Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma

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Received June 25, 2009; Accepted September 4, 2009

DOI: 10.3892/or_00000596

Abstract. Squamous cell carcinomas of head and neck (HNSCC) are a result of multiple genetic and epigenetic alterations. Epigenetic inactivation of tumor suppressor genes is an important event in head and neck carcinogenesis. Here we analyzed the promoter methylation of 15 genes (*RASSF1A*, *p16*, *MGMT*, *DAPK*, *RARβ*, *MLH1*, *CDH1*, *GSTP1*, *RASSF2*, *RASSF4*, *RASSF5*, *MST1*, *MST2*, *LATS1*, *LATS2*) in 54 HNSCC and in matching 23 normal tissues. Methylation of these tumor-related genes (TRG) was significantly more frequent in HNSCC (42%) compared to normal samples (23%; p<0.05). Particularly, methylation of *p16* (60%), *MGMT* (53%), *DAPK* (67%), *RARβ* (75%), *MLH1* (69%), *CDH1* (43%), *RASSF5* and *MST1* (96%) was often found in HNSCC. Methylation of *RASSF1A* (18%), *GSTP1* (4%), *RASSF4* (13%), *MST2* (4%), *LATS1* (24%) and *LATS2* (8%) was less frequently detected. A trend of increased TRG methylation in more advanced tumor stages and less differentiated HNSCC was observed. Methylation of *p16* was significantly higher in poorly differentiated HNSCC (p=0.037) and *RASSF5* methylation occurred preferentially in advanced tumor stages (p<0.05). Methylation of *RASSF4* was higher in patients with recurrent HNSCC (23%) than patients without relapse (0%; p=0.033). Methylation of TRG in head and neck cancer cell lines was observed at similar frequency as in primary HNSCC. In summary, frequent hypermethylation of tumor-related genes in HNSCC was detected and this epigenetic silencing event may have an essential role in head and neck carcinogenesis.

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Abbreviations: HNSCC, head and neck squamous cell carcinomas; TRG, tumor-related genes; RASSF, ras association domain family; MGMT, O-6-methylguanine-DNA methyltransferase; DAPK1, death-associated protein kinase 1; RAR, retinoic acid receptor; MLH1, mutL homolog 1; CDH1, cadherin 1; GSTP1, glutathione S-transferase pi 1; MST, mammalian sterile20-like kinase; LATS, large tumor suppressor; Hpo, hippo; Sav, salvador; MSP, methylation specific PCR; COBRA, combined bisulfite restriction analysis

Key words: head and neck cancer, tumor suppressor gene, epigenetics, DNA methylation, biomarker

Introduction

Squamous cell cancer of head and neck (HNSCC) is one of the most prevalent cancers in the world, with over 400,000 cases diagnosed annually (1). It accounts for 5% of all newly diagnosed cancers worldwide and is the sixth most common cancer in the world (2). Despite considerable improvements in diagnosis, treatment and understanding of the molecular mechanisms in HNSCC, the overall survival rate has remained constant at approximately 60% over the past 30 years in the United States (3). This lack of progress in prognosis is mainly due to the high loco-regional recurrence, distant metastases rate and high incidence of secondary primary tumors, which often located in the same or adjacent anatomical regions (4). Cancerous lesions are regarded as multifactorial diseases affected through multiple exogenous agents. The association between tobacco smoking and alcohol consumption and the development of HNSCC has been established (5-7).

Genetic and epigenetic alterations of tumor-related genes (TRG) caused by carcinogens contribute essentially to tumor development and tumor progression in head and neck cancer. Epigenetic silencing of TRG is mediated by aberrant methylation of CpG islands promoter (8). Promoter hypermethylation has been studied as a biomarker system for diagnosis and detection of early cancers and can be probably used as prognostic factor in malignant diseases (9). Hypermethylation of tumor suppressor gene is frequently found in HNSCC (10). In particular promoter methylation of *p16*, *RASSF1A*, *MGMT*, *DAPK*, *CDH1* and *RARβ* have been described (11-18).

The aim of our study was to clarify, whether promoter hypermethylation of several tumor-related genes correlates with clinical variables, and is prognostic value in HNSCC. Therefore, we analyzed TRG involved in DNA damage avoidance and repair (*GSTP1*, *MGMT* and *MLH1*), in signal

Table I. Demographic data of head and neck cancer patients.

	No. (%)
No. of patients	54
Mean age (years)	57 (41-77)
Male/Female ratio	48/6
Localization of HNSCC	
Larynx	20 (37)
Hypopharynx	8 (15)
Oropharynx	18 (33)
Tongue	8 (15
Tumor staging	
I	4 (7)
II	0
III	12 (22)
IV	38 (70)
T-stage	
T1	9 (17)
T2	8 (15)
T3	23 (43)
T4	14 (26)
N-stage	
N0	12 (22)
N1	7 (13)
N2	28 (52)
N3	7 (13)
Grading	
Well differentiated (G1)	7 (13)
Moderately differentiated (G2)	32 (59)
Poorly differentiated (G3)	15 (28)

transduction (*RASSFs*), in differentiation (*RARβ*), in cell adhesion (*CDH1*), in cell cycle control (*p16* and *LATSs*), and in apoptosis (*DAPK* and *MSTs*). Methylation of TRG occurred frequently in HNSCC and hypermethylation was correlated with clinical and histopathological data.

Materials and methods

Cell lines and tissue samples. All probes were obtained from patients of the Department of Otolaryngology (University of Halle-Wittenberg) by surgical resection and were stored at -80˚C until use. For this study 54 squamous cell carcinomas of the head and neck and 23 corresponding macroscopically normal tissues from opposite cheek mucosa of several patients were analyzed (Table I). The average age of the investigated population was 57 years. Each tumor was scored based on the current TNM classification (19). The local medical ethics committee approved the study and all patients gave their consent. Three head and neck cell lines Hep-2 (larynx carcinoma), RPMI-2650 (nasal septum SCC) and UM-SCC-14C (SCC) were obtained from Cell Lines Service (Eppelheim, Germany) and cultured in the recommended growth medium. DNA of patient samples and cell lines were isolated by a standard phenol/chloroform extraction and a NaAc/EtOH-precipitation. As a methylation control genomic DNA of HeLaS3 was *in vitro* methylated using the CpG methylase *SssI* (New England Biolabs, Frankfurt am Main, Germany) according to the recommendations of the manufacturer.

Methylation-specific PCR. Promoter methylation of *RASSF1A*, *p16*, *MGMT*, *DAPK1*, *RARβ*, *MLH1*, *CHD1*, *GSTP1*, *RASSF5*, *MST1*, *MST2*, *LATS1* and *LATS2* was analyzed by methylation-specific PCR (Table IV). Therefore, bisulfite-treated DNA samples were amplified with methylation- and unmethylation specific primers by a standard PCR protocol (20,21). All PCR products were analyzed on 2% TBE agarose gels.

Combined bisulfite restriction analysis. Methylation of *RASSF2* and *RASSF4* was analyzed using the combined bisulfite restriction analysis (22). Bisulfite treated DNA was amplified with primers specific for *RASSF2* or *RASSF4* (Table IV). For *RASSF2* a semi-nested PCR using an internal primer was performed. PCR products (20-50 ng) were restricted with 10 units of *BstUI* (New England Biolabs, Beverly, MA) according to the manufacturer's protocol and analyzed on 2% TBE agarose gels.

Statstics. All statistical correlations were performed by SPSS 15.0 (Chicago, IL, USA).

Results

Frequent methylation of tumor-related genes in head and neck cancer. Methylation status of 15 CpG island promoters of tumor related genes (*RASSF1A*, *p16*, *MGMT*, *DAPK*, *RARβ*, *MLH1*, *CDH1*, *GSTP1*, *RASSF2*, *RASSF4*, *RASSF5*, *MST1*, *MST2*, *LATS1* and *LATS2*) was analyzed in 54 head and neck squamous cell carcinoma (HNSCC) and 23 matching normal tissues. Representative data are shown in Fig. 1 and results are summarized in Table II. Combined methylation of TRG (n=15) was 42% in primary HNSCC (Table II) and this methylation was significantly higher compared to normal matching tissues (23%; p=0.04, Fisher's exact test). Hypermethylation of *p16* (60%), *MGMT* (53%), *DAPK* (67%), *RARβ* (75%), *MLH1* (69%), *CDH1* (43%), *RASSF*5 and *MST1* (96%) was frequently observed in HNSCC. Methylation of *RASSF1A* (18%), *GSTP1* (4%), *RASSF4* (13%), *MST2* (4%), *LATS1* (24%) and *LATS2* (8%) was less frequently detected. No significant correlation between methylation of TRG was found. Only methylation of *MST2* occurred more frequent in normal tissues (55%) compared to HNSCC (4%). Methylation of *RASSF1A* and *RASSF4* was not detected in normal matching samples (Table II). To confirm the aberrant methylation of TRG in established head and neck cancer, we analyzed the methylation status in three head and neck cancer cell lines (Table II). The methylation index of 15 TRG in cancer cell lines (44%) was similar compared to primary HNSCC (42%; Table II). Our results show that methylation of TRG is frequent in head and neck cancer.

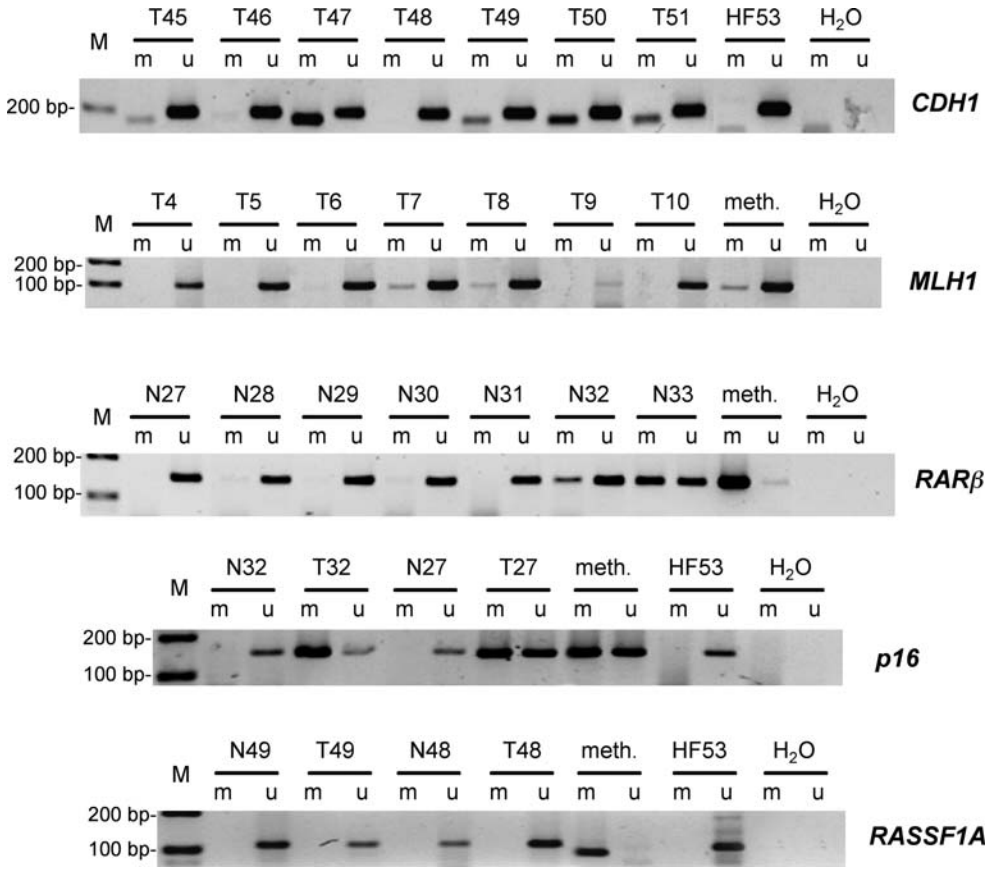

Figure 1. Methylation analysis in primary head and neck tissue samples. Representative results of the methylation specific PCR (MSP) of *CDH1*, *MLH1*, *RARβ*, *p16* and *RASSF1A*. Bisulfite treated DNA from HNSCC (T) and matching normal tissues (N) were amplified with methylation specific primers (m) and unmethylation specific primers (u) together with controls (human fibroblast DNA, HF53; *in vitro* methylated DNA, meth. and water, H_2O). PCR products were separated on 2% TBE agorose gels with a 100 bp marker (M).

Table II. Summary of methylation analyses.

	Primary HNSCC n=54 (%)	Normal tissues n=23 (%)	Cancer cell lines n=3 (%)	Recurrence n=30 (%)	No recurrence n=20 (%)
RASSF1A	18	0	66	17	24
p16	60	24	0	64	60
MGMT	53	29	100	48	59
DAPK1	67	33	66	59	72
RARβ	75	47	66	81	81
MLH1	69	45	66	68	71
CDH1	43	8	0	45	40
GSTP1	4	5	0	7	0
RASSF2	41	11	33	41	45
RASSF4	13	0	0	**23**[a]	**0**[a]
RASSF5	58	17	100	57	55
MST1	96	42	100	96	94
MST2	4	55	0	7	0
LATS1	24	23	0	17	25
LATS2	8	9	66	3	15
MI (n=15) (%)					
± SD	42±28	23±17	44±40	42±28	42±30

Significant changes are indicated in bold: [a]p=0.033 (Fisher's exact test); MI, methylation index; SD, standard deviation.

Table III. Correlation of methylation and clinicopathological characteristics.

		Staging				Differentiation			T-stage				N-stage			
	Age	I	II	III	IV	G1	G2	G3	T1	T2	T3	T4	N0	N1	N2	N3
RASSF1A																
m (%)		33	-	10	21	0	21	8	25	25	14	8	10	14	16	33
m	51±8	1	0	1	6	0	6	1	2	2	3	1	1	1	4	2
u	57±10	2	0	9	29	4	23	11	6	6	18	11	9	6	21	4
p16																
m (%)		66	-	50	57	50	**43**[a]	**83**[a]	63	75	48	54	50	43	64	57
m	58±11	2	0	5	20	2	13	10	5	6	10	7	5	3	16	4
u	54±7	1	0	5	15	2	17	2	3	2	11	6	5	4	9	3
MGMT																
m (%)		33	-	60	53	25	56	58	33	57	39	56	78	33	46	80
m	59±10	1	0	6	17	1	15	7	3	4	12	5	7	2	11	4
u	55±9	2	0	4	15	3	12	5	6	3	9	4	2	4	13	1
DAPK1																
m (%)		33	-	40	74	100	62	69	86	63	67	58	38	57	80	57
m	58±8	1	0	4	25	2	18	9	6	5	14	7	3	4	20	4
u	58±12	2	0	6	9	0	11	4	1	3	7	5	5	3	5	3
RARβ																
m (%)		100	-	60	75	50	78	67	89	83	74	75	71	83	78	67
m	58±9	3	0	3	24	4	18	8	8	5	14	6	5	5	18	4
u	55±8	0	0	2	8	4	5	4	1	1	5	2	2	1	5	2
MLH1																
m (%)		33	-	50	75	67	61	85	50	75	77	69	70	43	81	57
m	58±10	1	0	5	27	2	19	11	4	6	17	9	7	3	21	4
u	58±10	2	0	5	9	1	12	2	4	2	5	4	3	4	5	3
CDH1																
m (%)		0	-	36	51	0	53	36	33	50	50	38	18	57	56	29
m	59±8	0	0	4	18	0	16	5	3	4	10	5	2	4	14	2
u	55±10	3	0	7	17	3	14	9	6	4	10	8	9	3	11	5
GSTP1																
m (%)		0	-	0	6	0	3	7	0	13	6	0	0	0	8	0
m	64±18	0	0	0	2	0	1	1	0	1	1	0	0	0	2	0
u	57±9	3	0	9	34	4	28	13	8	7	21	12	11	5	24	7
RASSF2																
m (%)		33	-	50	38	25	45	31	33	25	33	64	50	43	32	50
m	56±12	1	0	5	13	1	13	4	3	2	7	7	5	3	8	3
u	58±8	2	0	5	21	3	16	9	6	6	14	4	5	4	17	3
RASSF4																
m (%)		0	-	0	17	25	8	23	17	0	10	23	0	0	12	50
m	53±10	0	0	0	6	1	2	3	1	0	2	3	0	0	3	3
u	58±10	2	0	7	29	3	23	10	5	6	19	10	9	4	23	3
RASSF5																
m (%)		67	-	55	57	24	60	67	**44**[b]	**38**[b]	**50**[b]	**91**[b]	55	57	54	60
m	58±10	2	0	6	20	1	18	8	4	3	11	10	6	4	14	3
u	56±9	1	0	5	15	3	12	4	5	5	11	1	5	3	12	2

Table III.Continued.

		Staging				Differentiation			T-stage				N-stage			
	Age	I	II	III	IV	G1	G2	G3	T1	T2	T3	T4	N0	N1	N2	N3
MST1																
m (%)		67	-	100	94	67	96	100	86	86	100	100	88	100	100	100
m	58±10	2	0	8	31	2	26	13	6	6	20	12	7	6	24	6
u	71±9	1	0	0	2	1	1	0	1	1	0	0	1	0	0	0
MST2																
m (%)		0	-	10	3	0	4	7	0	13	5	0	10	0	4	0
m	73±4	0	0	1	1	0	1	1	0	1	1	0	1	0	1	0
u	57±9	3	0	9	32	4	26	13	9	7	20	10	9	6	23	7
LATS1																
m (%)		0	-	9	26	0	20	23	0	13	39	9	9	14	33	14
m	62±9	0	0	1	9	0	6	3	0	1	9	1	1	1	8	1
u	56±10	3	0	10	25	4	24	10	8	7	14	10	10	6	16	6
LATS2																
m (%)		0	-	9	6	0	6	14	0	0	9	15	9	0	8	14
m	55.5±2	0	0	1	2	0	2	2	0	0	2	2	1	0	2	1
u	57±10	3	0	10	34	4	30	12	9	8	20	11	10	7	24	6
MI (n=15) (%) ± SD		31±31	-	36±28	44±28	29±30	41±28	45±31	37±31	41±30	41±28	44±32	37±30	36±30	45±30	45±28

Significant changes (Fisher's exact test) are indicated in bold: [a]p=0.037 (G2 vs G3) and [b]p<0.05 (T4 vs. T3, T2 and T1); MI, methylation index; SD, standard deviation.

Increased promoter methylation of tumor-related genes occurs in advanced HNSCC. Subsequently, we determined if promoter hypermethylation correlated with different clinicopathological parameters including recrudescence, age, stage, differentiation and TNM staging and lymph node status (Tables II and III). No significant methylation differences between recurrent and non-recurrent tumors were observed (42 vs. 43%, respectively; Table II). However, *RASSF4* methylation was preferentially detected in recurrences (p=0.033). Methylation of TRG was not associated with aging (Table III). In stage IV HNSCC combined methylation (44%) was higher compared to stage III (36%) and methylation of *RASSF1A*, *DAPK1*, *MLH1*, *CDH1*, *RASSF4* and *LATS1* increased considerably (Table III). Methylation index of TRG in poorly differentiated HNSCC (45%) was noticeably higher compared to well differentiated tumors (29%). In poorly differentiated HNSCC methylation of *p16*, *MGMT*, *RASSF5*, *MST1*, *LATS1* and *LATS2* was more pronounced compared to moderately differentiated HNSCC (Table III) and this trend was significant for *p16* (43 vs. 83%, respectively; p=0.037). Combined methylation of TRG in stage T4 (44%) was higher compared to stage T1 (37%). Methylation of *RASSF2*, *RASSF5* and *LATS2* occurred more frequent in stage 4 than in lower stages and this trend was significant for *RASSF5* (p<0.05). In HNSCC with increased involvement of cancerous lymph node (N2 and N3), a higher methylation index (45%) compared to N0 (37%) was found (Table III). Particularly hypermethylation of *RASSF1A* and *RASSF4* was observed in N3 compared to N0 (Table III). In summary, these data indicate a higher methylation frequency of TRG in more advanced HNSCC.

Discussion

HNSCC is one of the most frequent cancers and therefore new biomarkers for early diagnosis and to predict prognosis are important. Promoter hypermethylation of tumor-related genes (TRG) is a promising new tool for these issues (9,23). In our study, we evaluated the promoter hypermethylation of 15 TRG in primary HNSCC and matching normal tissues. Methylation of several TRG was preferentially found in HNSCC indicating a cancer relevant event of TRG silencing. Tumor specific methylation of several members of *RASSFs* (*RASSF1A*, *RASSF2*, *RASSF4* and *RASSF5*) and downstream effectors (*MST1*, *LATS1* and *LATS2*) was found (Table II). Methylation of certain TRG was neither co-segregating nor mutually exclusive.

In previous work, we have identified the *RASSF1A* tumor suppressor gene, which is frequently hypermethylated in primary tumors, including lung cancer (40-80%) (24-26). In HNSCC, *RASSF1A* methylation is less frequent (18%) and this was previously observed (12,27,28). Interestingly, *RASSF1A* methylation was only found in tumors and not in normal mucosa from cancer patients and increased in more

Table IV. Primers and conditions for methylation-specific PCR (MSP) and COBRA.

	Forward primer (5'-3')	Reverse primer (5'-3')	Length (bp)
RASSF1A	M: GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	94
	U: TTTGGTTGGAGTGTGTTAATGTG	U:CAAACCCCACAAACTAAAAACAA	108
p16	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	151
MGMT	M: TTTCGACGTTCGTAGGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	81
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U:AACTCCACACTCTTCCAAAAACAAAACA	93
DAPK	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	106
RARβ	M: TCGAGAACGCGAGCGATTCG	M: GACCAATCCAACCGAAACGA	146
	U: TTGAGAATGTGAGTGATTTGA	U: AACCAATCCAACCAAAACAA	146
MLH1	M: AACGAATTAATAGGAAGAGCGGATAGCG	M: CGTCCCTCCCTAAAACCGACTACTACCC	91
	U: TAAAAATGAATTAATAGGAAGAGTGGATAGTG	U: AATCTCTTCATCCCTCCCTAAAACA	102
CDH1	M: GGTGAATTTTTAGTTAATTAGCGGTAC	M: CATAACTAACCGAAAACGCCG	204
	U: GGTAGGTGAATTTTTAGTTAATTAGTGGTA	U: ACCCATAACTAACCAAAAACACCA	211
GSTP1	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	91
	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	97
RASSF2	F: GGTGTAGGGTTGGGGAGGGTTTGAT	R1: AACAAAACCCTCAATCTCCCTATAAAACCA	384
		R2: CCCAACCACCTCAAACACCAACTCC	220
RASSF4	F: GTAGCGGTTTTTGTTGGAAGTTTAGGAGTT	R: AGTTGAATAATGGTTTGGGGATATTTGGT	174
RASSF5	M: CGTCGTTTGGTACGGATTTTATTTTTTTCGGTTC	M: GACAACTTTAACAACGACGACTTTAACGACTACG	202
	U: ATTTATATTTGTGTAGATGTTGTTTGGTAT	U: ACTTTAACAACAACAACTTTAACAACTACA	215
MST1	M: GCGGGGCGGGTTTAGGAGGTTC	M: CCAATAACCCCTCACCGACGCG	120
	U: TTTGTGGGGTGGGTTTAGGAGGTTTGT	U: AACCAATAACCCCTCACCAACACAACAA	125
MST2	M: CGGGAGGGAGATTCGTCGCG	M: AAACCGAAACACCGACCGACCG	99
	U: TTTTAAGTGGGAGGGAGATTTGTTGTGG	U: AAAAACCAAAACACCAACCAACCAAACC	108
LATS1	M: GAACGATTAGAGTTGCGGGCGAC	M: TGAATGATTAGAGTTGTGGGTGATGT	126
	U: AACATTTCCCGACGTCGCTTACG	U: AAACATTTCCCAACATCACTTACACA	128
LATS2	M: TTCGTTCGGATTGGTATGCGGTC	M: CCATCTTCCCGAAACGCTCACG	137
	U: GGTGTTTTGTTTGGATTGGTATGTGGTT	U: CATCTTCCCAAAACACTCACACCACA	141

M, methylation specific primer; U, unmethylation specific primer; F, forward primer; R, reverse primer.

advanced tumor stages and when more affected lymph node were involved (Table III). Methylation of *RASSF4* was rather infrequent and this was also reported for nasopharyngeal cancer (29). However, a trend for higher *RASSF4* methylation was detected in recurrent HNSCC, stage IV and N3 HNSCCs. *RASSF1A* and *RASSF4* are tumor suppressors and participate in the proapopotic Hippo-pathway (Hpo; *drosophila* MST homologue) through a Sav-RASSF-Hpo domain, which is located at their C-terminus (30). This Sav-RASSF-Hpo domain is also found in *RASSF2* and *RASSF5* (NORE1). To our knowledge this is the first report of frequent methylation *RASSF2* and *RASSF5* in HNSCC (41 and 58%, respectively). Zhang *et al* have reported that *RASSF2* methylation occurs in 51% of nasopharyngeal carcinoma and correlates with lymph node metastasis (31). Methylation of RASSF-dependent proapopotic kinase *MST1* was frequently detected in HNSCC, however methylation of *MST2* was not tumor specific (Table II). We and others have reported that *MST1* is frequently methylated in soft tissue sarcoma and colorectal carcinoma (21,32). Loss of cytoplasmic *MST1* is associated with higher T and/or N stage, higher tumor grade and poor prognosis in colorectal cancer (32). Interestingly, methylation of *MST1* occurred in all poorly differentiated HNSCC (Table III). Also methylation of *LATS1* and *LATS2* was more pronounced in dedifferentiated tumors. The large tumor suppressors LATS1 and LATS2 are MST and RASSF

binding proteins that regulate apoptosis and mitotic exit (33,34). Hypermethylation of *LATS1* and *LATS2* was previously reported only in sarcoma, breast cancer and astrocytoma (21,35,36).

We detected frequent promoter methylation of the tumor-related genes *p16*, *MGMT*, *DAPK1*, *RARβ*, *MLH1* and *CDH1* in HNSCC (Table II). The revealed methylation frequency of these TRG is consistent with other studies (11-13,16-18,28,37). Methylation of *GSTP1* in HNSCC is rarely observed (13,17). Methylation index of TRG was more frequent in advanced tumor stages (IV, T4 and N3) and poorly differentiated HNSCC (G3; Table III). Methylation of *MLH1*, *CDH1* and *DAPK1* was higher in stage IV. Sanchez-Cespedes *et al* have reported that methylation of DAPK1 correlates with an advance disease stage (17). Methylation of *p16* and *MGMT* was more frequent in poorly differentiated tumors (Table III). Zuo *et al* have reported that MGMT hypermethylation correlated with impaired prognosis and increased tumor recurrences (38). Hypermethylation of *p16* was associated advanced oral squamous cell carcinoma and lymph node metastasis (13). Methylation of *RASSF1A*, *DAPK1* and *RASSF4* increased with increased involvement of regional lymph nodes (Table III). It has been shown that DAPK1 methylation is significantly associated with lymph node involvement and metastases (11,17).

Methylation of TRG (e.g. *p16* and *RARβ*) was also found in matching normal tissue (Table II). This result is consistent with previous reports that reveal frequent methylation of *RARβ* (50%), *p16* (27%), *MGMT* (41%) and other TRG in normal mucosa of HNSCC patients (14-16). Aberrant promoter methylation of TRG (e.g. *p16* and *DAPK1*) is also found frequently in normal oral mucosa and bronchial brushes from smokers and former cigarette smokers, respectively (39,40). These data indicate that promoter methylation of several TRG in pre-cancerous tissue is an early event and may occur as a result of exposure to different agents (e.g. alcohol, betel quid and tobacco) that were related to HNSCC (5-7,41). To confirm this hypothesis it would be interesting to analyze methylation of TRG in normal mucosa of healthy persons, who were exposed to these substances or not. Increased TRG methylation in agent exposed mucosa may represent first pre-cancerous alterations and this could be used as a biomarker for HNSCC. However, methylation of TRG in body fluids of normal controls may affect the utility of hypermethylation detection strategy (23). Methylation of certain TRG (e.g. *p16* and *MGMT*) could also be due to epigenetic field effects, which was reported for colorectal cancer and lung cancer (42-44). Interestingly, *RASSF1A* and *RASSF4* methylation was not detected in matching normal mucosa.

In summary, our data show frequent hypermethylation of TRG in HNSCC and hypermethylation was considerably increased in more advanced HNSCC. To our knowledge this is the first report of methylation of *RASSF2*, *RASSF4*, *RASSF5*, *MST1*, *LATS1* and *LATS2* in HNSCC. RASSFs, MSTs and LATSs are prominent tumor suppressors and regulate microtubule stability, cell cycle and apoptosis (30,33,34). Thus silencing of RASSF members and their downstream effectors may represent important events in head and neck carcinogenesis.

Acknowledgements

Reinhard Dammann received grant support from BMBF (FKZ 01ZZ0104), Deutsche Krebshilfe (107742) and DFG (DA552).

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