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Promising link of HLA-G polymorphism, tobacco consumption and risk of Head and Neck Squamous Cell Carcinoma (HNSCC) in North Indian population

Vertica Agnihotri [a], Abhishek Gupta [a], Rahul Kumar [a], Ashish Datt Upadhyay [b], Sadanand Dwivedi [b], Lalit Kumar [c], Sharmistha Dey [a,*]

[a] Department of Biophysics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India
[b] Department of Biostatistics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India
[c] Department of Medical Oncology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

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ABSTRACT

Human leukocyte antigen (HLA-G) is a potent immune-tolerant molecule and has a critical role in various pathological conditions of cancer. The aim of the study was to analyze the association of HLA-G polymorphism as a risk factor in Head and Neck Squamous Cell Carcinoma (HNSCC). The HLA-G polymorphism at 3′UTR *14bp INDEL* (rs371194629) and *+3142G/C* (rs1063320) were studied in 383 HNSCC patients and 383 ethnically similar-aged healthy controls in North Indian population. The genotyping study of two polymorphisms of HLA-G was documented using DNA-PAGE and RFLP-PCR method. *14bp INDEL Del/Ins*, *Ins/Ins* genotype and *Ins* allele were more pronounced in HNSCC patients in compared to controls. Whereas, *+3142 C/C* genotype and *C* allele were associated with risk factors in HNSCC. Furthermore, the dual effect of polymorphisms; both variants (*Del/Ins-Ins/Ins & G/C-C/C*) carrying loci was significantly (OR = 2.78) associated with the disease compared to one variant (*Del/Del-G/C or Del/Del-C/C or Ins/Ins-G/G*). Moreover, both polymorphisms showed promising link in terms of tobacco influence on HNSCC risk. It can be concluded that this study first time reports that *C/C*, *Del/Ins* and *Ins/Ins* genotype as well as *C* and *Ins* allele could be major risk factors with strong impact of tobacco for HNSCC in North Indian population.

1. Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is most prominent cancer in developing countries. HNSCC is the topmost cancer affected in Indian men and mostly due to the habit of tobacco [1]. Tobacco use and alcohol consumption are two major risk factors for HNSCC. There is an established relation between cancer occurrence and defect in the immune response. Immune surveillance of tumor cells depends on the recognition of antigens presented in the context of human leukocyte antigen (HLA) class I molecules by cytotoxic T cells (CTL) via their T-cell receptors and by natural killer (NK) cells via killer-cell immunoglobulin-like receptors (KIR) [2]. HLA-G has been reported as immunomodulatory molecule for many diseases as well as for cancer [3]. HLA-G has a role in inhibiting NK and T cells cytolytic response by binding to its inhibitory surface receptors (ILT2 & KIR2DL4). High expression of HLA-G has been observed in various cancers-breast, renal, ovarian, lung, gastric and colorectal cancer [4,5]. Several studies have regarded the polymorphic nature of HLA-G gene. Most polymorphic sites are present at 5′URR (upstream regulatory region) and 3′UTR (untranslated region) in comparison to protein coding regions. The polymorphism in the 5′UTR affects the transcription of the HLA-G gene, while that in the 3′UTR influences mRNA processing and stability [6]. Moreover, a 14bp Del/Ins polymorphism, present in the 3′UTR of the HLA-G gene, has been suggested to influence the mRNA stability and therefore HLA-G protein expression [7,8]. HLA-G polymorphism is associated with several types of malignancies [9–13] in different populations. Till date, no report has been put forward related to association between HNSCC and HLA-G polymorphism in North-Indian

Abbreviations: HNSCC, Head and Neck Squamous Cell Carcinoma; HLA-G, human leukocyte antigen-G; RFLP, restriction fragment length polymorphism.

* Corresponding author at: Department of Biophysics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India.
E-mail address: sharmistha_d@hotmail.com (S. Dey).

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population, hence, the present study would focus on association between 3′UTR *14bp INDEL* (rs371194629) and *+3142 G/C* (rs1063320) HLA-G polymorphism with HNSCC in North Indian population.

2. Materials and methods

2.1. Group selection

This retrospective study includes two groups: HNSCC patients and healthy controls. Three hundred and eighty three patients were recruited from Head and Neck Cancer OPD, Dr. B.R.A. Institute Rotary cancer Hospital (IRCH), All India Institute of Medical Sciences (AIIMS), New Delhi, India. Patients with following conditions were excluded from the study: any serious illness, chronic infection, any other inflammatory diseases, any histories of cancer or undergone any kind of treatment (such as surgery, chemotherapy or radiotherapy) previously. Patient's blood was collected at the time of diagnosis. Patient's clinical data includes tumor staging, tumor histopath and diseased site informations were collected from medical records. Patients were staged in the TNM classification according to the American Joint Committee on Cancer (AJCC).

Control group was interviewed for demographic data before considering for the study. Controls were excluded who had refused to participate in the study and had any chronic diseases. Demographic data include: age, gender, tobacco status and drinking status. Tobacco status includes both smoking and chewing habits of patients. Subjects who smoked 5–6 cigg/day for 6 months considered as smokers and who chewed 4–5 packets/day for 6 months considered as chewers. Subjects who consumed alcohol 3–4 times a week for 6 months were considered under alcohol status. The study protocol was approved by the ethics sub-committee of AIIMS (IESC/T-469.12.2014). After receiving written informed consent forms from both groups, 2 ml venous blood sample was withdrawn.

Geographically, the samples included in the study belong to urban population of North (N) Indian ethnic population of India which consider states of Punjab, Haryana, Uttar Pradesh and Delhi as per Indian Genome Variation Consortium [14].

2.2. DNA extraction and genotyping

Two ml of venous blood was collected in an EDTA containing vial. Genomic DNA was isolated from blood samples using Medi G Blood Genomic DNA (gDNA) Miniprep Kit (mdi Membrane Tech, Inc.) according to manufacturer's instructions.

Del/Ins polymorphism was analyzed by polyacrylamide gel electrophoresis (PAGE) analysis. PCR was run with 50ul of total reaction volume, which contains: 100 ng of genomic DNA, Taq buffer 5 ul (10X), dNTP mix 0.5 ul (10 mM), Taq DNA Polymerase 1.25 U (New England Biolabs), 0.5 ul (20 uM) of each primers, fwd primer: TGTGAAACAGCTGCCCTGTG and rev primer: AAGGAATGCAGTTCAGCATGAGG [15]. PCR reaction conditions were: initial denaturation at 95 °C for 3 min, 29 cycles of denaturation at 95 °C for 30sec, annealing at 55 °C for 30sec, extension at 72 °C for 40sec, final extension at 72 °C for 10 min. The amplified product was run on 6% DNA-PAGE and stained with Ethidium bromide for visualization of bands. Two sizes of amplified products were seen: a) 151bp for 14bp insertion allele, b) 137bp for 14bp deletion allele.

The genotyping of *+3142G/C* polymorphism was performed by restriction fragment length polymorphism (RFLP-PCR). PCR reaction conditions were similar as given for *14bp Del/Ins* polymorphism with an annealing at 55.7 °C for 30sec. Primers used for *+3142G/C* were: fwd primer: CATGCTGAACTGCATTCCTTCC and rev primer: CTGGTGGGACAAGGTTCTACTG [16]. The digestion of amplified product was performed according to previously described protocol [16]. Briefly, PCR products were digested with 3U of the restriction enzyme BaeGI (New England BiolabsInc, Ipswich, MA) according to manufacturer's instructions. Digested products were run on 3% agarose gel and stained with Ethidium bromide for visualization of bands. Two different patterns appeared: 406bp for the allele 'C', and 316bp and 90bp for the allele 'G'. To validate the results, 10% of the total samples were chosen randomly and further assessed by direct sequencing (ABI analyzer). Sequencing results were in agreement with our results. Wild type alleles are *Del* and *G* whereas; mutant alleles are *Ins* and *C*.

2.3. Statistical data

All statistical analysis was performed using STATA/SE 9.0 software. Fisher's exact test and chi-square tests were applied for comparison of demographic characteristics between patients and controls. Frequencies of genotypes and alleles were calculated by simple calculation. The odds-ratios were adjusted for age, gender, tobacco status and drinking status using multiple logistic regression analyses. LD parameters were calculated using SHEsis software. For sample size exploration, nQUERY advisor software was used. The chi-square test was performed to test Hardy-Weinberg equilibrium of genotypes among the control group. The significance level was considered for $P < 0.05$.

3. Results

In the present study, genotyping of two polymorphisms of HLA-G was documented using PAGE and RFLP-PCR method. We have considered the effect of these two polymorphisms on HNSCC risk, either alone or in association with demographic characteristics.

3.1. Distribution of demographic characteristics between HNSCC patients and controls

Total 383 patients and 383 controls were genotyped for both polymorphisms. Distribution of demographic data among patients and controls is presented in (Table 1). The power calculation analysis showed that the patient-control pair of 383 was sufficient to achieve 80% power for effective sample size. Male individuals (91.12%) were seen greater than female among the study population. The number of tobacco consumers in patients were significantly ($P < 0.001$) higher than controls. HNSCC patients had a considerably higher exposure of tobacco consumption (mean pack yrs = 19.68) in comparison to controls. Percentage of patients with III and IV stage, oral cavity site, node involvement and moderately defined histopath were observed more.

3.2. Genotypic and allelic frequencies of Del/Ins and +3142G/C polymorphisms in HNSCC patients and controls and the risk of HNSCC

The distribution of genotype and allele frequencies of HLA-G *Del/Ins* and *+3142 G/C* polymorphisms among patients and controls are shown in (Table 2). No deviations from Hardy-Weinberg equilibrium were seen for both polymorphisms among controls ($P > 0.2$). In *Del/Ins* polymorphism, genotype frequencies of *Del/Ins* (55.35%) and *Ins/Ins* (23.24%) in patients were higher than controls (Del/Ins = 45.69%; Ins/Ins = 22.45%). The individuals with genotypes *Del/Ins* (OR = 1.71) and *Ins/Ins* (OR = 1.81) have more risk of developing HNSCC than with *Del/Del* genotypes. Similarly, *Ins* allele (Table S1) was found increased in patients ($P = 0.03$) than controls. On the other hand, the combined effect of *Del/Ins + Ins/Ins* genotype (OR = 1.74, $P = 0.005$) was found to have more impact on comparing it with *Del/Del* genotype.