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DNA Content and Methylation of *p16, DAPK* and *RASSF1A* Gene in Tumour and Distant, Normal Mucosal Tissue of Head and Neck Squamous Cell Carcinoma Patients

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Abstract. *Long-term survival of head and neck squamous cell carcinoma (HNSCC) patients has not improved significantly during the last 20 years and recurrent disease is frequently observed. In this study, the potential presence of pre-malignant cells or rare malignant cells at the time of diagnosis in HNSCC was investigated. Patients and Methods: Fifty-nine biopsies obtained from 41 HNSCC patients were analysed. Eighteen of these biopsies were normal mucosal tissue, located at least 5 cm from the tumour margin. DNA content and DNA methylation of p16, DAPK and RASSF1A was examined. Results: Thirty-nine out of 41 (95%) tumour biopsies showed p16 methylation and 21 (51%) of them displayed aneuploidy. Of 18 distant normal mucosal biopsies, 6 (33%) of these showed evidence of aneuploidy and 15(83%) of them showed methylated p16 genes. Among paired samples, the highest frequencies of DNA methylation were found in tumours with aneuploidy. Regardless of DNA content, methylation at DAPK, RASSF1A or p16 were found in the corresponding distant mucosal biopsies. Conclusion: The cells with abnormal DNA content or DNA methylation in mucosal tissue were not detected clinically or by pathological macroscopic and microscopic examination. Thus, distant mucosal tissue DNA content and DNA methylation analyses in combination with histopathology will provide a better prognostic base for the evaluation and treatment of HNSCC patients.*

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Head and neck squamous cell carcinoma (HNSCC) is the fifth most common malignancy worldwide (1). The analysis of DNA content has previously been suggested to give an indication of tumour aggressiveness (2). Despite advances in therapy, long-term survival of HNSCC has not improved significantly over the last 20 years and recurrent disease is observed in almost 50% of these patients (3).

Epigenetic changes, such as altered patterns of DNA methylation or histone modification are key features of cancer pathogenesis (4, 5). Methylation of death-associated protein kinase (*DAPK*) and Ras association domain family 1A (*RSSF1A*) gene strongly contributes to carcinogenesis, metastasis and treatment failure in various types of cancer (6-8). In addition, methylation of the cell cycle regulator gene, *p16* has been detected in patients with squamous cell lung carcinoma 3 years prior to clinical diagnosis (9).

Cigarette smoke and alcohol are considered to be important factors in the aetiology of HNSCC (10, 11). Epithelial cells in the head and neck are the first in the body to be exposed to these environmental carcinogens. Nicotine, the addictive component of tobacco, has never been shown to have direct effects on experimental tumourigenesis. However, nicotine down-regulates certain anti-oncogenes by DNA methylation (12).

The reason for the high incidence of local recurrences in HNSCC is as yet unclear (13). It may be due to multiple malignant and pre-malignant lesions as a results of field cancerization (14). Alternatively, clonal spreading from/of progenitor cells or metastasis from the primary tumour have also been suggested (15).

Regardless of their origin, the recurrences of primary or secondary HNSCC are very relevant factors in treatment decisions and clinical outcome. The ability to detect the pre-malignant cells or rare malignant cells at the time of

Table I. *Summary of the primers used in the present study.*

Primers	Product	Sequences
DAPK Forward	227 bp	5'CGG TAG GGT TTG GGG TCG 3'
DAPK Reverse		5'AAA CCT CCC AAC TTC GAT CG 3'
Beta actin Forward	184 bp	5'AAG TTA AGT TTT GTT TTT ATT TTT 3'
Beta actin Reverse		5'CAA TAA TCT CCT TCT ACA TCC TAT C3'
RASSF1A Forward	169 bp	5'GTT TTG CGA GAG CGC G3'
RASSF1A Reverse		5'GCT AAC AAA CGC GAA CCG 3'
p16 Forward	75 bp	5'AGG GGT TGG TTG GTT ATT G 3'
p16 Reverse		5'CTA CCT ACT CTC CCC CTC TC 3'

diagnosis would be of great benefit in deciding treatment regimes. This study analysed the alteration of DNA content and DNA methylation of *p16, DAPK* and *RASSF1A* genes in primary tumour tissue and morphologically normal-distant mucosal biopsies from HNSCC patients.

Patients and Methods

Patients and biopsies. The local Ethical Committee approved this study. Primary tumour and morphologically normal mucosal tissue, located at least 5 cm from the tumour site, were collected at the diagnostic time from 41 patients, 30 males and 11 females, with median age 62 years (range 42-101 years).

The tumour samples were excised within the tumour mass but excluded the tumour margin. The distant mucosal biopsy samples with a normal phenotype were obtained, located at least 5 cm from the macroscopic tumour edge as judged by a ear nose and throat (ENT) specialist. The biopsy was placed on dry ice and then divided into two sections. One biopsy sample was placed in formalin and sent to the pathologist. The other biopsy sample was used for molecular analysis.

All of these distant macroscopically normal mucosa biopsies and tumour biopsies were anonymised. No information was given to the pathologist who examined the samples for histopathology.

DNA content analysis. The tumour and distant mucosal biopsies were fixed and bare cell nuclei were analysed (16). Briefly, the biopsies were incubated with Subtilisin Carlsberg solution (0.1% protease XXIV in 0.1 M Tris, 0.07 M NaCl pH 7.2; Sigma-Aldrich Inc, Stockholm, Sweden). The nuclei were directly stained with DAPI-SR 101 solution (8 M DAPI, 50 mM sulforhodamine 101, 0.1 M Tris, 0.07 M NaCl pH 7.5; Sigma-Aldrich Inc). The samples were analysed using a flow cytometer (BD Bioscience, Stockholm, Sweden). DAPI was excited at 365 nm and fluorescence was measured at 435 nm. A multicycle program applied for histogram analysis (Phoenix Flow Systems, San Diego, CA, USA) was used for calculating the percentage of nuclei after subtraction of sliced nuclei background.

DNA extraction and bisulphite modification of genomic DNA. Nuclear DNA was obtained from the biopsies by Phenol/Chisam extraction. Methylation-specific PCR (MSP) was used for detection of the aberrant methylation in DNA samples (17, 18). Briefly, 2 μg of genomic DNA were treated overnight at 50˚C with 2.5 M sodium bisulphite and 100 mM hydroquinone (Sigma-Aldrich Inc). The bisulphite treatment was stopped by equilibration with TE buffer and de-sulphonation in 0.2 M NaOH.

Detection of DNA methylation in the CpG promoter region of p16, DAPK and RASSF2A gene. The 200 ng of bisulphite-treated DNA was used as the template for MSP analysis. The primers were designed to amplify 75-300 bp regions of the CpG-rich gene promoters of the *p16, DAPK*, and *RASSF1A* genes. The specific primer sequences and products are summarised in Table I.

The optimized PCR amplification was run at 95˚C for three min, four cycles at 94˚C for one min, 60˚C for 30 s and 65˚C for 45 s. After this, 36 cycles at 94˚C for one min, 56˚C for one min and 65˚C for four min were run. The PCR products were analysed by 2.5% agarose gel electrophoresis.

Results

Patients, HNSCC tumour and distant mucosa tissue biopsies. Of the 41 patients included in the study, 20 (49%) were diagnosed with tongue cancer, 17 (41%) with tonsil cancer and 4 (10%) with pharyngeal cancer.

Fifty-nine biopsies obtained from these 41 patients were analysed. Forty-one tumour samples were assessed by an ENT specialist and confirmed by standard immunohistochemical analysis. The 18 macroscopically healthy mucosal tissue biopsies, as judged by this ENT specialist, were obtained at the time of the surgery. All of these 18 distant mucosal biopsies (100%), were confirmed to be normal tissue by pathological microscopic reviewing (data not shown).

DNA content in tumours and the distance mucosal biopsies. The DNA content of 41 tumours and the 18 distant mucosal biopsies from the HNSCC patients were analysed. The amount of abnormal DNA in the matched tumour and distant mucosal tissue biopsies obtained from the same patient differed notably (Figure 1).

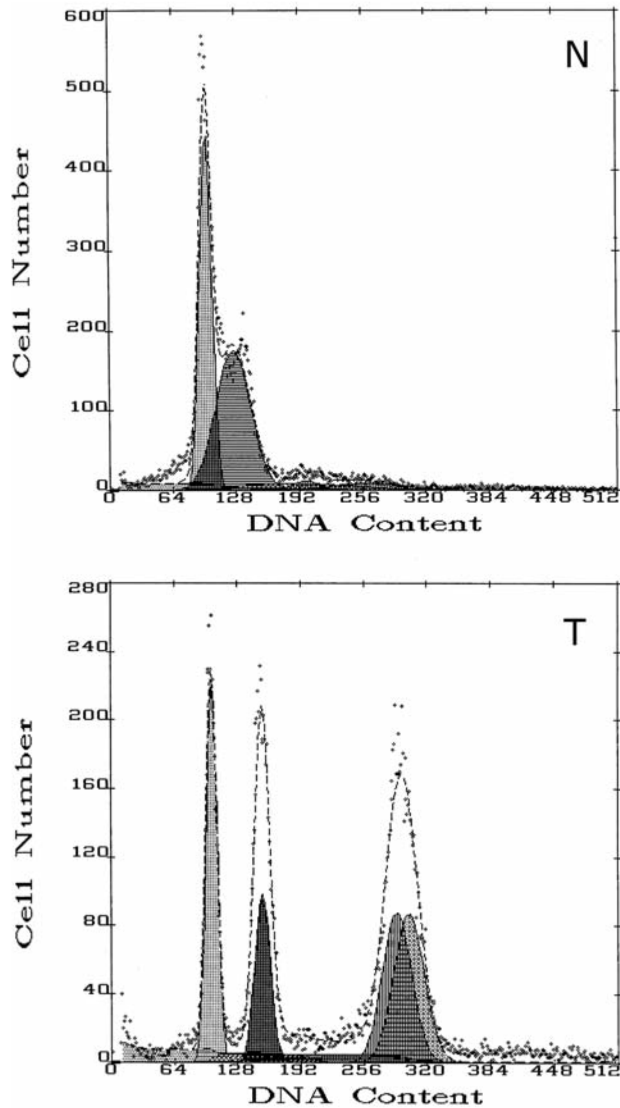

Figure 1. *DNA histogram of tumour (T) and distant normal phenotype morphology mucosa (N) biopsies from one representative patient.*

Table II. *The patient DNA alteration and methylation in paired biopsies with normal (N) and tumour (T) phenotype.*

Patient no. (n=18)	Biopsy	DNA content		DNA methylation		
		Diploidy	Aneuploidy	*DAPK*	*RASSF1A*	*p16*
2	N	+		-	-	+
	T		+	-	-	+
3	N		+	+	-	+
	T		+	+	+	+
7	N	+		-	+	+
	T	+		-	+	+
8	N	+		-	-	+
	T		+	-	-	+
10	N	+		-	-	+
	T		+	-	+	+
14	N	+		-	+	+
	T		+	+	+	+
15	N	+		-	+	+
	T	+		+	+	+
17	N	+		-	-	+
	T	+		+	+	+
18	N		+	-	-	-
	T		+	-	-	+
19	N	+		-	-	+
	T	+		-	-	+
20	N	+		-	-	-
	T	+		-	-	+
21	N		+	-	-	+
	T		+	+	+	+
25	N	+		-	-	-
	T	+		+	-	+
26	N		+	-	-	+
	T		+	-	-	-
28	N		+	-	-	+
	T		+	-	+	+
29	N		+	+	+	+
	T		+	+	-	+
30	N	+		-	-	+
	T	+		-	-	-
31	N	+		-	-	+
	T		+	-	-	+
Total	N	12		0	3	10
			6	2	1	5
	T	7		3	3	6
			11	4	5	10

Twenty-one out of the 41 (51%) HNSCC tumour biopsies contained of cells with aneuploid DNA content. Despite normal phenotypic morphology, as judged by an ENT specialist and verified by a pathologist, 6 of 18 (33%) of distant biopsies contained cells with aneuploid DNA content (Table II).

DNA methylation of p16, DAPK and RASSF1A genes. Single positive MSP of *DAPK, RASSF1A* and *p16* was performed with bisulphite-treated DNA from a nasopharyngeal carcinoma cell line, CNE1 (Figure 2 A). The corresponding multiplex MSP (Figure 2 B) was performed with the bisulphite-treated DNA from tumour-derived cell lines (CNE1, C666-1, Namalwa and MCF-7) and non-tumourigenic, immortalized transformed cell lines (HF and PBMC). Thus, *p16* methylation might be the early event in the disturbance of cell cycle control.

Methylation at *p16* was observed in 39 out of 41 (95%) tumour and 15 out of 18 (83%) distance mucosal biopsies. *DAPK* and *RASSF1A* methylation was also observed at to a lesser extent than *p16* methylation in these biopsies. Within paired tumours and their corresponding distant mucosal biopsies (Table II, Figure 3), *DAPK, RASSF1A* and *p16*

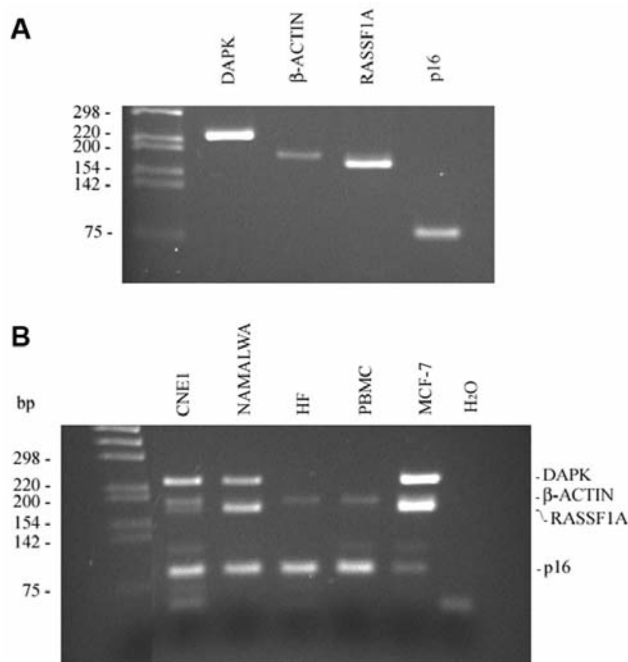

Figure 2. *DAPK, RASSF1A and p16 gene methylation analysis by single and multiplex methylation PCR. Single methylation PCR of a nasopharyngeal carcinoma cell line, CNE1 (A). Multiplex methylation PCR (B) of tumour derived cell lines (CNE1, Namalwa and MCF-7) and non-tumourigenic, immortalised transformed cell lines (HF and PBMC). The molecular marker is indicated in the left lane.*

methylation were detected more often in the tumour than in their corresponding distant mucosal biopsies. As expected, the highest degree of these DNA methylations was found in the aneuploid tumours.

Discussion

From the initiation of carcinogenesis, normal cells must proceed through various changes prior to becoming malignant. Long exposure to carcinogenic agents such as cigarette smoke might increase the possibility of such a transformation, as well as promote expansion of the progenitor cancer clone (19). To date, diagnosis of pre-malignant cells or second primary tumours is currently based on macroscopic or microscopic detection by pathologists. With such subjective detection, it is possible that a large proportion of pre-malignant or rare malignant progenitor cells are not detected (13-15).

The cell cycle control gene, *p16*, is of critical importance in controlling cell growth. The significance of *p16* methylation in carcinoma of the tongue has been reported (20). Methylation of *p16* might be an early event of transformation reflected by the disturbance of cell cycle control. This assumption was supported by the detection of p16 methylation in the immortalised cell lines and in distant mucosal biopsies with diploid DNA content. As a consequence of uncontrolled cell growth due to methylation of *p16*, a gross genetic abnormality might then arise, as indicated by DNA aneuploidy (21).

The methylation in *DAPK* and *RASSF1A* genes was also detected in some of the tumours. These epigenetic changes may be involved in a subset of HNSCC (7). Alternatively, it might occur later as a consequence of abnormalities or deregulation of cell cycle controls, as indicated by methylation at *p16* (8).

Methylation at *p16* was found in established immortalised non-tumourigenic cell lines, tumour derived cell lines and tumour biopsies. The *p16* methylation in distant mucosal tissue indicated the possibility of transformed, immortalized pre-malignant or malignant cells in these patients at the time of diagnosis. This may explain how second primary tumours and tumour recurrence occur at a high frequency, even after successful primary treatment of HNSCC patients (5, 13).

The presence of pre-malignant cells and malignant cells in morphologically normal mucosal tissue of HNSCC patients is a critical factor in terms of treatment outcome (21). Persistence of these abnormal cells could be detected by simple, low-cost and well-established methods using flow cytometry or MSP analysis. Such molecular analyses have the potential to be powerful adjuncts to standard histopathology assessment in the diagnosis and treatment of HNSCC. Since DNA methylation is reversible by pharmacological means (22), the use of anti-methylation agents in the clinical setting requires further investigation.

Treatment of HNSCC has long been a challenge due to its high rate of recurrence. This study indicated that more than half of the distant mucosal tissues of HNSCC patients harbour transformed, pre-malignant or malignant cells. These cells were undetected by conventional macroscopic or microscopic examination.

DNA methylation may be the first initiation of general oncogenesis. It is proposed that these simple, low cost methods of methylation-specific PCR and DNA content analysis by flow cytometry, should be added to the diagnosis armamentarium for HNSCC. This may well provide a better prognostic evaluation of HNSCC and could be used in addition to histopathological assessment. The use of anti-methylation agents for cancer prevention in high-risk groups or as additional treatment in HNSCC patients warrants further investigation.

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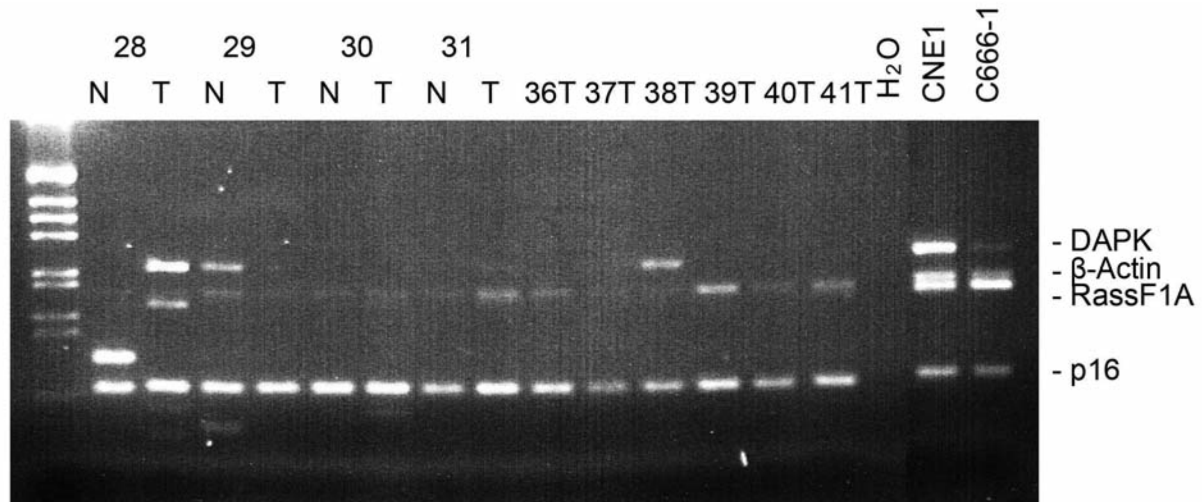

Figure 3. *DAPK, RASSF1A and p16 methylation of the individual HNSCC tumour biopsies (T), distant normal phenotype morphology mucosa biopsies (N) and tumour-derived cell lines, CNE1 and C666-1. Results obtained from 10 representative patients are shown. The molecular marker is indicated in the left lane.*

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