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# Prognostic value of O-6-methylguanine-DNA methyltransferase loss in salivary gland carcinomas

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**ABSTRACT:** *Background.* The purpose of this study was to determine the prognostic value of O-6-methylguanine-DNA methyltransferase (MGMT) inactivation in a group of 286 patients with salivary gland carcinoma and matched histologically normal tissues.

*Methods.* MGMT promoter methylation was studied in 36 patients with salivary gland carcinoma and 19 histologically matched normal tissues by pyrosequencing. MGMT protein expression was examined in 286 patients with salivary gland carcinomas and histologically matched normal tissues by immunohistochemistry on tissue microarrays. The results were correlated to demographic, clinicopathologic parameters, and disease follow-up data.

*Results.* MGMT hypermethylation was significantly ($p = .021$) associated with the protein loss. MGMT loss was found in 39.2% of salivary gland carcinomas and was predominant in aggressive tumors (poorly differentiated, grade III, regional lymph node involved). MGMT loss significantly ($p = .004$) predicted poor clinical outcome of salivary gland carcinomas and defined high-risk subgroups in clinically favorable tumor groups.

*Conclusion.* We suggest that immunohistochemical evaluation of nuclear MGMT protein might serve as a tool for the prediction of overall survival in patients with salivary gland carcinoma. © 2013 Wiley Periodicals, Inc. *Head Neck* **36:** 1258–1267, 2014

**KEY WORDS: salivary gland carcinoma, $O^6$-methylguanine-DNA methyltransferase, protein expression, prognosis, methylation**

## INTRODUCTION

Salivary gland carcinomas are a relatively uncommon type of malignancies comprising <0.5% of all carcinomas and 3% to 5% of head and neck cancers.[1] They represent a histopathologically heterogeneous group of tumors with different biological behavior and clinical features. Therefore, different classifications of salivary gland carcinomas have been introduced and are continuously updated.[2] The majority of malignancies arise in the parotid glands (80%), followed by the submandibular glands (10%) and sublingual glands (5%).[3] The main histopathological types are mucoepidermoid carcinoma, adenoid cystic carcinoma, acinic cell carcinoma, adenocarcinoma, not otherwise specified, and salivary duct carcinoma.[3] Rare salivary gland tumors include squamous cell carcinoma, myoepithelial carcinoma, malignant mixed tumor, polymorphous low-grade adenocarcinoma, basal cell adenocarcinoma, and epithelial-myoepithelial carcinoma. Acinic cell carcinomas, basal cell adenocarcinomas, and epithelial-myoepithelial carcinomas are considered low-grade neoplasms, whereas adenocarcinomas, not otherwise specified, salivary duct carcinomas, and squamous cell carcinomas in general behave much more aggressive. These high-grade neoplasms grossly invade the surrounding tissue and metastasize to the regional lymph nodes and distant organs, such as lungs and brain.[4] Mucoepidermoid carcinomas and adenoid cystic carcinomas are tumors with extremely variable behavior. In order to better predict the clinical course of the patients, a 3-tiered grading system is applied to mucoepidermoid carcinomas. Prognosis of the disease usually depends on the tumor stage, grade, and status of the surgical resection.[5] Salivary gland carcinomas are commonly treated by complete tumor removal followed by radiotherapy, if indicated by risk factors such as high-grade, positive or close resection margins, perineural or vascular invasion, and lymph node metastasis.[6] However, response rate to conventional treatment in salivary gland carcinomas remains limited.[1] New strategies for prediction of disease behavior are highly needed to improve the management of salivary gland carcinomas.

Alkylation of DNA is one of the most critical events leading to mutations in cancer-related genes.[5] O-6-methylguanine-DNA methyltransferase (MGMT) is a well known DNA repair protein that specifically removes alkyl adducts from the O-6-guanine and restores to its normal form without causing DNA strand breaks. Unrepaired

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adducts can miss-pair with T during replication, resulting into G to A mutation.[5,6] Therefore, the MGMT protein is important in maintenance of normal cell physiology, genome stability, and protection of the cell from malignant transformation. Recent studies have shown MGMT protein loss in various malignancies, including soft tissue sarcomas, gastric adenocarcinomas, gliomas, and oral tumors.[7–11] Usually the loss of MGMT protein occurs via *MGMT* promoter hypermethylation and correlates with clinicopathologic parameters.[12] Because of the rarity of salivary gland carcinomas, data on genetic and epigenetic silencing of *MGMT* in these tumors and especially in normal salivary gland tissues remain sparse.[13–15]

We analyzed the *MGMT* promoter methylation status and protein expression in a group of salivary gland carcinomas and histologically matched normal tissues from 286 patients. MGMT status was correlated to demographic clinicopathologic parameters and disease follow-up data in order to determine the prognostic value in salivary gland carcinomas. To our knowledge, this is the only study to date on such a large and homogenous set of salivary gland carcinomas. We suggest that immunohistochemical (IHC) evaluation of nuclear MGMT protein might serve as a tool for the prediction of overall survival in patients with salivary gland carcinoma.

## MATERIALS AND METHODS

### Study population and tissues specimens

The study population consisted of 286 patients with salivary gland cancer who underwent surgical resection at the Department of Otorhinolaryngology, University Hospital of Erlangen, Department of Otorhinolaryngology and Maxillofacial Surgery, Nuremberg City Hospital, and Department of Otorhinolaryngology, Regensburg University Hospital between February 1988 and July 2008. No radiotherapy or chemotherapy was administered before surgery. The removed tissues were collected after obtaining the appropriate institutional review board permission. Conventional hematoxylin-eosin-stained slides were reviewed by 2 pathologists experienced in salivary gland tumor pathology (A.A. and S.S.). The cases were then classified according to the World Health Organization classification of salivary gland tumors[2] and grouped into acinic cell carcinomas ($n = 41$), adenoid cystic carcinomas ($n = 46$), mucoepidermoid carcinomas ($n = 42$), squamous cell carcinomas/adenocarcinomas and not otherwise specified/salivary duct carcinomas ($n = 101$), myoepithelial carcinomas/malignant mixed tumors ($n = 21$), and basal cell adenocarcinomas/epithelial-myoepithelial carcinomas/polymorphics low-grade adenocarcinomas ($n = 17$). Rare categories included cystadenocarcinoma ($n = 2$), low-grade cribriform adenocarcinoma ($n = 2$), oncocytic carcinoma ($n = 4$), large cell carcinoma ($n = 2$), and small cell carcinoma ($n = 2$). For the rest of the 6 carcinomas, no agreement could be reached between the pathologists. These carcinomas were high-grade carcinomas without differentiation. Grading was based on a 3-tiered grading system, as recently described.[14,15] Acinic cell carcinoma, basal cell adenocarcinoma, epithelial myoepithelial carcinoma, cystadenocarcinoma, and polymorphous low-grade adenocarcinoma were considered low grade (G1) with the exception of dedifferentiated tumors, which were classified as high grade (G3). Salivary duct carcinoma, adenocarcinoma, not otherwise specified, squamous cell carcinoma, oncocytic carcinoma, malignant mixed tumor, undifferentiated carcinoma, small cell carcinoma, and large cell carcinoma were classified as high grade (G3). Mucoepidermoid carcinoma was graded according to the criteria proposed in the current World Health Organization classification.[16] Adenoid cystic carcinomas were divided into predominantly tubulocribriform (G2) and predominantly solid (G3) tumors. Grading of myoepithelial carcinoma was based on nuclear pleomorphism and mitotic activity similar to the Elston and Ellis[17] grading of breast cancer. The 27 cases of carcinoma ex pleomorphic adenoma were classified and graded according to the malignant component of the tumor. All cases of squamous cell carcinomas were classified as originating from the salivary glands after intensive staging procedures (CT or MRI of the head and neck, panendoscopy, X-ray or CT of the chest, and ultrasonography of the abdomen) and exclusion of a metastasis to the salivary gland. The median age of the patients was 63 years (range, 11–99 years). One hundred fifty patients (52.4%) were women and 136 (47.6%) were men. Eighty-eight of 286 tumors (30.7%) were low grade (grade I), 68 (23.7%) were grade II, and 130 (45.6%) were poorly differentiated grade III tumors. Status of distant metastases was recorded for 281 patients, of whom 23 (8.2%) were positive (pM+). Eighty of 270 tumors (29.6%) spread into the lymph nodes (pN+). In 16 patients – mainly patients with distant metastasis – reporting the nodal stage retrospectively failed because of inconsistencies in the clinical records. Tumors were staged according to the Union Internationale Contre le Cancer (UICC) criteria.[16] Of 283 patients, 66 (23.3%) had stage I, 62 (21.9%) had stage II, 62 (21.9%) had stage III, and 93 (32.9%) had stage IV tumors. For 3 cases, it was not possible to define the disease stage because clinical information was insufficient. The median survival of the entire study population was 41 months (range, 0–298 months). One hundred eight of 286 patients (37.8%) died of their disease.

### DNA preparation

Genomic DNA was extracted from formalin-fixed paraffin-embedded samples mounted on microscopic slides with strictly marked tumor borders using Nucleospin Tissue kit (Macherey–Nagel, Düren, Germany) in accord with the manufacturer's instructions. Bisulfite treatment was performed using CpGenome DNA modification kit (Millipore, Billerica, MA) and 1 µl of treated DNA aliquots were used as templates for polymerase chain reactions (PCRs) immediately after treatment.

### Quantification of DNA methylation by pyrosequencing

The level of DNA methylation of *MGMT* was assessed by pyrosequencing in 36 salivary gland carcinomas and 19 histologically normal salivary gland tissues. Pyrosequencing was followed by bisulfite treatment and PCR. Primers from commercially available kits (Biotage AB, Uppsala, Sweden) were used to amplify +17-39 bases in

TABLE 1. Baseline characteristics and association between O-6-methylguanine-DNA methyltransferase loss and various clinicopathologic factors in patients with salivary gland carcinoma.

| Variables | Total no. of cases | No. of cases (%) with MGMT loss, <30% | *p* value |
|---|---|---|---|
| Age | 286 | | |
| MGMT loss | 112 | 64.5 y | **.007** |
| MGMT expressed | 174 | 57.6 y | |
| Sex | 286 | | **.046** |
| Female | | 50/150 (33.3) | |
| Male | | 61/136 (44.9) | |
| Tumor localization | 285 | | .463 |
| Parotid glands | | 82/200 (41) | |
| Submandibular glands | | 13/42 (31) | |
| Minor salivary glands | | 16/43 (37.2) | |
| Histology | 268 | | **.002** |
| Acinic cell carcinoma | | 8/41 (19.5) | |
| Adenoid cystic carcinoma | | 11/46 (23.9) | |
| Mucoepidermoid carcinoma | | 15/42 (35.7) | |
| Squamous cell carcinoma, adenocarcinoma, not otherwise specified, salivary duct carcinoma | | 52/101 (51.5) | |
| Myoepithelial carcinoma, malignant mixed tumor | | 10/21 (47.6) | |
| Basal cell adenocarcinoma, epithelial-myoepithelial carcinoma, polymorphous low-grade adenocarcinoma | | 6/17 (35.3) | |
| Disease stage | 283 | | .106 |
| I | | 19/66 (28.8) | |
| II | | 22/62 (35.5) | |
| III | | 26/62 (41.9) | |
| IV | | 44/93 (47.3) | |
| Grade | 286 | | **< .001** |
| I | | 21/88 (18.9) | |
| II | | 25/68 (22.5) | |
| III | | 65/130 (50.0) | |
| pT | 281 | | .430 |
| 1 | | 24/76 (31.6) | |
| 2 | | 41/94 (43.6) | |
| 3 | | 27/68 (39.7) | |
| 4 | | 18/43 (41.9) | |
| Metastases | 281 | | .181 |
| No | | 104/258 (40.3) | |
| Yes | | 6/23 (26.1) | |
| Node involvement | 270 | | **< .001** |
| No | | 61/190 (32.1) | |
| Yes | | 46/80 (57.5) | |
| Death | 286 | | |
| No | | 59/178 (33.1) | **.012** |
| Yes | | 52/108 (48.1) | |

Abbreviations: MGMT, O-6-methylguanine-DNA methyltransferase.
The *p* values in boldface represent significant differences.

the promoter region of the *MGMT* gene, which was shown to correlate with MGMT protein loss previously.[18] PCR was set up in accordance with the manufacturer's recommendations and PCR products were analyzed by capillary electrophoresis (Qiaxcel, Hilden, Germany). Specific biotin-labeled PCR product was immobilized onto Streptavidin Sepharose High Performance beads (Biotage AB) purified, washed, and denaturated. Biotinylated strand was annealed to sequencing primer (0.3 μM final concentration) and was subjected to sequencing analysis using an automatically generated nucleotide dispensation order. For each pyrosequencing reaction, 20 μl of PCR product were used. Pyrosequencing was performed using the PyroMarkQ 24 instrument (BiotageAB) in conjunction to PyroGold reagents in accordance with the manufacturer's protocol. For validation of each run, commercially available CpGenome universal methylated DNA (Millipore) and deoxyribonucleic acid from human placenta (Sigma, Saint Louis, MO) served as positive and negative controls, respectively. The results were analyzed by PyroQ-CpG 1.0.10 software (Biotage AB). The methylation level was recorded as the percentage of the relative CT ratio. Average methylation level was calculated from methylation percentages for each CpG analyzed. A cutoff of 10% methylation (average methylation level at 5 CpGs,<10% vs ≥10%) was used as a threshold for describing the methylation status of a sample.

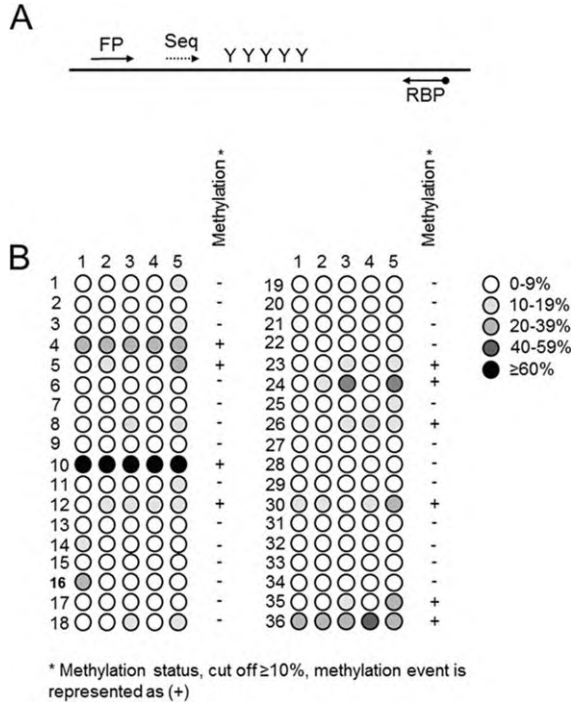


FIGURE 1. (A) Illustration of the O-6-methylguanine-DNA methyltransferase (MGMT) pyrosequencing assay. Polymerase chain reaction (PCR) primers are shown as solid arrows and the sequencing primer as a dashed arrow. FP, forward primer; RBP, biotinylated reverse primer; Seq, sequencing primer; Y, CpG site. (B) A representative DNA methylation profile of an individual CpG site in salivary gland tumor tissues. Each circle represents 1 CpG island in a panel of 5 studied.

### Immunohistochemical analysis

Two hundred eighty-six tumor samples and 70 normal salivary gland tissues taken from tumor-free margins of resected tumors and histologically confirmed as normal were studied for IHC MGMT protein expression. After construction of a tissue microarray from formalin-fixed paraffin-embedded tumor and normal tissue specimens (punch diameter, 2 mm), MGMT protein expression analysis was performed on 3-μm thin sections. Samples were analyzed in 1 slide containing a single core of each sample. Representative whole tumor slices showed that tumor heterogeneity for MGMT protein expression can be neglected.

Sections were dewaxed by xylene and rehydrated in descending concentrations of ethanol. Slides were incubated with the primary mouse monoclonal (SPM287) antibody (Abcam, Cambridge, MA) and then with the secondary antibody Envision+ (Dako, Glostrup, Denmark). Normal salivary gland tissue was used as control for positive antibody reactivity.

For the assessment of the MGMT protein expression, nuclear staining was considered. The slides were estimated by 3 investigators (S.S., A.H., and M.M.) blinded to the clinical history of the patients. The k value reached 0.66, thus demonstrating a substantial agreement between the 3 observers. In some cases, discrepancies in interpretation were resolved using a multihead microscope to reach consensus. Protein expression was quantified manually at ×200 magnification and scored as absent or low staining (<30% of cells stained) and present (≥30% of cells stained).

### Statistical analysis

Statistical analysis was performed using Fisher's or chi-square exact test in cross tables and 1-way analysis of variance (for comparisons of means) to evaluate significant differences between MGMT protein expression and clinicopathologic factors. MGMT protein expression in tumors and nontumors was analyzed by paired test. All statistical tests were 2-sided. Because of restrictions concerning the application of the chi-square test, categories with <5 cases were disregarded (Table 1). We used the log-linear model and the related chi-square test for analysis in multivariate contingency tables as well as the configuration frequency analysis for the identification of types (observed frequency was higher than the expected) and antitypes (observed frequency was lower than expected).[17] The Kaplan–Meier method and the log-rank test were used for survival curves. Multivariate analysis was performed after adjustment for age and sex and based on Cox regression analysis. Differences with $p$ values $< .05$ were considered statistically significant. All statistical calculations were performed using SPSS version 18.0.0 software package (SPSS, Chicago, IL).

## RESULTS

### O-6-methylguanine-DNA methyltransferase promoter methylation status and O-6-methylguanine-DNA methyltransferase protein expression

Methylation analysis was undertaken in a lower number of cases in which sufficient tissue material was available, including 36 tumor and 19 nontumor specimens (15 matched samples). Schematic presentation of the pyrosequencing assay and analyzed CpG sites in the promoter region of *MGMT* are given in Figure 1A. Aberrant *MGMT* promoter methylation (average methylation level at 5 CpGs ≥10%) was detected in 27.8% (10 of 36) of tumor specimens (Figure 1B). Considering all 19 normal salivary gland tissues, only 15.8% (3 of 19) were positive for methylation, whereas the rest were evaluated as unmethylated. After examining DNA methylation in the 15 matched pairs, hypermethylated DNA was detectable in normal salivary gland tissue only in case of promoter methylation in corresponding tumors. The average methylation level of *MGMT* in tumors was increased in comparison to histologically normal tissues and ranged from 2% to 35% and 1% to 10%, respectively. The methylation status in tumors did not correlate with any clinicopathologic and demographic variables.

MGMT protein expression showed a homogenous predominantly nuclear staining (Figure 2A and 2B). For 10 cases, we verified a homogeneous MGMT protein staining pattern in whole tumor slices (Figure 2B). Consequently, we suggest that the estimated punch was representative for the analysis. One hundred twelve of 286 salivary gland tumors (39.2%) showed MGMT loss, whereas the other tumors (60.8%) expressed protein immunohistochemically. Promoter hypermethylation was significantly correlated with MGMT protein loss ($p = .021$; Figure 3A). In tumors, the MGMT protein expression was

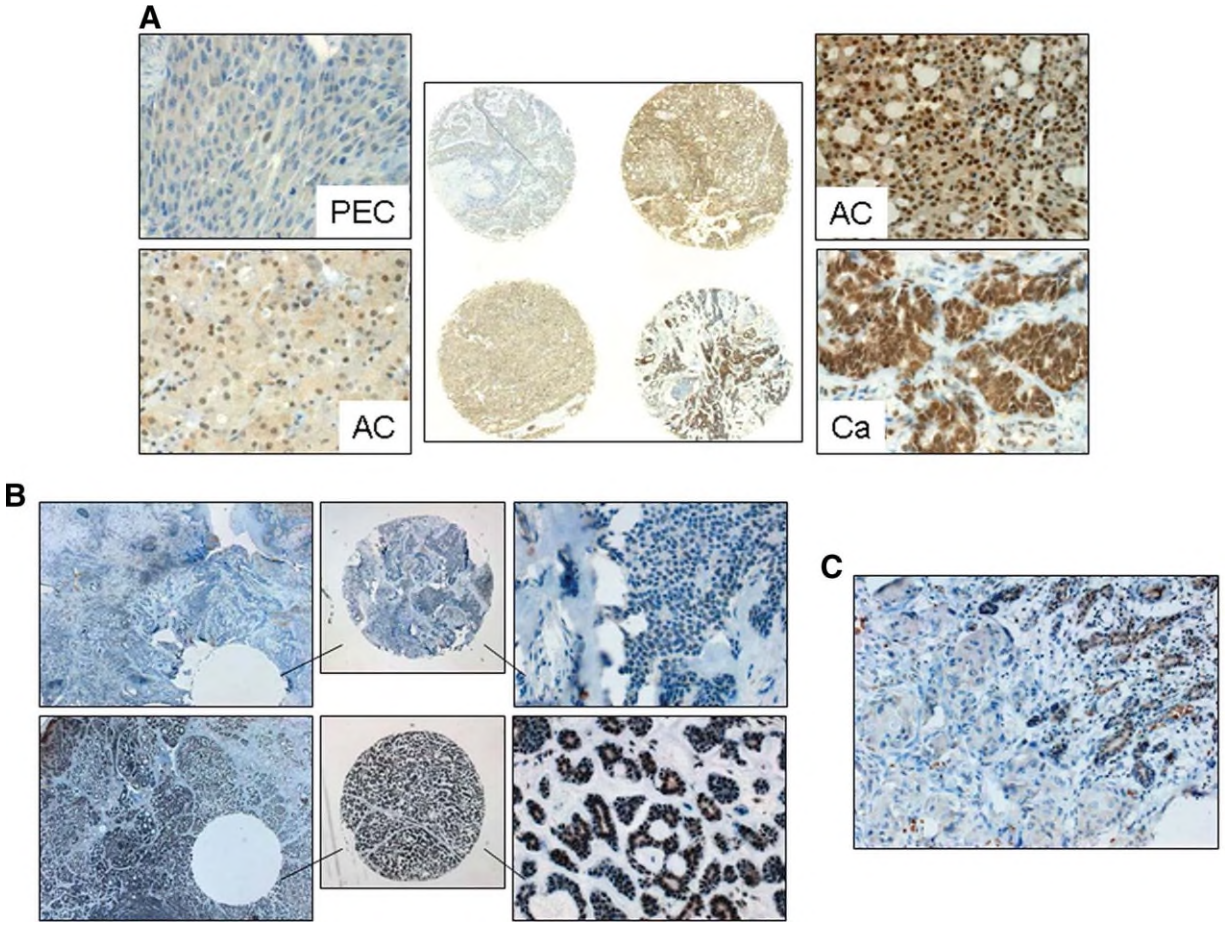


FIGURE 2. Immunohistochemical expression of O-6-methylguanine-DNA methyltransferase (MGMT) in salivary gland carcinomas. (A) Differential expression of MGMT protein. Left side: MGMT negative squamous cell carcinoma and weak expression in an adenocarcinoma not otherwise specified. Right side: strong nuclear and cytoplasmic expression in 2 carcinomas. Middle panel: punch biopsies of the tissue microarray. (B) Tissue microarray reflects the protein expression pattern of the whole tumor slice. Upper panel: no expression of MGMT within the entire tumor (left: 25-fold magnification; right: 400-fold magnification; middle: representative tissue punch from the tumor). Lower panel: strong immunohistochemical staining of MGMT protein (left: 25-fold magnification; right: 400-fold magnification; middle: representative tissue punch from the tumor). (C) Representative example of MGMT expression in salivary gland tissues with negative staining in the tumor (left side) and moderate staining in the normal tissue (right side).

significantly lower than in normal salivary gland tissues (31% vs 45%, respectively; $p = .001$; Figures 2B and 3B).

## O-6-methylguanine-DNA methyltransferase protein expression and correlation with clinicopathologic data

The group of poorly differentiated tumors, such as squamous cell carcinomas/adenocarcinomas and not otherwise specified/salivary duct carcinomas, showed MGMT loss (52 of 101; 51.5%) at higher frequency than did acinic cell carcinomas (8 of 41; 19.5%), adenoid cystic carcinomas (11 of 46; 23.9%), and mucoepidermoid carcinomas (15 of 42; 35.7%; $p = .002$; Table 1; categories with <5 cases were disregarded). Protein loss was observed more frequently in grade III tumors (50%) than grade I (18.9%) and grade II (22.5%) tumors ($p < .001$; Figure 4A; Table 1). Also, significant positive correlation was found between MGMT protein expression and regional lymph node involvement. Patients with pN+ tumors had a significant lower MGMT protein expression ($p < .001$; Figure 4B). Interestingly, patients having MGMT-negative tumors were significantly older in age ($p < .007$; Figure 4C; confirmed by a significant negative Spearman correlation coefficient with $r_s = -0.222$; $p < .001$). Men showed a higher frequency of MGMT-negative tumors (44.9% vs 33.3%). There was no statistical correlation between IHC MGMT protein expression and tumor size, distant metastasis, and UICC stage, although there was a tendency of prevalence of MGMT loss with advanced clinical stages (28.8%, 35.5%, 41.9%, and 47.3% for stages I–IV, respectively).

There was a significant correlation between MGMT loss and death of disease: 52 of 108 dead patients (48.1%) had MGMT-negative tumors, whereas only 59 of 178 yet alive patients (33.1%) had negative MGMT protein expression ($p = .012$; Figure 4D). Investigating single histological subgroups, we verified the worse prognosis for MGMT-negative tumors also for

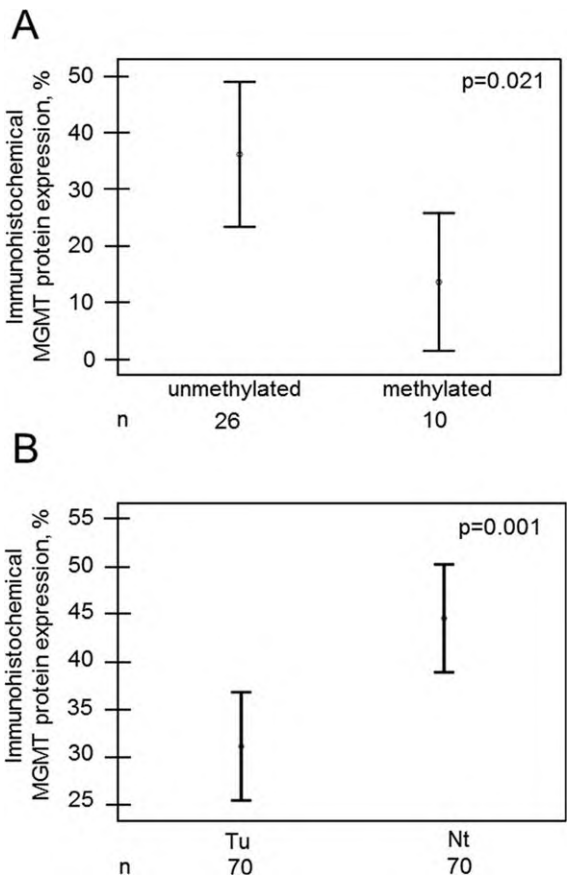


FIGURE 3. (A) The association between O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter hypermethylation and protein expression in salivary gland tumors. (B) The protein expression detected in tumors was lower in comparison to matched normal tissues.

mucoepidermoid carcinoma ($p < .08$; Figure 5A) and adenocarcinoma not other specified ($p < .04$; Figure 5B). All other subgroups always showed the same tendency but without reaching any statistical significance.

## Prognostic values of demographic and clinical variables

The Kaplan–Meier curves reflected the prognostic values of the classic clinical features demonstrating the accuracy of sampling of our tumor group. In the Kaplan–Meier analysis (log-rank test), the worse prognosis was seen in patients having higher pT ($p < .001$), pN+ ($p < .001$), pM+ ($p < .001$), high-grade ($p < .001$), and high UICC stage ($p < .001$) tumors, incomplete tumor resection (tumor margins, $p < .001$), and histological high-grade types (squamous cell carcinomas/adenocarcinomas, not otherwise specified/salivary duct carcinomas; $p < .001$). Patients with tumors localized in minor salivary glands showed the best prognosis in comparison to all other tumor localizations ($p = .005$). Using the univariate Cox regression analysis, we verified the high prognostic impact of all these factors (Table 2). When those factors showing a statistically significant ($p < .05$) correlation to outcome in univariate analysis were included in multivariate Cox regression analysis, sex, age, tumor grade, tumor resection in respect to tumor margins, and UICC stage retained their strong independent prognostic value (Table 2).

## Prognostic value of O-6-methylguanine-DNA methyltransferase protein loss

Patients having tumors with MGMT protein loss had a worse prognosis than those showing MGMT protein expression. Five-year overall survival in these 2 groups with and without MGMT loss was 55.9% and 69.9%, respectively. The 10-year survival magnifies the prognostic difference between both groups (38.0% vs 59.9%). There was a high predictive value for MGMT loss on the clinical outcome of these patients ($p = .004$; log-rank; Figure 4E). Fifty-two of 108 patients (48.1%) with MGMT-negative tumors but only 59 of 178 patients (33.1%) with MGMT-positive tumors died of disease. Thus, MGMT seems to be useful for defining a subgroup with unfavorable prognosis of patients with salivary gland tumors.

Univariate Cox regression analysis revealed a correlation between MGMT loss and overall survival (Table 2) showing a 1.7-fold risk of dying of disease. But MGMT loss did not retain an independent prognostic value in the multivariate Cox regression analysis.

In our study sex, age, lymph node involvement, tumor categories, distant metastases, tumor grade, UICC stage, tumor localization, histological subtype, and incomplete tumor resection were shown as significant predictors for worse prognosis in salivary gland carcinomas; thus, we assessed the prognostic value of MGMT loss in the prognostically favorable groups of tumors separately. MGMT loss defined a high-risk group in metastasis-free (pM-) patients (Figure 4F; $p = .006$) and showed a tendency to define a subset of tumors with a worse prognosis in lymph node-free (pN-) patients ($p = .08$), in lower UICC stages (I and II; $p = .07$) and in women ($p = .049$). Considering the group of grade I tumors, and tumors with lower tumor categories (pT1, pT2), the MGMT loss did not yield any additional predictive value ($p = .33$ and $p = .515$, respectively). Examining tumors with and without complete tumor resection separately, we found that MGMT loss was correlated with an unfavorable prognosis in both groups ($p = .04$ for both). Analyzing the tumor types, adenoid cystic carcinomas, mucoepidermoid carcinomas, and squamous cell carcinomas/adenocarcinomas, and not otherwise specified/salivary duct carcinomas separately, we found an additional value for MGMT loss that allowed us to select a subgroup with worse prognosis only in the squamous cell carcinomas/adenocarcinomas, not otherwise specified/salivary duct carcinomas ($p = .041$). The other tumor types have not been analyzed because of the low number of cases within the groups.

We performed the multivariate configuration analysis including 3 prognostic parameters: MGMT loss and died of disease together with a third variable: pN, grade, and pM, respectively. The table for multivariate

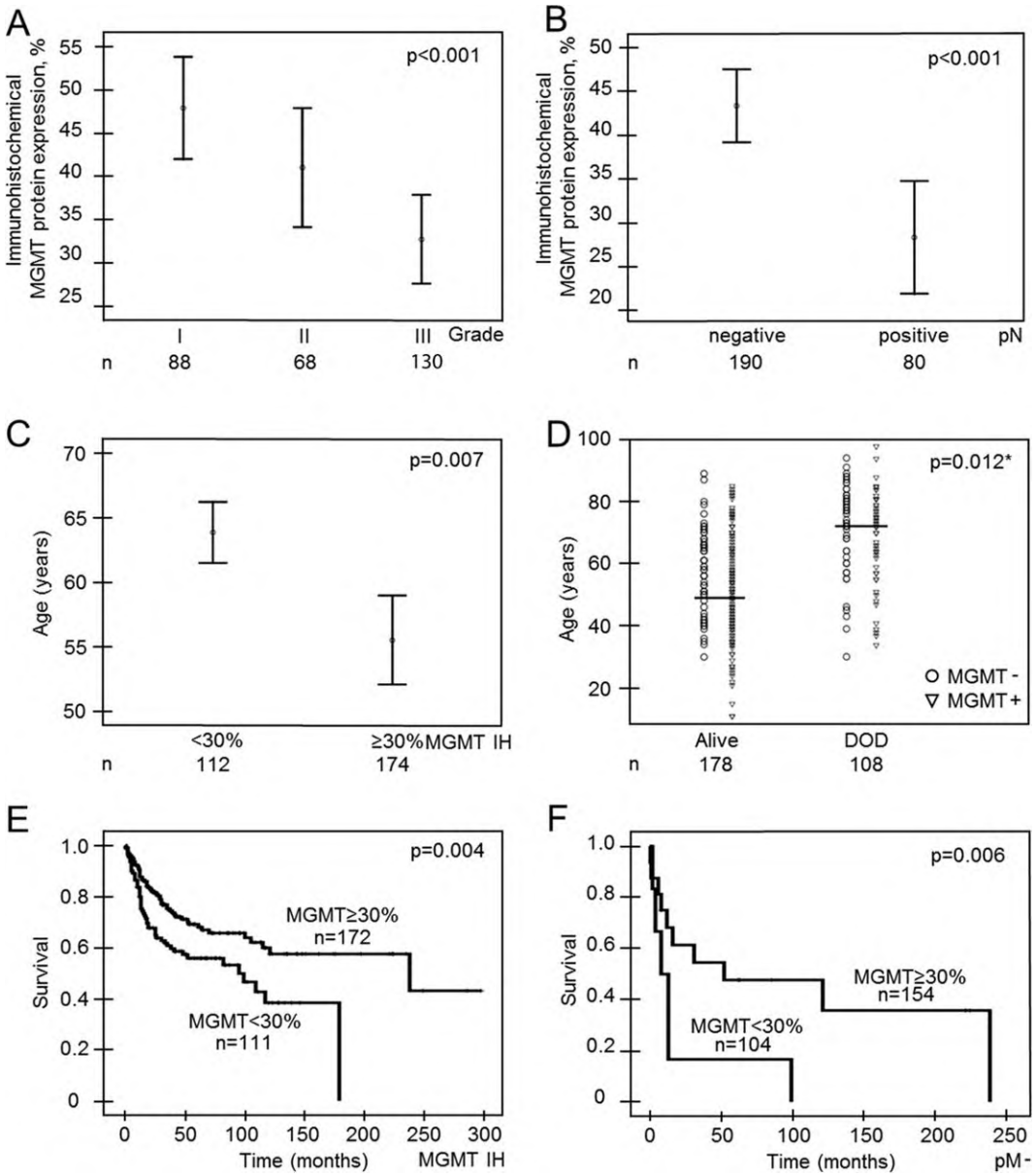


FIGURE 4. The association between O-6-methylguanine-DNA methyltransferase (MGMT) protein expression and: (A) tumor grade, (B) node involvement, (C) patients' age at diagnosis, (D) patients' age at diagnosis in died of disease group (DOD) and alive group (*$p$ value represents correlation between MGMT protein loss and DOD). (E) Kaplan–Meier curve for survival of patients with salivary gland carcinomas as a function of MGMT protein expression. (F) Kaplan–Meier curve for survival of patients with metastasis-free (pM-) salivary gland tumors.

configuration analysis revealed that loss of MGMT, pN+, and died of disease were highly associated prognostic variables showing types and antitypes (Table 2). The antitype reflected the underrepresented combination for the 3 variables. Exemplarily shown, patients with MGMT-positive and pN+ tumors can be rarely found alive (antitype: 16 cases observed, but 30 cases statistically estimated) whereas an overrepresented group of patients with MGMT loss and pN+ tumors who died of disease (type: 27 cases observed, but only 11 cases statistically estimated). Conversely, patients with MGMT expression and pN- tumors have more favorable outcome (type). Similarly, multivariate contingency table showed a significant association between MGMT loss and tumor grade in relation to death of disease. We identified the overrepresented "types" consisting of the combination of the following variables: MGMT loss, high-grade, died of disease and MGMT-positive, lower grade, and alive (Table 2). Otherwise there were 2 "antitypes" with MGMT expression, low grade and died of disease, verifying a favorable outcome in this patient group as well as MGMT expression, high-grade and alive documenting a rather unfavorable clinical course (Table 2). The variable combination consisting of MGMT loss, died of disease, and pM did not reveal types or antitypes.

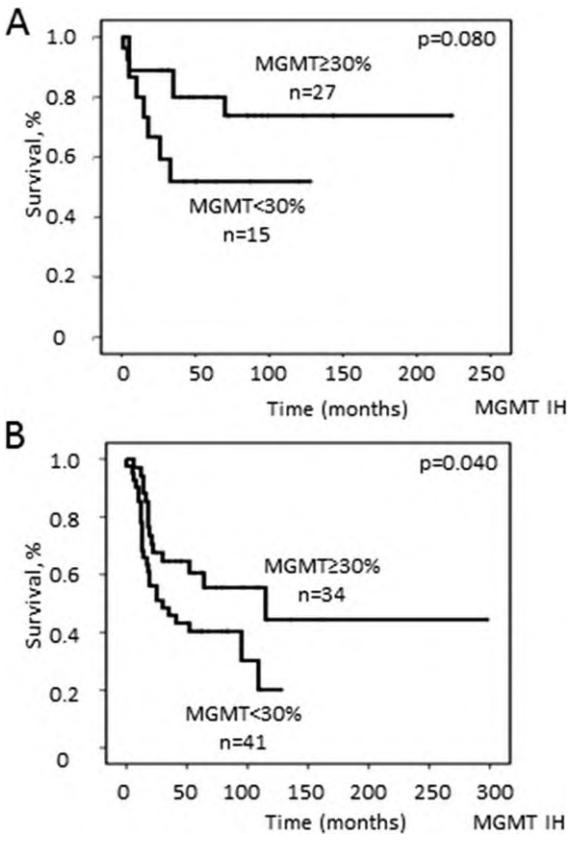


FIGURE 5. A) Kaplan-Meier curve for survival of patients with mucoepidermoid carcinoma and B) adenocarcinoma not other specified as a function of MGMT protein expression.

## DISCUSSION

This study is the first to evaluate the prognostic significance of MGMT in a large group of patients with well-characterized salivary gland carcinomas and long-term follow-up in which MGMT protein expression and promoter hypermethylation were simultaneously evaluated in matched tumor and nontumor tissues. Our results suggest that loss of MGMT protein in salivary gland carcinomas occurs via promoter hypermethylation, and downregulation of protein is more prevalent in tumors than in nontumor tissues. Furthermore, the absence of tumor MGMT is associated with worse overall survival in patients with salivary gland carcinomas. This is important for clinical and pathogenesis-related prospects because no epigenetic marker for salivary gland carcinomas, to date, has been validated for clinical utility.

Several reports have established that MGMT promoter hypermethylation and/or loss of *MGMT* gene expression are prognostic for poor survival in patients with various cancers.[12,19] Transcriptional silencing by promoter hypermethylation is postulated to be the predominant mechanism leading to the MGMT protein loss in different tumor types.[19,20] As indicated elsewhere,[9] the level of methylation within promoter and neighboring sequences may additionally regulate the *MGMT* gene expression. In this study, we used a highly sensitive pyrosequencing method to evaluate 5 CpGs in the MGMT promoter. We determined methylation status in tumor DNA and found significant correlation with the protein loss. Only 1 previous study investigated the relationship between aberrant MGMT promoter hypermethylation and protein loss in 42 salivary gland carcinomas and no association was recognized.[13] Potential explanation may lie in the low number of salivary gland carcinomas analyzed. However, larger investigations on head and neck cancer showed a significant link.[19] In a recent study by Durr et al[15] MGMT promoter hypermethylation was not detected in a group of 78 salivary gland carcinomas using a quantitative methylation specific PCR (MSP) method. The discrepancy to our study with a methylation frequency of 27.8% could be explained by the different CpG islands investigated in Durr et al[15] and our studies. In this respect, it is known that the methylation status of promoter regions may differ at single CpG sites and may have also different consequences for transcriptional silencing of the gene.[21] However, larger studies and mechanistic work is needed to clarify this point.

A better understanding of the molecular alterations associated with salivary gland carcinomas will help to improve the diagnosis, management, and outcomes of this patient population. In our study, the MGMT promoter hypermethylation was highly associated with loss of protein expression, and MGMT protein loss was shown to be of high prognostic value in a series of 286 salivary gland carcinomas. We found more frequent loss of MGMT protein with larger tumor sizes and in tumors metastasized to lymph nodes showing that the loss of this repair protein is also associated with tumor progression. MGMT loss was also useful for risk prediction in female patients but not in grade I and II tumors. It is known that the histological type itself contributes to the prognostic estimation, as acinic cell carcinomas, basal cell adenocarcinomas, and epithelial-myoepithelial carcinomas are often associated with an indolent course of the disease,[18,22,23] complicated by relapse but only rarely by dedifferentiation and metastasis. In contrast, squamous cell carcinomas, adenocarcinomas, not otherwise specified, and salivary duct carcinomas behave extremely aggressively. The clinical course of patients with adenoid cystic carcinomas and mucoepidermoid carcinomas is much more variable and often unpredictable. Because some tumors might be grouped into more than 1 category, caution should be taken. In our study, the loss of MGMT protein expression was predominant in high-grade tumors of squamous cell carcinomas/adenocarcinomas, not otherwise specified/salivary duct carcinomas, followed by myoepithelial carcinomas/malignant mixed tumors. Accordingly, the worst survival probability was observed for patients with these neoplasms. Nevertheless, analyzing the squamous cell carcinomas/adenocarcinomas, not otherwise specified/salivary duct carcinomas separately it was possible to select a more aggressive subpopulation with MGMT protein loss.

Several clinicopathologic studies of salivary gland carcinomas demonstrated that clinicodemographic parameters, such as age, tumor size, and presence of residual tumor could serve as prognostic parameters.[24–26] Diverse authors proved pN status to be of high prognostic

TABLE 2. Prognostic significance of O-6-methylguanine-DNA methyltransferase loss and clinical-demographic variables in univariate, multivariate and multivariate configuration analysis.

| | | Overall survival | |
|---|---|---|---|
| Variable | No. of cases | Relative risk | *p* value |
| **Univariate analysis** | | | |
| Sex | 284 | 0.587 | .007 |
| Age | 284 | 4.819 | .001 |
| pN | 271 | 1.681 | < .001 |
| pT | 279 | 1.561 | < .001 |
| pM | 281 | 1.575 | .023 |
| Grade | 283 | 2.548 | .001 |
| Stage | 283 | 1.703 | < .001 |
| Localization | 284 | 0.709 | .019 |
| Type | 266 | 1.344 | .001 |
| R | 270 | 2.347 | < .001 |
| MGMT loss | 283 | 1.742 | .004 |
| **Multivariate analysis** | | | |
| Stage | 270 | 0.024 | 1.298 |
| Sex | 270 | 0.638 | .042 |
| Age | 270 | 1.067 | < .001 |
| Grade | 270 | 1.606 | .006 |
| R | 270 | 1.964 | < .001 |

| **Multivariate configuration analysis** | | | | | | |
|---|---|---|---|---|---|---|
| MGMT | pN | DOD/alive | No. of observed cases | No. of estimated cases | *p* value | Type/antitype |
| Loss | Yes | DOD | 27 | 11.51 | < .001 | Type |
| Expressed | No | Alive | 98 | 73.07 | .004 | Type |
| Expressed | Yes | Alive | 16 | 30.77 | .008 | Antitype |
| MGMT | Grade | DOD/Alive | No. of observed cases | No. of estimated cases | *p* value | Type/antitype |
| Loss | High | DOD | 39 | 18.85 | < .001 | Type |
| Expressed | Low | Alive | 92 | 59.23 | < .001 | Type |
| Expressed | Low | DOD | 18 | 36.35 | .002 | Antitype |
| Expressed | High | Alive | 26 | 48.6 | .001 | Antitype |

Abbreviations: R, tumor margins; MGMT, O-6-methylguanine-DNA methyltransferase; DOD, died of disease.
Univariate analysis: prognostic significance of sex, age, pN, pT, pM, grade, stage tumor localization, type and tumor resection margins, and MGMT loss in salivary gland carcinoma using Cox regression model (univariate analysis). Multivariate analysis: prognostic significance of sex, age, grade, tumor resection margins and stage in salivary gland carcinoma using Cox regression model (multivariate analysis with reduction using all variables from the univariate Cox regression analysis). Multivariate configuration analyses, including the 3 variables: MGMT expression/loss, node involvement, DOD, and MGMT loss, grade, and DOD.

impact,[27,28] Also, grading guides in prediction of disease behavior, but, so far, its profit has consistently been demonstrated for myoepithelial carcinomas, whereas grading of adenoid cystic carcinomas remains controversial.[29] Other and our previous studies evaluated the use of IHC markers to predict outcome in patients with salivary gland carcinomas.[30–33] In the last years, genetic and epigenetic markers such as *RB1* or *RARB* promoter methylation were found to significantly contribute to characterization of tumor pathogenesis and estimating prognosis of this disease.[14,34]

MGMT represents one of the defense mechanisms critical to maintaining the genome integrity of the cell. In this study, MGMT loss was found to be correlated with older age of patients. Reduced repair capacity because of aging or polymorphisms known in the *MGMT* gene[12] may lead to an increased DNA error rate, and consequently cause cancer. It is well known that epigenetic alterations contribute to aging-associated pathologies and especially cancer.[35] The epigenetic changes may initiate aging and cancer or prime cells to make them more susceptible to subsequent genetic or epigenetic alterations.[35] Because tumors with MGMT loss are more aggressive ones, we can explain the observation that elderly patients more often have pN+ tumors in our tumor group. Our data finally reflect that a destroyed balance between DNA damage and repair has a major impact on aging.

Recent observations highlight the importance of MGMT methylation/protein loss as a specific predictive biomarker for the responsiveness to alkylating chemotherapy. Indeed, MGMT methylation/loss is associated with significantly prolonged overall and disease-free survival in glioma patients treated with alkylating agents, such as temozolomide.[36,37] Vice versa, the expression of MGMT results in the resistance of tumors to alkylating agents.[6] However, in a clinical study in patients with oligodendroglial tumors, MGMT promoter methylation was of prognostic significance but did not have predictive significance for outcome to adjuvant procarbacine and vincristine chemotherapy.[38] To date, nonalkylating chemotherapy is administered to patients with salivary gland carcinoma, however, MGMT loss could not be examined in correlation with therapy responsiveness in our tumor group. We suggest from our study that elderly female patients with high-grade salivary gland carcinomas could be promising candidates for an alkylating chemotherapy.

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