Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma

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Genetic alterations that lead to loss or changes in tumour-suppressor genes are known to contribute to oral carcinogenesis. Traditional molecular methods to detect such losses have relied on mutation analysis or deletion of the gene. However, epigenetic mechanisms could also contribute to silencing of tumour-suppressor genes. Methylation regions rich in CpG promoters prevent DNA transcription by changing the binding of histone complexes. The substantial contribution of methylation, specifically in oral squamous-cell carcinoma, is now being realised and investigated.

Lancet Oncol 2006; 7: 77–82

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Introduction

Squamous-cell carcinoma of the oral cavity has long been known to be the endpoint of many genetic changes (figure). A visible change in the histology of the oral mucosa is accompanied by mutations or loss of DNA. Various genetic events have been investigated in the context of mutations or disruptions in the DNA sequence, which lead to loss of function of tumour-suppressor genes or activate oncogenes. These cumulative changes can lead to cellular atypia and eventually cancer.[1]

Although many important genes and gene products have been identified through DNA changes, no single unifying pathway has been identified that accounts for all oral squamous-cell carcinomas. Rather, accumulation of many varied genetic modifications results in changes of crucial pathways that maintain cellular homoeostasis. Promoter methylation is an alternative form of gene silencing, which relies on epigenetic factors rather than direct DNA mutations.

Many regions rich in cytosine–guanine, also known as CpG islands, have been noted within the human genome. These islands are located upstream from the promoter region at the 5′ end of a gene, and up to half of all human genes seem to have CpG islands.[2] Three known DNA methyltransferases are known to have the ability to add methyl groups to the 5′ cytosine of a C–G dinucleotide. Addition of these methyl groups to the CpG islands silences genes by facilitating the association of methylated DNA with a methyl-binding complex. This methylation ultimately leads to histone deacetylation, which then prevents transcription of the subsequent gene.[2] Thus, gene products are not produced, even though the DNA coding sequence is maintained.

For many decades, methylation-induced gene silencing has been recognised as being needed for developmental regulation.[3] Methylation is involved in carcinogenesis, and many tumour-suppressor genes have been investigated in various tumours.[4–6] These studies suggest that methylation-induced gene silencing is as important as gene mutation or loss of heterozygosity in the progression to cancer.

Carcinogenesis might not be the result of just one mechanism of gene suppression. Rather than focus only on methylation of tumour-suppressor genes, many studies[7–10] have investigated the role of the combination of loss of heterozygosity and of methylation to establish the importance of each with the gene of interest. The incidence of promoter methylation is enhanced in those tumour suppressor genes that do not have loss of heterozygosity as a prominent mechanism. Because genes can be silenced through at least two mechanisms, promoter methylation might explain why studies have shown widely different results of methylation status, since loss of heterozygosity could vary within their study samples.

In this review, we discuss genes that have been implicated in oral squamous-cell carcinoma and the clinical implications and future possibilities.

Molecular techniques

The molecular techniques used to detect methylation have evolved from the Southern blot to more sensitive quantitative-PCR techniques. In the past, investigators used methylation-specific restriction enzymes followed

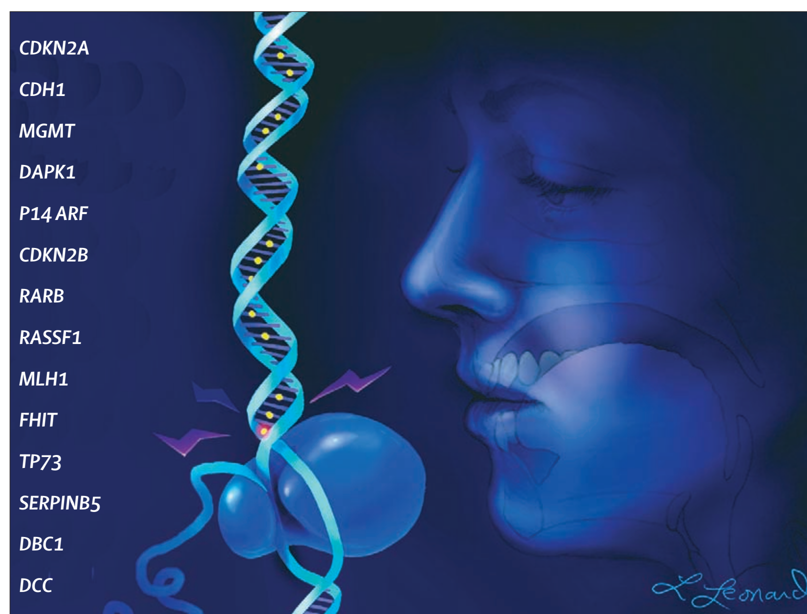

Figure: **Many genes are involved in development of oral cancer**

	Samples*	Samples methylated	Ref
CDKN2A			
PCR-based restriction assay	30	7 (23%)	7
Methylation-specific PCR	32	16 (50%)	8
Methylation-specific PCR	26†	15 (58%)	10
Methylation-specific PCR	50	14 (28%)	26
Methylation-specific PCR	80‡	26 (33%)	27
PCR-based restriction assay	96	28 (29%)	28
PCR-based restriction assay	99	23 (23%)	29
CDH1			
Methylation-specific PCR	60	40 (67%)	23
Methylation-specific PCR	48	41 (85%)	24
Methylation-specific PCR	32§	10 (31%)	25
Methylation-specific PCR	80‡	29 (36%)	27
PCR-based restriction assay	99	35 (35%)	29
PCR-based restriction assay	70¶	47 (67%), 5 (71%), 6 (67%)	30
Methylation-specific PCR	2||	0, 13 (72%)	31
MGMT			
Methylation-specific PCR	60	31 (52%)	23
Methylation-specific PCR	32§	10 (31%)	25
Methylation-specific PCR	48	12 (25%)	26
PCR-based restriction assay	99	41 (41%)	29
Methylation-specific PCR	10	4 (40%)	32
DAPK1			
Methylation-specific PCR	60	41 (68%)	23
Methylation-specific PCR	32§	6 (19%)	25
Methylation-specific PCR	46	7 (15%)	26
Methylation-specific PCR	80‡	19 (24%)	27
PCR-based restriction assay	96	7 (7%)	28
Methylation-specific PCR	10	5 (50%)	32

*Oral squamous-cell carcinoma (OSCC) unless otherwise specified. †Oral severe dysplasia. ‡Head and neck cancer samples, of which 37 are OSCC. §Head and neck cancer samples, of which 26 are OSCC. ¶7 recurrent OSCC, 9 lymph nodes. ||18 lymph nodes.

Table 1: **Promoter methylation status of well-described genes in primary oral cancer**

by Southern-blot analysis to probe for predicted fragments, which are produced by methylation-specific cutting of DNA fragments, to show the presence or absence of methylation.[11] This technique needed a large quantity of DNA and was not very sensitive. Similarly, PCR-based restriction assays took advantage of the fact that appropriately-located methylated cytosines would protect a segment of DNA from cleavage by methylation-specific restriction enzymes. Specific PCR primers were then designed to amplify across this region, and the presence of an amplifiable band of the appropriate length showed the existence of methylated DNA at the restriction site.[12]

New assays use sodium bisulphite treatment, a technique that deaminates non-methylated cytosines and converts them to uracil: methylated cytosines remain unconverted, and retain their original DNA sequence information. Thus, methylated genes can be differentiated from non-methylated genes by sequencing of promoter regions after sodium bisulphite treatment.[13] To extend this idea, investigators designed methylation-specific PCR primers that accounted for the altered sequence of the sodium bisulphite-treated DNA. Dual primer pairs were created so that researchers could ascertain the relative amounts of methylated and non-methylated strands in a semiquantitative way.[14] Real-time quantitative PCR was the next step, which has greater specificity with the use of a fluorescent probe, and allows calculation of a methylation index that shows the amount of methylated DNA in the sample.[15]

Other detection schemes look at genome-wide methylation, rather than focusing on specific primers and probes. These schemes include methylation-sensitive genome scanning,[16] restriction fingerprinting,[17] arbitrarily-primed PCR,[18] and representational difference analysis.[19] These techniques allow rapid screening of the whole genome, which provides information on global methylation and the discovery of new methylated genes.

A study[20] of 13 patients with metastatic head and neck cancer used genome-wide restriction landmark mapping to compare the methylation pattern of 1300 genes in matched samples of tumour and metastases. The investigators found that many of the loci methylated in the primary tumour were no longer methylated in the metastases, suggesting that methylation is a dynamic process. Furthermore, the methylation pattern differed between patients: some patients had low methylation overall and in others most of the genes investigated were methylated. This technique allows patterns of methylation to be investigated between patients with different clinical characteristics.

One widely used functional technique involves the treatment of cell lines with decitabine—a cytosine analogue that demethylates all genes.[21] Gene products that have been reactivated can then be identified because the methylation pattern differs before and after treatment. This technique is useful in confirming gene silencing when methylation is suspected on the basis of detection assays. However, such a process could reactivate many genes that have other subsequent effects, which could induce genes downstream from methylated targets that are not directly controlled by promoters with CpG-island methylation.

Methylated genes in oral cancer

Many genes in oral-cancer tissue have been tested for methylation. These tumour-suppressor genes all have a mechanistic basis for their role in carcinogenesis and are generally implicated in other tumour types. However, matched or paired healthy tissue should be tested in conjunction with affected tissue, since seemingly healthy tissue can have low rates of methylation, especially in patients who are smokers.[22] Tissue adjacent to tumours that is histologically normal[23–25] and premalignant lesions[10] can also have high levels of methylation of some genes, suggesting that methylation is an early event. Furthermore, it could be a way to assess margins that is even more sensitive than identification of cytological abnormalities. These findings also show that the definition of healthy control tissue can substantially affect their results and could help to explain the variability in methylation rate between studies.

Investigation of gene-methylation in tumours of the oral cavity is still quite new. Four important genes have been assessed in more detail (table 1): *CDKN2A*, *CDH1*, *MGMT*, and *DAPK1*.[7,8,10,23–32] Table 2 shows genes that are less well described but have been investigated in the context of oral carcinogenesis.[9,10,25,27–29,33–35]

CDKN2A

CDKN2A is located on chromosome 9p21 and was first identified as a putative tumour-suppressor gene. The gene was localised by microsatellite analysis, is involved in the retinoblastoma pathway to help control the cell cycle, and is commonly altered in head and neck cancer. *CDKN2A* was one of the first genes investigated in the head and neck that was associated with promoter methylation,[36,37] and is the most widely investigated in oral cancer. Several studies have looked at the effects of *CDKN2A* on oral squamous-cell carcinoma in primary tumours[7,8,10,23,25–29] and in cell lines.[7,8,28,38–40]

Data from cell lines showed that 17–43% of oral-cavity cancer cell lines have methylation of the *CDKN2A* promoter region. Furthermore, 23–67% of primary tumours have methylated *CDKN2A* (table 1), making methylation of this gene a very important component of marker panels for tumour screening and detection.

Timmermann and colleagues[38] showed that treatment of the cells with decitabine led to re-expression of *CDKN2A*, confirming that this gene is under the control of promoter methylation. Because the treated cells subsequently underwent senescence, simple demethylation of *CDKN2A* could help to re-establish cell-cycle control. However, such findings in cell lines should be interpreted with caution, in view of the artificial nature of the cellular milieu. Furthermore, studies have suggested that the gene-methylation status in cell lines could be changed after replication in culture conditions, leading to high levels of methylation throughout the genome.[41]

CDH1

CDH1 plays a part in cell–cell adhesion. When mutated or underexpressed, it leads to a greater probability of tumour invasion or metastasis. *CDH1* promoter methylation is found in 0–85% of oral-cavity tumours.[24,25,27,29–31,42,43]

Nakayama and colleagues[43] showed that 17 of 18 samples of primary oral cancer that did not express *CDH1* had methylated *CDH1*; one of five tumours that expressed *CDH1* did not have methylated *CDH1*.[43] These findings suggest that promoter methylation plays an important part in the silencing of *CDH1*. The 18 of 23 samples with methylated *CDH1* were higher than those from other reports in which none of the primary oral tumours showed *CDH1* methylation.[42] Part of this discrepancy might lie in the tumour stage, although both studies[42,43] seemed to have a mix of stages. Furthermore, the location of a tumour, even within the oral cavity, might be enough to determine the methylation pattern.

	Samples*	Samples methylated	Ref
DBC1			
Methylation-specific PCR	34	15 (44%)	9
P14ARF			
Methylation-specific PCR	26†	1 (4%)	10
Methylation-specific PCR	32‡	5 (16%)	25
PCR-based restriction assay	96	13 (14%)	28
CDKN2B			
PCR-based restriction assay	96	6 (6%)	28
PCR-based restriction assay	99	23 (23%)	29
RARB			
Methylation-specific PCR	32‡	15 (47%)	25
Methylation-specific PCR	5	5 (100%)	33
RASSF1			
Methylation-specific PCR	32‡	0 (0%)	25
Methylation-specific PCR	80§	6 (8%)	27
MLH1			
PCR-based restriction assay	96	0 (0%)	28
PCR based restriction assay	99	8 (8%)	29
P73			
Methylation-specific PCR	32‡	1 (3%)	25
DCC			
PCR-based restriction assay	96	16 (17%)	28
FHIT			
Sodium bisulphite then sequencing	29	8 (27%)	34
SERPINB5			
Sodium bisulphite then sequencing	12	3 (25%)	35

*Oral squamous-cell carcinoma (OSCC) unless otherwise specified.†Oral severe dysplasia. ‡Head and neck cancer samples, of which 26 are OSCC.§Head and neck cancer samples, of which 37 are OSCC.

Table 2: **Promoter methylation status in less-common genes in oral cancer**

Ogi and co-workers[28] noted that genes in samples from the tongue, lower gingival tissue, and the oral floor had different tendencies for methylation, further supporting the idea that methylation is dependent on many factors and is not a static state.

Two studies[30,31] have shown high rates of methylation in lymph-node metastases of primary oral cancers, suggesting that cells could develop the capacity for metastasis through the loss or decreased expression of *CDH1*.

MGMT

MGMT is a detoxifying agent of DNA adducts. It is important in preventing alkylation and, thus, could be predictive of chemosensitivity. In a large survey[44] of many tumour types, *MGMT* was methylated in up to 38% of primary colon cancers, gliomas, and head and neck cancers. In our survey of the published work, 25–52% of primary oral squamous-cell carcinomas had methylated *MGMT*.[23,25,26,29,32]

DAPK1

Death-associated protein kinase (*DAPK*) genes are tumour-suppressor genes involved in apoptosis. Early studies[45] in lymphoma and bladder cancer suggested that methylation of these genes was involved in abnormal gene silencing. *DAPK1* has also been implicated in metastatic lung cancer.[46] In oral cancers,[23,25–28,32] between 7% and 68% of primary tumours

have methylated *DAPK1*. Like *CDH1*, *DAPK1* has a wide range of prevalence, which could cause variability in patient samples and other unknown factors.

Other genes

Many other genes have been investigated with respect to promoter methylation in the tissue of primary cancer of the oral cavity (table 2).

An alternative splice of *CDKN2A*, previously called *P14ARF*, is involved in cell-cycle control via the ubiquitin-protein ligase E3, MDM2, and cellular tumour antigen P53 pathway. Its involvement in oral cancer seems less robust than that of *CDKN2A*,[10,25,28] but some studies have detected promoter methylation in oral cancer samples. Methylation of this alternative-reading-frame gene has been associated with better survival.[28]

CDKN2B is also involved in the cell-cycle pathway as a cyclin-dependent-kinase inhibitor. Although its role has been described in leukaemia and glioma,[47] it seems to have only a small role in oral cancer.[28,29]

Retinoids have been widely investigated as chemopreventive agents for inhibition of progression of premalignant lesions or prevention of second primary tumours in patients with a known history of malignant diseases of the head and neck. Retinoic-acid receptor (RAR) changes have been associated with cell immortalisation, and re-expression of *RARB* leads to growth inhibition in some circumstances. Studies[25,33] have suggested that some gene-silencing mechanisms cannot be overcome with retinoic acid, and several groups have thus turned to methylation as a possible mechanism for epigenetic control. Methylation of *RARB* was identified in 15 of 32 samples of primary oral malignant diseases in one study,[25] and in all five samples in another study.[33] Youssef and colleagues[33] also found that ten of 18 head and neck cancer cell lines showed methylation of *RARB* and treatment of the head and neck cell lines with tretinoin and decitabine led to re-expression of *RARB*. Their large study of premalignant lesions also showed that 66 of 124 (53%) lesions had methylated *RARB*, again suggesting that methylation is an early event.

RASSF1 is a RAS homologue located on chromosome 3p21.3 that has been implicated in lung cancer. In one study,[48] 40% of primary tumours showed methylation of this gene. However, the frequency of *RASSF1* methylation seems to be much lower in oral cancer, at between 0% and 8%.[25,27]

The *MLH1* gene is involved in mismatch repair and has been implicated in colon cancer and in other cancers prone to microsatellite instability. Methylation of this gene is also an inactivating factor in colon-cancer cell lines.[49] In the oral cavity, a low rate of *MLH1* methylation has been shown by two groups of investigators.[28,29]

The *FHIT* gene is important in maintaining genetic stability through control of the cell-cycle or of apoptosis. It has been implicated in head and neck cancers that have reduced gene expression, but has a rather low rate of mutation in primary tumours,[50] and could thus be under the control of methylation. Chang and co-workers[34] found that eight of 29 patients who use betel or tobacco showed methylation at the *FHIT* promoter. Thus, methylation plays a substantial part in silencing the *FHIT* gene although other unknown factors must be involved.

TP73 is a *P53* homologue and, thus a putative tumour-suppressor gene, and has a low mutation rate. The gene is implicated mainly in haematological malignant diseases, and methylation of this gene is involved in its silencing.[51] Only one study[25] has investigated the involvement of this gene in primary tumours of the oral cavity, and showed that the rate of *TP73* methylation was quite low (2%).

SERPINB5 is a putative tumour-suppressor gene that inhibits cell motility, invasion, angiogenesis, and metastasis and has been implicated in breast and prostate tumours. Murakami and colleagues[35] showed that three of 12 oral-cancer cell lines with low expression of *SERPINB5* had only limited methylation of the gene, which was re-expressed with decitabine treatment. The investigators resequenced these cell lines after treatment and found that the methylation pattern had not changed despite decitabine treatment and a subsequent increase in maspin production. They postulate that decitabine has a direct effect disruption of DNA methyltransferases and histone-associated proteins.

DBC1 is located on chromosome 9p33, has been implicated as a tumour-suppressor gene in bladder carcinoma, and is prone to methylation.[52] Investigators[9] showed that 15 of 34 primary oral-cell carcinomas showed methylation of *DBC1* by methylation-specific PCR. Furthermore, three of seven histologically normal margins and two of four dysplasias also showed a methylation pattern, further supporting the idea that this change is an early event in carcinogenesis.

DCC was originally implicated in colon cancer and also has a role in oral cancer. Only one study[28] has looked specifically at this marker for methylation, and 16 of 96 primary tumours showed promoter methylation of this gene. *DCC* methylation was correlated with bone invasion, and deep invasion of the tongue. Patients with primary oral cancer who had DCC methylation had a significantly shorter survival time than those who did not have DCC methylation.

Clinical importance

Despite few data for gene-promoter methylation in the oral cavity, measurement of such patterns has shown promise in cancer detection schemes. Because of the sensitivity and specificity of PCR-based assays (especially quantitative methylation-specific PCR) and the potential for use in high-throughput assays, determination of methylation status in oral cancers has great potential for early detection, monitoring, and treatment.

The creation of methylation gene panels could be useful for cancer screening. Ogi and colleagues[28] detected promoter methylation of at least one of 12 genes in 67 of 96 primary oral squamous-cell carcinomas. Viswanathan and co-workers[29] used five selected genes to detect oral squamous-cell carcinoma in 38 of 51 Indian patients. These studies show that a directed panel of markers could be designed to characterise and detect tumours of the oral cavity.

Sanchez-Cespedes and colleagues[26] first took advantage of methylation status as a clinical marker in their study of serum samples from 95 patients with primary tumours of the head and neck who had matched serum samples. Methylation-specific PCR was used for four markers: *CDKN2A*, *MGMT*, *GSTP1*, and *DAPK1*. 52 of 95 patients had methylation in at least one gene, and 21 of the 50 serum samples tested had corresponding methylation patterns. The investigators noted that serum could be a viable method of determining tumour recurrence in patients after oncological treatment.

Rosas and co-workers[32] investigated 30 patients with head and neck cancer who gave oral-rinse samples. At least one gene was methylated in 17 of 30 primary tumour samples by use of methylation-specific PCR analysis with *CDKN2A*, *MGMT*, and *DAPK1* as markers. 11 of 17 matched saliva samples contained the same methylation pattern. Patients with tumours of the oral cavity were more likely to have the tumour detected in saliva than were those patients with tumours further down the aerodigestive tract.

Lopez and colleagues[53] also used saliva samples; however, their study was in patients with clinical leucoplakias. They used methylation-specific PCR on a panel of three genes, and 28 of 34 patients were found to have had at least one gene with promoter methylation. Thus, methylation is an early event in carcinogenesis and even premalignant lesions can be detected by this technique. Future studies on population screening and studies that show a correlation with clinical outcome will further elucidate the use of this promising method of detection of suspicious oral lesions.

The assessment of margin status during surgery has relied on rapid histological study of a frozen section. Although molecular studies have investigated margin analysis, none have discussed the feasibility of real-time analysis. Because of the potential for rapid analysis of methylation status, Goldenberg and colleagues[54] did a feasibility study in which margins were sampled during surgery and tested by use of quantitative methylation-specific PCR for methylation of two markers, *CDKN2A* and *MGMT*. The investigators discovered that the assay could be done during surgery, allowing a new application of molecular analysis to direct surgical treatment.

Conclusion

Promoter methylation is a powerful and ubiquitous mechanism of gene silencing. Initially discovered to be a mechanism for developmental control, it plays an important part in the development of many tumour types. Various genes have been implicated in squamous-cell carcinoma of the oral cavity with differing results. However, methylation is important in the development of oral squamous-cell carcinoma, and other tumour-suppressor genes targeted by promoter methylation will no doubt be described in the future. Determination of promoter-methylation status has shown promise in detecting oral squamous-cell carcinoma from saliva and serum samples and in real-time analysis of margins during surgery. The techniques used at present to detect methylation provide good sensitivity, specificity, and speed. Future studies will focus on creating the best possible panel of markers for the prediction of outcome, sensitivity to chemotherapy, and disease status as investigators steadily bridge the gap between the research laboratory and the clinic.

Search strategy and selection criteria

A Medline search from January, 1966 to July, 2005 was done with the keywords “methylation” and “oral cancer”. Studies identified were screened for those that focused on tissues with primary oral cancer and that looked directly at DNA methylation by use of methylation-specific PCR, PCR-based restriction enzyme assay, and sequencing after sodium bisulphite treatment. We included only a few studies of oral squamous-cell carcinoma cell lines, since the focus of the review is primary tissue.

Conflict of interest

We declare no conflicts of interest.

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