Human leucocyte antigen – G gene polymorphism in laryngeal squamous cell carcinoma patients in Mansoura University Hospitals

Ghada Barakat¹, Asser Elsharkawy² and Yasmin NAbiel³

¹Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt; ²Otorhinolaryngology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

ABSTRACT
This study aimed to detect the genetic association of HLA-G gene polymorphism with LSCC. It was a comparative study estimating the prevalence of both HLA-G 14-bp insertion versus deletion polymorphism and HLA-G*01:05 N null allele using PCR-RFLP. HLA-G 14-bp deletion allele was highly recorded in patients with LSCC (63.0%) whereas insertion allele (37.0%) was lower. The −14/-14 and −14/+14 genotypes were significantly higher in LSCC cases whereas +14/+14 genotype was not. HLA-G*01:05 N null allele was found in 39 LSCC cases (43.8%) and 47 control (52.8%), (p = 0.23). Distribution of studied alleles and genotypes showed no significant correlation to the clinic-pathological features of included cases. There was a significant association between HLA-G 14-bp deletion allele (versus insertion allele) and the risk of LSCC but no clear association between null allele and occurrence of LSCC was detected.

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Introduction

According to the National Cancer Institute (NCI), laryngeal cancer comes first among respiratory cancers (27.8%) and the tenth among all total cancers (1.7%) in Egyptian patients [1]. The genetic basis and the potential genetic predisposition for the development of head and neck squamous cell carcinoma are not completely known and needed to be further studied. Epigenetic variations, mutations in somatic genes, divergent patterns of gene-expression in addition to tumor-infiltrating lymphocytes are widely investigated as diagnostic, prognostic, and predictive biomarkers [2,3]. There are a lot of carcinogenic risk factors such as tobacco, alcohol consumption, or infection [4]. These carcinogenic risks are modified by genetic polymorphisms [5,6].

Several pathological states such as autoimmune disorders, transplantation, infections especially viral, and malignancies have been linked to aberrant human leukocyte antigen-G (HLA-G) expression [7]. Previous studies evolved the occurrence of upregulation of such aberrant expression in the tumor cells [8]. There are evidences that HLA-G is overexpressed in lung carcinoma [9], cutaneous melanoma [10], breast carcinoma [11], ovarian carcinoma [12], and many other malignancies [9]. HLA-G is one of the non-classical HLA class I family of genes that is found at chromosome 6p21 [13]. The HLA-G gene has eight exons. These exons code for the signal sequence, the extracellular, the trans-membrane, and the cytoplasmic domains [14].

In exon 8, a 14-bp insertion/deletion polymorphism occurring in the 3’ untranslated
region (UTR) of HLA-G was found to be in association with the stability and patterns of splicing of the mRNA isoforms of HLA-G [14]. Soluble HLA-G (sHLA-G) levels were higher in homozygous 14 bp deletion (DD genotype), which suggested possible clinical significance in the pathophysiology of many malignancies [15].

Alternative splicing of the primary transcripts of HLA-G gene causes the formation of 7 isoforms, which includes four membrane-bound ones (HLA-G1, -G2, -G3, and -G4) and other three soluble isoforms (HLA-G5, -G6, and -G7) [16].

The null allele termed HLA-G*0105 N is one of the fifteen HLA-G gene alleles. It represents a single base pair deletion in exon 3 [17] where the deletion of cytosine at codon 130 disrupts the open reading frame, and then inhibits the translation of HLA-G1 and G5. But it can translate HLA-G2, HLA-G3 (membrane-bound isoforms) and HLA-G6, HLA-G7 (soluble isoforms), such variable effects over HLA-G translation are found to be a hindering factor for pregnancy occurrence through affecting fertilization and implantation, and show some effect over tumor development [18,19].

The action of HLA-G is emphasized mainly through the inhibition of cytotoxic effect of natural killer and CD8+ cells, the down regulation of the immune response of T helper cells and the full maturation of dendritic cell besides activating regulatory T cells [7]. Immunosuppressive effect of the HLA-G occurs through interacting with inhibitory receptors over immune cells [20,21].

Soluble HLA-G interacts with a co-receptor to induce apoptosis of natural killer and T lymphocytes [22]. Thus, an immune tolerance state happens due to the increased expression of HLA-G. Although such tolerogenic properties are beneficial in pregnancy, transplantation, and inflammatory diseases, they have deleterious effects in cancer and viral infections [13]. Although beneficial in transplant, elevated HLA-G expression is found to be detrimental in cancer; cells utilize HLA-G as a potential escape mechanism favoring disease progression [23].

The polymorphisms of HLA-G were discovered to be common with multiple types of malignancies such as urinary bladder transitional cell carcinoma, hepatocellular carcinoma, cervical cancer, esophageal cancer, neuroblastoma of childhood and breast cancer [24–27]. The relation of the HLA-G polymorphism and the laryngeal squamous cell carcinoma (LSCC) has not been widely studied yet.

The undertaken study aimed to investigate the possible genetic linkage of HLA-G genes polymorphism with the laryngeal carcinoma in patients confirmed to have LSCC by pathological analysis at Otolaryngology Department in Mansoura University Hospital, Egypt.

**Materials and methods**

This comparative study was enrolled over 178 subjects that were admitted to the Department of Otolaryngology at the Mansoura University Hospital, Mansoura, Egypt. It was conducted during a time period of 24 months extending from January 2018 to January 2020. A complete medical history including the medications they received, clinical and radiological evaluation was conducted for all study participants.

Eighty-nine patients (case group) were proved to have LSCC. These patients were subjected to laryngoscopy for biopsy. All patients underwent total laryngectomy at the Otolaryngology Department at Mansoura University Hospital with further confirmation by pathological investigations. As regard the control group (89); it included 35 participants who were admitted for functional rhino-plasty besides 54 healthy volunteers. The rhino-plasty group was included in the study immediately before the procedure. All case and control group subjects were heavy tobacco smokers.

For staging of laryngeal carcinoma, the guidelines of The International Union against Cancer (UICC), 2002 were applied [28]. They included clinical, imaging, and endoscopic results. TNM staging was as applied by the American Joint Committee on Cancer staging.
system followed by tumor differentiation scoring using an ordinal scale of the hierarchy of tumor cell differentiation to be classified into well, moderately, poorly differentiated or sarcomatous lesion [29].

Inclusion criteria for studied patients were those diagnosed with laryngeal carcinoma and having complete medical records. They showed no other severe organ disease, received no treatment prior to being diagnosed with LSCC, and they received neither radiotherapy nor chemotherapy before being included in the current study.

Exclusion criteria included any history or evidence of associated allergic diseases, having positive skin test, any benign lesions, or infections especially HPV or EBV. The protocol of this current study had an approval from the faculty review board (R.18.10.303). An informative consent was recruited from study members.

**Samples collection and genotyping**

Five ml venous blood samples were obtained on EDTA under complete aseptic precautions from all study members. Blood samples were then kept at a temperature of −20°C to be targeted for the extraction of DNA and then genetic testing.

DNA isolation and genotyping of HLA-G gene variants: Whole Blood Genomic DNA Extraction Kit (QIAamp DNA Mini Kit only) (QIAGEN Hilden, Germany) was used to extract DNA followed by genotyping steps.

Aiming for genotyping of HLA-G 14-bp insertion/deletion polymorphism in its 3’ UTR (rs66554220), the following set of primers was used: the base sequence of the forward one was: 5’ GTGATGGGCTGTATAAGTGTCCAC −3’ and that of the reverse was 5’ GGAAGGATGCAGTTCCAGCATG −3’ in a reaction mixture of 25 µL total volume containing PCR Master Mix (12.5 µL), 0.2 µL of each primer (10 pmol), 5 µL of extracted DNA and 8.1 µL of sterilized clear nuclease – free water.

PCR conditions were carried out in Norwall, CT (USA) thermal cycler, where it started by incubation at 95°C for a time period of 3 min, then a total of 30 turns where denaturation occurred at 95°C for 1 min, followed by annealing at 64°C for 1 min, and the elongation step that occurred at 72°C for a duration of 1 min then final elongation which needed incubation at 72°C for 10 min. The products were then run in 3% agarose gel for 20 min to be demonstrated under UV-light in the presence of 1000 bp DNA ladder. PCR outcomes of 224 bp indicated the presence of 14 bp insertion variant whereas 210 bp fragments evidenced deletion variant. The appearance of two bands at both 224 bp and 210 bp indicated insertion/deletion genotypes [30].

The detection of HLA-G*01:05 N null allele was conducted by PCR-based restriction fragment length polymorphism assay (PCR-RFLP) using another set of primers which was; forward 5’ CAGTTTCTCACACCTCCAG −3’ and reverse 5’ CCTCCACTCCCTCAGAGACTTCATC −3’.

The reaction was also undertaken in volume of 25 µl with similar thermal cycling conditions except for annealing step that was at 63°C for 30 s to produce a DNA piece of 504 bp. Then, the products were digested using Fast Digest PpuM1 restriction enzyme (Thermo Scientific, EU, Lithuania) where an incubation at 37°C for 3 h was needed to yield either an intact PCR fragment indicating the presence of HLA-G*01:05 N allele or two fragments of 389 bp and 115bp showing the absence of studied allele [31] as the absence of the restriction site for used enzyme in the exon 3 of HLA-G indicated the existence of the studied null allele [32].

**Statistical analysis**

The resulting data were analyzed by SPSS version 23 (SPSS, Inc., Chicago, IL, USA). Obtained
quantitative parametric data were described as means and standard deviation; on the other hand, the qualitative results were shown in the form of frequency (number-percent). Quantitative parametric data were compared by Student’s t-test. For comparing qualitative data, we used Pearson’s chi-square ‘χ2’ or Fisher’s exact tests for (2-by-2 tables) and monte-carlo for more than (2-by-2 tables) as required.

Frequencies of genotypes were evaluated using gene counts. The goodness of fit between the observed and expected frequencies of genotype was tested ('χ2' test). On fitting to Hardy-Weinberg equilibrium, ‘χ2’ test (2-by-2 tables) was carried out to calculate the significant distribution of genotype among studied patients and controls. Also risk ratio was detected on calculating odd’s ratio (OR) and confidence interval 95%. P < .05 was significant.

Results

The current study was undertaken over a period of 24 months extending from January 2018 to January 2020 including 178 participants who were admitted to Otolaryngology Department at the Mansoura University Hospital. Eighty-nine male patients (case group) proved to have LSCC had the mean age of 53.5 ± 7.7 years, and their BMI had a mean of 30 ± 2.3, besides 89 male cancer-free subjects who were included as a control group, the mean of their ages was 49 ± 8.5 and their BMI was 28 ± 2.4 (Table 1). There was no significant difference between both groups (P-values were 0.76 and 0.81, respectively).

Cases were further classified according to clinical TNM staging followed by tumor differentiation scoring; T1: 2 cases (2.2%), T2: 4 (4.5%), T3: 62 (69.7%) and T4: 21 cases (23.6%). According to degree of lymph nodes involvement; 28 cases (31.5%) were graded as N0, 8 (9%) as N1, 30 (33.7%) as N2 and 23 (25.8%) as N3. Twenty three cases (25.8%) had been pathologically identified with well-differentiated tumor, 47 (52.8%) had moderately differentiated tumor, 16 (18%) suffered from poorly differentiated tumor and only 3 patients (3.4%) had sarcomatoid lesions. According to the position of the tumor, supraglottic and transglottic tumors were detected in 35 cases each, whereas the remaining 19 studied cases had glottic tumors (Table 1).

The resulting frequencies of genotypes of HLA-G 14-bp insertion/deletion polymorphism in the 3’ UTR (rs66554220) in healthy group were all on line with Hardy–Weinberg equilibrium. PCR analysis showed that the deletion of allele was higher in patients diagnosed with LSCC (63.0%) whereas insertion of allele was lower (37.0%). The results were statistically significant as compared to control group (p = <0.001*, OR = 2.74 & 95% CI = 1.78– 4.21) showing a significant association between HLA-G 14-bp deletion allele (versus the insertion allele) and the risk of LSCC (Table 2).

The −14/-14 and −14/+14 genotypes were significantly more in LSCC patients (34.8% & 56.2%) whereas the +14/+14 genotype (9.0%) was not (p values for both −14/-14 and −14/+14 genotypes were < 0.001*), the calculated ORs were 8.66 & 6.98, 95% CI = 3.3–22.7 & 2.9–16.8, respectively, these recorded results showed that −14/-14 and −14/+14 genotypes had higher association with LSCC with a risk that increased 8.66 & 6.98 folds, respectively. (−14/-14) + (−14/+14) distribution also gave significant results assuring the previously detected association (p <0.001*, OR =7.5 & 95% CI = 3.3–17.5 (Table 2).

On studying HLA-G*01:05 N null allele distribution in study participants; it was present in 39 LSCC cases (43.8%) and absent in the other 50 members (56.2%). Control group showed this null allele in 47 members (52.8%) and was absent in 42 (47.2%). The results were not statically significant showing no clear association between HLA-G*01:05 N null allele and the occurrence of LSCC (p = 0.23, OR = 1.4 & 95% CI = 0.79–2.58) (Table 2).
On studying the relationship between different studied alleles and genotypes & the clinic-pathological features of included cases, no statistically significant association was recorded except for the lymph node staging with regard to HLA-G*01:05 N null allele when comparing its distribution between patients categorized as N0 and those with lymph node metastasis (p = 0.004*) (Table 3).

**Discussion**

This study investigated the association between HLA-G genes polymorphism and LSCC. HLA-G binds to many receptors on the immune cells like T, B, natural killer, and antigen presenting cells [26]. HLA-G suppresses the natural killer cells and the cytotoxic T cells [33]. It also stimulates apoptosis [34]. Thus, it may facilitate tumor escape from the immune-surveillance [35]. Expression of HLA-G protein in multiple types of malignancies, as detected by immunohistochemistry in colorectal carcinoma, breast carcinoma, ovarian carcinoma, and others may suggest its role in tumor growth and progression [36].

HLA-G 14bp deletion allele may act as one of the risk factors for laryngeal carcinoma as there was a statistical significance for our results when compared to the control group (p = <0.001*, OR = 2.74 & 95% CI =1.78–4.21). The study results are in concordance with some researches that had dealt with different types of cancers like hepatocellular carcinoma [34] and renal cell carcinoma [37]. In an Egyptian study that tested the association between Non-Hodgkin lymphoma and the HLA-G 14bp gene polymorphism, its results suggested that HLA-G 14bp ins/del gene polymorphism was a risk factor for such malignancy in the Egyptian population and was associated with poor clinical pathological features [38].

The data pointed out that −14/-14 and −14/+14 genotypes had higher risk association with LSCC by 8.66 & 6.98 folds, respectively. In line with some previous studies, the risk of the esophageal carcinoma increases more in 14-bp del/del individuals than 14-bp ins/ins individuals by 2.69-fold [21,24,39].

The results analysis of HLA-G*01:05 N null allele revealed no statistically significant presence whether in the LSCC nor the control groups (p = 0.23).

In order to precisely view the differences in the frequencies of HLA-G*01:05 N allele throughout different world populations, a comparison of habitats and other population circumstances, like nutritional conditions now and in the past is mandatory.

On splicing the transcripts with 14bp sequence by removing 92 bases from around the sequence region, mRNA stability is greatly affected. This was evidenced, as such transcripts were having better stability than the completed ones [34]. Linkage disequilibrium parameters of HLA-G alleles and existence or nonexistence of the 14bp sequence was compared between the case group of patients and the control group.

No statically significant association was concluded between different studied alleles and genotypes & the clinic-pathological features of included cases except only for the lymph node staging with regard to HLA-G*01:05 N null allele. Statically significance was present when comparing its distribution between patients categorized as N0 and those with lymph node metastasis (p = 0.004*).

This research suggests that HLA-G alleles may participate in LSCC pathophysiology. The −14/-14 and −14/+14 alleles may have an effect on the biological function of the expressed HLA-G protein. Thus, their presence may act as a genetic risk factor that may predispose to LSCC pathogenesis. Murdaca and his coworkers illustrated that HLA-G expression is associated with poor survival in stage III gastric cancer patients and so it can represent a possible immune
escape mechanism of cancer cells [40]. Further studies with larger population are recommended in this locality or may be with other ethnic groups. HLA-G alleles and the 14bp sequence may be proven to be predictive markers for the LSCCs.

To the best of our knowledge, this current study is the first to link between the HLA-G alleles and the 14 bp sequence and LSCC in our locality. However, we recommend enlarging the study and the control groups in the subsequent researches with deeper analysis of their physiologic functions and molecular polymorphisms with their effects on the pathogenesis mechanisms of the LSCC.

**Conclusion**

It is important to reveal that a significant association of HLA-G 14-bp deletion allele in contrast to HLA-G 14-bp insertion allele with the risk of LSCC in Egyptian patients was demonstrated. However, no clear association between HLA-G*01:05 N allele and occurrence of LSCC was revealed. HLA-G alleles and the 14bp sequence may be proven to be predictive markers for the LSCCs.

**Disclosure of potential conflicts of interest**

The authors have no relevant financial or non-financial interests to disclose.

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