RARß2 suppression in head and neck squamous cell carcinoma correlates with site, histology and age

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Abstract. Retinoids as important growth and differentiation regulating agents have a potential role in the chemoprevention of head and neck squamous cell carcinoma (HNSCC). Despite the promising preclinical and early clinical findings, limitations of application are raised by intrinsic resistance acquired during carcinogenesis. Retinoic acid receptor ß2 (RARß2) is one of the proximate mediators of retinoid signalling and its expression is often diminished in early stages of head and neck carcinogenesis. One form of retinoid resistance has been associated with the methylation-induced silencing of the RARß gene. We studied primary HNSCC samples of different anatomical sites in respect of methylation, expression and allelic loss of RARß gene. A strong correlation ($p<0.01$) was found between hypermethylation and reduced expression of RARß2, however the allelic loss at 3p24, the locus of RARß, did not considerably influence its mRNA level. Hypopharynx tumors showed significantly lower hypermethylation ($p<0.05$) and higher mRNA expression levels of RARß2 compared to the tumors located at other sites of the head and neck. We could also provide evidence that poorly differentiated grade 3 tumors had significantly higher RARß2 expression and lower methylation levels ($p<0.05$) than better differentiated grade 1 and grade 2 tumors. In addition, we found a good correlation between the methylation degree of the RARß2 promoter and the ages of patients. Collectively, our results suggest that evaluation of several factors such as tumor location, age, histology and methylation state of the RARß gene might contribute to the selection of patients for retinoid-based chemoprevention.

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; RARE, retinoic acid responsive element; UICC, International Union Against Cancer

Key words: retinoic acid, RARß2 expression, promoter hypermethylation, chemoprevention, retinoid resistance, HNSCC, squamous differentiation

Introduction

Retinoids, natural and synthetic vitamin A analogues, play fundamental roles in regulation of cell growth, differentiation and apoptosis (1,2). Retinoic acid (RA) can stimulate or inhibit growth and differentiation depending on the cell type (3). One of the main functions of vitamin A analogs is to prevent squamous differentiation with keratinization of non-keratinizing epithelial tissues (2,4). In animal models (5,6) and cell culture systems (7-9) retinoids can suppress or reverse epithelial carcinogenesis. Several clinical studies demonstrated that retinoids can arrest or reverse the process of carcinogenesis in premalignant lesions of the skin, oral cavity, larynx and cervix and prevent the development of second primary tumors in patients with head and neck cancer, lung cancer and hepatocellular carcinoma (10). Nevertheless, the use of retinoids to prevent or treat human cancer has achieved only modest success, because of prevalent retinoid resistance (11,12) and considerable toxicity of natural retinoids (13,14).

The nuclear retinoid receptors that mediate the actions of retinoids belong to the steroid/thyroid hormone receptor superfamily. Similar to other members of this family retinoid receptors are ligand-activated transcriptional regulators (15,16). There are two types of nuclear retinoid receptors, RARs and RXRs (17). Each of the receptor types has three subtypes, α, ß and γ. Various receptor isoforms exist through alternative splice sites and promoters. There are two major isoforms for RARα and RARγ and four major isoforms for RARß (16). In the human RARß2 and RARß4 transcript variants have been identified in adult cells (16).

The RARs bind RA and 9-cis RA whereas the RXRs bind 9-cis RA but not RA. RXRs and RARs can form heterodimers and bind to specific retinoic acid response elements (RAREs) of target genes (18,19). As nuclear retinoid receptors are the direct mediators of retinoid actions, changes in their expression

may cause aberrations in the response of cells to retinoids. Especially, the supression of RARß2 expression has been found in various cancer cell types (20), including head and neck squamous cell carcinoma (HNSCC) cell lines (21,22), premalignant oral lesions (23) and malignant head and neck tissues (24,25). Consequently it is plausible that silenced RARß2 expression may be partially responsible for the limited clinical activity of classical retinoids (12). RARE has been identified in the RARß2 promoter (26), thus normally RARß2 can be induced by RA treatment; however, there are several cancer cell lines which are resistant to RA-mediated RARß2 induction (27,28).

RARß has been mapped at chromosome band 3p24 which shows frequent loss in head and neck cancer (29,30). It is therefore presumable that LOH at 3p24 may partly account for reduced expression of RARß2. In addition, recent studies have demonstrated that hypermethylation of CpG islands in RARß2 promoter correlates with transcriptional repression in several types of cancer, including HNSCC (31-33).

The aim of this study was to examine the genetic and epigenetic factors for RARß2 inactivation in head and neck cancer and identify patients who are more likely sensitive to retinoid therapy and chemoprevention.

Materials and methods

Patients and samples. Eighty-nine primary HNSCC and corresponding normal tissue samples of the oral cavity (34), oropharynx (15), hypopharynx (23) and larynx (17) were obtained from patients operated at the Head and Neck Surgery Department of the National Institute of Oncology, Budapest, between 1997 and 1999. UICC stages (34) and grades of tumors were defined (Table I). The sample collection was approved by the institutional ethics committee. All tissue samples were snap-frozen in liquid nitrogen and stored at -80˚C before DNA/RNA isolation.

DNA isolation. DNA was isolated from 89 cancer and corresponding normal tissue samples with the standard phenol-chloroform extraction and ethanol precipitation following proteinase K (Sigma-Aldrich Co., St. Louis, MO, USA) digestion.

Loss of heterozygosity (LOH) analysis. Fluorescently labelled microsatellite markers were used for the loci D3S1283 and D3S1286 at the chromosome locus 3p24 (Table II) (52). Following PCR amplification of paired tumor and normal DNA samples, fragments were electrophoretically separated and detected by ABI-PRISM 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). LOH was stated when the relative peak height of one allele showed >50% decrease in the tumor sample compared to the normal one in heterozygous samples. Samples homozygous for both alleles were assessed as non-informative.

Bisulfite modification. Sodium bisulfite conversion of the DNA from the 89 tumor samples and the matching normal samples was performed as described previously (35). Briefly, 2 μg of genomic DNA was denatured in 0.3 M NaOH, for 15 min at 37˚C, then incubated in dark at 55˚C for 16 h in modification medium (2.6 M $NaHSO_3$, 0.5 mM hidroquinon, 0.1 M NaOH). The converted DNA was purified with Wizard DNA clean-up system (Promega Co., Madison, WI, USA) and desulfonated (0.3 M NaOH, 37˚C, 10 min), then precipitated with ethanol and ammonium acetate and finally pellets were resolved in distilled water.

Methylation analysis. The methylation analysis was performed by the fluorescence-based real-time PCR assay, MethyLight as described previously (36,37). Primer and probe sets (Table II) (53) were designed specifically for bisulfite converted DNA, a methylated set for the RARß gene and a reference set for ß-actin, to normalize for input of modified DNA. Human sperm DNA was used as negative control and SssI methylase (New England Biolabs, Ipswich, MA, USA) treated human lymphocyte DNA was used as positive control. The percentage of fully methylated reference (PMR) at the RARß2 promoter was calculated by dividing the RARß2:ACTB ratio of the sample by the RARß2:ACTB ratio of the positive control and multiplying by 100 (37). The real-time PCR assay was performed by the use of ABI PRISM 7900 sequence detection system (Applied Biosystems). The PCR reaction mix contained 600 nM of each primer, 200 nM of TaqMan probe, 5 mM $MgCl_2$ and 1X JumpStart Taq ReadyMix (Sigma-Aldrich Co.) in a final volume of 25 μl.

RNA isolation. Thirty-eight tumor samples (Table I) were available for isolation of intact RNA. Total RNA was isolated by MagNa Pure LC instrument applying the RNA isolation kit III (tissue) (Roche Diagnostics GmbH, Mannheim, Germany).

Quantitative RT-PCR. Two-step RT-PCRs were performed. RNA (250 ng) per sample was transcribed by M-MLV reverse transcriptase (Promega Co.). cDNA (1-1 μl) was used as template in the subsequent real-time PCRs; reactions were run in LightCycler instrument using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. RARß2 mRNA expression level was determined in terms of percentage of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as endogenous control. The relative RARß2 expression values were calculated from standard curves drawn for RARß2 and GAPDH in each run. The primer sequences are indicated in Table II.

Statistical analysis. Associations of the LOH data with the pathological parameters (tumor, node, stage, grade) and also with the tumor sites were assessed using Chi-square tests. The methylation levels (PMR values) of normal and corresponding tumor samples were compared by the Wilcoxon matched-pairs signed-ranks test. The relative RARß2 expression and PMR values were related to the tumor locations and the pathological parameters above by the use of the Kruskal-Wallis test with Dunn's post test and Mann-Whitney test.

The relationship between the methylation level and RARß2 expression and correlation of each with the ages of patients were examined by performing Spearman tests. The above statistical tests were performed by GraphPad Instat 3 software. The results were considered statistically significant at $p \leq 0.05$ significance level.

Table I. Patient data: results of loss of heterozygosity and methylation analysis.

Location	Patients	*T*	*N*	*S*	*Grade*	Age at diagnosis (years)	Deletion at 3p24	RARß2 methylation (PMR)	RARß2 expression (GAPDH%)
Oral cavity	4	3	0	3	1	53	LOH	38.14	NA
	22	2	2	4	1	48	NI	31.10	NA
	42	3	0	3	1	68	LOH	11.98	3.89
	52	2	0	2	1	70	LOH	18.77	NA
	60	4	2	4	1	60	-	7.37	0.83
	72	2	0	2	1	63	-	31.55	5.43
	110	3	2	4	1	44	LOH	13.80	0.83
	125	2	1	3	1	61	-	23.97	2.79
	9	2	0	2	2	72	-	25.06	NA
	18	3	2	4	2	59	LOH	34.41	NA
	31	3	0	3	2	57	LOH	19.79	NA
	49	2	0	2	2	59	-	12.71	0.82
	50	2	0	2	2	57	LOH	1.70	NA
	56	3	2	4	2	72	-	14.80	NA
	57	4	1	4	2	48	-	6.67	0.24
	59	3	2	4	2	62	-	3.83	NA
	79	4	1	4	2	48	-	5.62	NA
	83	2	2	4	2	66	-	17.72	NA
	85	2	0	2	2	39	-	0.42	14.06
	90	2	1	3	2	52	-	10.56	NA
	109	3	1	3	2	39	-	64.83	1.23
	120	4	2	4	2	56	LOH	38.40	0.00
	123	2	0	2	2	47	LOH	26.59	NA
	124	3	0	3	2	58	LOH	0.01	34.31
	126	3	0	3	2	38	LOH	32.17	NA
	131	2	0	2	2	52	LOH	2.40	NA
	133	3	1	3	2	71	-	5.60	NA
	61	2	1	3	2	55	LOH	3.42	NA
	16	4	2	4	3	42	LOH	25.05	NA
	33	3	0	3	3	47	-	2.68	3.32
	97	3	0	3	3	64	-	15.12	2.04
	101	4	0	4	3	62	NI	26.72	0.72
	108	4	3	4	3	40	-	6.00	5.20
	116	2	0	2	3	49	NI	0.99	NA
Oropharynx	27	4	0	4	1	46	LOH	13.23	0.93
	127	4	2	4	1	45	LOH	2.75	NA
	38	3	1	3	2	61	LOH	10.30	NA
	41	2	2	4	2	54	-	22.64	NA
	148	4	2	4	2	57	-	23.97	0.00
	70	3	1	3	2	55	LOH	29.93	3.29
	105	4	1	4	2	61	-	23.60	0.00
	114	3	2	4	2	42	-	8.13	1.55
	119	4	2	4	2	47	LOH	22.54	0.00
	128	2	2	4	2	61	-	43.96	NA
	135	4	0	4	2	48	LOH	41.28	NA
	39	4	1	4	3	44	LOH	2.19	4.76
	73	2	0	2	3	38	NI	23.18	NA
	92	1	1	3	3	59	-	31.54	NA
	113	2	2	4	3	42	-	2.40	4.96

Table I. Continued.

Location	Patients	*T*	*N*	*S*	*Grade*	Age at diagnosis (years)	Deletion at 3p24	RARß2 methylation (PMR)	RARß2 expression (GAPDH%)
Hypopharynx	19	2	0	2	1	46	LOH	3.10	NA
	62	4	0	4	1	74	NI	4.39	0.03
	82	3	1	3	2	64	LOH	36.08	NA
	3	3	2	4	2	45	LOH	7.47	NA
	8	3	1	3	2	50	-	16.93	NA
	25	3	1	3	2	48	-	13.38	NA
	54	3	1	3	2	44	-	0.49	NA
	84	4	2	4	2	44	LOH	0.00	0.16
	98	4	2	4	2	49	LOH	9.96	0.27
	112	3	0	3	2	46	-	3.16	14.04
	134	3	0	3	2	67	-	7.40	NA
	5	3	2	4	3	45	LOH	0.00	NA
	43	1	2	4	3	57	-	3.44	5.44
	51	4	1	4	3	56	-	4.74	NA
	55	3	2	4	3	47	-	5.41	4.24
	104	3	3	4	3	44	LOH	6.66	14.66
	106	4	2	4	3	45	LOH	1.80	45.00
	117	3	2	4	3	56	-	1.98	28.89
	121	2	2	4	3	45	LOH	8.13	NA
	129	3	2	4	3	39	-	8.39	NA
	143	4	2	4	3	52	-	2.30	16.01
	150	3	2	4	3	74	-	5.77	NA
	6	2	0	2	3	52	LOH	3.33	NA
Larynx	44	1	0	1	1	49	LOH	31.28	NA
	76	3	2	4	1	59	LOH	100.00	2.03
	87	3	0	3	1	46	-	6.40	NA
	32	3	0	3	2	65	-	8.40	NA
	34	3	0	3	2	46	-	7.40	NA
	58	4	0	4	2	63	LOH	13.39	1.31
	80	3	2	4	2	70	LOH	4.08	NA
	81	3	2	4	2	50	-	17.14	NA
	95	3	2	4	2	58	-	26.44	1.68
	107	3	0	3	2	59	-	16.43	NA
	122	3	0	3	2	47	-	58.60	0
	149	3	1	3	2	49	-	21.10	0
	12	3	0	3	3	65	LOH	14.43	NA
	21	4	0	4	3	42	-	10.00	NA
	37	4	0	4	3	54	LOH	5.60	NA
	71	4	2	4	3	48	-	0.69	NA
	94	4	0	4	3	52	LOH	4.93	NA

T-N-S, tumor size - lymph node metastases - stage according to the UICC classification; LOH, loss of heterozygosity; NI, not informative; NA, not available; PMR, percentage of methylated reference.

Results

Loss of heterozygosity analysis. We found loss of heterozygosity in 38 of 84 informative cases (45.2%) for at least one marker of 3p24 band (Table I). Oropharynx tumors showed the highest incidence of LOH (50%), but the differences were not significant among different location groups. There were no significant associations between allelic loss and clinicopathological parameters such as tumor size, lymph node metastasis or stage. It can be noted that the highest frequency of LOH

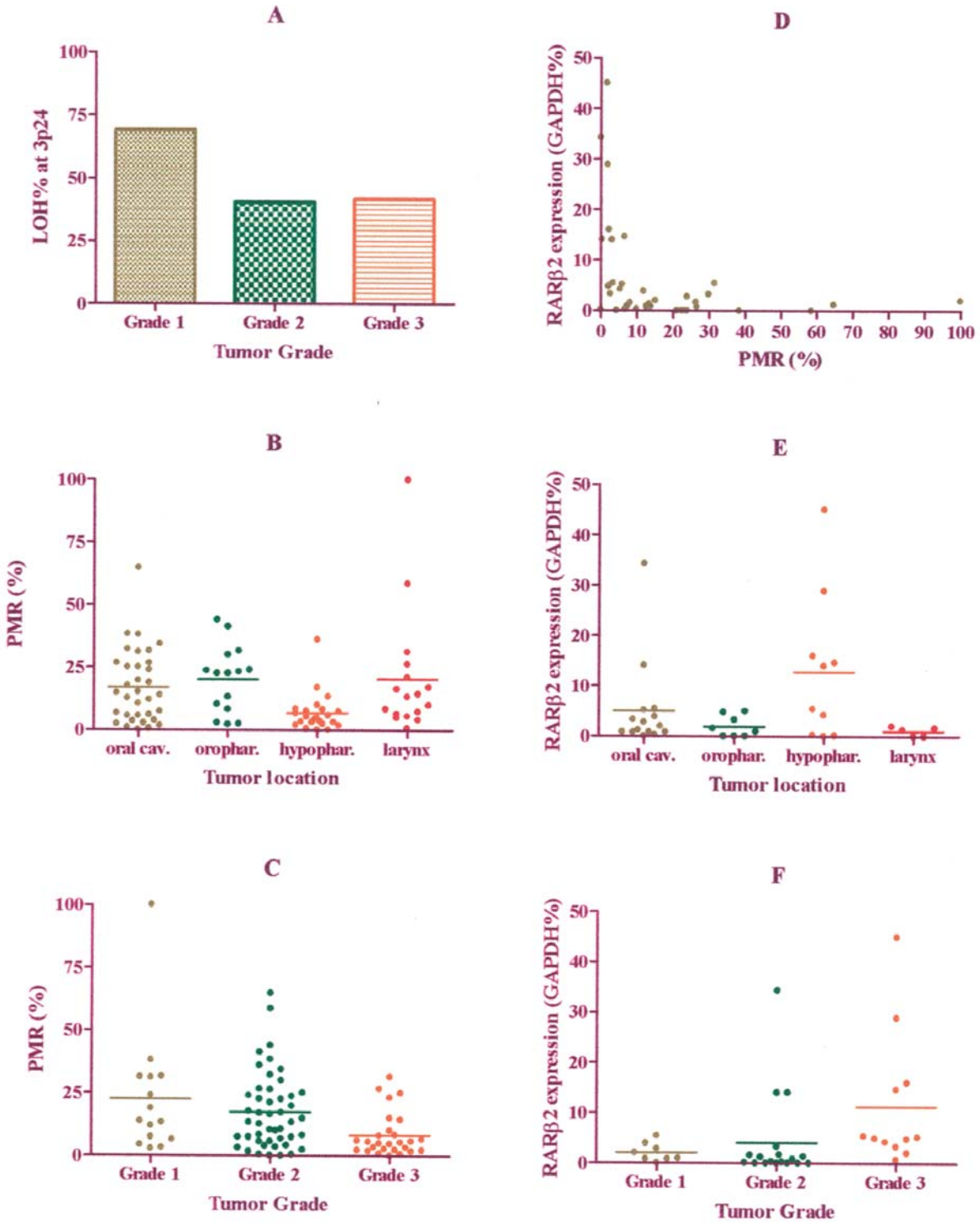

Figure 1. Associations of allelic loss, promoter methylation and RARß2 expression with the differentiation grade and location of the tumor. (A) Loss of heterozygosity at locus 3p24 in distinctly differentiated tumors. (B) Methylation degree of RARß2 promoter in tumors of different sites. (C) Methylation degree of RARß2 promoter in tumors of different grades. (D) RARß2 mRNA expression in the function of promoter methylation. (E) RARß2 mRNA levels in tumors of different locations. (F) RARß2 mRNA levels in tumors of different grades. LOH, loss of heterozygosity; PMR, percentage of methylated reference; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Mean values are indicated by horizontal lines.

was seen in grade 1 tumors (69.2%) in contrast to grade 2 and 3 tumors (40.4% and 41.7%, respectively); however, the difference was not quite significant (p=0.07) (Fig. 1A). No correlation was seen between LOH and the ages of patients.

Methylation analysis. The quantitative MethyLight analysis of matched tumor - normal sample pairs revealed that the tumor samples had significantly higher methylation levels than their normal counterparts ($p<0.01$). The average methylation level of RARß2 promoter was found to be the lowest in the hypopharyngeal tumors (Fig. 1B) and these had a statistically similar mean methylation value as normal samples (p=0.59). The tumors of oral cavity, oropharynx and larynx had significantly higher methylation degrees as

Table II. Primer and probe sets applied for LOH, methylation, and expression analyses.

Analysis	Target (ref.)	Forward primer (5'-3')	Reverse primer (5'-3')
LOH	*D3S1286* (52)	FAM-GCAGTGACTTCAGAGGGGC	ATTGACGGGGCTAGGGC
	D3S1283 (52)	HEX-GGCAGTACCACCTGTAGAAATG	GAGTAACAGAGGCATCGTGTATTC
RT-PCR	*RAR_2* (NM_000965)	GGGAGGAAGTGGAGATGG	ATGCTTCAGTGGATTGACC
	GAPDH (NM_002046)	TGAACCATGAGAAGTATGACAACA	AGTCCTTCCACGATACCAAA
MethyLight	*RAR_2* (X56849)	TGAGGATTGGGATGTCGAGAA	CCTTCCGAATACGTTCCGAAT
	TaqMan probe (5'-3')	6FAM-CGACGATACCCAAACAAACCCTACTCGAAT-TAMRA	
	ACTB (Y00474) (53)	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA
	TaqMan probe (5'-3')	6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA	

compared to hypopharynx tumors ($p<0.05$). The largest difference was observed between the mean PMR (percentage of methylated reference) values of oropharynx and hypopharynx samples ($p<0.01$) (Fig. 1B). The methylation data of oral cavity, oropharynx and larynx did not vary significantly from each other. The promoter methylation status did not correlate with the clinicopathological parameters, but was found to be related to the histological grade. Although grade 1 and 2 tumors did not differ statistically, the tumors had significantly higher methylation levels than grade 3 tumors (Dunn's $p<0.05$, $p<0.05$) (Fig. 1C). The grade 3 group did not differ considerably from the normal samples ($p=0.34$). Spearman test resulted in a positive correlation between methylation levels and the age of the patients ($r=0.22$; $p=0.04$).

RARß2 expression analysis. RARß2 mRNA expression had very strong negative correlation with methylation level ($r=-0.55$; $p=0.0003$) (Fig. 1D), but showed independence from loss of heterozygosity at 3p24. In line with methylation results, hypopharynx tumors were observed to have considerably higher RARß expression levels than oropharynx ($p=0.04$) and larynx ($p=0.06$) and moderately higher expression than oral tumors ($p=0.2$) (Fig. 1E). Grade 1 and 2 samples expressed significantly less RARß2 mRNA than grade 3 tumors ($p<0.05$ and $p<0.001$, respectively) (Fig. 1F), whereas grade 1 and 2 samples did not differ considerably in this respect. Similar to LOH and methylation results, expression levels proved to be independent from clinicopathological characteristics. We could not demonstrate a significant relationship between age and expression level.

Discussion

Retinoids are able to reverse carcinogenesis through multiple mechanisms including the modulation of epithelial differentiation and proliferation by regulating gene expression. This regulation is mediated by nuclear retinoid receptors. One of these receptors is the retinoic acid receptor-ß2 (RARß2), expression of which is suppressed in early stages of head and neck carcinogenesis (24,25). The restoration of RARß2 expression has been shown to be associated with clinical response, suggesting it as a marker in chemoprevention trials (24).

We investigated the role of allelic loss and epigenetic silencing of RARß2 expression in tumors located at different sites of the head and neck region and we also examined the associations of these molecular features with clinico- and histopathological parameters.

In line with other studies (33) we found a frequent allelic loss (45.2%) at 3p24 region, where RARß is located. It is of interest that loss of heterozygosity did not show significant correlation with either the clinical stage or RARß2 mRNA expression. These findings are in line with observations in breast carcinoma (38) and esophageal cancer (39) where other groups also reported no correlation between LOH at 3p24 and RARß2 expression. It can be due to the retained allele, which remains inducible by retinoids if otherwise intact. In contrast to that, the methylation degree of RARß2 promoter was negatively correlated with the RARß2 mRNA level ($r=-0.55$; $p<0.01$), suggesting hypermethylation as the main reason for RARß2 suppression. Significantly higher methylation levels were detected in tumor samples than their normal counterparts ($p<0.01$). RARß2 methylation was found rather frequently by others in normal squamous epithelium (40) and resection margins (41). It is likely because RARß2 methylation is an early event in carcinogenesis, and resection margins frequently have such an epigenetic alteration owing to field cancerization. Although RARß2 methylation occurs in normal mucosa, the reverse quantitative correlation of RARß2 methylation and mRNA expression suggests its higher expression level in normal tissue. Similar to loss of heterozygosity, promoter hypermethylation and RARß2 expression were not correlated with clinicopathological parameters such as tumor size and lymph node involvement. These results support that LOH and epigenetic alterations at the locus of RARß are both early events and independent prognostic factors in the carcinogenesis of the head and neck region.

Promoter methylation and RARß2 mRNA levels proved to have significant correlation with the histological grade (Dunn's $p<0.05$ in both cases). Namely, grade 3 tumors had lower methylation and higher expression levels of RARß2 than more differentiated tumors. Similar correlation was found between grade and methylation status by Liu *et al* in esophageal squamous carcinoma (42). Unfortunately, differentiation grade is not accurate in reflecting the agressiveness of HNSCC (43). Prognosis is influenced by many factors, such as tumor

size, site, vascularity, lymphatic drainage, host immune response, age, sex, nutritional and performance status (44). According to the traditional grading criteria developed by Broders (45) keratinization is the major determinant of grade. Better differentiated tumors that produce more keratin are thought to be less likely to metastasize (46). Nuclear receptors for retinoic acid and thyroid hormone regulate transcription of keratin genes (46). In squamous carcinoma cells of head and neck retinoids were found to inhibit squamous cell differentiation and suppress keratin expression (e.g. K1, K5) by the implication of RARß2 (47). Accordingly, expression of RARß2 is inversely related to squamous cell differentiation and cell growth (48). We first found correlation between expression status and histological grade in HNSCC tumors, as it was suspected from the cell culture experiments (47,48). Moreover, the methylation degree of grade 3 tumors did not differ significantly from normal samples (p=0.34). Considering these facts, grade 3 tumors can be predicted to express higher levels of RARß2 and therefore seem more sensitive to retinoid treatment.

Comparing tumors of different locations, we found that the methylation of RARß2 promoter was significantly lower in hypopharynx tumors than tumors of the oral cavity, oropharynx and larynx ($p<0.05$; $p<0.01$; $p<0.05$), but it was statistically indistinguishable from the normal samples (p=0.59). Concordant results were determined for mRNA expression. RARß2 mRNA levels were significantly higher in hypopharynx tumors compared with oropharyngeal and laryngeal tumors ($p<0.05$; p=0.055), although the differrence was not found significant in the case of oral tumors (p=0.22), probably because of the limited sample sizes. It should be noted that these results may be related to the fact that grade 3 tumors were the most frequent in the hypopharyngeal group, though the Chi-square test for independence did not result in significant association. Based on this, it is also presumable that the carcinogenesis of the hypopharyngeal region follows different pathways in some aspects compared to the other sites of the head and neck. We have not found any similar or supportive findings in the published literature yet, therefore further in-depth investigations are needed.

We also revealed a positive correlation between the methylation degree of the RARß gene and the ages of the patients (r=0.22; p=0.04). We can suppose that continous exposure of the upper aerodigestive tract to carcinogens leads to gradually expanding methylation of several gene promoters, such as RARß2. It is also supported by the findings of Zöchbauer-Müller and co-workers, who showed aberrant methylation of multiple genes in the upper aerodigestive epithelium of heavy smokers (49).

Being a mediator of RA response, RARß2 can serve as a marker in chemoprevention. Our results suggest that promoter hypermethylation is the main reason for RARß2 suppression, which is a frequent event in head and neck carcinogenesis. Thus, methylation of RARß gene can be not only a marker for early carcinogenesis, but also a putative indicator for resistance to retinoids. Sensitive methylation-specific PCR-based techniques enable to detect DNA methylation even in serum, and saliva (50,51). On one hand, the success of retinoid therapy, could be improved by the application of a new generation of receptor selective, synthetic retinoids and the use of combination regimens including demethylating agents (11). On the other hand, multistep evaluation of several factors, including tumor location, age, histology and methylation state of RARß2 promoter may contribute to the selection of patients promising for retinoid-based therapy or post-operative chemoprevention. These considerations support the idea that grade 3, hypopharynx tumors of younger patients may have better results in retinoid therapy.

Nevertheless, the high frequency of RARß2 repression has not excluded the presence of other potential mechanisms of retinoid resistance (11). Further investigations are still needed to reveal other (possibly downstream) targets of retinoid signalling which might play roles in resistance mechanisms.

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