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ORIGINAL ARTICLE

Alterations of $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ Genes in Primary Laryngeal Squamous Cell Carcinoma

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Abstract The 9p21 gene cluster, harboring growth suppressive genes $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$, is one of the major aberration hotspots in head and neck cancers. We try to elucidate specific aberrations affecting this region, throughout methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay. Methylation of the gene was investigated by MS-MLPA in a well-characterized series of 27 laryngeal squamous cell carcinomas and 20 samples of healthy mucosa. Aberrant promoter hypermethylation was confirmed using and methylation-specific. All samples studied except 3 (11 %) presented losses at 9p21 segment. The most common finding was the small deletion (exon 1α) of the $p16^{INK4a}$ locus (44 %). Deletion of the 9p21 gene cluster was identified in 5 cases (18 %). We only found methylation in 8 samples (30 %) for $p15^{IK4b}$-exon 1. Promoter methylation of $p14^{ARF}$, $p15^{IK4b}$ and $p16^{INK4a}$ was not detected in any tumor sample. Methylation-specific polymerase chain reaction confirmed the results. Our data indicate that there may be a subgroup of patients in which epigenetic regulation of 9p21 segment might have little relevance. Nevertheless, MS-MLPA could not be suitable for the study of methylation at this region and further research is required.

Keywords DNA methylation · Larynx squamous cell carcinoma · Head and neck squamous cell carcinoma · MS-MLPA · *CDKN2B* · *CDKN2A*

Introduction

Genetic alterations at the 9p21 locus have been linked to malignant progression in head and neck squamous cell carcinomas (HNSCC) [1, 2]. Within a short distance of 50 kb, this region harbors a gene cluster consisting of 3 genes, cyclin-dependent kinase 2 A (*CDKN2A)*, which encodes $p14^{ARF}$ and $p16^{INK4a}$, and *CDKN2B*, which encodes $p15^{INK4b}$ (Fig. 1). The 9p21 gene cluster have functional importance in regulating cell proliferation. Beside this, it is associated with multiple tumor suppressor activities, which makes it a target of selective inactivation during carcinogenic process [3].

To date, a vast amount of data has demonstrated multiple types of genetic alterations on the complex genomic 9p21 region, which varies with the type of tumors [4]. Large homozygous deletions, point mutations and transcriptional-inactivating promoter methylation are common in HNSCC [1]. Despite the ample evidence of the 9p21 gene cluster as a frequent tumor target, it is not known whether all 3 genes in the cluster are indiscriminately affected in a tumor, and whether all of the genes are uniformly disrupted by the same mechanism. Some reports state that *CDKN2A* was primarily affected by hypermethylation and less frequently subject to deletion, whereas *CDKN2B* was frequently homozygously deleted and occasionally methylated [5]. This pattern is different from other reports identifying *CDKN2A* as the main deletion target [6]. Beside this, the physical proximity between $p14^{ARF}$ and $p16^{INK4a}$ genes, hinders understanding the role of $p15^{INK4b}$ gene, which possibly plays a role like tumor suppressor gene. Moreover, although inactivation of $p16^{INK4a}$ has been reported as the most common genetic

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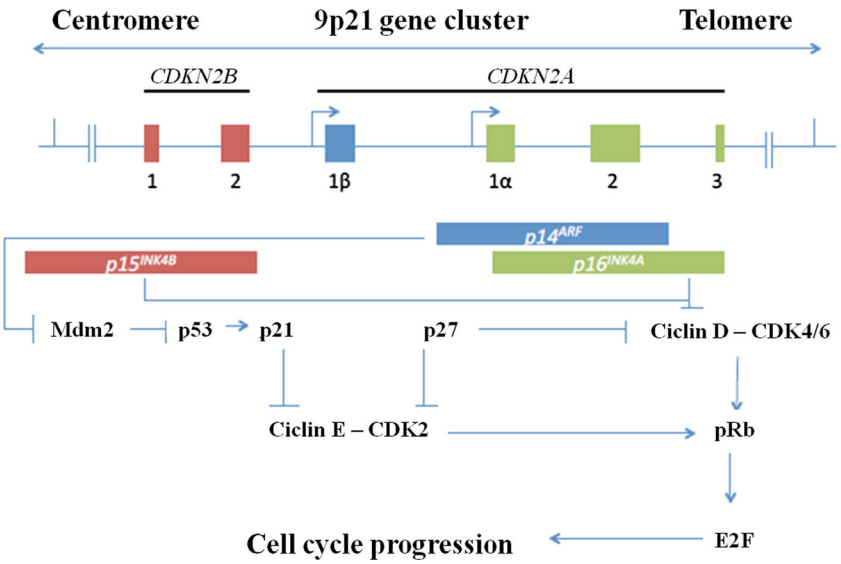

Fig. 1 Genomic organization of the 9p21 gene cluster and schematic description of the involvement of $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ gene products in the pRb and p53 antitumorigenetic pathways. $p14^{ARF}$ and $p16^{INK4a}$ (*CDKN2A*) transcripts are produced via utilization of a common coding sequence for exons 2 and 3, together with distinct sequences for promoter and exon 1. However, the resulting proteins are completely different because different reading frames are used for the respective translation processes. $p15^{INK4b}$ (*CDKN2B*) is highly homologous to $p16^{INK4a}$, particularly in exon 2, where they share 91 % sequence identity, indicating their origination by a gene duplication event

alteration in HNSCC, making it an ideal target for gene replacement, the extent to which $p14^{ARF}$ is independently altered, or the frequency with which $p14^{ARF}$ and $p16^{INK4a}$ are affected, has not been examined thoroughly. Because these proteins are completely unrelated, bearing no amino acid homology to one another, each of these targets has the potential to independently serve as therapeutic interventions in HNSCC.

We reported previously, using a MS-MLPA assay, an alteration pattern of methylation at the 9p21 region in human laryngeal squamous cell carcinoma (LSCC) [7]. Our data suggest that hypermethylation of the promoter region of *CDKN2B* is a frequent event in LNSCC. However, unlike previous reports we did not find DNA methylation of *CDKN2A*. In light of the publish results so far, herein we try to construct a map of the 9p21 gene cluster by analyzing the aberrant methylation of the $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ genes individually in 27 resected LSCC samples. For our purpose we have used a multicandidate gene assay. Then our results were confirmed using conventional procedures.

Methods

Samples

Between 2005 and 2009, 27 surgical tissue specimens from patients who were diagnosed of LSCC were collected at our Department of Otolaryngology. Patients had to meet the following criteria to be included in the study: (1) access to a complete clinical history of the patient, (2) pathologic diagnosis of LSCC, (3) with glottic or supraglottic lesions, (4) without distant metastases at the time of diagnosis, (5) with a sample of tissue in optimal conditions for genetic analysis, (6) no prior history of head and neck cancer, and (7) no prior chemotherapy or radiotherapy. Representative tissue sections were obtained from archival paraffin-embedded blocks.

All 27 LSCC samples proceeded from paraffin embedded tissue after histopathological analysis. All patients who were enrolled in the study provided written informed consent for the collection, storage, and analysis of specimens; and the study had received prior approval from our institutional ethical committees. After biopsy, all patients were treated with curative intent.

Normal controls for methylation assays are run using DNA from paraffin-embedded squamous epithelium from non-smoker patients and active adult smokers. Non-smoker group consisted of 10 patients not exposed to risk factors who underwent a nononcologic surgery (phonosurgery). The samples were obtained from an area of the normal mucosa from the aryepiglottic fold and were obtained from paraffin embedded blocks after confirming the absence of histopathological lesions. Smoker group consisted of 10 active adult smokers undergoing tympanoplasty. The samples were obtained from an area of the normal mucosa from the aryepiglottic fold and were obtained from paraffin-embedded blocks after confirming the absence of histopathological lesions.

Clinical Variables

Patient clinical data are summarized in Table 1. None of patients without risk factors or smokers with normal mucosa

Table 1 Characteristic of the clinical groups

	No.	Age, y	Sex	Tobacco	Alcohol	Macroscopic lesion
Without risk	10	48 (34–60)	13 M 2 F	0 %	0 %	Normal/healthy
Smoker	10	58 (40–62)	14 M 1 F	100 %	66 %	Normal/healthy
LSCC	27	62 (34–80)	24 M 3 F	93 %	37 %	Exophytic carcinoma (100 %)

M male, *F* female, *LSCC*, laryngeal squamous cell carcinoma

developed an epithelia precursor lesion or an LSCC during the follow-up period.

Of 27 LSCC patients, 18 had tumours localized in the supraglottic region (66 %), and 9 in glottic region (34 %). The series comprised of 6 well differentiated LSCC (22 %), 13 moderately differentiated LSCC (48 %), and 8 poorly differentiated LSCC (30 %). According to the 7th AJCC TNM Staging criteria, the series consisted of 4 stage I (15 %), 8 stage II (30 %), 9 stage III tumours (33 %), 6 stage IV tumours (22 %). All patients underwent surgery and 23 patients (85 %) received postoperative. The median follow-up was 27 months (range, 50–183 months).

DNA Extraction

Tumor purity was evaluated using an hematoxilin-eosin-stained section of the sample, and accepted when tumor cells were present at 70 % or more to minimize contamination by normal cells. Tumour DNA was extracted from paraffin embedded tissue samples using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. MS-MLPA on DNA from paraffin tumor tissue was analyzed by comparing it with normal reference DNA obtained from paraffin blocks of normal tissue (internal control). The technique was performed as described in detail previously [8].

MS-MLPA

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), a modification of the conventional MLPA assay [9], allows for the simultaneous detection of changes in methylation status as well as copy number changes in a single reaction requiring only 20 ng of human DNA. The standard use of the MLPA technique to observe quantitative changes in copy number has been outlined in other studies [7].

The MS-MLPA technique was performed as described previously in detail [10]. The SALSA MLPA probemix ME024-A1 9p21 CDKN2A/2B region (MRC-Holland, Amsterdam, The Netherlands) was used to perform promoter methylation analysis on a total of 27 tumors and 20 healthy samples. Probe sequences, gene loci and chromosome locations are shown al at Table 2. The experimental procedure was carried out according to the manufacturer's instructions. This kit contains 11 MS-MLPA probes, which recognized different HhaI restriction sites in their regions. Nine of them and it allows to analyze the methylation status of the promoter region of *CDKN2A* y *CDKN2B*. Such genes are frequently silenced by methylation in tumor cells and unmethylated in blood-derived DNA of healthy individuals. In addition, reference probes are included which are not influenced by the methylation-sensitive restriction endonuclease HhaI. Besides detecting aberrant methylation, this kit contain 20 probes which give us information on *CDKN2A* and *CDKN2B* copy number changes in the analyzed sample.

Validation of MS-MLPA Results

Validation of MS-MLPA results was possible using methylation-specific PCR (MSP). Immunohistochemistry was employed to analyze the expression of p16 and p15 (Table 5).

MSP was carried out using primers that had been designed for unmethylated or methylated DNA in previous studies [11]. The MSP was performed in a 25 m L reaction mixture containing 0.3 mmol/L of each primer, 2.5 mL of 10X Gene Taq Universal buffer (15 mmol Mg2+ Wako, Osaka, Japan), 2.5 mL of 2.5 mmol dNTP Mixture (Wako), 0.15 mL of Taq polymerase (5 U/mL; Wako) and 2.0 mL of template DNA. The initial temperature was 95 °C for 5 min, followed by 35 cycles consisting of 45 s at 95 °C, 45 s at each annealing temperature, and 1 min at 72 °C, with a final extension for 10 min at 72 °C. Amplified DNA was visualized on 2 % agarose gel using UV transillumination after ethidium bromide staining.

For the immunohistochemical the formalin-fixed, paraffin-embedded tissues were cut into 4 μm and dried on Flex IHC microscope slides (DakoCytomation). Sections were deparaffinized with standard xylene and hydrated through graded alcohols into water. Antigen retrieval was done using Envision Flex Target Retrieval solution, high pH (Dako). Immunohistochemistry was performed with monoclonal antibody anti p16INK4 (clon E6H4;CINtec Histology Kit. Mtm lab AG) and monoclonal antibody anti-p15INK4B (clon SPM303, predi¬luited (ab54324) (Abcam), using an automatic staining workstation (DakoCytomation) with the Envision system with

Table 2 Multiplex ligation-dependentprobe amplification gene probe panel

Length of PCR	Gene	Probe	HhaI restriction site	Location
124	Reference	9176-L09350		17q11
130	*CDKN2B*	11,867-L12664	+	p15 promoter
136	*CDKN2A*	11,868-L13885	+	p14 promoter
142	Reference	9789-L10204		15q21
148	*CDKN2A*	8658-L13470		intron1
157	*CDKN2A*	1524-L00962	+	p14 promoter
166	MLLT3	1286-L00847		9p22
172	*CDKN2A*	11,869-L12666	+	p16 promoter
178	Reference	8698-L08710		13q32
184	MLLT3	1287-L00846		9p22
193	Reference	3217-L02642		10q25
202	*CDKN2B*	1525-L00960		p15 promoter
211	*CDKN2B*	0607-L00591	+	exon 1; p15
220	*CDKN2B*	11,871-L13741	+	p15 promoter
229	*CDKN2B*	10,337-L10874		exon2;3'utr p15
238	*CDKN2A*	1289-L00834	+	exon 2α; p16
242	*CDKN2A*	10,333-L13471		exon 2α; p16 promoter
247	Reference	11,627-L12384		15q26
256	*CDKN2A*	1290-L00166		exon 3 (p14 + p16)
261	*CDKN2A*	10,334-L13472		exon 4 (3'utr; p14 + p16)
269	*CDKN2A*	2238-L13277		p14 promoter
274	*CDKN2A*	1291-L13280		exon 4 (p14 + p16)
283	GLDC	6857-L13502		9p24.1
292	MTAP	1292-L00839		9p21
301	Reference	2558-L02021		3q23
310	MTAP	1293-L00838		9p21
319	DOCK8	1130-L00688		3p24.3
328	MTAP	1294-L13278		9p21
337	GLDC	6869-L13503		9p24.1
346	Reference	0550-L00372		16p11
355	*CDKN2A*	1528-L06031		intron1
364	KLHL9	1296-L13281		9p21
373	Reference	5288-L04644		14q22
382	IFNW1	1297-L13886		9p21
391	*CDKN2A*	8659-L00958		exon 2α; p16 promoter
400	Control	10,357-L10895	+	22q12
409	Reference	6756-L06360		8q12
419	*CDKN2B*	10,336-L13473	+	p15 promoter
427	*CDKN2A*	1530-L00955	+	exon 1; p14
436	Control	9167-L09460	+	11q13
445	Reference	9612-L09907		20p12
454	*CDKN2B*	1531-L13742		Between p14 and p15
463	Reference	9980-L10439		19p13

diaminobenzidine chromogen used as the substrate. Negative controls with an omission of the antiserum from the primary incubation were also included. Immunostaining was scored blinded to clinical data by 2 independent observers. To score the staining pattern, we used the criteria proposed by Geradts and Wilson [12]. Only cells with evidence of nuclear staining were considered positive. If all cells within a lesion exhibited nuclear staining for p15 or p16, the pattern was considered normal or positive; any reduction of the number of stained cells in the presence of

positive staining of stromal cells was considered an abnormal pattern or negative. The immunostaining was regarded as inconclusive when stromal cells lacked nuclear reactivity.

Human Papillomavirus DNA Detection

Human papillomavirus (HPV) detection analysis was performed in all samples with LSCC as described in detail previously [13]. HPV genomic sequences were detected by polymerase chain reaction (PCR) amplification by use of consensus degenerate primers MY09/MY1119 and GP5?/6? complementary to the conserved L1 region. All tumor DNAs were also tested for HPV16 and HPV18 by amplification of the viral E6 and E7 region by use of type-specific primers (HPVONC 1/HPVONC 2). Positive control samples and negative control samples were applied in all analyses. The products were analyzed by electrophoresis on 2 % agarose gels, stained with ethidium bromide.

Statistical Analysis

Possible correlations between genetic and clinical parameters were analyzed statistically by using SPSS 15.0 software for Windows (SPSS Inc., Chicago, Ill) with the Fisher exact test. Kaplan-Meier analysis was performed for estimation of survival, and distributions of survival were compared using the logarithmic range test (log-rank test). P values <.05 were considered significant.

Results

Follow-up Data in Patients with LSCC

All follow-up clinical results are presented in Table 3. The mean time to recurrence was 38 months (range 4–124 months). At the time of the current report, a total of 13 patients (48 %) remained disease free. The overall 5-year survival rate was 48 %, and the median survival time was 60 months (range 1–134 months). The 5-year disease-free survival rate was 42 %. The main causes of death in our series were intercurrent causes (30 %) and distant metastasis (11 %).

Genetic Data

Without Risk Normal Mucosa and Smoker Normal Mucosa Loss of entire segment 9p21 was identified in 2 of 10 cases (20 %) of smoker normal mucosa. In the healthy mucosa group and in smoker normal mucosa group, we did not observe gene methylation.

LSCC All samples studied except 3 (11 %) presented losses at 9p21 segment. The most common finding was the small deletion (exon 1α) of the $p16^{INK4a}$ locus (44 %). Deletion of the 9p21 gene cluster was identified in 5 cases (18 %) (Table 4).

We only found methylation in 8 samples (30 %) for $p15^{IK4b}$-exon 1. Promoter methylation of $p14^{ARF}$, $p15^{IK4b}$ and $p16^{INK4a}$ was not detected in any tumor sample.

Table 3 Follow-up clinical results

	Supraglottic LSCC (n = 18)	Glottic LSCC* (n = 9)	Total (n = 27) (%)
Recurrences			
Recurrence			
Local	3	0	3 (11)
Regional	1	1	2 (7)
Second primary tumor			
Hypopharynx	1	0	1 (4)
Oral cavity	1	0	1 (4)
Esophagus	0	1	1 (4)
Lung	1	2	3 (11)
Distant metastasis			
Lung	2	1	3 (11)
Bone	0	1	1 (4)
Causes of death			
Loco (regional) recurrence	2	0	2 (7)
Distant metastasis	2	1	3 (11)
Second primary tumor	1	0	1 (4)
Intercurrent disease	5	3	8 (30)

*Laryngeal squamous cell carcinoma

Table 4 Losses as detected by MLPA

Point deletion	
•$p14^{ARF}$	6 (22 %)
•$p16^{INK4a}$	12 (44 %)
•$p15^{IK4b}$	5 (18 %)
Deletion of 9p21 segment	5 (18 %)
Absence of alterations	3 (11 %)

Expression of the p15 and p16 Gene

Immunohistochemical analysis revealed positivity at the nucleus in 12 of 27 tumors (44 %) for p15 and in 13 of 27 tumors (48 %) for p16 (Table 5). Overall, there was no correlation between protein expression and gene or methylation status. Nevertheless, when $p15^{IK4b}$-exon 1 was methylated, there was not p15 overexpression (Fisher exact test; $p = 0.05$). Normal appearing epithelium displayed nuclear staining for p15 and p16.

Correlation with Clinicopathological Characteristics and Follow-up Data

We found that when 9p21were lost, the second primary tumor free survival was lower (Log-rank test; $p = 0.001$). No significant correlation was found between the gene or methylation status and other clinicopathological profiles.

p15 overexpression was more frequent in alcohol drinkers (Fisher exact test; $p = 0.05$) and p16 overexpression was related to supraglottic tumors (Fisher exact test; $p = 0.02$) and early stage tumors (Fisher exact test; $p = 0.04$). Those patients with p16 overexpression showed a lower disease free survival (Log-rank test; $p = 0.013$). No correlation was observed between protein overexpression and other clinicopathologic parameters.

Validation of MS-MLPA Results

Alternative analyses confirmed MS-MLPA results in all occurrences. As is evident from the comparative analyses reported in Table 6, there was a good concordance between MS-MLPA and MSP. When $p15^{IK4b}$-exon 1 was methylated, there was not p15 overexpression.

HPV-DNA Detection

No HPV DNA was seen in any of the 27 samples of LSCC studied.

Discussion

The 9p21 region harbors 3 genes: $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$, with putative growth-suppressive activities. On the basis of the unusual genomic organization of this gene cluster and the functional relevance of these genes in both the Rb and p53 pathways, we reasoned that genes within this compact cluster can be differently regulated and are potentially susceptible to distinctive inactivating mechanisms, rather than a single deletion or methylation event occurring across the whole region. In the present study, we investigated the genetic and epigenetic alterations of chromosome 9p21 gene cluster in 27 resected LSCC. To our knowledge, this is the first study to look at 9p21 region in LSCC and not in the whole HNSCC.

Genetic alterations of chromosome band 9p21 ± 22 is one of the most frequent genetic aberrations in HNSCC and have been linked to malignant progression [1, 2, 14]. Two mechanism have been postulated as the primary cases of inactivation of the potential tumor suppressor genes on this area: homozygous deletion and promoter hypermethylation [15].

Herein, using a specific MS-MLPA kit to test the 9p21 region. The number of tumors in this study may be low and may be present statistical limitations, but considering that only 2 studies [1, 16] have used this method to study the methylation status in LSCC, we believe our results offer several interesting and important observations and may serve as a validation of the findings obtained by other authors through other techniques. To date, our series is the only one that attempts a fine-mapping architecture at genes located at 9p21 in LSCC, using MS-MLPA. The epidemiologic, clinical, and pathologic variables studied in our patients are similar to those described in previous series published in the literature.

In previous studies we have established a promoter methylation profiling of LSCC using MS-MLPA [7]. Unlike previous reports [17] we did not find DNA methylation of *CDKN2A*. Chen et al. [6] using the MS-MLPA assay also failed to find methylation of this gene. This may be due to

Table 5 Immunohistochemical analysis

	Monoclonal antibody	Expression
CDKN2A (p16)	Monoclonal antibody anti-p16 (clone E6H4; CINtec Histology Kit. Mtm lab AG) Working dilution 1/200	13 of 27 tumors (48 %)
CDKN2B (p15)	Monoclonal antibody anti-p15 (clone SPM303, prediluited (ab54324) (Abcam) Working dilution 1/200	12 of 27 (44 %)

Table 6 Validation of MS-MLPA results using methylation-specific polymerase chain reaction (MSP)

Gene	Method	Primer sequences/monoclonal antibody	No. of LSCC samples	Overall concordance with MS-MLPA results
$p14^{ARF}$	Methylation-specific PCR (MSP)	MET- Fw: 5′-GTGTTAAAGGGCGGCGTAGC-3′ MET- Rv: 5′-AAAACCCTCACTCGCGACGA-3′ UNMET- Fw: 5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′ UNMET - Rv: 5′-CACAAAAACCCTCACTCACAACAA-3’	27	100 %
$p15^{IK4b}$		MET-Fw: 5′-GCGTTCGTATTTTGCGGTT-3′ MET-Rv: 5′-CGTACAATAACCGAACGACCGA −3′ UNMET-Fw: 5′-TGTGATGTGTTTGTATTTTGTG-GTT-3′ UNMET-Rv: 5′-CCATACAATAACCAAACAACCAA-3′	27	100 %
$p16^{INK4a}$		MET-Fw: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ MET-Rv: 5′-GACCCCGAACCGCGACCGTAA-3′ UNMET-Fw: 5′-TTATTAGAGGGTGGGGGTGGATTGT-3′ UNMET-Rv: 5′- CAACCCCAAACCACAACCATAA −3′	27	100 %

the methylation occurs at regions different than those analyzed with this kit. Moreover, although a distinct advantage of MS-MLPA is the ability to examine methylation in multiple cancer genes in a single assay run, multiplex PCR of a large number of gene probes inherently encounters competitive amplification. In addition, because MS-MLPA interrogates and detects promoter hypermethylation in multiple genes simultaneously, the utility of MS-MLPA as a high throughput application to interrogate small amounts of DNA seems to be limited in its ability to assess sensitivity issues between different methods; such an assessment would be best addressed by larger studies.

Inactivation of the *CDKN2B* is a frequent event in HNSCC. Our data suggest that hypermethylation of the promoter region of *CDKN2B* could contribute to the observed inactivation. We found medium levels (28 %) of methylated *CDKN2B*. These figures are lower than those reported by Wong et al. [17] who evaluated the methylation status of the *CDKN2B* in 73 HNSCC surgical specimens and they found high levels (60 %) of methylated *CDKN2B*. Differences may be explained by the different methodology used. Lower rates (up to 15 %) than ours have been found with MS-MLPA [1, 16].

We identified losses at 9p21 in up to 88 % of cases. Whole region loss was seen in a 18 % of cases and point deletion of $p16^{INK4a}$ in a 44 % of samples. This figures are similar to those reported by Worsham et al. [1] who identified exon 1α-$p16^{INK4a}$as the smallest region of loss in the *CDKN2A*. This is a region of overlap for loss in the *CDKN2A* region (Fig. 1). Studies reporting homozygous deletions of *CDKN2A* may, therefore, underestimate the frequency of this region depending on the extent to which deletion mapping was performed.

Moreover, to attempt to validate the data found in previous studies, we designed a study that checks the methylation status of 9 different points within the 9p21. Our methylation study shows that only a 30 % of samples have altered DNA methylation pattern at exon 1 of $p15^{INK4b.}$ We failed to find methylation of $p14^{ARF}$, $p15^{INK4b}$, *and* $p16^{INK4a}$ promoters. Recently, Pierini et al. [18] assessed promoter methylation status of these selected genes, including $p16^{INK4a}$, using Methylation-Sensitive High Resolution Melting (MS-HRM) in 100 LSCC patients. The prevalence of promoter methylation in $p16^{INK4a}$ was 48 %. These authors suggest an association between deregulation of p16 because of promoter hypermethylation with increased cancer cell migration, tumor invasiveness, and, thus, aggressive phenotype. We did not find association between the methylation status and clinicopathologic parameters. Otherwise, we found that when whole 9p21 region was lost, the second primary tumor free survival was lower. In addition, we observed an increase in the rate of loss of this cluster in the mucosa of healthy smokers. This alteration could be related to the field cancerization and would increase the risk of developing of second primary tumors.

Simultaneous analysis of gene methylation and expression levels has hardly ever been performed [5]. We found expression in 44 % and 48 % of samples for p15 and p16, respectively. Although HPV infection, especially infection due to the high-risk type HPV-16, was found to be significantly associated with the risk of LSCC [19], none of our 27 LSCC specimens showed HPV DNA, suggesting, once more, a low prevalence of HPV infection in our geographic area [20]. Preliminary results suggest that these high-risk HPV infections seem to be biologically relevant in laryngeal carcinogenesis, manifested as having viral DNA integration in the cancer cell genome and increased expression of the p16 protein. Despite these data, the significance of these infections and the implications on disease prevention and treatment of LSCC are still unclear and require further investigation. As Demokan et al. [5] stated, promoter methylation is responsible for $p16^{INK4a}$ gene inactivation in 44 % of the patients with HNSCC, and therefore, there is a concordance between promoter hypermethylation and loss of gene expression. However, lack gene expression may also result from deletions or inactivating mutations of the $p16^{INK4a}$ suggesting that these events may play an equally significant role in silencing of the $p16^{INK4a}$ as has been shown for oral SCCs [21] or endometrial

carcinomas [22] and pleural mesothelioma [23]. Due to the absence of $p16^{INK4a}$ methylation in our series, the lack of expression of p16 in our samples would be due to a deletion rather than a methylation. There was concordance between $p15^{IK4b}$-exon 1 methylation positivity and lack of expression. This result suggest that, silencing of $p15^{IK4b}$ is associated with methylation.

Hence, our data lead to 2 conclusions. First, probably, there might be a group of patients in whom the most recurrent alteration at the 9p21 region is due to deletions rather than methylation of the gene promoters. Although the number of samples is not very large, the consistency of the results can be an endorsement to justify their validity and reliability. Second, MS-MLPA could not be suitable for the study of methylation at this region. Probes designed to test the degree of methylation of this region are not adequate.

Most of the published epigenetic data in LSCC comes from methylation-specific polymerase chain reaction (MSP) after bisulfite treatment. Currently, MSP analysis is the most convenient, rapid and cost effective [24]. However, this technique allows to assess 1 gene at a time and it is inclined to false positive results [25]. Also, it has been demonstrated that MSP cannot detect methylation of less than 5 % and it has lower sensitivity compared to other methods. In the present study, we used a MS-MLPA assay, that allows the simultaneous assessment of gains and losses and promoter methylation of multiple genes in a single experiment [9]. In contrast with high-throughput methods, MS-MLPA requires only small quantities of short fragments, which makes it very suitable for diagnostic application using formalin-fixed and paraffin-embedded DNA. In addition, epigenetic signatures from MS-MLPA profiling, after subsequent validation as diagnostic or prognostic biomarkers, can be reduced to a more definitive candidate gene panel of only a few key genes. The latter would be amenable for increased detection sensitivity by a targeted MS-MLPA or by MSP. Furthermore, MLPA gene panels can be updated on an ongoing basis to include newer gene targets as their involvement in LSCC becomes established to achieve comprehensive targeted gene panels. In recent years, many studies have compared MS-MLPA with other methods used for DNA methylation analysis demonstrating a very good concordance with these assays [26]. A disadvantage of the MS-MLPA is the absence of specific genes in pre-designed MLPA kits.

In conclusion, our experience indicates that there may be a subgroup of patients in which epigenetic regulation of 9p21 segment might have little relevance. A limitation of this study remains the relatively small number of patient samples and its retrospective analysis. Moreover, MS-MLPA could not be suitable for the study of methylation at this specific region and further research is required. Validation and clarifying the results in larger cohorts can provide impetus for exploitation of these targets as therapeutic biomarkers.

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