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Expression of the nonclassical HLA-G and HLA-E molecules in laryngeal lesions as biomarkers of tumor invasiveness

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Summary. Introduction: HLA-G and HLA-E are two nonclassical class I molecules, which have been well recognized as modulators of innate and adaptive immune responses, and the expression of these molecules in virus infected cells has been associated with subversion of the immune response. Objective: In this study we performed a cross-sectional study, systematically comparing the expression of HLA-G and HLA-E in benign, pre-malignant and malignant laryngeal lesions, correlating with demographic and clinical variables and with the presence of high-risk and low-risk HPV types. Materials and methods: Laryngeal lesions were collected from 109 patients and stratified into 27 laryngeal papillomas, 17 dysplasias, 10 *in situ* laryngeal carcinomas, 27 laryngeal carcinomas without metastases, 28 laryngeal carcinomas with metastasis along with their respective draining cervical lymph nodes, and 10 normal larynx specimens. The expression of HLA-G and HLA-E molecules was determined by immunohistochemistry. HPV DNA detection and typing was performed using generic and specific primers. Results: HLA nonclassical molecules showed a distinct distribution pattern, according to the larynx lesion grade. HLA-G expression increased in benign and premalignant lesions, and gradually decreased in invasive carcinomas and in respective draining cervical lymph nodes. Conversely, HLA-E expression increased as far as lesion grade increased, including increased molecule expression in the draining lymph nodes of malignant lesions. Only 17 (15.6%) patients were HPV DNA positive. Conclusions: Overexpression of HLA-E and underexpression of HLA-G appear to be good markers for malignant larynx lesion.

Key words: HLA-G, HLA-E, Laryngeal lesions, HPV, Biomarkers

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common forms of cancer worldwide, with a total of 640,000 new cases per year. The larynx carcinoma primarily affects males, with a total of 159,000 new cases per year (Parkin et al., 2005; Koskinen et al., 2007). Among the Brazilian population, laryngeal tumors corresponding to approximately 8,000 new cases annually (Wünsch-Filho, 2004; Miranda et al., 2009).

Tumors can induce the generation and accumulation of immunosuppressive molecules, such as the nonclassical class I human leukocyte antigen (HLA-E, HLA-F and HLA-G) in the tumor microenvironment, contributing to tumor escape from immunological responses. Compared to classical class I antigens (HLA-A, HLA-B and HLA-C), HLA-G presents a low number of variants, limited tissue distribution, and each variant may present several membrane-bound and soluble isoforms (Moreau et al., 1995; Le Bouteiller, 2003). The HLA-G molecules were first detected on cytotrophoblast cells at the maternal-fetal interface, where HLA-G

modulates maternal immune response cells contributing to the maintenance of tolerance to the semiallogenic fetus (Le Bouteiller, 2003). HLA-G is also observed in non-physiological conditions such as organ transplantation, inflammatory diseases, viral infections and hepatitis (Onno et al., 2000; Cardili et al., 2010; LeMaoult et al., 2003, Souto et al., 2010) and tumours (Singer et al., 2003; Favier et al., 2007; Rouas-Freiss et al., 2007). Unlike HLA-G, the expression and function of HLA-E in physiological and pathological processes remain poorly established. HLA-E is constitutively expressed in T and B lymphocytes, NK cells, monocytes and macrophages (Sullivan et al., 2006; Coupel et al., 2007). There is strong evidence supporting the role of HLA-G and HLA-E in immune system regulation (Carosella et al., 2008), since some viruses and tumor cells may down-regulate HLA classical class I molecules and up-regulate nonclassical molecules, allowing the escape from the action of cytotoxic and NK cells (Tomasec et al., 2000; Marin et al., 2003; Sheu et al., 2005).

Epidemiological and experimental evidence indicate the relationship of human papillomavirus infection and HNSCCs (Tran et al., 2007). The most frequent HPV types associated with the development of HNSCC are HPV-16 and -18, which have been considered as high-risk types. The role of the HPV infection in laryngeal carcinomas has not been defined, since its prevalence varies greatly according to the study (reviewed by Kreimer et al., 2005). On the other hand, the low oncogenic HPV-6 and -11 types have been associated with the development of benign laryngeal lesions such as papillomas and recurrent respiratory papillomatosis (Herrero et al., 2003; Madkan et al., 2007). In pre-malignant lesions of the oral cavity, there is a strong association between HPV-16 and -18 with the development of highly dysplasic lesions (Donofrio et al., 1995).

Specific biomarkers such as HLA have been proposed for the evaluation of cancer progression (Singer et al., 2003; Yie et al., 2007a,b); however, immunological biomarkers are still needed to predict the clearance of the HPV infection during cancer progression. Recently, we described the expression of HLA-G in specimens obtained from patients with pre-neoplastic (Gonçalves et al., 2008) lesions or with invasive uterine cervical cancer, which were not associated with the presence of HPV infection (Guimarães et al., 2009).

Considering that larynx cancer is the second most commom between the head and neck tumors and nonclassical HLA molecule was not evaluated solely in larynx biopsies, we performed a cross-sectional study, systematically comparing the qualitative and quantitative expression of the soluble HLA-G and HLA-E in laryngeal lesions, stratified according to demographic and clinical variables, HPV type, and the severity of the lesions; i. e, benign lesions (laryngeal papillomas), premalignant lesions (dysplasias), and malignant lesions (in situ carcinomas and invasive laryngeal carcinomas presenting or not metastasis).

Materials and methods

Specimens

The study protocol was approved by the Brazilian Institutional Ethics Committee on Human Experimentation (protocol #512206/04). Between 1995 and 2004, 137 laryngeal lesions from 109 patients were selected from the archives of the Pathology Department, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Brazil. Patients were stratified into five groups according to the type of lesion: benign lesion-BL (laryngeal papilloma; n=27/137), premalignant lesion-PL (low, moderate and severe dysplasia; n=17/137), *in situ* laryngeal carcinoma-LSCC^{S} (n=10/137), laryngeal invasive carcinoma without metastasis-LSCC^{WT} (n=27/137) and laryngeal invasive carcinoma with metastasis-LSCC^{W} (n=28/137). The draining cervical lymph nodes-LN (n=28/137) from LSCC^{W} were also evaluated.

Five μm sections were cut, placed on organosilane-pretreated slides and submitted to immunohistochemical assays (HLA-G and HLA-E). Additionally, a 10 μm section was cut for DNA extraction and HPV typing. Demographic and clinical variables such as age, gender, history of smoking, history of alcohol consumption, histological grade, TNM stage, tumor treatment and recurrence was obtained from patient medical files. To classify tumor invasiviness, two criteria was considered: how depth was the tumor invasion into the estroma and the break or lack of the basal membrane.

Immunohistochemistry (HLA-G and HLA-E)

Immunohistochemistry using the streptavidin-biotin system (EP- USA/500, Signet, USA) was performed for the detection of HLA-G and HLA-E antigens. Specimens were dewaxed in xylene, rehydrated in graded alcohol and rinsed in water. For antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer, pH 6.0. Endogenous peroxidase blocking was performed using immersions in a hydrogen peroxide bath in absolute methanol (15 minutes each change) and nonspecific binding was performed with 3% low fat dried milk diluted 1:100 in PBS. Slides were incubated with the primary monoclonal antibody for HLA-G (5A6G7, diluted 1:50; EXBIO, Prague, Czech Republic) and for HLA-E (MEM-E/02, diluted 1:50; EXBIO, Prague, Czech Republic) in a humidified chamber at 4°C overnight, and then incubated with the streptavidin-peroxidase complex at 37°C for 30 min. Sections were incubated in a solution containing 5 mg of diamino-benzidine (GIBBICO, Gaithersburg, Maryland, USA) dissolved in 5 ml of PBS, and 100 μL of a fresh peroxidase solution (450 μL of PBS and 50 μL of hydrogen peroxide) for 10 min, lightly counterstained

with Carrazzi's hematoxylin without acid for 60 sec, exhaustively rewashed with tap water, air dried, and mounted with Permount mounting medium (MERCK; Darmstadt, Germany).

All scoring and qualitative interpretation of the immunohistochemical results was carried out by an experienced pathologist and the results were classified as negative, discrete, moderate or intense immunolabeling. In the present study, we considered HLA-G and HLA-E expression to be of low intensity when laryngeal biopsies showed discrete immunostaining, and to be of high intensity when the biopsies showed moderate or intense immunolabeling.

To validate the anti-HLA-G and anti-HLA-E mAb and the immunohistochemical method, we systematically analyzed a paraffin-embedded section of trophoblastic tissue from a third-trimester human placenta (positive control). The secondary antibody control consisted of histological sections of the same human trophoblast, in which both primary antibodies were replaced with PBS. Basal HLA-G and HLA-E expression was evaluated in normal larynx biopsies obtained at autopsy from 10 previously healthy individuals who had died from violent trauma.

Image acquisition and quantification of HLA- G and HLA-E by image analysis

Positive cytoplasms were automatically quantified by a computer-assisted system (Image-Pro Plus Cybernetics, MD, USA). A mean of ten random microscope fields were selected in order to analyze 1,000 cytoplasm fields per biopsy in all patient sections. The image acquisition of all biopsies was performed on an electron photomicrograph and the image was then processed and analyzed by the software. For each slide, the digitized image segmentation was interactively controlled by the red-green-blue color filter of the software. The automatic cytoplasm count was determined and expressed in percentage, and was considered as negative expression when HLA-G and HLA-E expression was below 20% immunostaining cells in laryngeal specimens immunolabeling.

HPV detection and typing

Genomic DNA was obtained from 2 sections of paraffin blocks (10 μm each) according to the protocol proposed by Bettini et al. (2003) with minor modifications. For generic HPV typing, DNA was amplified using the GP5+ and GP6+ primers (Ting and Manos, 1990), which amplify a shared 150 bp DNA fragment. Amplification was performed together with a set of primers for a beta globin (PCO3 and PCO4) gene as an internal control. The generic HPV-positive samples were amplified with specific primers for HPV16, HPV18, HPV31, HPV33, HPV6 and HPV11 types as described by Walboomers et al. (1999) (Table 1). The amplification procedure for HPV detection and typing was carried out using a final volume of 25 μl. The reaction mixture contained 20 ηg of genomic DNA, 0.20 mM deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 0.6 μM of each primer (IDT, IA, USA), 1.25 U *Taq* polymerase (Invitrogen, Brazil), 3 mM $MgCl_2$, and 1x buffer (Invitrogen, Brazil).

The HPV positive control and beta globin control were cervical samples collected by cytologic cytobrushing. As a negative control, all PCR reagents were added to an Eppendorf tube containing no DNA.

Statistical analysis

Age, reported as mean ± SEM, was compared between groups by one-way analysis of variance (ANOVA) followed by the Tukey post-test. The distribution of epidemiologic variables (gender, skin color, tobacco and alcohol use) was compared between the different groups of laryngeal lesions (BL, PL, $LSCC^S$, $LSCC^{WT}$ and $LSCC^W$) using the two-sided Fisher exact test for 2x2 contingency tables, which was also used to estimate the odds ratio (OR) and its 95% confidence interval (95% CI). Data regarding the anatomic site of the tumor were analyzed by the Chi-square test.

Comparisons of the expression of HLA-G and HLA E between groups stratified according to clinical variables (TNM classification, histological grade, tumor treatment and recurrence) were evaluated by means of Fisher's exact test (in case of 2x2 tables) using the GraphPad InStat software or an exact test that uses the Metropolis algorithm to obtain an unbiased estimate of the exact p value and its standard error (in more-complex RxC tables) using the RXC software (http://www.marksgeneticsoftware.net/rxc.htm). The TNM system is the most widely used means for classifying the extent of cancer spread (UICC, 1997).

The intensity distribution between different subgroups of laryngeal lesions (BL, PL, $LSCC^S$, $LSCC^{WT}$, $LSCC^W$ and LN) was compared by the Chi-square test. Since the HLA-G and HLA-E quantitative expression data did not reach the normality assumption, non-parametric analyses were performed. The Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison post-test was applied to compare the median expression levels of both HLA-G and HLA-E molecules among subgroups of laryngeal lesions. Comparisons of the median expression levels of HLA-G and HLA-E molecules observed in invasive carcinomas with metastasis ($LSCC^W$) and respective draining cervical lymph nodes (LN) were performed using the Wilcoxon matched-pairs signed-ranks test. The correlation between $LSCC^W$ and LN was estimated using the Spearman rank correlation test.

The association between the positivity for HPV and the expression of HLA-G and HLA-E molecules was determined by the two-sided Fisher exact test for 2x2 contingency tables.

Two sided P-values were considered to be

significant at P<0.05. All data were analyzed using the GraphPad Instat software (San Diego, CA, USA).

Results

Demographic and clinical variables

Overall, patients of this series were predominantly older males (>40 years) who used tobacco, exhibiting laryngeal lesions predominantly located in the supraglottic region. According to clinical and epidemiological variables, the presence of laryngeal lesions (BL, PL, $LSCC^{S}$, $LSCC^{WT}$, and $LSCC^{W}$) was significantly associated with age, alcohol and tobacco consumption, particularly for patients exhibiting laryngeal carcinomas. No significant differences were observed regarding gender, skin color or anatomic site of the lesion, i. e., glottis, supraglottis or subglottis (Table 2).

HLA-G and HLA-E expression in biopsy specimens from laryngeal lesions

Based on the tolerogenic functions of HLA-G and HLA-E, we investigated possible associations between the qualitative and quantitative expression of HLA-G and HLA-E with demographic and clinical variables. To qualitative and quantitative HLA-G and HLA-E expression, no significant associations were observed regarding gender, skin color, tobacco and alcohol use, and anatomic site of the lesion, histological grade, TNM stage, tumor treatment and recurrence. In the qualitative analyze, HLA-G expression was negatively associated with age (P<0.0414), indicating that low levels of HLA-G was observed in patients over 40 years old. Considered as a whole, HLA-G molecules were detected in 45.3% (62/137) of specimen biopsies, being observed in 74% (20/27) of BL, 76.5% (13/17) of PL, 60% (6/10) of LSCCS, 18.5% (5/27) of LSCCWT, 32.1% (9/28) of LSCCW, and 32.1% (9/28) of LN (P<0.0001). We observed an association (P<0.0001) between the low intensity of HLA-G and the high grade lesions (laryngeal carcinomas). Overall, HLA-E molecules were detected in 62.04% (85/137) of biopsies, 40.74% (11/27) of BL, 29.41% (5/17) of PL, 40% (4/10) of LSCCS, 70.37% (19/27) of LSCCWT, 85.71% (24/28) of LSCCW, 78.57% (22/28) of LN (Table 3). A positive association between the qualitative expression of HLA-E and the lesion grade was also observed (P<0.0002), indicating that high intensity of HLA-E expression was associated

Table 1. Oligonucleotide sequences of HPV E7 type-specific PCRs (Walboomers et al., 1999).

Primer	Sequence (5'-3')	Probe	Sequence (5'-3')
HPV16E7.667 HPV16E7.774	gatgaaatagatggtccage gctttgtacgcacaaccgaage	PROHPV16E7	cggacagagcccattacaatattgtaacct
HPV18E7.696 HPV18E7.799	aagaaaacgatgaaaatagatgga ggcttcacacttacaacaca	PROHPV18E7	cccgacgagccgaaccacaacgtcacacaa
HPV31E7.811 HPV31E7.890	gggctcatttggaatcgtgtg aaccattgcatcccgtcccc	PROHPV31E7	tacctgctggatcagccattgtagttacag
HPV33E7.671 HPV33E7.761	tgaggatgaaggcttggacc tgacacataaacgaactgtg	PROHPV33E7	tgtgacaacaggttacaatgtagtaatcag
HPV35E7.674 HPV35E7.752	ctattgacggtccagct tacacacagacgtagtgtcg	PROHPV35E7	caacaggacttacaatattataattggag
HPV39E7.601 HPV39E7.723	ccaaagcccaccttgcagga atggtcgggttcatctatttc	PROHPV39E7	tcctaattgctcgtgacatacaaggtcaac
HPV45E7.741 HPV45E7.822	cccacgagccgaaccacag tctaaggtcctctgccgagc	PROHPV45E7	agctcaattctgccgtcacacttacaacat
HPV51E7.718 HPV51E7.841	tacgtgttacagaattgaag aaccaggcttagttagttcgcccatt	PROHPV51E7	tcaagtgtagtacaactggcagtggaaagc
HPV52E7.691 HPV52E7.776	gcagaacaagccacaagcaa tagagtacgaaggtccgtcg	PROHPV52E7	atagccgtagtagtgtgctatcacaactgtgac
HPV56E7.784 HPV56E7.886	ggtgcagttggacattcagag gttacttgatgcgcagagtg	PROHPV56E7	caaagaggacctgcgtgttgtacaacagct
HPV58E7.98 HPV58E7.761	cgaggatgaaataggcttgg acacaaacgaaccgtggtgc	PROHPV58E7	tgttgttcaatgttacatcattaatcgaca
HPV59E7.646 HPV59E7.749	ctccgagaatgaaaaagatgaa gctgaagttgattattaca	PROHPV59E7	gtcgagcagatcgatcatcgtttcctacta
HPV66E7.641 HPV66E7.742	aattgcaatgagcaattggacag cttatgttgttcagcttgtc	PROHPV66E7	aggatgaaatagaccatttgctggagcggc
HPV68E7.4604 HPV68E7.4704	aacaacagcgtcacacaattca agttgtacgttccgcaggt	PROHPV68E7	agtgtaacaacctactgcaactagtagtag

with high grade of lesions (invasive carcinoma independent of the metastasis in cervical lymphonodes).

Apparently, the reduced number of patients in premalignant group (n=17) and *in situ* carcinoma group (n=10) is the consequence of late diagnosis. Thus, the early diagnosis of laryngeal cancer is rare and the invasive lesions without or with metastasis is more commonly found, in our hospital service.

Figure 1 shows the quantitative expression of HLA-G and HLA-E, stratified according to the lesion grade. HLA-G and HLA-E expression was primarily detected in the cytoplasm of squamous epithelial cells.

Normal laryngeal biopsies showed low intensity and diffuse HLA-G expression in all specimens studied, which was present in 100% of the laryngeal cells (Fig. 2A). In contrast to normal tissue, laryngeal lesions exhibited a different pattern of HLA-G distribution, irrespective of the lesion grade. Benign lesions exhibited low HLA-G expression concentrated all over the lesion (Fig. 2B). Premalignant lesions exhibited patchy and low expression of HLA-G (Fig. 2C). Malignant lesions (such as LSCCS, LSCCWT and LSCCW) and draining cervical lymph nodes exhibited high intensity of HLA-G

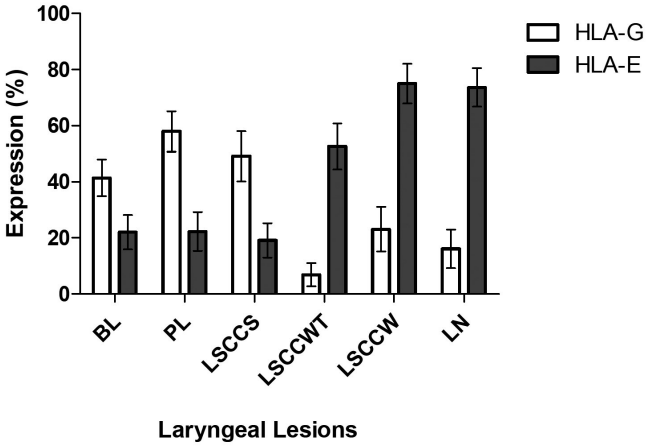

Fig. 1. Quantitative expression of HLA-G and HLA-E. HLA-G expression: Analysis of variance (Kruskal-Wallis test with Dunn's post test)- P values: P<0.0004; BL *versus* LSCC^{WT} (P<0.05); PL *versus* LSCC^{W} (P<0.05). HLA-E expression: Analysis of variance (Kruskal-Wallis followed by Dunn's post test)- P values: P<0.0001; BL *versus* LSCC^{W} (P<0.05); BL *versus* LN (P<0.05); PL *versus* LSCC^{W} (P<0.05); PL *versus* LN (P<0.05); LSCC^{S} *versus* LSCC^{W} (P<0.05); LSCC^{S} *versus* LSCC^{W} (P<0.05).

Table 2. Demographic and clinical variables of the 109 patients with laryngeal lesions stratified according to age, gender, tobacco, alcohol consumption, and anatomic site.

	Age[a,c]	Gender		Tobacco[b,d]		Alcohol[c]		Skin color		Anatomic site		
Lesions	M ± SEM	Male	Female	Yes	No	Yes	No	White	Non-white	Glottis	Supraglottis	Subglottis
BL (n=27)	28.0±3.26	19	8	3	24	4	23	25	2	11	16	0
PL (n=17)	69.4±2.04	14	3	16	1	13	4	14	3	5	12	0
LSCC^{S} (n=10)	59.2±2.56	6	4	6	4	3	7	9	1	4	6	0
LSCC^{WT} (n=27)	59.18±1.47	18	9	25	2	17	10	24	3	6	20	1
LSCC^{W} (n=28)	56.07±1.78	23	5	28	0	20	8	22	6	9	18	1

[a]: Data are reported as mean (M) and standard error of the mean (SEM); [b]: Fisher exact test; OR: Odds ratio; BL: benign lesion; PL: premalignant lesion; LSCC^{S}: in situ laryngeal carcinoma; LSCC^{WT}: invasive laryngeal carcinoma without metastasis; LSCC^{W}: invasive laryngeal carcinoma with metastasis. P-value: Age[c] (BL vs PL: P<0.001; BL vs LSCC^{S}: P<0.001; BL vs LSCC^{WT}: P<0.001; BL vs LSCC^{W}: P<0.001; PL vs LSCC^{WT}: P<0.05; PL vs LSCC^{W}: P<0.001). Tobacco[d] consumption (BL vs PL: P=0.001 OR=0.0078 (0.0007-0.0819); BL vs LSCC^{S}: P=0.0053 OR=0.8333 (0.1456-0.4768); BL vs LSCC^{WT}: P=0.0001 OR=0.0100 (0.0015-0.0652); BL vs LSCC^{W}: P=0.0001; PL vs LSCC^{S} P=0.0437 OR=10.667 (0.9830-115.75); LSCC^{S} vs LSCC^{WT} P=0.0347 OR=0.1200 (0.0176-0.8165) LSCC^{S} vs LSCC^{W} P=0.0028 OR=0.0253 (0.0012-0.5320)). Alcohol[c] (BL vs PL: P=0.0001 OR=0.5351 (0.1142-0.2507); BL vs LSCC^{WT}: P=0.0006 OR=0.1023 (0.0273-0.3824); BL vs LSCC^{W}: P=0.0001 OR=0.6957 (0.1818-0.2662); PL vs LSCC^{S}: P=0.0402 OR=7.583 (1.309-43.941); LSCC^{S} vs LSCC^{W} P=0.0302 OR=0.1714 (0.3524-0.8340)).

Table 3. Qualitative expression of HLA-G and HLA-E in the lesions of the larynx stratified according to histological diagnosis.

Lesions	HLA-G		HLA-E	
	Low Intensity[a] n (%)	High Intensity[a] n (%)	Low Intensity[a] n (%)	High Intensity[a] n (%)
BL n=27	20 (74)	0	8 (29.63)	3 (11.11)
PL n=17	13 (76.5)	0	4 (23.52)	1 (5.88)
LSCC^{S} n=10	5 (50)	1 (10)	4 (40)	0
LSCC^{WT} n=27	2 (7.40)	3 (11.11)	10 (37.03)	9 (33.33)
LSCC^{W} n=28	5 (17.85)	4 (14.28)	11 (39.28)	13 (46.43)
LN n=28	3 (10.71)	6 (21.43)	16 (57.14)	6 (21.43)

BL: benign lesion; PL: premalignant lesion; LSCC^{S}: in situ laryngeal carcinoma; LSCC^{WT}: invasive laryngeal carcinoma without metastasis; LSCC^{W}: invasive laryngeal carcinoma with metastasis; Cervical lymph nodes: LN. Chi-square test: HLA-G (P<0.0001); HLA-E (P<0.0002).

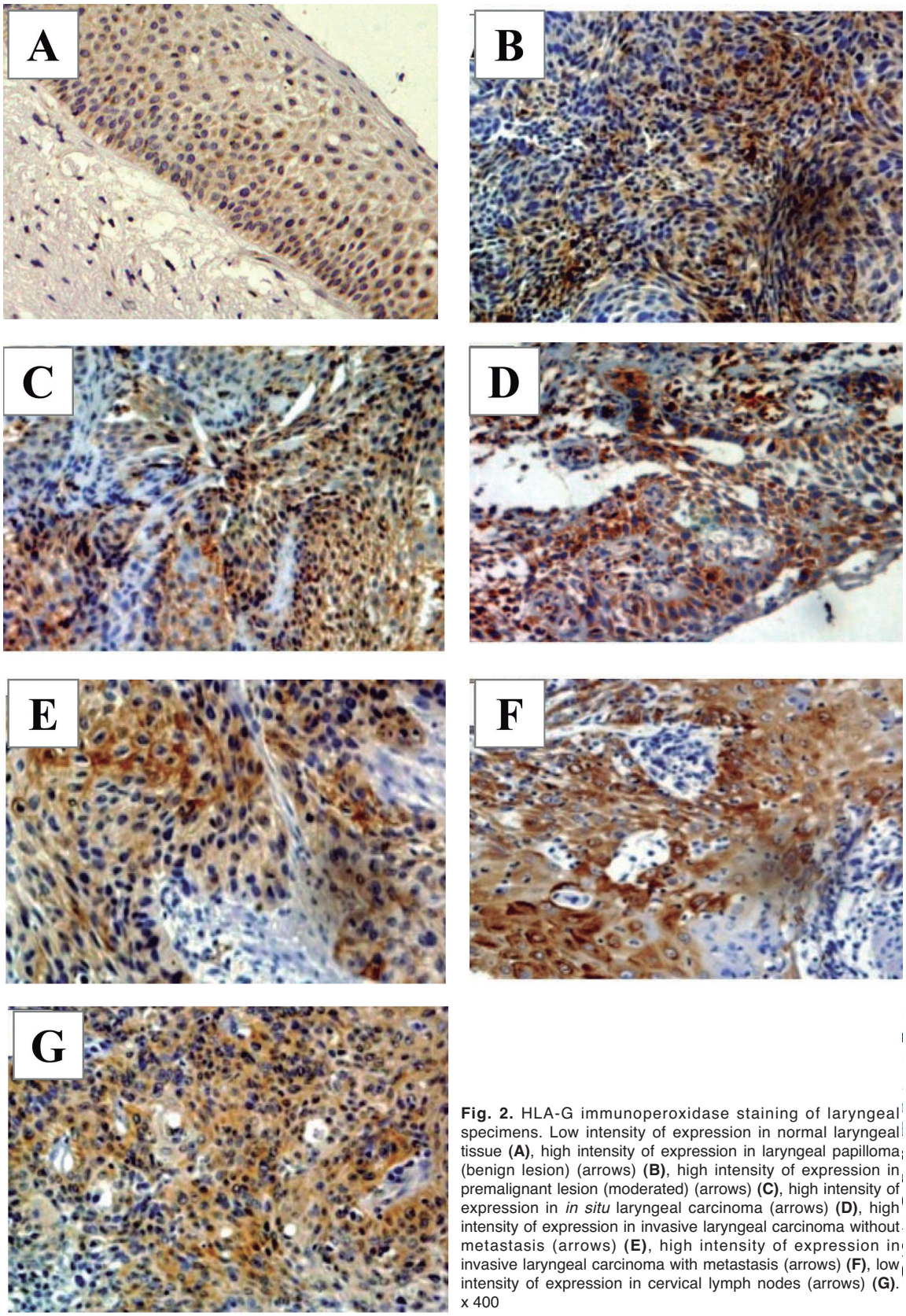

Fig. 2. HLA-G immunoperoxidase staining of laryngeal specimens. Low intensity of expression in normal laryngeal tissue **(A)**, high intensity of expression in laryngeal papilloma (benign lesion) (arrows) **(B)**, high intensity of expression in premalignant lesion (moderated) (arrows) **(C)**, high intensity of expression in *in situ* laryngeal carcinoma (arrows) **(D)**, high intensity of expression in invasive laryngeal carcinoma without metastasis (arrows) **(E)**, high intensity of expression in invasive laryngeal carcinoma with metastasis (arrows) **(F)**, low intensity of expression in cervical lymph nodes (arrows) **(G)**. x 400

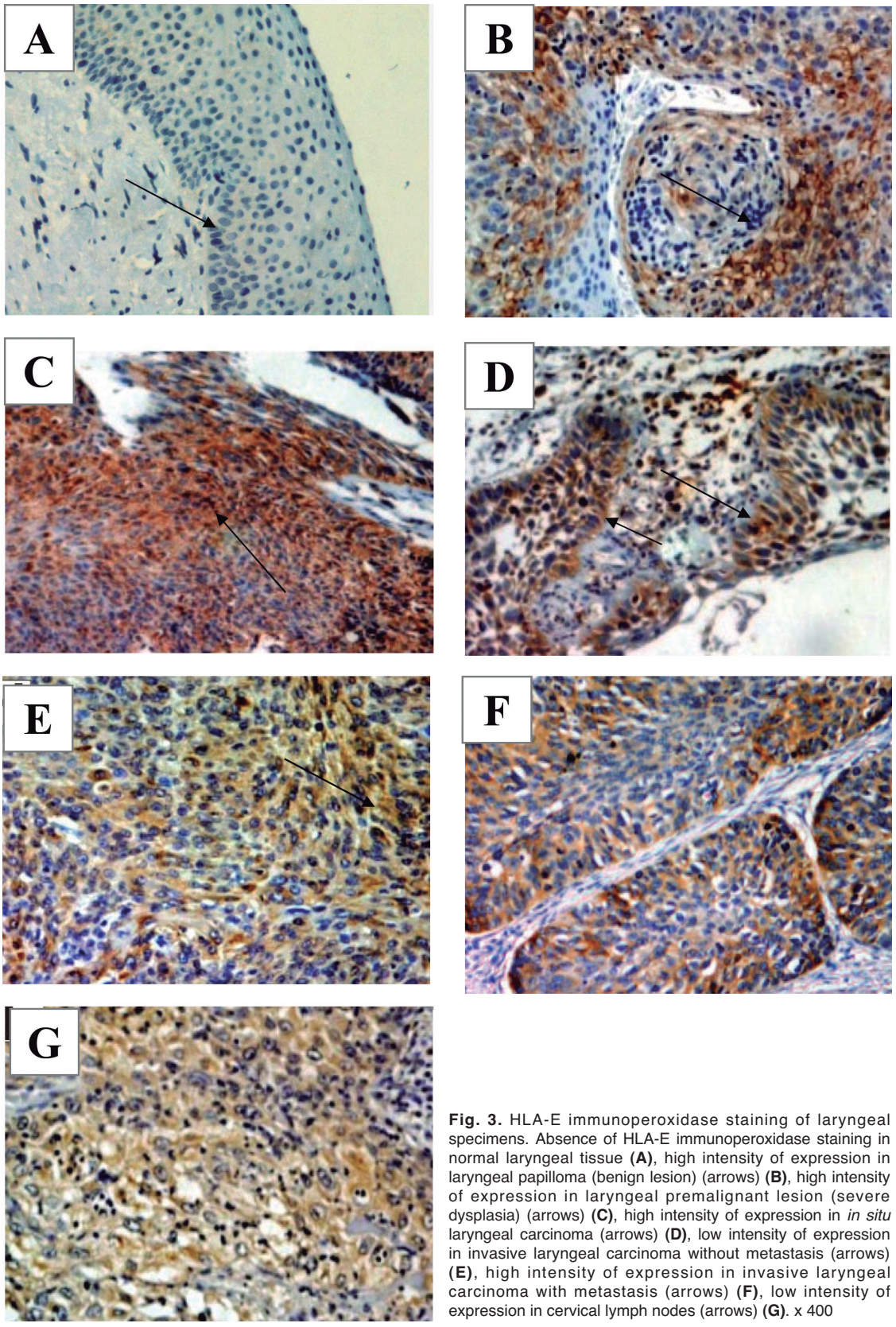

Fig. 3. HLA-E immunoperoxidase staining of laryngeal specimens. Absence of HLA-E immunoperoxidase staining in normal laryngeal tissue **(A)**, high intensity of expression in laryngeal papilloma (benign lesion) (arrows) **(B)**, high intensity of expression in laryngeal premalignant lesion (severe dysplasia) (arrows) **(C)**, high intensity of expression in *in situ* laryngeal carcinoma (arrows) **(D)**, low intensity of expression in invasive laryngeal carcinoma without metastasis (arrows) **(E)**, high intensity of expression in invasive laryngeal carcinoma with metastasis (arrows) **(F)**, low intensity of expression in cervical lymph nodes (arrows) **(G)**. x 400

in two different patterns; patchy and diffuse (Fig. 2C-F respectively). In contrast to HLA-G, no expression of the HLA-E molecule was observed in normal laryngeal tissue (Fig. 3A). Benign and premalignant lesions exhibited mainly low and diffuse HLA-E expression (Fig. 3B,C, respectively). Malignant lesions and draining cervical lymph nodes presented intense and diffuse HLA-E expression (Fig. 3C,F, respectively).

Overall, qualitative and quantitative analyses of nonclassical HLA molecules showed different patterns of distribution, according to the laryngeal lesion grade. In terms of qualitative analysis HLA-G expression increased in benign and premalignant lesions, and gradually decreased in invasive carcinomas and draining cervical lymph nodes. Conversely, HLA-E over-expression was observed in invasive cancer independent of the presence of metastasis and in the draining lymph nodes (Figs. 2 and 3).

Detection and typing of human papillomavirus

Among 109 patients studied, 12 did not present amplification for viral and internal control primers, and in 17 (15.6%) HPV was detected. Among HPV+ patients, 11 (64.7%) patients primarily presented benign lesions, harboring low risk HPV types. Twelve patients (70.6%) harbored high risk HPV types, associated or not with low risk types. High risk types were observed in association with both benign and malignant lesions. Because of the low frequency of HPV in patients, particularly in the PL group, it was not possible to perform associations between HPV type and the magnitude of HLA-G and HLA-E staining in all lesions. Notwithstanding, the presence of HPV was statistically associated with the expression of HLA-G molecule ($P<0.0335$) in benign lesions, particularly in laryngeal papillomas harboring low risk HPV types.

Discussion

Tumors can induce the generation and accumulation of immunosuppressive molecules such as nonclassical class I antigens in tumor microenvironment, contributing to tumor escape from immunological response. The expression of nonclassical HLA class I molecules by malignant cells can evade immune system control by inhibiting natural killer (NK) cells, T-lymphocytes and antigen-presenting cells (Carosella et al., 2008). HLA-G may directly interact with killer immunoglobulin-like receptors (KIRs) in NK cells, and may also provide the signal peptide that stabilizes the HLA-E molecule on cell surface, which in turn interacts with the inhibitory receptors CD94/NKG2A on NK cells (Braud et al., 1998).

In the present study, HLA-G molecule expression was constitutively present in normal laryngeal specimens, exhibiting a weak and diffuse staining. In contrast, HLA-G expression in benign, pre-malignant and malignant lesions, together with the respective draining cervical lymph nodes, exhibited a different expression pattern and different intensity. Conversely, HLA-E was not constitutively expressed in normal larynx, and the expression increased as far the severity of the lesion increased. It is interesting to observe that in pre-malignant and malignant uterine cervical lesions harboring HPV infection, HLA-G expression was observed in atypical glandular cells of undetermined significance (AGUS) and in cervical intraepithelial neoplasia (CIN-1, CIN-2, and CIN3), whereas normal cervix did not constitutively express HLA-G (Gonçalves et al., 2008). Furthermore, HLA-G expression was also observed in uterine cervical cancer and draining lymph nodes with metastasis, but not in lymph nodes without metastasis (Guimarães et al., 2009). It is worth mentioning that HLA-G expression gradually increased from pre-malignant to malignant lesions; however, no more than 20% of lesions were stained even in the more severe lesions (Gonçalves et al., 2008; Guimarães et al., 2009). In contrast, expression of HLA-E gradually increased from CIN-1 to invasive cancer, particularly in specimens harboring HPV16/18 infection (Gonçalves et al., 2008). Although we have not performed classical HLA class I and class II molecules, literature studies report the presence of classical HLA-A,-B,-C and HLA-DR, -DQ, -DP molecules in normal larynx and decreased expression of HLA-A,-B-C molecules in laryngeal malignant lesions (Esteban et al., 1990, 1996; Rees et al., 2003; Hobbs et al., 2006).

To date, no report has focused on the study of HLA-G and HLA-E expression in laryngeal biopsies stratified according to the lesion grade. Both molecules have been implicated on the modulation of cells of the innate and adaptive immune response; however, the role of the HLA-G molecule has attracted more attention than HLA-E molecules. HLA-G interacts with leukocyte immunoglobulin like receptors type 1 and 2 (LILR1 and LILRB2), with more avidity than the interaction with classical HLA class I molecules (Shiroishi et al., 2003; Gonen-Gross et al., 2005), inhibiting T and NK cells (Contini et al., 2003). In addition, HLA-G may be transferred from cancer cells to immune system cells by trogocytosis, further inhibiting the cytotoxic function of these cells (Joly and Hudrisier 2003; Caumartin et al., 2007). Although HLA-G expression was not expressed in all cells of benign, pre-malignant and malignant lesions, the patchy and intense HLA-G staining in these lesions suggests that the molecule is modulating the immune response effects. On the other hand, the increasing expression of HLA-E as far lesions progressed from benign to malignant ones is also indicative of NK cells inhibition, since HLA-E also inhibits NK cell cytotoxic action. Taken together, the neoexpression of HLA-E and altered expression of HLA-G in laryngeal lesions may act in concert to subvert immune system cells, impairing immune surveillance against these lesions, permitting lesion progression.

Besides upregulating nonclassical HLA molecules as

described by cytomegalovirus, rabies, HIV, hepatitis B viruses and Epstein-Barr viruses (Onno et al., 2000; Lozano et al., 2002; Lafon et al., 2005; LeMaoult et al., 2005; Gazit et al., 2007), certain viruses may also down-regulate classical class I HLA molecules further inhibiting the function of T cytotoxic cells (Kanodia et al., 2007; Madeleine et al., 2008). Although there is no study on the role of HPV proteins on HLA classical and nonclassical molecules, HLA-A-B-C molecules are reported to be gradually decreased from pre-malignant to malignant cervical lesions, particularly in patients harboring the HPV-16 and HLV-18 types (Gonçalves et al., 2008). The role of HPV infection in laryngeal lesions has not been completely elucidated, being well-defined in recurrent papillomatosis of the larynx and contributing to malignant transformation (Muenscher et al., 2008); however, in other lesions, the presence of HPV has greatly varied. A systematic review of the literature showed a significantly higher prevalence of HPV in squamous cell carcinoma of the oropharynx than the oral cavity or larynx (Kreimer et al., 2005). Other studies have shown that the presence of HPV in the larynx is variable, ranging from 0% (Gorgoulis et al., 1999) to 19% (Rihkanen et al., 1994). In the present study, HPV was observed in approximately 15% of the laryngeal lesions, irrespective of the lesion grade. Moreover, no association between HPV infection (high-risk HPV types and low-risk types) and the expression of HLA-G and HLA-E was observed. Since the percentage of HPV detection in laryngeal lesions has greatly varied among the reported studies, and considering that the frequency is quite low (0-19% of the lesions), larger studies are necessary to evaluate the role of HPV on laryngeal lesion development and nonclassical molecule expression: HLA-G and HLA-E.

Few studies have been addressed with HLA-G protein levels and demographics parameters. Previous study demonstrated no association between to age and HLA-G 14-bp -/- polymorphism in the asthmatic patients and normal controls (Xiao-Qun Zheng et al., 2010). In esophageal squamous cell carcinoma and gastric carcinoma, a positive association was demonstrated between HLA-G immunolabelling and histologic grade, nodal status and advanced disease stage. In addition, these studies have found a positive correlation between HLA-G and tumor invasion. (Yie et al., 2007a,b) In both tumor type Yie et al. (2007a) and Yie et al. (2007b) have not verified any significant association between HLA-G levels, age and gender (Yie et al., 2007a,b). In contrast, our findings demonstrated a significant association between low levels of HLA-G, in patients with more than 40 years old and high grade laryngeal tumor. To HLA-E expression, the present study demonstrated association between high intensity of HLA-E expression and laryngeal high grade of lesions However, at our knowledge, no study was found, comparing HLA-E levels, clinical variables and tumor grade.

Concluding, in the present study we observed distinct patterns of HLA-G expression and HLA-E. Interestingly, HLA-E molecule overexpression was associated with the presence of carcinoma and invasive lesions, indicating that HLA-E could be a good biomarker for laryngeal cancer and mucosal invasion. On the other hand, HLA-G high expression was associated with benign and premalignant lesions of the larynx, decreasing the expression in malignant lesions. Therefore, the absence or diminished expression of HLA-G may be a biological marker for malignant lesions.

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