylation in the promoter regions of *p16*, *p15*, *p14*, *GSTP1*, *E-cadherin* and *VHL* genes has been well described[11–13] and histone acetylation is closely associated with transcriptional activation of various genes. Deacetylation possibly induces a tight chromatin structure that is inaccessible to transcription factors[14] and recently, a histone hypoacetylated status was detected in invasive and metastatic cancer cells and inhibition of histone deacetylase activity by trichostatin A found to induce apoptosis and inhibit cell growth of gastric cancer cell lines.[14] Therefore, the identification of genes susceptible to hypermethylation and histone acetylation status may provide insights into cancer development.

Little information has so far been reported regarding genetic and epigenetic alteration in SGCs. We therefore here examined 36 cases using methylation-specific PCR (MSP), loss of heterozygosity (LOH) assays, mutational analysis and immunohistochemistry (IHC) in the *RB1*, $p21^{Waf1}$, $p27^{Kip1}$, *PTEN*, *p53* and O^6-*MGMT* tumour associated genes. We also assessed the levels of histone H3 and H4 acetylation by IHC.

Materials and methods

Tumour samples and DNA extraction

Thirty-six tumour specimens were obtained from patients undergoing radical surgery or biopsy at the Department of Oral and Maxillofacial Surgery at Nara Medical University. Tumours were graded according to the WHO classification criteria.[15] The samples were from 17 adenoid cystic carcinomas (ACC), 7 mucoepidermoid carcinomas (MEC), 3 squamous cell carcinomas (SCC), 3 acinic cell carcinomas (Acinic CC), 2 carcinomas in pleomorphic adenomas (Ca in PA), 2 adenocarcinomas (ADC), 1 salivary duct carcinoma (SDC), and 1 basal cell adenocarcinoma (BCA). Sections from each sample were fixed in 10% neutral buffered formalin, paraffin-embedded, and cut at 5 μm for hematoxylin and eosin (H & E) staining. Semi-serial sections were cut at 3 μm and mounted unstained for IHC. DNA was extracted from paraffin-embedded tissues as previously described.[16]

Methylation-specific PCR (MSP)

DNA methylation status in the CpG islands of the *RB1*, $p21^{Waf1}$, $p27^{Kip1}$ *PTEN* and O^6-*MGMT* genes was determined by MSP. Sodium bisulfate modification was performed using a CpGenome™ DNA Modification Kit (Intergen, Oxford, UK) according to the manufacturer's protocol. The primer sequences and MSP conditions for *RB1*, $p21^{Waf1}$, $p27^{Kip1}$, *PTEN* and O^6-*MGMT* were as previously reported.[5,9,12,17] Amplified products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Loss of heterozygosity (LOH) assays for the INK4a/ARF and RB1 loci

The markers D9S157, D9S1748 and D9S171 were used for the *INK4a/ARF* gene, and D13S153 for the *RB1* gene locus. D9S157 marker lies teromeric to exon 3, D9S1748 lies between exon 1α and exon 1β, and D9S171 lies centromeric to exon 1β. The marker D13S153 is located in intron 2 of the *RB1* gene. The primers for specific chromosomal maps and sequences were obtained from the Whitehead Institute (http://www-genome.wi.mit.edu) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gor). A PCR reaction was carried out for 33 cycles of amplification at 60 °C annealing temperature, and amplified products were size-separated on an automated DNA sequencer (ABI 310 Genetic Analyzer, Applied Biosystems). A tumour was judged as positive for LOH when 40% of a target allele was lost. An LOH value of <0.60 indicates the tumour sample to show significant loss of the longer allele, whereas an LOH value of >1.7 indicates significant loss of the shorter allele.[16]