Pathol. Oncol. Res. (2014) 20:923–929
DOI 10.1007/s12253-014-9775-9

RESEARCH

Frequency of Genetic and Epigenetic Alterations of p14ARF and p16INK4A in Head and Neck Cancer in a Hungarian Population

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Received: 21 October 2013 / Accepted: 27 March 2014 / Published online: 9 April 2014

Abstract Occurrence of genetic and epigenetic alterations affecting p14ARF and p16INK4A were investigated in tumour samples of 37 oral (OSCC) and 28 laryngeal squamous cell cancer (LSCC) patients, and compared to exfoliated buccal epithelial cells of 68 healthy controls. Presence of deletions and mutations/polymorphisms affecting exons were examined using sequencing. Methylation status of promoters was assessed by methylation-specific PCR. Chi-square and Fisher's exact tests were used to compare frequency of events. Exon deletions were found in four controls, one OSCC and 22 LSCC patients; the latter significantly differed from controls ($p<0.001$). Only two mutations (T24610A and C24702A) were in p16 exon 1 of two OSCC patients. Polymorphisms G28575A (Ala140Thr), G31292C (C540G) and G28608A were found in both patient groups. The p14 promoter was unmethylated in 86.7 % of OSCC and in 85.7 % of LSCC patients; for the p16 promoter these rates were 69.0 % and 76.2 % for OSCC and LSCC patients, respectively. Combining the two patient groups, unmethylated promoter was significantly less frequent in case of both p14 and p16 ($p=0.043$ and $p=0.001$, respectively) compared to the control group. In summary, exon deletion may be important in LSCC, while promoter methylation was relatively frequent in both patient groups.

Keywords Oral squamous cell cancer · Laryngeal squamous cell cancer · Tumour suppressor gene · Promoter methylation

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Introduction

Head and neck cancer is a heterogeneous group of malignant diseases. It is the sixth most common malignancy and accounting for more than 500,000 new cases annually and approximately 350,000 deaths per year [1–3]. Though it is widely accepted that mainly chemical carcinogens (especially smoking and alcohol consumption) are involved in the aetiology of head and neck squamous cell cancer (HNSCC) [3–6]; a portion (approximately 15–20 %) of HNSCC develops in non-smoker and non-drinker patients [7, 8]. This suggests the role of additional factors such as dietary habit, genetic predisposition as well as oncogenic viruses, e.g. human papillomaviruses (HPVs) or the Epstein-Barr virus (EBV) [9–13]. As the mentioned viruses interact with the tumour suppressor pathways involving the retinoblastoma protein (pRB) and the p53 tumour suppressor proteins, the concerted action of these viruses with genetic/epigenetic variations or alterations in the genes of these pathways offers a likely explanation for carcinogenesis.

Such tumour suppressor genes in these pathways are the p16INK4A and the p14ARF, encoded by the INK4A/ARF locus containing four exons (1α, 1β, 2 and 3) localized on chromosome 9p21, which is one of the major sites of chromosomal abnormalities in human tumours. The p16INK4A is encoded by exons 1α, 2 and 3, while p14ARF is encoded by exons 1β, 2 (and possibly also by exon 3); the two proteins use the second exon with alternative reading frames, thus

sharing a common second [14] and possibly also a third exon [15, 16]. The p16INK4A is a cyclin-dependent kinase inhibitor and can inhibit cyclin D-Cdk4/6 thus preventing hyperphosphorylation of pRB. The p14 interacts physically with MDM-2 and stabilizes the p53 tumour suppressor protein in the nucleus by blocking its cytoplasmic transport and MDM-2-mediated degradation [14, 15]. In this manner, both p16INK4A and p14ARF plays a role in inhibition of G1 to S transition in the cell cycle.

Genetic and epigenetic alterations of tumour suppressor genes, including p16INK4A and p14ARF, were found to contribute to tumourgenesis in various types of cancer [17–20]. Polymorphisms G28575A (Ala140Thr) in the p16 gene is generally regarded as neutral [21]; G31292C in the non-coding region (C540G at mRNA level) and C580T shown to be protective in cervical and ovarian cancer, respectively [22, 23]. However, C580T or both was shown to be associated with faster progression in pancreatic cancer [24, 25] or melanoma [35], respectively. The polymorphisms C540G and C580T were shown to be neutral for squamous head and neck cancers [27].

The aim of this study was to determine the frequency of the genetic alterations and promoter inactivation through methylation of p16INK4A and p14ARF tumour suppressor genes in patients with HNSSC of known virological (HPV and EBV) status in an Eastern Hungarian population [28, 29].

Materials and Methods

Patients, Specimens and DNA Extraction

Patients and controls were recruited between 2001 and 2007 from Department of Dentoalveolar and Maxillofacial Surgery (oral squamous cell cancer patients) and Department of Periodontology (healthy controls), Faculty of Dentistry, as well as from the Clinic of Otorhinolaryngology and Head and Neck Surgery (laryngeal squamous cell cancer patients) at the University of Debrecen, Hungary. All participants signed an informed consent; the study was conducted under the supervision of the local Ethics Committee (No. of Ethics Committee approval 2273–2004).

Thirty-seven patients with oral squamous cell carcinoma (OSCC) (28 males; 9 females; mean age 54.5; age-range 39–80) and 28 patients with laryngeal squamous cell carcinoma (LSCC) (27 males; 1 female; mean age 56.8; age-range 43–71) were enrolled. Individuals of both groups were newly diagnosed and none of the patients received neoadjuvant chemo- or radiotherapy before the surgical intervention and specimen collection. All individuals fulfilling the inclusion criteria and agreeing to participate were enrolled. Fresh tissue samples were obtained from the central part of the tumours during operation.

As an age-matched control population, 68 healthy individuals (16 males; 52 females; mean age 52.4; age-range 22–77) without history of oral cancer and with healthy mucosa attending the Faculty of Dentistry for regular oral screening were sampled. Exfoliated buccal epithelial cells were collected from the controls using cytobrush after a thorough rinse of the mouth with physiological saline. Importantly, control individuals lived in the same geographical area (Eastern Hungary) where the patients came from.

Occurrence of human papillomaviruses and Epstein-Barr virus in these samples has been reported elsewhere [28, 29].

All samples were frozen immediately after collection at −70 °C and stored at this temperature until use. From tumour tissue (OSCC and LSCC) samples, the DNA was isolated using TRI Reagent (Sigma, St Louis, MO, USA) according to manufacturers' recommendations. Exfoliated cells were treated with proteinase K-SDS, proteins were removed by 5 M NaCl treatment, and finally, DNA was precipitated with 96 % ethanol.

Polymerase Chain Reaction and Single Strand Conformation Polymorphism Analysis (PCR-SSCP)

Quality of the DNA was confirmed by PCR-amplification of the β-globin gene. Exon deletions in the p16INK4A/p14ARF locus were analysed by means of PCR assays described earlier using primers complementary to intron sequences close to the exon boundaries [30–32]. Briefly, the 25 μl PCR mixture was composed 1× PCR buffer containing 250–250 μM of each dNTP, 25 pmol of each primer, 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI, USA) and 2 μl (100–300 ng) template DNA. PCR conditions were 94 °C for 3 min, followed 35 cycles of 94 °C denaturing for 1 min, annealing at temperatures 56–63 °C depending on the primers used (see Table 1) for 1 min, 72 °C elongation for 1 min with a final extension of 7 min at 72 °C. Sensitivity of the four exon-specific PCR assays was determined on serial dilutions of DNA extracted from primary keratinocyte cell culture and human fibroblast cells to exclude bias caused by differences in PCR sensitivity. All assays were run in triplicates. Samples repeatedly not yielding PCR product was considered as with a deleted respective exon.

Single nucleotide polymorphisms/point mutations were sought for by means of SSCP analyses of the amplified exons digested with different restriction enzymes. p16INK4A exon 1 and 2 PCR products were digested with SmaI (Fermentas, Vilnius, Lithuania) while in case of p14ARF exon 1 DdeI (Promega, Madison, WI, USA) was applied. Human fibroblast cells were used as a wild-type reference. PCR products were diluted in a buffer containing 95 % formamide, 0.05 % bromophenol blue and 0.05 % xylene cyanol, heat denatured at 95 °C for 5 min and then loaded onto a denaturing 18 % polyacrylamide gel. Electrophoresis was performed at 300 V for 4–6 h at 4 °C. After electrophoresis, the gel was stained by

Table 1 Primers used for amplification and sequencing of tumour suppressor gene exons

Primer ID	Primers	Product size (bp)	Annealing temperature (°C)
PCR-SSCP			
p14 exon 1β	F: CTGCTCACCTCTGGTGCCAA	367	62
	R: TCTCCTCCTCCTCCTAGCCT		
p16 exon 1α	F: GGAGGAAGAAAGAGGAGGG	316	63
	R: ACTTCGTCCTCCAGAGTCG		
p16 exon 2	F: GCTCTGACCATTCTGTTCTC	355	56
	R: CTCAGATCATCAGTCCTCAC		
p16 exon 3	F: GTAGGGACGGCAAGAGA	159	60
	R: ACCTTCGGTGACTGATG		
Methylation-specific PCR			
p14 U	F: TTTTTGGTGTTAAAGGGTGGTGTAGT	132	61
	R: CACAAAAACCCTCACTCACAACAA		
p14 M	F: GTGTTAAAGGGCGGCGTAGC	122	61
	R: AAAACCCTCACTCGCGACGA		
p16 U	F: TTATTAGAGGGTGGGGTGGATTGT	151	63
	R: CAACCCCAAACCACAACCATAA		
p16 M	F: TTATTAGAGGGTGGGGCGGATCGC	150	63
	R: GACCCCGAACCGCGACCGTAA		

SSCP: single strand conformation polymorphism; bp base pairs; F: forward primer; R: reverse primer; U: unmethylated; M: methylated

silver precipitation. Fragments with electrophoretic mobility different from the wild type were analysed by direct sequencing to confirm and characterize the nature of the alteration.

Exons of tumour suppressor genes were amplified using the abovementioned primers and conditions. PCR products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., East Markham Ontario, Canada) and sequenced by the Sanger chain termination method using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3100-Avant Genetic Analyser. Resulting sequences were compared to the GenBank reference sequence (Accession Number NG007485). When sequencing suggested heterozygosity, this was confirmed by cloning and sequencing of ten clones. TA cloning of the PCR fragments was performed with pGEM-T Easy vector (Promega, Madison, WI, USA). Transformed cells were recovered on duplicate LB agar plates supplemented with ampicillin (100 μg/mL). Ten colonies were tested further; plasmids were isolated by PureYield Plasmid Miniprep System (Promega, Madison, WI, USA) kit according to the protocol provided. Sequencing of the inserted PCR fragments was carried out as described above.

Methylation Analysis of the p16INK4A and p14 ARF Promoters

Promoter hypermethylation of the p16INK4A and p14ARF genes was determined by methylation-specific PCR as described Herman et al. [33]. First, genomic DNA was modified with sodium bisulphite. Briefly, 1 μg DNA was treated with NaOH (final concentration 0.3 M) for 20 min at 42 °C. Freshly prepared 3.8 M sodium bisulphite and 1 nM hydroquinone solution (pH 5.0) were added and incubated at 55 °C for 16 h. Modified DNA was purified on Wizard DNA Clean-Up system (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer, ethanol precipitated and resuspended in water. The methylation-specific PCR was performed using primers (Table 1.) and conditions as described earlier with minor modifications [33]. Briefly, the 25 μl PCR mixture contained 1× AmpliTaq Gold PCR buffer, 250 μM of each dNTP, 25 pmol of each primer, 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 2 μl template DNA. PCR conditions were as follows: 95 °C for 5 min, the 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s and finally 72 °C for 4 min. Methylation status was determined based on the PCR patterns seen. BL41 (methylated for p16INK4A; CRL-2323) and Ramos (methylated for p14ARF; CRL-1596) as well as Namalwa cell lines (CRL-1432) were used as methylated and unmethylated controls, respectively.

Statistical Analysis

Frequency of genetic differences or epigenetic alterations was compared between study populations using chi-square test or Fisher's exact test, survival was analysed with Kaplan-Meier test by means of SPSS for Windows 15.0.

Results

Sensitivities of the exon-specific PCR assays were uniformly as low as 1 ng total DNA. Among the controls four individuals

were found lacking one or more exon-specific amplimers; one showed p16 exon1α deletion, another exhibited lack of p16 exon2 amplimer, two individuals has deletion in two exons, one in p16 exon1α and 2, another in p14 exon1β and p16 exon3. Out of the 37 patients with OSCC, only one patient showed lack of p16 exon1α, all other exons were detected in all other patients. In patients with LSCC, deletion of at least one of the three exons (exon1α, 2 and 3) of p16INK4A was observed in 21 cases (75.0 %), while 10 cases (35.7 %) showed p14 exon1β deletion; ten of 28 LSCC samples showed deletion in p14ARF exon1β; 19 in exon1α; nine in exon2 and only two samples in exon3 of p16INK4A. Regarding inactivation by exon deletion, p14 is inactivated in three controls, none of the OSCC, and 14 of the LSCC patients; p16 is inactivated in four controls, one OSCC and 21 LSCC patients; both are lost in three controls, none of the OSCC and thirteen of the LSCC patients. This corresponds to a significantly different distribution of deletions in LSCC as compared to the controls or to OSCC patients ($p<0.001$ in both comparisons).

The SSCP alterations confirmed the presence of two mutations, a homozygous T24610A nucleotide change in the non-coding region of p16 exon1α and a heterozygous C24702A change in the coding region of p16 exon1α, leading to an Ala13Asp acid change. Three polymorphisms were identified. A G28575A polymorphism in exon2 corresponding to alanine and threonine variants at codon 140, all present heterozygously. A G31292C polymorphism was found in the non-coding region of exon3 found in homozygous and heterozygous forms in six and seven patients, respectively; this correspond to the C540G polymorphism at the mRNA level. The third polymorphism G28608A was detected in the non-coding region of exon2, always heterozygously. The occurrence of mutations/polymorphisms in the patients and controls is shown in Table 2.

Examining the promoter methylation patterns, bisulphite modification was successful in case of the p14 promoter for all 68 controls, for 30 of 37 OSCC and for all 28 LSCC samples; in case of the p16 promoter success rates were 68 of 68, 29 of 37, and 21 of 28 for control, OSCC and LSCC samples, respectively.

Neither p14 nor p16 promoter was found to be completely methylated in samples obtained from healthy individuals; the p14 and the p16 promoters were unmethylated in 97.1 % (66/68) and 95.6 % (65/68) of the controls, respectively. Two and three individuals showed partial methylation of p14 and p16 promoters, respectively.

In OSCC tumour samples, p14 promoter was unmethylated in 86.7 % (26/30) of the patients; complete and partial methylation was found in one and three patients, respectively. The p16 promoter was unmethylated in 69.0 % (20/29) of patients, which correspond to complete and partial methylation in three and six patients. Thus, unmethylated promoters were significantly less frequent in case of p16 promoter ($p=0.001$) as compared to the control group. In case of the p14ARF unmethylated promoters were also less frequent, but this was not significant statistically ($p=0.069$).

In case of LSCC, the p14 promoter was unmethylated in 85.7 % (24/28) of patients; one and three patients had completely methylated promoters and partial methylation, respectively. The p16 promoter was unmethylated in 76.2 % (16/21) of the patients, five patients showed partial methylation of the promoter; complete promoter methylation was not found. Similarly to OSCC, these data differ significantly from the healthy controls regarding the methylation status of the p16 ($p=0.016$) but not of the p14 ($p=0.058$) promoter. Between the methylation status of the two patient groups there was no statistically significant difference in either comparison.

Combining the two patient groups to a group of head and neck cancer patients, unmethylated promoter was significantly

Table 2 Distribution of mutations and polymorphisms in p16INK4A exons of patients. Patients not shown did not carry mutations and polymorphisms; mutations or polymorphisms in the p14ARF were not found. In case of heterozygous alterations the nucleotides of both strands are shown separated by a slash

Exon		p16 exon1α		p16 exon2		p16 exon3
Nucleotide position		24610	24702	28575	28608	31292
Reference (Accession number NG007485)		T	C	G	G	G
Control	K36			G/A		
OSCC patients	M03	A				
	M54		C/A			
	M20			G/A		
	M23			G/A	G/A	
	M33				G/A	
	M68				G/A	
	M37					C
	M65					C
	M76					G/C
	M29					G/C
	M30					C
	M71					G/C
LSCC patients	T67			G/A		
	T38				G/A	G/C
	T12					G/C
	T16					C
	T17					C
	T35					G/C
	T47					G/C
	T54					C
Amino acid change in coding regions			Ala13Asp	Ala140Thr		

OSCC: oral squamous cell cancer; LSCC: laryngeal squamous cell cancer

less frequent in case of both p14 and p16 ($p=0.043$ and $p=0.001$, respectively) compared to the control group.

Table 3. summarizes the number of individuals in each group showing mutational inactivation of the tumour suppressor genes or promoter hypermethylation. Association of genetic events or promoter hypermethylation with presence or absence of human papillomaviruses or Epstein-Barr virus was not found.

Mean tumour-free survival time was 870 (93–1,807) days and 951 (167–2,988) days for OSCC and LSCC patients, respectively. Exon deletions in case of LSCC and p16 promoter methylation in case of OSCC led to poorer tumour free survival, but neither was statistically significant ($p=0.054$ and 0.108, respectively).

Discussion

Major inactivating mechanism of p14ARF and p16INK4A gene is deletion, mutation and/or promoter methylation. Promoter methylation of p16INK4A was shown to be a relatively early event in the development of OSCC [34]. A number of authors reported data on the prevalence of genetic as well as epigenetic alterations (mostly on promoter methylation) affecting these genes in head and neck cancer, but the occurrence of these alterations varies widely among the studies; e.g. promoter methylation rates vary from 5 to 68 % and 14–34 % in case of p16INK4A and p14ARF, respectively, as reviewed by Demokan et al. [3]. As the majority of these studies concentrated on prevalence and used few or no healthy controls or other means to allow for statistical evaluation, the importance of genetic or functional inactivation of p16INK4A and/or p14ARF remains controversial in head and neck cancers.

According to the hereby presented data, major deletions may be important inactivation mechanisms for both genes in LSCC but not in OSCC; deletions in p16 may even affect survival. This is in agreement with a number of earlier studies on OSCC or head and neck cancer [18, 35, 36]. In contrast, some studies reported relatively high deletion rates in OSCC [20, 36, 37]. The difference between the present results and the cited Japanese and Indian data may represent geographical differences, while the contrast with data derived from studies of mainly Caucasian patients may be due to differences in exposure to chemical carcinogens (smoking or dietary habits), which were unfortunately unrecorded in the cited studies. Published studies reporting deletion rates specifically in LSCC were not found.

Curiously, results suggesting major deletions were also found in a small number of healthy individuals. These may be regarded as individuals with higher risk of tumours, or as results due to less important genetic events, e.g. polymorphism, mutation or deletion in primer binding sites. This also points to a potential limitation of studies using such an approach (including the present one), i.e. a repeatedly negative PCR assay may not only be due to lack of amplifiable sequences. This study tried to minimize such a possibility by running the assays in triplicates and by assessing PCR sensitivity to exclude negative results due to low sensitivity caused e.g. by mutations affecting primer binding sites. Another limitation of the approach is that only homozygous deletions can be detected.

The role of p14ARF and p16INK4A mutations in tumourgenesis seems to be small, as only two mutations were found. Most alterations found correspond to well-known polymorphisms of the exons involved. Though such polymorphisms were shown to play a role in some cancers [23, 26], in the study population they do not seem to be important, as all alterations found in the coding region were heterozygous and mutations consistently associated with tumour tissue were not found. Previous studies report similarly low mutation carriage rates in the two genes in head and neck cancer patients [36, 38, 39]. Occurrence of mutations was shown to be slightly higher in recurrent tumours [40]. Moreover, two of the three polymorphisms detected were previously shown to be neutral in head and neck cancer [27].

Table 3 Distribution of genetic and epigenetic alterations in the different study groups

		p14ARF			p16INK4A		
		Control	OSCC	LSCC	Control	OSCC	LSCC
Exon deletions		4.4 % (3/68)	ND	50.0 % (14/28)	5.9 % (4/68)	2.7 % (1/37)	75.0 % (21/28)
Mutations		ND	ND	ND	ND	5.4 % (2/37)	ND
Polymorphisms		ND	ND	ND	1.5 % (1/68)	27.0 % (10/37)	28.6 % (8/28)
Promoter methylation status	m	ND	3.3 % (1/30)	3.6 % (1/28)	ND	10.3 % (3/29)	ND
	m/u	2.9 % (2/68)	10.0 % (3/30)	10.7 % (3/28)	4.4 % (3/68)	20.7 % (6/29)	23.8 % (5/21)
	u	97.1 % (66/68)	86.7 % (4/30)	85.7 % (4/28)	95.6 % (3/68)	69 % (20/29)	76.2 % (16/21)

OSCC: oral squamous cell cancer; LSCC: laryngeal squamous cell cancer; m: methylated promoter; m/u: partially methylated promoter; u: unmethylated promoter; ND: not detected

Methylation status of the promoters suggests an at least moderate importance of promoter methylation in functional inactivation of p16INK4A less in case of p14ARF. Many of these samples exhibited partial methylation similarly to the findings of Shintani et al. and Kulkarni et al. [18, 41]. This may be caused not only by partial methylation of the promoter and consequent false priming, but also by heterogeneity of the sample tissue, e.g. the bulk or a part of the tumour is hypermethylated, while the normal tissue present in the excised section (or part of the tumour) shows very low or no methylation level [18, 21].

Methylation of the p16INK4A and the p14ARF promoters is a generally recognized epigenetic event in the literature in many cancer types including squamous cell cancer of the head and neck [39, 40] or the oesophagus [42], as well as in lung cancer [17]. Association of promoter methylation with dietary habits characteristic to certain geographical regions was demonstrated in case of oesophageal squamous cell cancer [42]. High frequency of hypermethylation of p16 promoter was even shown in oral epithelial dysplasia [43]. In the present study, p16 promoter methylation was significantly more frequent both in OSCC and LSCC than in healthy individuals; in OSCC it may also affect survival unfavourably. Methylation of the p14 promoter was also more frequent, but statistical significance was seen only in case of OSCC.

It was shown that promoter methylation at critical CpG islands is the main epigenetic silencing mechanism; hypermethylated promoters are always inactive [44]. Acetylation and methylation of histone proteins modify gene expression only in case of promoters where most or all CpG islands are unmethylated [44]; micro RNAs play an exclusively inhibitory role by promoting degradation of mRNA [45]. Consequently, the gene expression levels suffer some decrease even in case of partially methylated promoters; therefore our data represent a conservative estimate of the importance of epigenetic inactivation.

These data suggest that the importance of different genetic events as well as of promoter methylation affecting the p16INK4A and p14ARF tumour suppressor genes differs in different types of head and neck cancer. Exon mutations seem to be infrequent and consequently unimportant events both in LSCC and OSCC. In OSCC, promoter methylation seems to be the most frequent event, especially in case of the p16 promoter. In LSCC, both promoter methylation (mainly affecting the p16 promoter) and exon deletions seem to play a role in gene inactivation. The effect of these events on survival needs to be confirmed in larger cohorts. The findings are strengthened by the low rate of these events in the healthy control population.

Acknowledgements The work was supported by National Scientific Research Fund (OTKA) F046749, Mecenatura Mec-13/2011 and TÁMOP-4.2.2/B-10/1-2010-0024 grants. The TÁMOP project is co-financed by the European Union and the European Social Fund. The funding sources had no role in study design, data collection and interpretation or writing of the manuscript.

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