MGMT Activity in Mucosal Epithelium and Squamous Cell Carcinoma of the Head and Neck

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Abstract. *Smoking and alcohol abuse cause squamous cell carcinoma of the head and neck (SCCHN) through smoke-induced mutations, which are counteracted by O^6-methylguanine-DNA methyltransferase (MGMT). This study aimed at elucidating the role of MGMT in SCCHN and its precursor lesions (SIN). MGMT was also determined in the normal mucosa (NM) and blood lymphocytes (PBLCs). Results: a) MGMT was lower in NM than in PBLCs. b) Smoking reduced MGMT in NM but had no effect in PBLCs. c) MGMT activity increased in the sequence NM<SIN II and III<CIS. d) There was no correlation between MGMT and prognostic parameters or clinical course in SCCHN. The data suggest that MGMT becomes down-regulated due to smoking in non-cancerous pharyngeal mucosa. The low MGMT activity in early dysplastic mucosal lesions may increase the risk for tumour development. Since some advanced carcinomas showed low MGMT activity, chemotherapy with O^6-alkylating agents might be an alternative option.*

Squamous cell carcinoma of the head and neck (SCCHN) is a common form of cancer in industrial countries. Carcinogenesis in SCCHN shows several stages (mild, moderate, severe dysplasia and carcinoma *in situ*), and deregulation of tumour growth leads to invasive carcinoma. In established carcinomas, great tumour cell heterogeneity has been reported (1) and tumour growth rate, the degree of aneuploidy, expression of surface markers/receptors and resistance to apoptosis vary within the tumour, whose most aggressive part determines the individual prognosis.

SCCHN commonly occurs with multiple primaries within the head and neck area. This is due to 'field cancerization' or 'condemned mucosa' (2). Smoking and alcohol consumption are important aetiological factors, which have been shown to act in a dose-related manner (3). The incidence of second primaries ramges between 10-40%, occurring simultaneously (within 6 month) or metachronically (within 5 years). Patients with second primaries have been shown to consume more cigarettes and alcohol than patients exhibiting only a single tumour (4).

The processes of DNA damage and repair, as well as detoxification rates of carcinogenic substances, play a crucial role in early steps of cancer development. Smoking is an important source of alkylating carcinogens, including *N*-nitrosamines (5). Ethanol contributes to smoking-related carcinogenesis in different ways, acting as a tumour promoter by causing chronic inflammation that stimulates proliferation and mediates oxidative DNA damage (6). It may also enhance microsomal activation of carcinogens through phase I enzyme induction (7).

N-Nitrosamines induce alkylated DNA bases, including the highly pro-carcinogenic adduct O^6-methylguanine (O^6MeG) (8, 9). If this adduct is not removed from DNA, it causes mutations due to mispairing with thymine (10). The adduct is also genotoxic and cytotoxic, causing replication and mismatch repair-mediated DNA double-strand breaks (11) that trigger the formation of chromosomal aberrations and apoptosis (11-13).

The main mechanism counteracting these responses is O^6MeG repair by the suicide enzyme O^6-methylguanine-DNA methyltransferase (MGMT), which transfers the methyl group from O^6MeG to a cysteine residue in the MGMT protein (14). Since this is a stochiometric reaction, MGMT activity correlates with the number of pre-existing MGMT molecules per cell. MGMT is regulated in a complex manner, which includes activation of transcription factors by genotoxic stress (15, 16) and epigenetic regulation *via* promoter methylation (17, 18). In this study, MGMT activity in SCCHN was determined and compared with tumour differentiation, aneuploidy, proliferation rate and the patients' alcohol and cigarette consumption. It was also compared with MGMT in the normal mucosa and peripheral blood lymphocytes

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(PBLCs). The data revealed a remarkable variability of MGMT expression in normal mucosa and SCCHN, low MGMT levels in the normal mucosa of smoking individuals, and lack of correlation with PBLC MGMT.

Materials and Methods

Tumour and normal tissue. PBLCs and mucosal biopsies from patients with head and neck cancer were examined. For comparison, biopsies were taken from patients with sleep apnoea who underwent pharyngeal operations. All patients gave informed consent to the additional examination of tissue and blood (according to the declaration of Helsinki 1993). Patients were asked about their smoking and drinking habits. Relevant alcohol consumption was assumed when daily use was reported. Twenty-three patients (21 male, 2 female) were recruited with advanced stages (UICC 1997, III+IV) of cancer of the oropharynx (n=13), hypopharynx (n=6), larynx or floor of the mouth (n=2 each). Biopsies were obtained from the primary tumour and the surrounding mucosa at a distance of 1-2 cm. Previous studies revealed that the most malignant and invasively growing cells are located at the tumour margins (tumour front). Therefore, specimens (approx. 0.5 cm^3) were taken from the tumour margin. The assessment of tumour biological factors included tumour front grading, quantitative DNA analysis, along with immunohistochemical identification of the proliferating cell nuclear antigen (PCNA). Mucosal samples were also obtained from 26 patients with no cancer (16 male and 10 female) who underwent surgery for other reasons (tonsillectomy, palatoplasty).

Histology and immunohistochemistry. For histology, 4 μm slices were cut from the paraffin-embedded tumour material and stained with haematoxylin-eosin. For immunohistochemical identification of the PCNA a monoclonal antibody was used (PC10, Clone Lane, Oncogene Science, Uniondale, N.Y, USA). The stained slides were assessed by counting of at least 1,000 tumour cells. The scores were determined as the percentage of positive cells per 1,000 cells (19).

Quantitative DNA measurements. For quantitative DNA measurements, cytological smears were obtained from each specimen. The slides were Feulgen stained. Quantitative DNA analysis was then performed using a computerised image analysis system (Cytometer CM1; Hund Cie, Wetzlar, Germany). Several DNA indices were calculated by measuring more than 300 tumour cells (1).

MGMT activity assay. For MGMT activity assay, deeply frozen tissue was homogenised by an UltraTurrax homogeniser in buffer containing 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 5% glycerol and a cocktail of protease inhibitors (10 μg/ml aprotinin, 10 μM bestatin, 10 μM leupeptin, 1 μM pepstatin and 0.1 mM PMSF). Thereafter, the homogenate was sonified by a Branson sonifier 250 (2×10 pulses, duty cycle 40%, intensity 4.5, on ice). The sonication product was centrifuged to remove debris and the supernatant was snap frozen in liquid nitrogen and stored at –80˚C until further use. This procedure did not result in loss of MGMT activity. For positive control, HeLa S3 cells were used which express MGMT at a high level (750 fmol/mg protein). HeLa MR cells deficient in MGMT served as a negative control, which was included in each assay. MGMT activity was determined essentially as previously described (20). For the assays, at least 100 μg of cell extract protein were used.

Statistics. All data were analyzed by SPSS/PC+ (Statistical Package for Social Sciences, Fa. SPSS GmbH/ München, Germany). Non-parametrical tests (Mann-Whitney test, Chi^2 test, Kruskal-Wallis test, Spearman correlation) were used to check significance. Data were considered to be significant at $p \leq 0.05$.

Results

The average age of cancer patients in this study was 52±8 years; the age of the control group (patients operated for sleep apnoea) was 30±23years. All cancer patients (n=23) were smokers who at the same time regularly consumed alcohol. Among the controls, only 27% (n=7) smoked, and regular alcohol use was only reported for 46% of them (n=12). The histological examinations of tumour-surrounding mucosa showed mild dysplasia in 26% of cases, moderate dysplasia in 39%, severe dysplasia in 13% and carcinoma *in situ* in 9%. The PCNA score from tumour-surrounding mucosa was 29%±10.5% (range 15-45%) and was much less than in the tumour tissue (54.2±26.0%; range 8-100%; Mann-Whitney test: $p=0.001$). The results of DNA cytometry showed aneuploidy in dysplastic mucosa and marked aneuploidy in the tumour tissue.

MGMT in mucosa and PBLCs in healthy volunteers. The MGMT activity in the mucosa of the oropharynx (lateral pharyngeal wall or velum) and PBLCs of healthy control individuals were determined. Data are shown in Table I. The average MGMT activity in the mucosa of the non-cancer patients was 152 fmol/mg protein. A significantly higher activity was found in PBLCs, on average 764 fmol/mg protein; Wilcoxon test: $p<0.001$). MGMT activity in mucosa and PBLCs was compared in the same non-cancer patients (Figure 1). In most cases MGMT activity is higher in PBLCs than in the mucosa. Overall, there was no correlation between MGMT activity in PBLCs and mucosa of the control individuals (Spearmann-correlation: r=0.173; $p=0.399$) (Figure 1).

Interestingly, in the non-cancer control group, smokers had significantly reduced mucosal MGMT activity than non-smokers (50 *vs*. 180 fmol/mg protein; Wilcoxon test: $p<0.0195$). There was no significant difference between smokers and non-smokers in MGMT activity in PBLCs (671 *vs*. 798 fmol/mg protein) (Table I).

MGMT in SCCHN. The MGMT activity in SCCHN specimens and in biopsies of surrounding 'normal' and dysplastic mucosal tissue was determined in 23 patients with head and neck cancer. The data shown in Table II revealed significant differences in dysplasias and tumours, with 254 fmol/mg protein in the dysplastic mucosa and 373 fmol/mg protein in the tumour tissue. The 'normal' mucosa of the same patients exhibited an activity of 145 fmol/mg protein. The differences between the groups were statistically

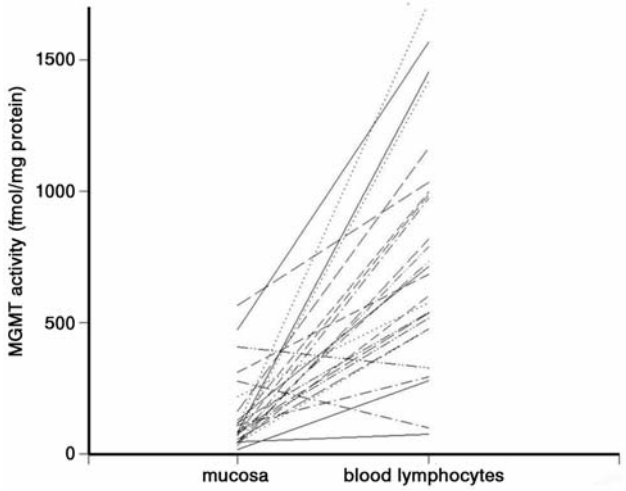

Figure 1. *MGMT activity (fmol/mg protein) in biopsies of pharyngeal mucosa in control (non-cancer patients) and peripheral blood lymphocytes (PBLCs) of the same individuals. PBLCs show increased MGMT activity in comparison to epithelial cells, but there was no correlation. There is a large inter-individual heterogeneity for MGMT activity both in mucosa and PBLCs. Paired samples are connected by lines.*

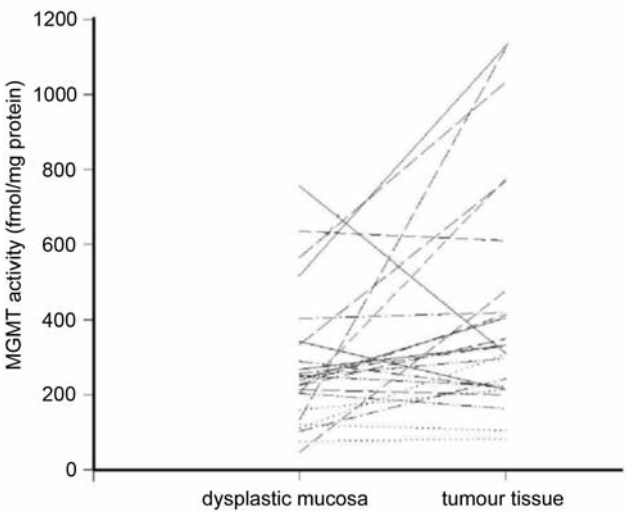

Figure 2. *MGMT activity (fmol/mg protein) in biopsies of tumour tissue and tumour-surrounding mucosa (dysplastic mucosa) of the same patient. Paired samples are connected by lines.*

Table I. *MGMT activity (fmol/mg protein) in pharyngeal mucosa and periheral blood lymphocytes (PBLC) in non-cancer patients: comparison with smoking habits.*

		MGMT in mucosa		MGMT in PBLC		
Smoking	n	Mean±SD	Range	Mean±SD	Range	*p*-Value
Yes	7	50±17	31-78	671±412	75-1421	0.0195
No	19	180±156	16-566	798±455	98-1720	0.5062
All	26	152±147	16-566	764±440	75-1720	0.001

significant (see Table II; p=0.0022). In the tumour tissue, the MGMT activity showed marked heterogeneity in the samples obtained from different patients, ranging from 80-1125 fmol/mg protein.

Gender and age of the patients showed no correlation to MGMT activity in the tumour tissue. There was no correlation of MGMT with tumour biological factors such as TN stage, DNA cytometry, PCNA score, or tumour front grading. However, the MGMT activity in the tumour tissue did correlate with the mean DNA content of tumour cells (r=0.534; p=0.015).

A comparison of MGMT activity in tissue samples with different levels of dysplasia revealed that the MGMT activity in the tumour-surrounding mucosa was lowest in moderate dysplastic mucosal biopsies and increased with further dysplastic changes in severe dysplasia and carcinoma *in situ* (Table III). Of note, the MGMT level found in any dysplastic tissue was significantly higher than in the normal mucosa (Mann-Whitney test p=0.0022). In Figure 2, MGMT activity of dysplastic mucosa (n=23) and SCCHN cancer tissue from the same patient are shown, demonstrating the great variability of MGMT expression and, with very few exceptions, an increase of MGMT activity in the tumour compared to the corresponding dysplastic tumour surrounding tissue.

Discussion

MGMT in biopsies from SCCHN and the surrounding mucosa was examined in 23 cancer patients and the activity levels were compared with the normal mucosa of 26 non-cancer patients. In addition, the MGMT level was determined in PBLCs of all non-cancer patients and compared with the level in the mucosa of the same patients. MGMT in the

Table II. *MGMT activity (fmol/mg protein) in primary and tumour surrounding mucosa of cancer patients: comparison with healthy mucosa of the control group.*

Tissue	MW±SD	Median	Min/Max
Normal tissue	145±145	83,5	16-566
Dysplast. *i.e.* mucosa	254±166	227	45-756
Tumour tissue	373±245	310	80-1125

Significance test (Mann-Whitney test): dysplastic mucosa *vs.* tumour tissue: p=0.0456; dysplastic mucosa *vs.* controls: p=0.0022; tumour tissue *vs.* controls: p<0,0001.

Table III. *MGMT activity (fmol/mg protein) in tumour and surrounding mucosa: comparison with histological grading.*

Grading	MW±SD	Median	Min/Max
Mild dysplasia	242±69	253	120-333
Moderate dysplasia	206±169	159	75-634
Severe dysplasia	280±107	224	213-404
Carcinoma *in situ*	522±330	522	288-756

normal mucosa and PBLCs is highly variable, with an inter-individual range of 16-566 fmol/mg protein (average 152 fmol/mg) in normal mucosa and 75-1720 fmol/mg protein (average 764 fmol/mg protein) in PBLCs. Two individuals had a very low level of MGMT in PBLCs of <100 fmol/mg protein. In two individuals, the MGMT level in PBLCs was lower than in the mucosa. The reason for this high variability of MGMT activity in pharyngeal mucosa and PBLCs is unknown. There was no clear correlation between MGMT in the mucosa and PBLCs, indicating that MGMT in PBLCs does not reflect the MGMT level in other tissues. High variability of MGMT expression in PBLCs was reported previously in another cohort (21). For MGMT activity in pharyngeal mucosa, according to the Authors' best knowledge, other data have not yet been reported. The lack of correlation of MGMT activity in PBLCs with other tissues of the same individual makes the use of PBLCs as a MGMT biomarker doubtful.

In the non-cancer control group, 7/26 of the individuals smoked (age 16-71 years; average 48 years). Interestingly, the MGMT activity in the pharyngeal mucosa (50 fmol/mg protein) of smokers was significantly lower than in the pharyngeal mucosa of non-smokers (180 fmol/mg protein) (Table I; p=0.0195). This is an interesting finding that suggests that smoking depletes MGMT in the pharyngeal mucosa. A reasonable explanation is that smoking-induced DNA damage in mucosa cells is repaired by MGMT, which thereby becomes depleted (22). Alternatively, smoking might reduce the level of gene expression. In fact, a hypermethylated *MGMT* promoter was found in tumour surrounding mucosa (23). It is reasonable to propose that depletion of MGMT by DNA damage and/or attenuation of MGMT gene expression promotes cancer formation by increasing mutation rates.

MGMT in 'normal' pharyngeal mucosa was then compared with the tumour-surrounding dysplastic mucosa, which showed significantly higher activities (145 *versus* 254 fmol/mg protein, Table II). It is important to note that all SCCHN patients were smokers. It is also important to note that the tumour-surrounding 'normal mucosa' is histologically different from the mucosa of non-cancer patients, which might explain the difference in the MGMT level in mucosa of smokers in the control group and in cancer patients. The high MGMT level in the tumour tissue may be due to up-regulation of MGMT as a consequence of smoking. This hypothesis is, however, unlikely since in the pharyngeal mucosa in non-cancer patients, MGMT up-regulation by smoking was not observed. More likely therefore is the hypothesis that MGMT up-regulation is a result of cellular dysregulation in dysplastic cells. This view is further supported by data obtained in carcinomas *in situ* and severe dysplasia, coming close to those for established carcinomas, in which the highest MGMT activity was found. In contrast, lowest MGMT activity levels were seen in moderately dysplastic pharyngeal mucosa. This is interesting because the risk of cancer development in moderately dysplastic tissue is considerably high (24). Therefore, it can be speculated that a low MGMT activity in premalignant moderately dysplastic mucosa may be a contributing factor for SCCHN development, at least in smokers. This is supported by data reported by others who showed that MGMT activity was reduced in early precancerous lesions of the oral mucosa, which correlated with their increased cancer risk (22).

Up-regulation of MGMT with increasing tumour stage or tumour de-differentiation has also been observed in ovarian carcinomas (25), glioblastomas, where MGMT increased in recurrent disease (20, 26), and lung cancer (27). Established carcinomas displayed not only increased MGMT activity but also a wide range of variation (see Table II). This might be taken as an indicator of severe dysregulation of *MGMT* gene expression. DNA cytometry revealed marked aneuploidy in tumour cells (10- to 15-fold rise compared to diploid cells) and mild to moderate aneuploidy in tumour surrounding tissue (3- to 5-fold rise), while the pharyngeal mucosa of the normal control showed diploidy (28). The proliferation rate (as determined by PCNA expression) was increased in tumour tissue, less in dysplastic epithelium and hardly detectable in healthy pharyngeal mucosa (data not shown), which confirms previous reports (29). There was no correlation between MGMT activity and proliferation rate. There was also no correlation of MGMT activity to tumour stage (TN stages). Only the mean DNA content correlated with MGMT activity, which may be taken to indicate that increased DNA content due to progressive aneuploidy may increase the copy number of the *MGMT* gene.

In the patients of the current study, there was no correlation between MGMT activity in the normal pharyngeal mucosa or the carcinomas *in situ* with the treatment response determined by the overall survival, lymph node metastasis or recurrence-free interval. It should be noted that patients were treated by surgical resection followed by radiochemotherapy (including cisplatin) for which MGMT does not act as a resistance factor. The same was found for ovarian cancer treated with a cisplatin-based therapy, the response of which did not correlate with MGMT (25). This contrasts with malignant gliomas treated with the methylating agent temozolomide, for which the therapeutic response was dependent on the MGMT activity level (20).

The high MGMT activity in SCCHN indicates that for therapy of SCCHN, methylating and chloroethylating anticancer drugs are most likely ineffective, unless MGMT is inactivated by continuous temozolomide administration or the use of an MGMT inhibitor such as O^6-benzylguanine (30). It should be noted that MGMT activity in some SCCHN patients was low (<100 fmol/mg protein), which might make this subgroup responsive to O^6-containing anticancer agents. It is clear that further studies are needed in order to explore the treatment response of SCCHN cells to anticancer drugs for which MGMT is the main factor of tumour cell resistance.

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