

Promoter methylation of *TIMP3* and *CDH1* predicts better outcome in head and neck squamous cell carcinoma treated by radiotherapy only

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Abstract. As with other solid tumor types, head and neck squamous cell carcinoma (HNSCC) has been identified as an epigenetic, as well as genetic, disease. Consequently, promoter hypermethylation, being the most important aberrant epigenetic characteristic, has been intensively investigated for its biomarker potential in this cancer type. As many of these evaluations are obscured by a heterogeneity of treatments, the current study aimed to evaluate the incidence and prognostic value of the promoter hypermethylation of *TIMP3*, *CDH1*, *DAPK*, *RASSF1A*, *p16^{INK4A}* and *MGMT* in HNSCC treated solely by radiotherapy. In 46 patients with advanced HNSCC treated with a hybrid accelerated fractionation radiotherapy schedule, DNA extracted from pretreatment paraffin-embedded tumor biopsies was used to determine the methylation status of the genes of interest by methylation-specific PCR (MSP). The detected epigenetic silencing was related with outcome in terms of locoregional control (LRC), and overall (OS), disease-free (DFS) and disease-specific survival (DSS). Tumor biopsies revealed the epigenetic silencing of *MGMT* in 42.5% (17 of 40) of patients and of *TIMP3* in 40.5% (17 of 42) of cases. For the remaining investigated genes, a lower methylation percentage was detected: 13.2% (5 of 38) for *CDH1*, 11.4% (4 of 44) for *DAPK*, 4.8% (2 of 42) for *p16^{INK4A}* and 2.4% (1 of 41) for *RASSF1A*. The promoter hypermethylation of *TIMP3* and *CDH1* was significantly related with better LRC ($p=0.009$ and $p=0.02$, respectively), OS ($p=0.005$ and $p=0.002$, respectively), DFS ($p=0.02$ and $p=0.004$, respectively) and DSS ($p=0.12$ and $p=0.007$, respectively). In conclusion, in

this representative group of 46 patients with advanced HNSCC treated by radiotherapy only, the epigenetic silencing of *TIMP3* and *CDH1* predicted a better outcome.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common solid tumor, with a worldwide incidence of >600,000 patients annually. This disease originates at several subsites in the head and neck region and typically affects older men with a history of tobacco and/or alcohol consumption. Partly due to the late onset of symptoms, HNSCC is often diagnosed in an advanced stage. Treatment for these malignancies mainly consists of surgery or radiotherapy, sometimes supplemented with chemotherapy. Despite considerable progress in therapy, outcome is still poor with 30-40% control rates in advanced stage disease.

As a result, research efforts focus on improving existing treatment modalities aiming to achieve better tumor control combined with limited normal tissue toxicity and maximal organ preservation. Concerning radiotherapy, these goals can be achieved by implementing altered fractionation and/or combination with chemotherapy. Altered fractionation schedules such as hyperfractionation and acceleration have been developed to improve the tumor control/normal toxicity ratio and have shown their efficacy in several randomized trials (1-7). However, the balance between improving outcome and limiting toxicity remains fragile (8-10). Due to this, our institute recently implemented a hybrid fractionation schedule. Favourable locoregional tumor control rates were obtained in the absence of significant toxicity, suggesting that this fractionation is feasible and can eventually be combined with chemotherapy (11).

Another strategy in the optimization of HNSCC therapy is the search for molecular markers that may help stratify patients to the most optimal treatment protocols. Researchers and clinicians have been evaluating markers originating from the tumor itself and its microenvironment to a variable success. One of the latest developments in this field has been derived from the acknowledgement that cancer is an epigenetic disease. Hematological and solid malignancies suffer from an altered epigenetic balance, of which the presence of promoter hypermethylation and histone modifications are the most

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important features. These alterations appear to cooperate in the transcriptional silencing of genes involved in a variety of processes (12,13). In HNSCC, the hypermethylation of CpG islands has been described in the promoter region of genes involved in cell cycle control [cyclin A1 (14), *p16^{INK4A}* (14-18)], apoptosis [*DAPK* (16-18)], cell adhesion [E-cadherin (*CDH1*) (14,16-19), *TIMP3* (14,16)], DNA repair [*MGMT* (14-17,20) and *ATM* (21)], cell proliferation and growth [*RASSF1A* (16,18,22)] and other cellular processes (23). Notably, the aberrant methylation profile has been observed in bodily fluids such as serum (24) or plasma (25), and for HNSCC in particular, in saliva (16,26) or oral scrapings (27). Whether these bodily fluid alterations actually have the potential to act as a tumor biomarker and predict recurrence during follow-up remains unclear. The presence of promoter hypermethylation in the tumor itself has also inconsistently been linked with outcome. Some groups have described epigenetic silencing as being associated with poor prognosis (20,21,28), while others have found no correlation (16,17) or an inverse one (14,29). These differences may be partly attributed to the heterogeneity of the studies including different HNSCC subsites, methodologies and treatments.

The present study used methylation-specific PCR (MSP) to investigate the methylation status and paired prognostic value of a set of six genes in 46 patients treated with hybrid fractionated radiotherapy. Promoter hypermethylation was investigated for *TIMP3*, *CDH1*, *p16^{INK4A}*, *MGMT*, *DAPK* and *RASSF1A*. Righini *et al* (16) recently showed that at least one of these 6 genes was methylated in >75% of primary HNSCC without additional positive samples when other genes were investigated.

Materials and methods

Patients and samples. Our group recently published a study on the feasibility and outcome of hybrid accelerated fractionation radiotherapy in a group of 73 patients with advanced HNSCC (11). Based on the availability of a paraffin-embedded pre-treatment tumor biopsy, promoter hypermethylation was assessed for 46 of these cases. The patients of this subgroup were treated between December 2000 and January 2004. Briefly, they received 20 daily fractions of 2 Gy (40 Gy) followed by 20 fractions of 1.6 Gy twice daily (32 Gy) to a total dose of 72 Gy. No patient received chemotherapy. The patients had HNSCC with primary site in the oral cavity, oropharynx, larynx or hypopharynx and did not have any evidence of distant metastasis at the time of treatment. Patients did not undergo routine neck dissections after primary RT, although surgery was considered if patients had evidence of residual disease or locoregional recurrence. Patient characteristics (age, gender, primary tumor site, histology, TNM classification and clinical stage) were recorded at the start of treatment.

In addition, a group of five controls was selected consisting of healthy individuals from whom fresh-frozen normal head and neck mucosa biopsies were obtained. Upon removal, the mucosa biopsies were immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C. Of these healthy control patients, two were active smokers, one had recently quit smoking and the other two were non-smokers.

DNA extraction and bisulfite modification. Of the paraffin blocks with embedded tumor biopsies, one section of 5 µm and 10 serial sections of 10 µm each were cut. The 5 µm section of each series was used to perform a hematoxylin and eosin staining from which the tumor region was delineated from the surrounding tissue. Upon removal of the paraffin, the 10-µm slices were dissected manually in order to extract DNA from at least 70% of tumor cells. From the five fresh-frozen normal mucosa biopsies, a sample of ±25 mg was used for direct extraction.

DNA extraction was performed using the Qiagen DNA mini kit (Qiagen) following the supplier's protocol and DNA yield and purity were determined by a spectrophotometer (Nanodrop Technologies). As a negative control, DNA extracted from the peripheral lymphocytes of healthy individuals was used. This DNA was treated with M. SssI CpG methylase (New England Biolabs) in the presence of S-adenosylmethionine (New England Biolabs) to serve as a positive control. DNA concentrations were adjusted to 50 ng/µl. To convert unmethylated cytosines into uracil while leaving methylated cytosines unaltered, 500 ng of each DNA sample was bisulfite-modified with the EZ DNA methylation kit (Zymo Research) according to the provided protocol.

Methylation-specific PCR (MSP). To determine the methylation status of the promoter regions of *TIMP3*, *CDH1*, *p16^{INK4A}*, *MGMT*, *DAPK* and *RASSF1A*, bisulfite-treated DNA was amplified in separate reactions using specific primers for the methylated (M) and unmethylated (U) promoters. A water control was added to each PCR series. Each PCR reaction was performed in a total 25 µl reaction volume containing 2 µl of the samples or controls, 1X buffer [16.6 mM (NH₄)₂SO₄, 67 mM Tris pH 8.8, 6.7 mM MgCl₂·6H₂O and 10 mM β-mercaptoethanol], 62.5 µM dNTPs, 0.4 µM primer sense and antisense and 0.5 units of Jump start Red Taq polymerase (Sigma). PCR runs were performed on a thermocycler (Biometra TProfessional, Westburg) under the following conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at the appropriate temperature and 30 sec elongation at 72°C. A final elongation step of 4 min at 72°C was added. Ten microliters of each PCR amplification were loaded directly onto a 3% ready agarose wide-mini precast gel with ethidium bromide (BioRad). Primer sequences and corresponding annealing temperatures are listed in Table I. Sequences for *TIMP3*, *CDH1*, *DAPK*, *MGMT* and *RASSF1A* were adapted from Righini *et al* (16) while sequences for *p16^{INK4A}* were available in-house and self-created.

Statistical analysis. A correlation between the clinicopathological characteristics and the methylation status of the genes was investigated by applying Fisher's exact test for discrete variables, or the Mann Whitney U-test for relations between continuous and discrete variables. Follow-up data were retrospectively collected: information was gathered on the date of first recurrence (local-regional) and/or poor outcome. Locoregional relapse, succumbing to the disease and succumbing to other causes were used as endpoints. The close-out date for survival analysis was March 2008. Survival curves were constructed using the Kaplan-Meier method, with

Table I. MSP primers and annealing temperatures.

Gene	U/M	Sense primer, 5'→3'	Antisense primer, 5'→3'	bp	AT (°C)
<i>TIMP3</i>	U	TGTGTTGGAGGTTAAGGTTGTTTT	CCTCTCCAAAATTACCATACACACC	97	59
	M	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	95	59
<i>CDH1</i>	U	TAATTTTAGGTTAGAGGGTTATTG	CACAACCAATCAACAACACA	97	53
	M	TTAGGTTAGAGGGTTATCGCGT	TAATAAAAAATTCACCTACCGAC	115	60
<i>p16^{INK4A}</i>	U	GTTGGTTATTAGAGGGTGGGGTGGATTGT	AACCAAAAACTCCATACTACTCCCCACCA	124	60
	M	TTATTAGAGGGTGGGGCGGATCGC	GAAAACTCCATACTACTCCCCGCCG	115	60
<i>MGMT</i>	U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	58
	M	TTTCGACGTTTCGTAGGTTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	58
<i>DAPK</i>	U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	106	60
	M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60
<i>RASSF1A</i>	U	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCACAACTAAAAACAA	108	60
	M	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	94	60

U, unmethylated sequence; M, methylated sequence; bp, fragment length in base pairs and AT, annealing temperature.

time intervals being calculated from the date of biopsy-proven diagnosis. Individual factors were evaluated for their predictive value by the log-rank test. Tests were two-sided, using a significance level of $p < 0.05$. Statistics were calculated using Statistica software 8.

Results

Patient characteristics. Patient and tumor characteristics are summarized in Table II. The study included 42 men and 4 women, with a mean age of 59 years (range 43-76). The majority of patients had a tumor of either oropharynx ($n=25$, 54.3%) or larynx ($n=14$, 30.4%). Staging was performed according to the TNM classification system of the UICC and was based on clinical and radiological criteria. Most patients presented with locally ($>T2$ tumors: $n=37$, 80.5%) and regionally (N^+ : $n=32$, 69.6%) advanced tumors, while 91.3% ($n=42$) had stage III or IV disease. Median follow-up was 34 months (range 3-85).

Promoter hypermethylation. The MSP analyses revealed aberrant hypermethylation of *MGMT* in 42.5% (17 of 40) of HNSCC patients and of *TIMP3* in 40.5% (17 of 42) of cases. For the remaining investigated genes, a lower percentage of promoter hypermethylation was detected: 13.2% (5 of 38) for *CDH1*, 11.4% (4 of 44) for *DAPK*, 4.8% (2 of 42) for *p16^{INK4A}* and 2.4% (1 of 41) for *RASSF1A*. At least one of the six investigated genes was methylated in 26 of 46 patients (56.5%). By evaluating the concordant methylation, it appeared that the tumors methylated for *CDH1*, were also methylated for *TIMP3*. No other concordant methylation patterns were observed.

The healthy control patients were free from epigenetic silencing of *MGMT*, *p16^{INK4A}* or *RASSF1A*. Weak methylation was detected in the promoter region of *DAPK* in the two

controls who were active smokers. For *TIMP3*, weak methylation was present in one active and one former smoker. In the active and former smokers, MSP for *CDH1* showed weak methylation.

Correlation with clinicopathological characteristics. We investigated the correlation between a) clinicopathological characteristics such as T (T1-2 vs. T3-4) and N classification (N^+ vs. $N0$), clinical stage (stage IV vs. other stages), tumor site and age, and b) the methylation status of the most frequently methylated genes (*TIMP3*, *MGMT*, *CDH1* and *DAPK*). Other variables such as differentiation and gender were not tested because of the low proportions in some of their subgroups. Similarly, *p16^{INK4A}* and *RASSF1A* methylation were not included in these analyses because of the low methylation percentage.

TIMP3 and *CDH1* methylation were more frequent in smaller tumors. For *TIMP3*, 6 of 8 T1-T2 tumors were methylated compared with 11 of 34 T3-T4 tumors ($p=0.04$). Similarly, 3 of 8 T1-T2 tumors were methylated for *CDH1* compared with 2 of 30 T3-T4 tumors ($p=0.05$). Patients whose tumors showed *DAPK* hypermethylation were older (median age 63 vs. 57 years, $p=0.06$), which was also the case for patients with *CDH1*-methylated tumors (median age 70 vs. 58 years, $p=0.003$). No other significant correlations were observed.

Outcome analyses (Table III)

Locoregional control (LRC). Locoregional control of the entire group of 46 patients was 56.6% at 2 years. In the univariate analysis, the T classification (T3-T4 vs. T1-T2) predicted a poorer LRC (47.5 vs. 89% at 2 years, $p=0.03$). *TIMP3* methylation predicted a better LRC rate (76.5 vs. 37.5% at 2 years, $p=0.009$) (Fig. 1), as did *CDH1* methylation (100 vs. 46% at 2 years, $p=0.02$).

Table II. Patient characteristics.

	Patient no. (%) (n=46)
Gender	
Male	42 (91.3)
Female	4 (8.7)
Primary tumor site	
Larynx	14 (30.4)
Oropharynx	25 (54.3)
Hypopharynx	5 (10.9)
Oral cavity	2 (4.3)
Tumor grade	
I	3 (6.5)
II	17 (37.0)
III	17 (37.0)
Unknown	9 (19.6)
T classification	
T1	2 (4.3)
T2	7 (15.2)
T3	13 (28.3)
T4	24 (52.2)
N classification	
N0	14 (30.4)
N1	10 (21.7)
N2	18 (39.1)
N3	4 (8.7)
Clinical stage	
I	0 (0)
II	4 (8.7)
III	8 (17.4)
IV	34 (73.9)

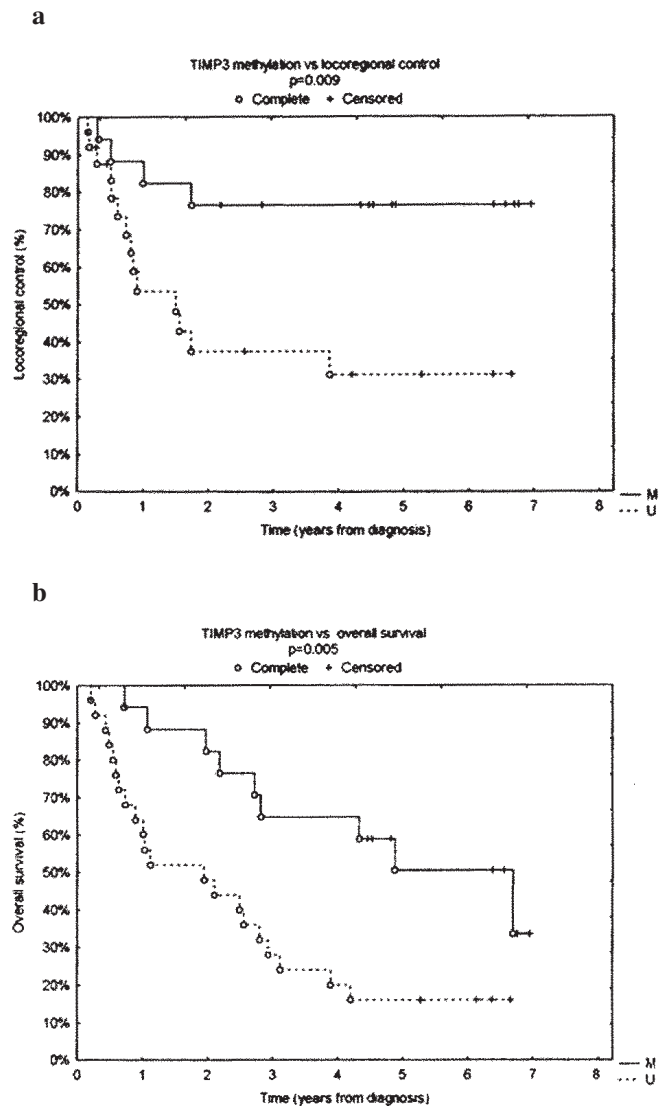
Figure 1. Kaplan-Meier curves of the impact of the *TIMP3* promoter hypermethylation on (a) locoregional control (LRC) and (b) overall survival (OS), as determined by the log-rank test are shown.

Table III. Survival analyses.

	No. of cases/ total no. (%)	Locoregional control	Overall survival	Disease-free survival	Disease-specific survival
Clinicopathological features					
T3-T4	37/46 (80.5)	0.03 (-)	0.004 (-)	0.002 (-)	0.0009 (-)
N ⁺	32/46 (69.6)	NS	0.09 (-)	NS	0.04 (-)
Stage IV	34/46 (73.9)	NS	NS	NS	0.02 (-)
Methylation					
<i>TIMP3</i>	17/42 (40.5)	0.009 (+)	0.005 (+)	0.02 (+)	0.12 (+)
<i>CDH1</i>	5/38 (13.2)	0.02 (+)	0.002 (+)	0.004 (+)	0.007 (+)

P-values in the univariate analysis are shown for clinicopathological and methylation data towards locoregional control, and metastasis-free, overall, disease-free and disease-specific survival. NS, not significant. (-) or (+) indicate whether the investigated parameter has a negative or positive predictive value towards outcome, respectively.

Overall survival (OS). For the patients investigated, the survival rate was 60.9% at 2 years and 43.5% at 3 years. In the univariate analysis, a higher T classification (T3-T4 vs. T1-T2) was related to poorer survival (52 vs. 100% at 2 years, $p=0.004$), while the methylation of *TIMP3* (Fig. 1) and *CDH1* predicted a better OS (*TIMP3*: 82.5 vs. 48.5% at 2 years, $p=0.005$ and *CDH1*: 100 vs. 52% at 2 years, $p=0.002$). Nodal invasion was weakly associated with a poorer OS (71.5 vs. 56.5% at 2 years, $p=0.09$).

Disease-free survival (DFS). Disease-free survival for the patients investigated was 50.8% at 2 years and 46.0% at 3 years. A better DFS was predicted for tumors with a lower T classification (T1-T2 vs. T3-T4: 89 vs. 56% at 2 years, $p=0.002$), *TIMP3* methylation (77 vs. 35% at 2 years, $p=0.02$) and *CDH1* methylation (100 vs. 41.5% at 2 years, $p=0.004$).

Disease-specific survival (DSS). The disease-specific survival for the whole patient group was 66.1% at 2 years and 53.4% at 3 years. Of the investigated clinicopathological parameters, a poorer DSS was significantly associated with a higher T classification (T3-T4 vs. T1-T2: 56 vs. 100% at 2 years, $p=0.0009$), nodal invasion (61 vs. 77.5% at 2 years, $p=0.04$) and a higher clinical stage (stage IV disease 53% vs. other stages 92% at 2 years, $p=0.02$). Patients with tumors methylated for *CDH1* had a better DSS (100 vs. 56.5% at 2 years, $p=0.007$). The same was true for tumors with *TIMP3* methylation (82 vs. 58% at 2 years), although this difference did not reach significance ($p=0.12$).

Discussion

In the search for new molecular markers which can predict the response of HNSCC patients to radiotherapy, this study aimed to investigate promoter hypermethylation as an epigenetic tumor marker in this cancer type. The epigenetic silencing of *TIMP3*, *CDH1*, *DAPK*, *RASSF1A*, *p16^{INK4A}* and *MGMT* was assessed in a group of 46 patients with advanced HNSCC, who were treated with hybrid accelerated fractionation radiotherapy as recently published (11).

In the pretreatment paraffin-embedded tumor biopsies, higher methylation rates were found for *MGMT* (42.5%) and *TIMP3* (40.5%), and lower levels were detected for *CDH1* (13.2%), *DAPK* (11.4%), *p16^{INK4A}* (4.8%) and *RASSF1A* (2.4%). For *MGMT*, *TIMP3*, *CDH1* and *DAPK*, these rates are within the range that has been published in the literature. Methylation percentages that have been previously described are between 14 and 56.4% for *MGMT* (14-17,20,24,26,29-32), 3-71.8% for *TIMP3* (14,16,33), 2-78% for *CDH1* (14,16-19, 28,30-32,34,35) and 7-74.2% for *DAPK* (16-18,24,26,30, 31,33,36). For *RASSF1A*, the methylation rate in our patient population was somewhat lower than in the literature [7.5-26% (16,18,22,30,31)]. The largest differences between previous reports and the current study were for *p16^{INK4A}*, with published percentages of between 23 and 51% (14-18,24,26,29-32, 34,36) in contrast to 4.8% in the present study. It is clear that for the investigated genes, a broad range of methylation rates has been described. Reasons for these discrepancies may be found in differences in techniques and primers [MSP (18), pyrosequencing (34), quantitative MSP (33) and restriction-

based PCR (32)], materials [fresh-frozen (18) vs. paraffin-embedded (17)], HNSCC subsites and patient numbers. For this study we only had the availability of paraffin-embedded material limiting us to the use of techniques that are not too demanding on DNA amount and quality such as MSP.

Concordant methylation, as we observed for *CDH1* and *TIMP3*, has previously been described (33), occasionally in the context of so-called CpG island methylaton profiles (CIMP) (14,34). However, besides the relationship between the methylation of these genes and involvement in tumor invasion, no other significant overlap in epigenetic silencing was observed. The limited number of patients and genes included in this study makes conclusiveness on the presence of a CIMP in this series impossible.

The normal control samples of non-smokers were free of the promoter hypermethylation of *p16^{INK4A}*, *RASSF1A* and *MGMT*, as expected (15,16,26). The detection of methylation of *DAPK*, *TIMP3* and *CDH1* to some extent in the normal mucosa of former or current smokers has yet to be described but would not be surprising given the (debated) association of smoking with epigenetic silencing in HNSCC and other solid tumor types (18,37,38).

The detected promoter hypermethylation was also related with the clinicopathological data of the patients. A more frequent methylation of *TIMP3* and *CDH1* was observed in smaller tumors. The connection of *TIMP3* and *CDH1* hypermethylation with early stage tumors may be contradictory to several reports suggesting that epigenetic silencing is more common in advanced stage disease (18,24,36). However, the absence of such a relationship (29,32), or even the presence of an inverse one (14,17) has also been described.

The finding that the epigenetic silencing of *DAPK* and *CDH1* occurred preferentially in older patients is in concordance with the fact that epigenetic signaling becomes altered during aging (39).

Finally, our study investigated the clinicopathological and methylation parameters for their influence on the outcome of patients treated by radiotherapy only. As shown in Table III, advanced disease indicated by a higher T or N classification or higher tumor stage significantly correlated with the poorer outcome. These observations are in agreement with what has been published for the original group of 73 patients treated with the hybrid radiotherapy schedule (11). More importantly, the fact that the classic clinicopathological features relevant in the outcome of HNSCC retained their significance in the current selection of 46 patients supports the representativeness of our patient group.

Further analyses showed that *TIMP3* and *CDH1* promoter hypermethylation was predictive of better radiotherapy response, as indicated by a better LRC. The potential of methylation of the two genes as biomarkers for better outcome in HNSCC was also reflected by their significant association with OS, DFS and DSS. Being aware of the limitations to the observations for *CDH1*, given its small methylation percentage, we concluded that the markedly significant effects on outcome merited further attention, due to our findings for *TIMP3*. A positive correlation between epigenetic silencing of one or more genes and tumor response has already been published (14,29). Concerning *TIMP3* and *CDH1* in particular, most studies in HNSCC have been performed on patients

treated with heterogeneous treatments largely based on surgery. Epigenetic silencing of *CDH1* has been related with poor survival in oral tongue carcinoma (28), while other studies did not reveal any prognostic or predictive value (16,17). For *TIMP3*, two groups found no relationship with outcome in HNSCC (16,33). Other publications are in support of our results: methylation analyses of *TIMP3* and *CDH1* were included in the CIMP phenotype described by Shaw *et al* which correlated with better survival (14). Promoter hypermethylation of the two genes has also been connected with better outcome in non-small cell lung (NSCLC) and bladder cancer (40-42). Notably, as with HNSCC, NSCLC and bladder cancer are two malignancies strongly associated with tobacco smoking. It may be possible that smoking modifies the effect of methylation of cell adhesion molecules such as *CDH1* and *TIMP3*, on tumor behavior.

This is the first study to report on the impact of epigenetic silencing of *CDH1* and *TIMP3* in HNSCC patients treated homogeneously with radiotherapy only. For *CDH1*, several arguments support the relationship between promoter hypermethylation and better outcome. A low expression of E-cadherin is normally associated with a decrease in adhesion and an enhancement of metastatic behavior. However, the observation that E-cadherin loss can also inhibit terminal differentiation and preserve the ability of cell proliferation may explain the better response to ionizing radiation (43). This idea is supported by a Danish study in supraglottic larynx squamous cell carcinoma that showed the benefit of accelerated radiation for tumors with a low E-cadherin expression (44). Similar findings have yet to be described for *TIMP3* but are plausible given its function as compared to that of *CDH1*.

Taken together, we have shown, largely in concordance with the literature, the hypermethylation of genes involved in several processes in a representative group of 46 patients with advanced HNSCC. Of the investigated genes, epigenetic silencing of *TIMP3* and *CDH1* was associated with better outcome as indicated by LRC, OS, DFS and DSS. In view of the contradictory reports on the prognostic impact of the hypermethylation of *TIMP3* and *CDH1*, we believe the relative homogeneity of the group of HNSCC patients, who were treated with the same radiotherapy regimen, is in favor of our study. These findings need confirmation from larger, preferably prospective clinical trials.

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