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P14 methylation: an epigenetic signature of salivary gland mucoepidermoid carcinoma in the Serbian population

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Objective. To investigate the prevalence of $p16^{INK4\ a}$, $p14^{ARF}$, tumor protein p53 (TP53), and human telomerase reverse transcriptase (hTERT) promoter hypermethylation in mucoepidermoid carcinomas (MECs) and search for a possible association between methylation status and clinicopathological parameters.

Study design. DNA extracted from 35 formalin-fixed and paraffin-embedded MEC samples and 10 normal salivary gland (NSG) tissue samples was analyzed for the presence of promoter hypermethylation using methylation-specific polymerase chain reaction testing.

Results. The percentages of gene hypermethylation in MECs versus NSGs were the following: p14: 100% versus 20% ($P < .001$); p16: 60% versus 20% ($P = .035$); hTERT: 54.3% versus 20% ($P = .078$); and TP53: 31.4% versus 30% ($P = .981$). Multiple sites were found to be methylated in 86% of MECs compared with 10% in NSGs ($P < .001$). TP53 and hTERT were more often methylated in lower clinical stages ($P = .033$ and $P = .005$, respectively).

Conclusions. Hypermethylation of p14 appears to be an important event in the development of mucoepidermoid carcinoma. High frequency of gene hypermethylation and high incidence of methylation at multiple sites point to the importance of epigenetic phenomena in the pathogenesis of MECs, although with modest impact on clinical parameters. (Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:52–58)

Mucoepidermoid carcinoma (MEC) accounts for approximately 35% of salivary gland cancers, ranging from nonaggressive low-grade to aggressive high-grade tumors.[1-5] Several malignant and benign tumors can histologically mimic MEC, such as Warthin tumor, adenoid cystic carcinoma, and squamous cell carcinoma, among others, leading to misdiagnosis.[6,7] MEC pathogenesis studies have mainly been focused on the reciprocal chromosomal translocation t(11;19)(q21;p13), which gives rise to a fusion oncoprotein MECT1-MAML2. This fusion appears to be one of the drivers of MEC development through Notch signaling disruption.[8] It also represents an important prognostic parameter[9,10]; namely, it was found that patients with MEC harboring the translocation have a less aggressive form of tumor and a more favorable outcome.[11,12] Recently, using whole exome sequencing, several new alterations have been found in MECs, such as tumor protein p53 (TP53) and POU6F2 mutations.[9] Still, these genetic changes cannot explain all MEC cases.

The latest molecular studies have broadened the knowledge on the importance of epigenetic changes associated with salivary gland cancer (SGC) development, in particular DNA methylation, as a mechanism of gene silencing. Kishi et al.[13] found, for instance, that RB1 was hypermethylated in 42% of cases of SGC, and Nikolic et al.[14] reported that approximately 70% of pleomorphic adenomas harbored hypermethylated p14 and p16. Using a microarray approach, Bell et al.[15] also found several highly methylated genes in adenoid cystic carcinoma. Williams et al.[16] found 48% of methylated RASSF1A in salivary duct cancers, whereas Zhang et al.[17] correlated RASSF1A promoter hypermethylation with poor survival in patients with salivary adenoid cystic carcinoma in a Chinese population. In the same type of tumors, on the other hand, Tan et al.[18] found that AQP1 was hypomethylated in 75% of cases.

Nonetheless, the number of published studies is still relatively limited and mostly focused on gene methylation in adenoid cystic carcinomas. Findings on DNA methylation status in mucoepidermoid carcinomas are

Statement of Clinical Relevance

Methylation of the p14 tumor suppressor gene seems to be a critical event in the pathogenesis of mucoepidermoid carcinoma and consequently p14 could be potentially used as a target in novel therapeutic approaches based on DNA epigenetic modifications.

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extremely scarce,[13,19] and that fact prompted us to explore the potential role of methylation in the pathogenesis of this type of tumor. The following genes known to be implicated in the etiology of different malignancies were selected for the present study: CDKN2A or INK4 a-ARF locus, which encodes 2 tumor suppressors ($p16^{INK4\ a}$ and $p14^{ARF}$), TP53, and human telomerase reverse transcriptase (hTERT). p16 is a potent cell cycle regulator, and when absent or nonfunctional, the progression of the cell cycle into the S phase is enabled.[20] p14 is one of the key p53 regulators; thus methylated p14 would mean inactive p53 tumor suppressor.[21] The TP53 gene, the most commonly mutated gene in human cancer, encodes the p53 suppressor, which promotes cell cycle arrest or apoptosis under replication stress; epigenetic modifications affect TP53 expression and p53 function.[22] The hTERT gene encodes the protein subunit of the enzyme telomerase. Telomere maintenance by telomerase has a key role in carcinogenesis, and hTERT expression is known to be the limiting step in telomerase function.[23]

With the aim of assessing their respective contribution to MEC development, progression, and outcome, the methylation status of 2 gene promoters analyzed in the past (p14 and p16) and 2 gene promoters not examined before (TP53 and hTERT) has been determined.

MATERIALS AND METHODS

This retrospective study included 45 formalin-fixed, paraffin-embedded samples originating from 35 patients with mucoepidermoid carcinoma (surgically treated between 2000 and 2010 at the Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade) and 10 controls. The control specimens were normal salivary glands (NSGs) obtained from patients with oral cancer who underwent radical neck dissection, which included the submandibular glands. Only tissue histopathologically confirmed as tumor free was used as control tissue. Initial histopathologic findings obtained from the Department of Pathology, School of Dental Medicine, University of Belgrade, were confirmed at the Institute of Hematopathology Hamburg, Hamburg, Germany. Patients with MEC with distant metastases were excluded from the study. MECT1-MAML2 translocation analysis on these cases was done in a previous study.[11] Relevant clinicopathologic data are given in Table I. The research was conducted in full accordance with the World Medical Association Declaration of Helsinki (Version 2002).

The promoter methylation status was determined by methylation-specific polymerase chain reaction (PCR). An adaptation of the protocol suggested by Herman et al.[24] was implemented. Genomic DNA was extracted from formalin-fixed, paraffin-embedded samples using phenol-chloroform extraction and was modified by sodium bisulfite treatment with an EZ-DNA Methylation Kit, according to manufacturer's recommendations (Zymo Research, Orange, CA, USA). Modified DNA was purified and resuspended in 20 μL of elution buffer provided in the kit. Subsequent methylation-specific PCR was used to distinguish unmethylated alleles, based on DNA sequence alterations after bisulfite treatment of DNA. Two separate PCR reactions were performed for each gene, using primers designed for methylated and unmethylated CpG islands in the promoter region. Primers used in this study were reported previously[24-27] and the sequences are listed in Table II. The modified genomic DNA samples were PCR amplified in a total volume of 50 μL, and the reaction mix contained 1 × PCR buffer (10 mM Tris-hydrochloride, 50 mM potassium chloride, 0.1% Triton X-100), 8 mM magnesium chloride, 1.25 mM deoxynucleoside triphosphates, 0.6 μM primers (Invitrogen, Life Technologies, Carlsbad, CA, USA), 0.4 μg/μL bovine serum albumin, 5% dimethyl sulfoxide, 1.5 U *Taq* polymerase (Thermo Scientific, Hudson, NH, USA), and 3 μL of bisulfite-treated DNA template. Amplification was performed in a PeqStar 96 Universal thermal cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles 95°C denaturation for 30 s, annealing for 30 s (primer-specific temperatures are listed in Table 2), 72°C extensions for 30 s, and final extension at 72°C for 4 min. Genomic DNA extracted from lymphocytes of healthy donors was used as control for unmethylated genes. The same DNA, treated in vitro with Spiroplasma sp. strain MQ1 (SssI) methyltransferase, was used as a positive control for methylated genes. PCR products were loaded on 8% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light.

Table I. Clinicopathologic features of patients with mucoepidermoid carcinoma (MEC) and demographic data for patients with MEC and normal salivary glands (NSG)

Characteristic		*MECs*	*NSGs*
Mean age, y		53.03 ± 17.53	56.27 ± 12.05
Male:female ratio		0.9:1	1.4:1
Mean duration of disease, y		4.47 ± 3.57	
Tumor site	Parotid	18	
	Submandibular	6	
	Sublingual	4	
	Palate	5	
	Retromolar	2	
Tumor size	>2 cm	15	
	<2 cm	20	
Positive lymph nodes		10	
Clinical stage	I and II	25	
	III and IV	10	
Perineural invasion		10	

Table II. Primer sequences, product lengths, and annealing temperatures

Primer	*Primer sequence*	*Length (bp)*	*Annealing temperature*
$p14^{ARF}$ U1	TTTTTGGTGTTAAAGGGTGGTGTAGT		
$p14^{ARF}$ U2	CACAAAAACCCTCACTCACAACAA	132	53°C
$p14^{ARF}$ M1	GTGTTAAAGGGCGGCGTAGC		
$p14^{ARF}$ M2	AAAACCCTCACTCGCGACGA	122	53°C
$p16^{INK4\,a}$ U1	TTATTAGAGGGTGGGGTGGATTGT		
$p16^{INK4\,a}$ U2	CAACCCCAAACCACAACCATAA	151	60°C
$p16^{INK4\,a}$ M1	TTATTAGAGGGTGGGGCGGATCGC		
$p16^{INK4\,a}$ M2	GACCCCGAACCGCGACCGTAA	150	65°C
TP53 U1	TTGGTAGGTGGATTATTTGTTT		
TP53 U2	CCAATCCAAAAAAACATATCAC	247	58°C
TP53 M1	TTCGGTAGGCGGATTATTTG		
TP53 M2	AAATATCCCCGAAACCCAAC	193	58°C
hTERT U1	TTGAGAATTTGTAAAGAGAAATGATG		
hTERT U2	ACTAAAAACAAACCCAAAAACACA	133	55°C
hTERT M1	TTGAGAATTTGTAAAGAGAAATGAC		
hTERT M2	TAAAAACGAACCCGAAAACG	131	55°C

bp, base pair; *hTERT,* human telomerase reverse transcriptase; *M,* methylated sequence; *TP53,* tumor protein p53; *U,* unmethylated sequence.

Table III. Distribution of methylated gene promoters in mucoepidermoid carcinoma (MEC) and normal salivary gland (NSG) groups

		p16 (%)	*p14 (%)*	*TP53 (%)*	*hTERT (%)*
No. of cases (%)	MEC 35 (100)	21 (60)	35 (100)	11 (31.4)	19 (54.3)
	NSG 10 (100)	2 (20)	2 (20)	3 (30)	2 (20)
χ^2 (Fisher's exact) *P* value		.035*	<.001*	.931	.078

hTERT, human telomerase reverse transcriptase; *TP53,* tumor protein p53.
*Statistically significant.

The genes were considered as (1) nonmethylated, when only the reactions with the unmethylated target-specific (U) primers had amplification, and (2) methylated or partially methylated, when a positive amplification was obtained only with the methylated target-specific (M) primer pair or with both (U and M). Reamplification of 20% of randomly chosen samples was done to confirm the findings, and there were no discrepancies between methylation statuses determined in duplicate.

Statistical analysis

All statistical analyses were done using Statistical Package for Social Science (SPSS software package, Version 17.0; SPSS Inc., Chicago, IL, USA). The differences of gene methylation status between different tumor types were assessed using Pearson's χ^2 test unless the smallest expected value was <5, in which case Fisher's exact test was used. *P* value < .05 was considered statistically significant.

RESULTS

Methylation status

The most remarkable finding was that 100% of MECs exhibited p14 promoter hypermethylation, versus only 20% of control specimens ($P < .001$). p16 hypermethylation was also statistically more common in MECs than in controls (60% vs. 20%, $P = .035$). No statistically significant difference was identified for the presence of TP53 hypermethylation between MECs and NSGs (31.4% vs. 30%, $P = .931$), or for the presence of hTERT hypermethylation (54.3% vs. 20%, $P = .078$). The frequencies of methylated promoter regions are summarized in Table III.

In all 35 analyzed cancer cases at least 1 of the 4 examined promoters had hypermethylation. A total of 30 (86%) MECs exhibited simultaneous hypermethylation of 2 or more genes, versus only 1 control sample (10%) with more than 1 methylated gene ($P < .001$) (Table IV).

Methylation status and clinical stage/histologic grade

All MEC cases harboring TP53 hypermethylation had been classified as lower clinical stages ($P = .033$). Similarly, hTERT hypermethylation dominated in stages I and II compared with stages III and IV ($P = .002$). There was no association between methylation and histologic grades (Table V).

Methylation status and overall survival

Promoter methylation status did not have a statistically significant association with survival rates, although patients with methylated p16 promoter appeared to have

Table IV. Methylation on multiple sites in mucoepidermoid carcinomas (MEC) and normal salivary glands

	1 Methylation (%)	*2 Methylations (%)*	*3 Methylations (%)*	*4 Methylations (%)*	*No methylations (%)*	P
MECs	5 (14.3)	13 (37.1)	13 (37.1)	4 (11.4)	0 (0)	
NSGs	7 (70)	1 (10)	0 (0)	0 (0)	2 (20)	<.001*

*Statistically significant.

Table V. Distribution of methylated gene promoters in mucoepidermoid carcinomas in relation to histologic grade and clinical stage

	Histologic grade				*Clinical stage*		
	1 (%)	*2 (%)*	*3 (%)*	*P*	*1&2 (%)*	*3&4 (%)*	*P*
N	12 (100)	11 (100)	12 (100)		26 (100)	9 (100)	
p16	6 (50)	7 (64)	8 (67)	.676	15 (58)	6 (67)	.712
p14	12 (100)	11 (100)	12 (100)		26 (100)	9 (100)	
TP53	6 (50)	4 (36)	1 (8)	.081	11 (42)	0 (0)	.033*
hTERT	7 (58)	8 (73)	4 (33)	.156	18 (69)	1 (11)	.005*

hTERT, human telomerase reverse transcriptase; *N,* number of patients per histologic grade or clinical stage; *TP53,* tumor protein p53.
*Statistically significant.

poorer survival than those with unmethylated p16. On the contrary, patients with methylated TP53 and hTERT promoter had a trend of better overall survival compared with patients with unmethylated TP53 and hTERT ($P = .120$ and $P = .151$, respectively) (Figure 1).

Methylation status and MECT1-MAML2 fusion

No correlation was found between the presence of the translocation and methylation of either of the genes ($P = .778$, $P = .197$, and $P = .778$ for p16, hTERT, and TP53, respectively).

DISCUSSION

DNA methylation, an important mechanism of gene expression regulation, is known to be involved in tumor development and progression. Indeed, it was suggested that hypermethylation is one of the main mechanisms of tumor suppressor gene (TSG) inactivation.[28]

Our results indicate that silencing of $p14^{ARF}$, a crucial regulator of p53 activity, might be a significant event in MEC pathogenesis because this TSG was methylated in all 35 tumor samples. Although only qualitative analysis of methylation was performed, the fact that 100% of cases harbored this epimutation points to its relevance. With either hypermethylated or in any other way inactivated p14, the cell will not be able to correct damages, even with intact (wild type) p53. The present study on the importance of p14 epigenetic inactivation in the pathogenesis of MECs is in agreement with our previous findings on pleomorphic adenoma and carcinoma ex pleomorphic adenoma.[14] Such a high frequency of methylation is not an uncommon phenomenon. For instance, the E cadherin gene was found to be hypermethylated in 95% of cutaneous squamous cell carcinomas.[29] It must be emphasized that only 1 study was previously published on p14 methylation in mucoepidermoid cancer. Nishimine et al.[30] analyzed 7 cases of MEC for the presence of p14 gene alterations and found 1 deletion and no methylation at all. When considering their entire SGC sample, in which adenoid cystic carcinomas dominated, 19.4% of methylated p14 promoter was found.[30] Quite low rates for p14 methylation were found by Weber et al. (2.5%)[31] in pleomorphic adenomas. Ishida et al.[32] reported p14 promoter hypermethylation in 20% of oral squamous cell carcinomas but found a significant association with later clinical stages and suggested that it could be a key molecular event in tumor progression.

The $p16^{INK4\ a}$ tumor suppressor gene encodes a cyclin-dependent kinase inhibitor with an important role in the cell-cycle regulation at the G1/S phase checkpoint. The absence of functional p16 protein leads to aberrant cell-cycle control and promotes cancer cell proliferation. In the present study, 60% of MEC cases had p16 hypermethylation, which is fairly in line with some previous findings on the importance of this epigenetic event in salivary gland carcinogenesis, with frequencies varying between 29% and 47%.[19,31,33] Only 2 papers analyzed p16 methylation in MECs—Guo et al.[19] reported 34% of methylated samples, whereas Nishimine et al.[30] did not find any methylated p16 promoter in MECs. In accordance with our findings, Guo et al.[19] indicated that the methylation rate of p16 in MECs increases with progression of tumor stage, but without statistical significance. Although their frequencies of p14 and p16 methylation were lower than ours (19% and 32%, respectively), Weber et al.,[34] however, concluded that the disruption of the INK4a-ARF/p53 pathway, by different mechanisms, was a very common event (84%) in head and neck cancer squamous cell carcinomas.

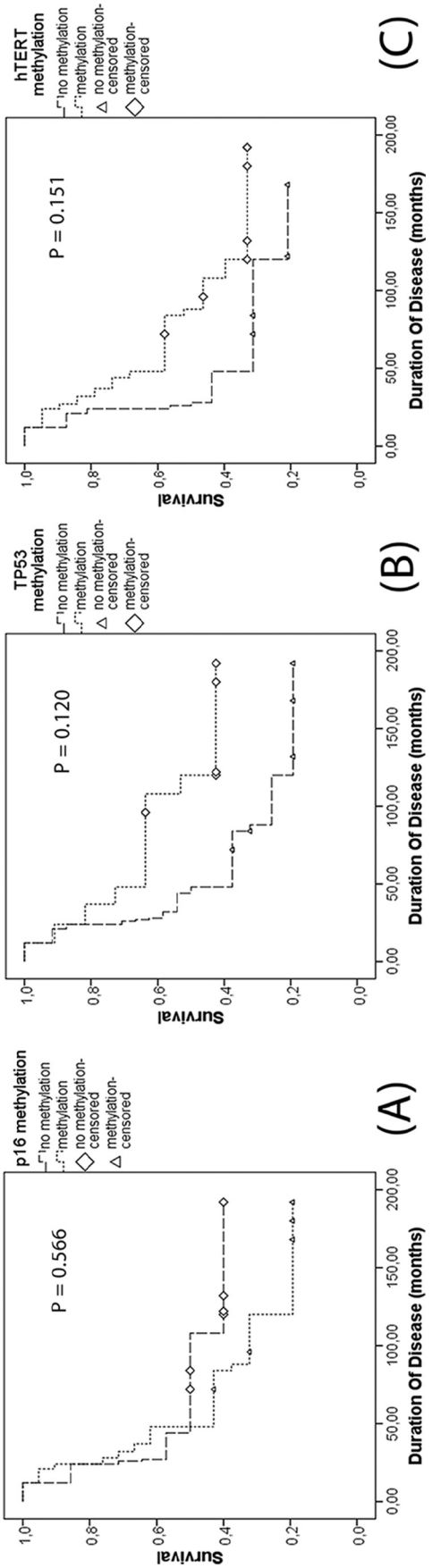

Fig. 1. Mucoepidermoid carcinoma (MEC) patients overall survival in relation to (**A**) p16, (**B**) tumor protein p53 (TP53), and (**C**) human telomerase reverse transcriptase (hTERT) methylation using Kaplan-Meier analysis with log-rank test.

The conflicting findings related to p14 and p16 methylation frequencies may be due to different factors, such as gender, diet, stress, environment, and so on.[35-37] Ethnicity is also known to greatly affect DNA methylation patterns[38,39] (and consequently gene expression). Finally, the use of detection assays with different sensitivity might in part explain the differences between the results.

Altered TP53 methylation patterns have been associated with multiple malignancies, including oral cancer.[40] According to the present study, hypermethylation of TP53 is not a common event in salivary gland malignancies, and this could be a logical finding in line with high frequency of p14 methylation. Namely, infrequent methylation of TP53 does not exclude p53 from the pathogenic process but rather supports the hypothesis that p53 inactivation is preferentially achieved via p14 silencing. Some studies have found TP53 methylation in cancer tissues,[41] whereas others state that TP53 hypermethylation could be a normal event in healthy cells,[40] which is also in agreement with our results.

One of the most important regulatory mechanisms of hTERT gene activity, and therefore of telomerase activity, is considered to be its promoter methylation, although sometimes in contrast to the general model of DNA methylation as a gene-silencing mechanism.[42] Renaud et al.[43] have proposed that the purpose of CpG island methylation in hTERT promoter is to prevent the binding of hTERT inhibitors and to allow transcription. In the present study, a statistically higher frequency of hTERT hypermethylation was found in tumors than in controls, suggesting that this molecular event might also play a role in the development of MECs. This is in accordance with some other studies that reported hypermethylation in cancerous tissue but not in normal tissue.[44,45] The association of hTERT methylation with lower histologic grades and clinical stages would imply its role in the earlier phases of tumorigenesis. It also might suggest the existence of a molecular subtype of low-grade MECs with methylated hTERT and good survival.

Generally in the present study none of the methylation events had significant association with patients' survival, which is not an infrequent situation. Although p16 methylation has been associated with poorer survival in head and neck cancer,[46] results similar to ours regarding the lack of relevance of p16 and/or p14 methylation on survival have been found in oral, oropharyngeal,[47] and head and neck squamous carcinomas.[48]

Neoplastic cells often exhibit aberrant methylation profiles of genes involved in cell-cycle regulation, DNA repair, and/or angiogenesis.[28] A so-called CpG island methylator phenotype was proposed by Toyota et al.[49] The authors categorized all cancers into those with genome-wide methylation and those with rare methylation events. The former are more at risk of transcriptional

silencing of numerous TSGs by promoter methylation.[49] In the present study, we found that 2 or more genes were simultaneously hypermethylated in 86% of cases, which implies that hypermethylation at multiple sites might be very important in salivary gland carcinogenesis.

CONCLUSIONS

Hypermethylation of p14 appears to be a critical event in the development of mucoepidermoid carcinomas. Although in general no important association was found with clinical features, high frequency of hypermethylation of 3 of 4 studied gene promoters points to the importance of epigenetic phenomena in the pathogenesis of salivary gland neoplasms.

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