Research Article

Laryngopharyngeal Reflux and Laryngeal Squamous Cell Carcinoma

Deganello A¹*, Meccariello G¹, Parrinello G² and Gallo O¹

¹Department of Surgical Sciences, University of Florence, Italy

²Department of Head and Neck Surgery, Netherlands Cancer Institute, Netherlands

*Corresponding author: Alberto Deganello, Department of Translational Surgery and Medicine, 1st Clinic of Otolaryngology/Head and Neck Surgery University of Florence, Largo Brambilla, 3, 50134 Florence, Italy, Tel: 0039 055 7947054; Fax: 0039 055 435649; Email: adeganello@hotmail.com

Abstract

Background: Patients with squamous cell carcinoma (SCC) of the larynx are usually heavy smokers, and tobacco exposure has been clearly correlated with p53 tumor suppressor gene damages. In this prospective study we investigated the possible independent carcinogenic role of laryngopharyngeal reflux (LPR) by correlating the development of laryngeal SCC, with the presence of LPR and p53 alterations.

Materials and Methods: Eighty-eight consecutive patients with laryngeal SCC treated at the ENT University Clinic of Florence preoperatively underwent a 24h dual-probe-pH-monitoring and laryngeal endoscopy. Then, all patients underwent a direct microlaryngoscopy with tumor biopsy and assessment of p53 gene mutations. Statistical analysis was performed correlating the presence of LPR, smoking status and mutations of p53, by chi-squared test and logistic regression analysis.

Results: In 40% of the cases TP53 mutations were encountered. Sixteen transition mutations, 10 transversions, and 9 insertion-deletions were found. Furthermore, 22 missense, 4 frame deletions, 7 frame-shifts and 3 non-sense mutations were identified. In 34% of the patients pathologic LPR was recorded at 24h dual-probe-pH-monitoring and laryngeal endoscopy. A positive correlation between smoke and p53 mutations (p=.024, $\chi2$ test) was found, while no correlations was recorded for LPR and p53 mutations (p>.05, $\chi2$ test). LPR patients were at highest risk for tumors at the posterior glottic region (p=0.037, Fisher test).

Conclusion: Our study highlighted a potential key role of LPR in the pathogenesis of laryngeal SSC in non smokers and non drinkers, especially for glottic cancer of the posterior region. Moreover, our findings seems to suggest the presence of different genetic mechanisms of biomolecular damage beside those due to smoking exposure, according to the low rate of the p53 tumor suppressor gene mutations in laryngeal SCC from patients with LPR.

Keywords: Laryngopharyngeal reflux; Laryngeal squamous cell carcinoma; p53 mutation; Larynx; Cancer immunohistochemistry; Lpr gerd

Abbreviations

SCC: Squamous Cell Carcinoma; LPR: Laryngopharyngeal Reflux; IHC: Immunohistochemistry; SSCP: Single-Strand Conformational Polymorphisms; GER: Gastroesophageal Reflux; UAD: Upper Aero-Digestive; LES: Lower Oesophageal Sphincter; UES: Upper Esophageal Sphincter; PCR: Polymerase Chain Reaction

Introduction

Smoking, alcohol consumption and exposure to viral and toxic agents are well recognized risk factors for laryngeal squamous cell carcinoma (SCC). However, none of the above mentioned risk factors are present in the medical history of 5% laryngeal SCC patients [1]. The gastroesophageal reflux (GER) has been pointed out as a carcinogen for upper aero-digestive (UAD) tract cancers [2-6]. Koufman codified the term laryngopharyngeal reflux (LPR), describing a reflux that affects roughly 40–60% of patients with various voice disorders [7]. The majority of LPR-suffering patients, in fact, usually do not report classical GER symptoms like heartburn and regurgitation; instead they suffer of dry or sore throat, globus sensation, hoarseness, chronic cough, dysphagia, or buccal burning. Furthermore LPR has been correlated with posterior laryngitis, laryngeal granuloma, chronic hoarseness, pharyngitis, asthma, pneumonia, nocturnal choking, and dental diseases [8,9].

Many studies have reported a high prevalence of reflux in patients with laryngeal cancer [4-6], however smoking and alcohol consumption are not only risk factors for laryngeal SCC but usually increase reflux episodes. In fact, tobacco smoke causes a significant reduction of basal tonicity of the lower oesophageal sphincter (LES) and increases gastric acid secretion, while alcohol consumption may reduce LES tonicity and alter oesophageal motility [10,11].

Acting as carcinogens tobacco smoke and alcohol alter the repairing mechanisms of the DNA by damaging cell cycle proteins (i.e.: p53) as revealed by biomolecular studies [12-14]. The aim of our study was to evaluate the role of LPR in the carcinogenesis of laryngeal SCC, the association of reflux with tobacco and alcohol

makes it difficult to separate the relative contribution of reflux in cancer development. In order to discriminate the role of LPR we assessed its prevalence in laryngeal cancer patients using 24-hour double probe pH measurements and we analyzed TP53 mutations in smoking and in not smoking LPR patients.

Materials and Methods

Patient selection

We prospectively examined 94 consecutive patients with suspect laryngeal cancer at the Otolaryngology and Head-Neck surgery department of University of Florence from January 2010 to December 2011. In all patients we recorded smoking and alcohol habits. Patients were requested to sign an informed consent and institutional board approval was obtained. All patients underwent a 24h dual-probe-pH-monitoring, laryngeal videoendoscopy under local anaesthesia and direct laryngoscopy under general anaesthesia to collect biopsies for diagnosis and biomolecular studies. From the initial cohort of 94 enrolled suspect lesions, the study cohort resulted in 88 consecutive patients with biopsy proven squamous cell carcinoma. Specifically, we correlated immunostaining with p53 gene mutations as assessed by means of single-strand conformational polymorphisms (SSCP). This simple method for mutation screening recognizes sequences variations through differences in the migration properties of single stranded DNA in non-denaturing polyacrylamide gels [15]. The efficacy of mutations detection with this technique has been reported to be as high as 90% [16].

24h-dual probe-pH-monitoring

24 hours dual-probe (pharyngeal and esophageal) pH monitoring was performed in all cases for diagnosing LPR. Two monocrystalline antimony pH sensors were positioned along a single catheter (diameter, 2.1 mm) with sensors 15–20 cm apart and a silver-silver chloride cutaneous reference electrode (Digitrapper Mark III, Medtronic Synectics, Maastricht, The Netherlands). Both probes (pH sensors) were calibrated simultaneously in buffer solutions pH 7 and pH 1 before monitoring. Under fibroscopic control, the proximal probe was placed in upper esophageal sphincter (UES). The second probe (distal pH sensor) was positioned at the lower esophageal sphincter (LES) using pH transition point (withdrawal technique). The distal probe was useful to detect true pharyngolaryngeal reflux episodes (pH drop at the distal probe preceding pH drop at the UES). Patients were encouraged to eat their regular meals without restrictions and to continue their usual daily routines. Pathologic laryngo-pharyngeal reflux event was defined by an abrupt decrease in pH to below 4 in the upper probe, with an accompanying or preceding decrease in pH to below 4 in the lower probe, except while eating food [17]. Pathologic LPR was defined when more than three episodes of LPR occurred. At distal probe, levels of pH <4 were considered pathologic if they were registered in more than 5.5% of total recording time, in more than 8.2% of time in upright position, and in more than 3% of time in supine position. At proximal probe, any reflux rate equal or more than 0.2% of the total time in upright position was considered pathologic as well as any reflux rate equal or more than 0.1% of the total time in supine position.

Laryngeal endoscopy

All patients underwent high definition videolaryngoscopy under local anesthesia. The findings were scored according to the reflux finding score (RFS) as described by Belafsky et al. [18]. The scale ranges from 0 (no abnormal findings) to a maximum of 26 (worst score possible). According to these authors, we considered an overall score ≥ 7 suggestive of the LPR.

Sample preparation

Formalin-fixed, paraffin-embedded tumor specimens were obtained from all of 88 laryngeal SSC patients. Tissue sections (7 to 8 μm in thickness) were placed on standard microscope slides. Specimens were deparaffinazed with xylene, rehydrated in serial graded (100%, 90%, 70%, 50%) water-ethanol solution, rinsed in deionized water, and stained with hematoxylin and eosin. The samples were dehydrated again, but slides were not fitted with a coverslip. The stained, unmounted sections were examined by microscopy. The portion of normal tissue from each specimen was identified and scraped away with sterilized blade, leaving only tumor cells for DNA extraction. If discordant results between SSCP and IHC were obtained, different areas of tumor were sampled to confirm original data.

DNA isolation

DNA from paraffin-embedded tumor sections was extracted by means of overnight incubation at 55ºC in an extraction buffer: 50 mmol/L potassium chloride, 10 mmol/L tromethamine (tris) at pH 8.3, 2.5 mmol/L magnesium chloride, 0.1 mg/mL gelatin, 0.45% NP40, 0.45% polysorbate 20 (Tween 20), and 0.5 mg/mL proteinase K. The samples were boiled for 8 minutes, and centrifugated for 15 minutes at 14,000 rpm. One to 5 μL of the supernatant was used in polymerase chain reaction (PCR) mixture.

Polymerase chain reaction and single-strand conformation polymorphism analysis

Exons 5 to 8 of p53 gene were subjected to PCR with intron primers. Exon 5 was amplified with two pairs of primers, which resulted in partially overlapping fragments. Amplification consisting of 32 cycles was carried out in 25 μL total volume with 1.5 mmol/L MgCl buffer (Perkin-Elmer, Branchburg, NJ), 1 μmol/L of exon flanking primer set, 50 μmol/L each of deoxyribonucleoside triphosphate, and 0.5 units of AmplitTaq (Perkin-Elmer). Temperature and time during reaction cycles were 95ºC for 1 minute, 62ºC for 30 seconds, and 72ºC for 30 seconds. PCR products were heat denatured and subjected to SSCP analysis by electroforesis on 6% polyacrylamide gel with 5% to 10% glycerol. Electrophoresis was carried out at 40 W for 4 hours at 4ºC using the Pharmacia (Piscataway, NJ) apparatus. The gels were silver stained and dried on filter paper. All cases were amplified and run independently at least twice, with consistent results. To exclude silent CGA/CGG dimorphism in codon 213, PCR products from samples showing SSCP abnormalities in exon 6 were subjected to restriction analysis with Taq1.

Sequence analysis

PCR-amplified DNA strands were tested on 3% agarose gels (NuSieve; FMC Bioproducts, Rockland, Me), eluted from gel by phenol extraction, and concentrated by ethanol precipitation. Purified products were sequenced on an automatic sequencer (Model 373A; Applied Biosystems, Foster City, Calif) using dideoxy dye terminator method with Taq polymerase and PCR primers. Mutations were assessed on both strands.

Table 1: Relationship between Do-7 reactivity and SSCP analysis by exon in 88 laryngeal squamous cell carcinomas.

DO-7 Immunohistochemistry				
	-	+	++	+++
SSCP-negative (n=52)*	27	13	4	8
SSCP-positive (n=36)*	11	2	3	20
Exon 5[n]	4	1	1	6
Exon 6	0	0	0	1
Exon 7	2	0	2	7
Exon 8[n]	6	1	0	6

SSCP = single-strand conformation polymorphism.
Degree immunostaining: + = <10%; ++ = 10% to 50%; +++ =>50%.
* P= 0.013 calculated by Fisher exact test. X2 =15.74; df = 3.
[n] one IHC-negative sample demonstrated SSCP shifts in both exon 5 and exon 8.

DO-7 Immunohistochemistry

Five-micrometer paraffin-embedded sections were mounted on silane-coated slides and dried at 56°C for 30 minutes. After dewaxing and blocking endogenous peroxidase, sections were rinsed in water and then placed in 10 mmol/L of citrate acid at pH 6.0. The sections were microwaved at 750 W for 20 minutes while covered with liquid. After microwave heating, the sections were transferred to phosphate-buffered saline solution. Specimens were stained with mouse anti-p53 protein antibody DO-7 (Dako, Carpinteria, Calif) at 1:40 in phosphate-buffered saline solution. This antibody recognizes both mutant and wild type p53 protein. Primary antibody bound to antigen was detected with standard streptavidin-biotin technique (LSAB kit; Dako) and visualized with diaminobenzidine. A light hematoxylin nuclear counterstain was used. Control slides without primary antibody were normal in all cases. Immunostaining was graded as follows: +, less than 10%; ++, 10% to 50%; +++, more than 50% stained cells.

Statistical analysis

The statistical analysis was carried out with the Fisher's exact, χ^2 tests and multivariate regression analysis using STATA software (College Station, TX). Statistical significance was set at p<.05.

Results

Clinical characteristics

Of 88 patients enrolled, 79 (89.8%) were males and 9 (10.2%) females. The mean age was 60.1 years old (range 45-78). According to 7th edition of American Joint Committee on Cancer (AJCC) [19], 59(67%) T1, 22(25%) T2, 4 (4.5%) T3, 3 (3,5%) T4a with 77 (87.5%) N0 and 11 (12.5%) N1-3 were found. The most involved subsite was the glottis (46 cases, 52.2%), followed by the supraglottis in 35(39.8%) cases. The posterior supraglottic and glottic larynx was involved in 3 (3.4%) patients and 4 (4.5%) transglottic tumors were found. Tobacco smoke exposure was recorded in 75 (85.2%) patients. Most patients (n=67, 76.1%) daily consumed 2-3 drinks, whilst 17(19.3%) patients had positive medical history of an alcohol-abuse (more than 4 drinks) and only 4(4.5%) patients were abstemious. All heavy drinkers were also heavy smokers (more than 30 packs per year).

24h-dual probe-pH-monitoring outcomes

Thirty (34%) patients showed positive 24h-dual probe-pH monitoring for LPR. In these patients, values of pH <4 at proximal probe were registered as a mean rate of 0.6% (range 0.35% - 0.8%) of time in upright position; while a mean rate of 0.93% (range 0.4% - 1.5%) of time in supine position was recorded. The average number of reflux episodes was 9 (range 5 – 13) and the mean duration of reflux episodes was 21 min (range 19 – 25).

Laryngeal endoscopy outcomes

Twenty-four out of 30 LPR-suffering patients (80%) documented also a RFS more than 7 with a mean of 12.3 ± 4.7. The most common endoscopic finding was posterior commissure hypertrophy (n=23); only in 1 case a concomitant small granuloma of the posterior commissure was encountered. No patients with negative LPR at 24h-dual probe pH-monitoring scored more than 7 at RFS.

p53 Immunohistochemistry and gene status

Among 88 laryngeal SCC, IHC-positive moderate (++) and abundant (+++) staining was found in 35 cases (40%). Thirty-six (41%) cases demonstrated mutations of TP53 by SSCP analysis in exon 5,6,7,8. One sample was altered in both exon 5 and 8. As shown in Table 1, the 88 tumors in this series were stratified into distinct categories according to IHC and SSCP results. Twenty-three (26%) lesions were positive in both techniques. Twenty-seven (31%) cases neither contained mutations detectable at SSCP nor stained for p53 protein and additional 13 (15%) SSCP-negative specimens demonstrated fewer than 10% stained tumor cells. These 13 lesions were tentatively scored as IHC negative, because multiple tumor sampling failed to produce a positive SSCP assay. Thus, a total correlation between IHC and SSCP methods was attained in 63 (72%) (p=.0013, Fisher exact test). Twelve (14%) lesions displayed moderate (++) or abundant (+++) p53 staining despite negative SSCP patterns. In contrast, 11 lesions without nuclear reactivity and 2 with focal (+) staining (15%) showed SSCP shifts. These latter cases were subjected to sequence analysis, and mutation was always found, confirming the good analytical power of SSCP [Table 2]. Ten mutations were either nonsense or frameshift alterations, thus generating a truncated protein. Two were base substitutions, leading to conservative amino

Table 2: Sequence analysis of IHC-negative SSCP-positive samples.

Exon	Codon	Nucleotide Change	Amino Acid Change
5	144	C to T	Gln-stop
5*	155	C to A	Thr to Asn
5	164	Del 10pb	Frame shift
5•	175	G to A	Arg to His
5	184	Del A	Frame shift
7	244	Del G	Frame shift
7	255	Ins T	Frame shift
8*	264	Del CTA	In frame deletion
8	264	Del T	Frame shift
8•	280	A to G	Arg to Gly
8	283	Del G	Frame shift
8	285	G to T	Glu-stop
8	298	G to T	Glu-stop
8	298	G to T	Glu-stop

IHC = immunohistochemistry; SSCP= single-strand conformation polymorphism.
*IHC-negative and double mutated.
• Demonstrated focal immunostaining (<10% positive cells).

Table 3: Distribution of p53 mutation in LPR-positive and negative patients.

		P53 mutation		Total
		Absent	Present	
LPR	No	26 (44.8%)	32 (55.2%)	58
	Yes	**27 (90%)***	3 (10%)	30
Total		53 (60.2%)	35 (39.8%)	88

*P<0.01 (χ² test)

Distribution of p53 mutation in smoking and non-smoking patients.

		P53 mutation		Total
		Absent	Present	
Tobacco smoke exposure	No	12 (92.3%)	1 (7.7%)	13
	Yes	**41 (54.7%)**	**34 (45.3%)***	75
Total		53 (60.2%)	35 (39.8%)	88

*P=0.024 (χ² test)

acid changes (Arg to His, and Thr to Asn), whereas remaining base substitution was of non conservative type (Arg to Gly) and involved codon 280. Sixteen (45.7%) transition mutations, 10 (28.57%) transversions (from G:C to A:T in 8 of 10), and 9 (25.71%) insertion-deletion were found. Further 22 missense, 7 frame shift and 3 non-sense mutations and 4 frame deletions were identified. In this series, 13 mutations only involved DNA binding portion of p53 whilst the remnants 24 mutations determined conformational alterations resulting in an abnormal structure of this protein.

Correlations among p53 gene alterations, smoke and LPR status

According to p53 gene status, laryngeal cancer patients with documented LPR showed a lower incidence of p53 gene alterations (p<.001, χ² test); in fact the mutation was absent in 27 (90%) of 30 LPR-suffering patients. Conversely, a positive tobacco smoke exposure was statistically associated to mutations of p53 (p=.024, χ² test) [Table 3]. Despite the small number (n=3) of posterior laryngeal cancers, a correlation to LPR was statistically found (p=0.037, Fisher exact test) as shown in Table 4. The multivariate regression analysis showed a high correlation between presence of LPR and the development of laryngeal SCC tumor of posterior region (p=.031, multivariate regression) [Table 5].

By combining risk factors and p53 gene status we found that non smokers PLR positive patients showed no mutations at p53 [Table 6].

Discussion

The p53 is a critical protein for cell cycle control, DNA repair, and apoptosis [14]. This protein is tightly regulated and, physiologically,

Table 4: Distribution of laryngeal subsites involved by tumor in LPR-positive and negative patients.

Subsites of larynx	LPR		Total
	Present	Absent	
Glottis	14(30.4%)	32 (69.6%)	46
Supraglottis	12(34.3%)	23(65.7%)	35
Transglottis	1(25%)	3(75%)	4
Posterior region	**3(100%)***	0	3
Total	30(34.1%)	58(65.9%)	88

*P=0.037 (Fisher exact test)

Table 5: Multivariate regression analysis for development of posterior laryngeal SCC.

	Coef.	Std. Err.	T	P>[t]	[95% Confidence Interval]	
smoke	0.93	0.057	1.64	0.104	-0.02	0.208
p53 status	-0.058	0.045	-1.27	0.208	-0.148	0.033
LPR	**-0.117**	**-0.053**	**-2.20**	**0.031**	**-0.224**	**-0.011**
age	-0.076	0.049	-1.53	0.130	-0.174	0.023
gender	0.043	0.063	0.67	0.503	-0.083	0.168
alcohol	0.064	0.057	1.13	0.263	0.177	0.049

LPR= Laryngopharyngeal Reflux
SCC= Squamous Cell Carcinoma

Table 6: Correlations among LPR status, smoke, alcohol, and p53 gene mutations.

LPR	Smoke	Alcohol	Mutation p53
Yes	Yes	Yes	11% (3/26)
No	No	Yes	11% (1/9)
Yes	No	No	0% (0/4)
No	Yes	Yes	63% (31/49)

LPR= Laryngopharyngeal Reflux
SCC= Squamous Cell Carcinoma

is expressed at low levels. Mutations of the p53-encoding onco-suppressor gene (TP53) usually results in expression of high levels of abnormal non functioning p53 protein, which has been clearly associated with the onset of cancer. The nature and distribution of TP53 mutations are still under investigation, and some evidences may indicate that the type of mutation may reflect a specific mutating capacity of a certain etiologic factor; for example, chemical carcinogens in tobacco smoke generally produce transversion mutations, whereas endogenous mutagens, such as nitric oxide, mostly cause transition mutations [20-22]. Furthermore, a strong correlation exists among missense mutations in TP53 and high levels of protein accumulation [23].

LPR and laryngeal cancer share the same risk factors (smoke and alcohol exposure). Our group documented specific ultrastructural dilation of intercellular spaces in laryngeal epithelium exposed to acid LPR [24] comparable to what happens on mucosa of lower esophageal third injured by GER [25]. The main neoplastic histology at the distal esophagus is adenocarcinoma while SCC is characteristic of the larynx; recent studies have shown that esophageal adenocarcinomas and squamous cell carcinomas are both related to TP53 gene mutations [26].

Our study, in contrast, seems to indicate a non p53-dependent carcinogenesis in LPR-related laryngeal squamous cell carcinomas, since none of the non smoking non drinking LPR patients showed mutations on TP53.

Some studies have analyzed the difference of p53 protein expression in smokers and non-smokers patients with Head-Neck SCC [27-29]. These studies showed that TP53 mutations were an early event of the tobacco smoke carcinogenesis and among smokers TP53 mutations were demonstrated in 80% of tumors [6]. Moreover, TP53 mutations were frequently located between exons 5 and 8 of this gene, and the most frequent is transversion mutation as it occurs in laryngeal and lung cancers [30,31].

Our study confirms that the incidence of TP53 mutations was clearly correlated with cigarette smoking exposure (p=.024, χ^2 test) while TP53 mutations did not appear to be associated with documented presence of LPR (p<.001, χ^2 test). No differences in type of mutation site have been also documented between mutated and wild-type p53 protein in LPR-positive patients. In conclusion, our study suggests possible role of LPR in laryngeal SCC pathogenesis, particularly for tumors involving the laryngeal posterior region (p=.037, Fisher exact test). LPR might be directly responsible of other genetic mechanisms of biomolecular damage than those related to tobacco smoke carcinogens. A huge limitation of our study is represented by the small cohort, but the use of 24h-dual probe-pH-monitoring allowed us to truly assess the incidence and the role of LPR excluding GER. Further studies investigating on laryngeal cancer-patients without smoke and alcohol exposure will help in classifying this hypothesis. A thoughtful comprehension of the role of LPR in laryngeal carcinoma might refine antireflux treatment in order to decrease the onset of laryngeal cancer and the incidence of post-treatment recurrences.

References

1. Muir C, Weiland L. Upper aerodigestive tract cancers. Cancer. 1995; 75: 147-153.

2. Biacabe B, Gleich LL, Laccourreye O, Hartl DM, Bouchoucha M, Brasnu D. Silent gastroesophageal reflux disease in patients with pharyngolaryngeal cancer: further results. Head Neck. 1998; 20: 510-514.

3. Harrill WC, Stasney CR, Donovan DT. Laryngopharyngeal reflux: a possible risk factor in laryngeal and hypopharyngeal carcinoma. Otolaryngol Head Neck Surg. 1999; 120: 598-601.

4. Copper MP, Smit CF, Stanojcic LD, Devriese PP, Schouwenburg PF, Mathus-Vliegen LM. High incidence of laryngopharyngeal reflux in patients with head and neck cancer. Laryngoscope. 2000; 110: 1007-1011.

5. Galli J, Cammarota G, Calò L, Agostino S, D'Ugo D, Cianci R, et al. The role of acid and alkaline reflux in laryngeal squamous cell carcinoma. Laryngoscope. 2002; 112: 1861-1865.

6. El-Serag HB, Hepworth EJ, Lee P, Sonnenberg A. Gastroesophageal reflux disease is a risk factor for laryngeal and pharyngeal cancer. Am J Gastroenterol. 2001; 96: 2013-2018.

7. Koufman JA, Aviv JE, Casiano RR, Shaw GY. Laryngopharyngeal reflux: position statement of the committee on speech, voice, and swallowing disorders of the American Academy of Otolaryngology-Head and Neck Surgery. Otolaryngol Head Neck Surg. 2002; 127: 32–35.

8. Poelman J, Feenstra L, Demedts I, Rutgeerts P, Tack J. The yield of upper gastrointestinal endoscopy in patients with suspected reflux-related chronic ear, nose, and throat symptoms. Am J Gastroenterol. 2004; 99: 1419–1426.

9. Tauber S, Gross M, Issing WJ. Association of laryngopharyngeal symptoms with gastroesophageal reflux disease. Laryngoscope. 2002; 112: 879-886.

10. Stanciu C, Bennett JR. Smoking and gastro-oesophageal reflux. Br Med J. 1972; 3: 793-795.

11. Vitale GC, Cheadle WG, Patel B. The effect of alcohol on nocturnal gastroesophageal pH value: influence of study center, pH electrode, age and gender. Dig Dis Sci. 1992; 37: 849-854.

12. Gallo O, Chiarelli I, Boddi V, Bocciolini C, Bruschini L, Porfirio B. Cumulative prognostic value of p53 mutations and bcl-2 protein expression in head-and-neck cancer treated by radiotherapy. Int J Cancer. 1999; 84: 573-579.

13. Deganello A, Gitti G, Mannelli G, Meccariello G, Gallo O. Risk factors for multiple malignancies in the head and neck. Otolaryngol Head Neck Surg. 2013; 149: 105-111.

14. Deganello A, Franchi A, Sardi I, Pignataro L, Leemans CR, Gallo O. Genetic alterations between primary head and neck squamous cell carcinoma and recurrence after radiotherapy: recurrence, genetically related cancer, or second primary? Cancer. 2010; 116: 1291-1297.

15. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst. 1993; 85: 200-206.

16. Hayashi K, Yandell DW. How sensitive is PCR-SSCP? Hum Mutat. 1993; 2: 338-346.

17. Richter JE, Bradley LA, DeMeester TR, Wu WC. Normal 24-hr ambulatory esophageal pH values. Influence of study center, pH electrode, age, and gender. Dig Dis Sci. 1992; 37: 849-856.

18. Belafsky PC, Postma GN, Koufman JA. The validity and reliability of the reflux finding score (RFS). Laryngoscope. 2001; 111: 1313-1317.

19. Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. AJCC Cancer Staging Manual 7th edn. New York: Springer. 2010.

20. Soussi T, Caron de Fromentel C, May P. Structural aspects of the p53 protein in relation to gene evolution. Oncogene. 1990; 5: 945-952.

21. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991; 351: 453-456.

22. Bennet WP, Hussain SP, Vahakangas KH, Khan MA, Shields PG, Harris CC. Molecular epidemiology of human cancer risk: gene environment interactions and p53 mutation spectrum in human lung cancer. J Pathol 1999; 187: 8-18.

23. Olivier M1, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: new online mutation analysis and recommendations to users. Hum Mutat. 2002; 19: 607-614.

24. Franchi A, Brogelli B, Massi D, Santucci M, De Campora E, Gallo O. Dilation of intercellular spaces is associated with laryngo-pharyngeal reflux: an ultrastructural morphometric analysis of laryngeal epithelium. Eur Arch Otorhinolaryngol 2007; 264: 907-911.

25. Caviglia R, Ribolsi M, Maggiano N, Gabbrielli AM, Emerenziani S, Guarino MP, et al. Dilated intercellular spaces of esophageal epithelium in non erosive reflux disease patients with physiological esophageal acid exposure. Am J Gastroenterol. 2005; 100: 543–548.

26. Pandilla R, Kotapalli V, Gowrishankar S, Chigurupati M, Patnaik S, Uppin S, et al. Distinct genetic aberrations in oesophageal adeno and squamous carcinoma. Eur J Clin Invest. 2013; 43: 1233-1239.

27. Figueroa JD, Terry MB, Gammon MD, Vaughan TL, Risch HA, Zhang FF, et al. Cigarette smoking, body mass index, gastro-esophageal reflux disease, and non-steroidal antiinflammatory drug use and risk of subtypes of esophageal and gastric cancers by p53 overexpression. Cancer Caus Cont. 2009; 20: 361-368.

28. Smith BD, Haffty BG, Sasaki CT. Molecular markers in head and neck squamous cell carcinoma: their biological function and prognostic significance. Ann Otol Rhinol Laryngol. 2001; 110: 221-228.

29. Cruz I, Snijders PJ, Van Houten V, Vosjan M, Van der Waal I, Meijer CJ. Specific p53 immunostaining patterns are associated with smoking habits in patients with oral squamous cell carcinomas. J Clin Pathol. 2002; 55: 834-840.

30. Sarkar FH, Sakr WA, Li YW, Jacobs J, Crissman JD. Tumor suppressor p53 gene mutation in squamous cell carcinoma of the larynx. Diagn Mol Pathol. 1996; 5: 201-205.

31. Chiba I, Takahashi T, Nau MM, D'Amico D, Curiel DT, Mitsudomi T, et al. Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Lung Cancer Study Group. Oncogene. 1990; 5: 1603-1610.