Polymorphism of *Interleukin-6* Is Not Associated with the Presence or Absence of High HPV E6/E7

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Abstract. *The present study evaluated the frequency of the polymorphism of Interleukin-6 (IL6) in women positive for E6/E7 Human Papillomavirus (HPV) (n=152) and women negative for HPV (n=238), 390 women in total. Material for analysis was obtained at the Federal University of São Paulo. Interleukin-6 polymorphism was detected by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) and analyzed in 3% agarose gel. Results: No significant associations between the frequency of the polymorphism of IL6 in patients expressing E6 and E7 with HPV-positive and -negative reactions were found. There was no statistically significant difference between the case and control group for genotype distribution (p=0.280). Conclusion: Genotypic analysis showed a striking similarity of IL6 polymorphisms in both cases and controls. The allelic distribution in cases and controls for G and C of IL6 were very similar (p=0.186), which could point to similar IL6 functionality for both groups.*

Cervical cancer is one of principal causes of death in women worldwide, competing with breast cancer in developing countries (1). The oncogenic human papillomavirus (HPV) is the obligatory etiological cause of cervical cancer development. A number of co-factors were described such as host immune characteristics, and number of sexual partners presumably infected, which increase the chances for HPV infection and viral load. HPV infection affects men and women, and HPV can be found in the perianal region, anal canal, scrotum, penis, semen, oral cavity and pharyngeal and esophageal regions (2, 3). Importantly, persistent high-risk HPV infection is the major risk factor for neoplastic progression (4-7).

There are almost 200 types of HPV biologically identified as oncogenic and non-oncogenic types, with an estimation that 75% of all humans will be infected during their life-time, although only a small percentage of these infections will develop cancer (8).

The long period between HPV, infection and the diagnosis of invasive carcinoma (approximately 15 years) indicates that multiple factors and multiple steps may be required for carcinoma-development (7, 9 and 10). Recently, several studies indicated that the detection of persistent high-risk HPV DNA in cervical cytological samples predicts the presence of precursor lesions of cervical cancer (11).

Carcinogenesis induced by oncogenic HPV depends on the activity of the viral oncoproteins E6 and E7 in cellular repository of the cervix, that culminates with the integration of the HPV genome into human DNA. Briefly, E6 is responsible for the degradation of p53, and E7 interferes with the retinoblastoma protein (pRb) function (12). Both p53 protein and pRb act as tumor growth suppressors; consequently, it is predictable that cell-cycle regulation is critically damaged upon infection by HPV, which induces an altered S phase distribution and consequently provokes errors in cellular DNA replication. Not surprisingly, the high-grade lesions have high rates of E6 and E7 transcripts in all layers of the epithelium, which is associated with genetic abnormalities and malignant transformation (13).

Co-factors have been identified for the development of cervical carcinoma, including exogenous influences such as smoking, use of oral contraceptives, and micro-organisms such as *Chlamydia trachomatis*, among others (14-16). Most of these factors are believed to enhance carcinogenesis, including high-risk HPV types, immunosuppression, and host genetic polymorphisms that

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Key Words: Gene polymorphism, IL6, HPV, E6/E7.

could modulate the effect of HPV behavior. Polymorphism of interleukin-6 (*IL6*), a pro-inflammatory cytokine that acts on different cells and that is released in almost all cases of acute infection, is supposedly involved in cervical cancer aggressiveness. *IL6* was studied by Woodworth and Simpson in 1993 (17), who described that the normal mucosa produces cervical *IL6*, but the presence of HPV influences the local immune response. It was also observed that high levels of IL6 are expressed in invasive cervical carcinomas, compared to normal cervix (18).

Considering that specific mutations of certain genes can be associated with the incidence of some types of cancer, polymorphisms can affect gene expression and play a role in oncogenesis, according to recent advances in molecular genetics, which includes genome-wide association studies (19).

The *IL6* gene is located on chromosome 7p21-24 and *IL6* polymorphism is located in the promoter region (-174G/C); this polymorphism has been previously correlated with increased concentration of IL6 in plasma; however, the individual genetic variation is believed to be related to the pathogenicity of cervical cancer (20, 21).

The study herein reported is a case–control evaluation comparing the frequency of *IL6* polymorphism in patients with E6 and E7 of high-risk HPV-positive *versus* -negative women in order to determine if this polymorphism plays a role in cervical lesions.

Materials and Methods

Study Population. A total of 390 women were evaluated, 152 formed the case group (HPV-positive, E6- and E7-positive) and 238 controls (HPV-negative, negative for E6 and E7). The average age was 30.2±7.6 years in the control group and 29.9±9.8 years in the case group (p=0.778). This work was performed with scraped cervical samples analyzed at the Laboratory of Molecular Gynecology, Federal University of São Paulo. The local Ethical Committee approved the project, n˚ 1275/10.

DNA extraction. The extraction of DNA was performed as per the Kit ilustra™ blood genomic Prep protocol of GE (Firfield, Connecticut, EUA) for extraction of the material from blood and epithelial cell scrapes. The purified DNA was stored at −80˚C until utilization. The amount of DNA in each sample was measured by spectrophotometry in a NanoDrop® 2000 (Thermo fisher Scientific-San Jose, California, USA)

Restriction Fragment Length Polymorphism assay (RFLP). The polymorphism of *IL6* was analyzed by Polymerase Chain Reaction (PCR). The primer pairs used were: *IL6* (174 position of the promoter region) - sense: 5'- ATG CCA AGT TCT GAG TCA CTA 3', - antisense: 5'- GGA AAA TCC CAC ATT TGA TA 3'.

Ten microliters of Master Mix (PCR Master Mix 2x; Promega - Madison, Wisconsin, USA) were used, with 1 μl of each primer (10 pmol/μl), 100 ng genomic DNA and Nuclease-Free Water, (Promega) for 25 μl reaction.

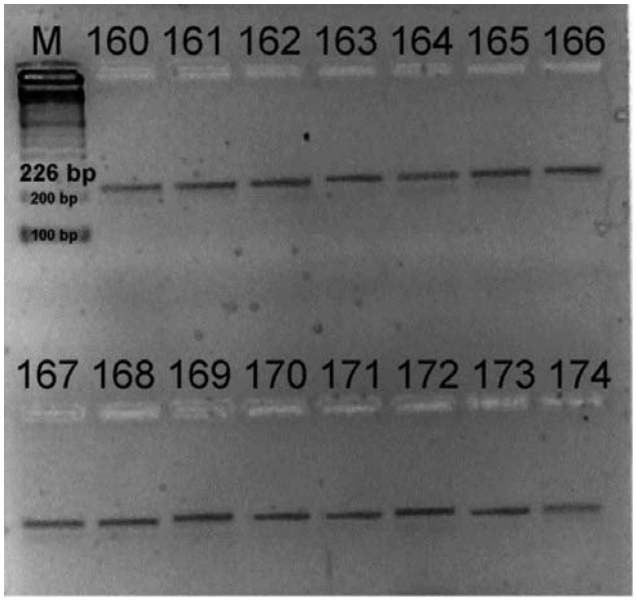

Figure 1. *Agarose gel after amplification of the target segment of interleukin-6 (IL6) in the DNA of certain patients. The 2.0% agarose gel was stained with ethidium bromide, showing single fragment of 226 bp of the gene. The numbers 160-174 represent a small portion from our sample group and M represents a molecular weight standard of 100 bp.*

For optimization of the tests, a reaction was carried-out with PCR using a temperature gradient in order to obtain the best conditions for the annealing temperature of the primers.

The amplification for the detection of *IL6* polymorphism was carried-out with the following cycling conditions: 94˚C for 5 min for initial denaturation, followed by 94˚C for 30 s, 54˚C for 45 s and 72˚C for 1 min for 40 cycles for denaturation, annealing and extension, with final extension of 72˚C for 7 min to finish the reaction. All material post-PCR were analyzed on an agarose gel for confirmation and documented for analysis of viability of each sample. The products of the PCR amplifications were applied to a 2.0% agarose gel (Amersham Pharmacia Biotech - Piscataway, New Jersey, USA) and stained with ethidium bromide (1 mg/ml - Promega) and subjected to electrophoresis for 20 min at 110 V in a horizontal tank containing running buffer of 0.04 M Tris-acetate and 1 mM EDTA (pH 8). The visualization of the amplicon was carried out on an ultraviolet transilluminator and documented by the Kodak Digital Science 1D photo system (Rochester, New York, USA) (Figure 1).

IL6 polymorphism. Viable samples were digested with restriction enzyme (NlaIII; Thermo Scientific - San Jose, California, USA) for 2 h at 37˚C in a water bath following the manufacturer's protocol. Polymorphism analysis was performed on an agarose gel (3.0%) staining with ethidium bromide. The human gene for *IL6* is located on chromosome 7p21-24 and its phosphorylated glycoprotein contains 185 amino acids. The target of this study was a polymorphism located in the promoter (174 G/C). Digestion with the enzyme *Nla*III generated a fragment of 226 bp for the wild-type allele (G) and 117 bp and 109 bp for the polymorphic allele (C). In heterozygous (G/C) patients fragments of 226 bp, 117 bp and 109 bp are generated (Figure 2).

Table I. *Distribution of genotypic polymorphism for interleukin-6.*

	Genotype				
Group	GG n(%)	CG n(%)	CC n(%)	Total n(%)	*p*-Value*
Case	89 (58.6)	51 (33.6)	12 (7.9)	152 (100)	0.280
Control	158 (66.4)	63 (26.5)	17 (7.1)	238 (100)	

*Chi-square test.

Table II. *Distribution of cases and controls by interleukin-6 G-carrying genotype.*

	Groups		
Genotype	Case n(%)	Control n(%)	*p*-Value*
GG	89 (58.6)	158 (66.4)	0.144
Non-GG	63 (41.4)	80 (33.6)	
Total	152 (100)	238 (100)	

*Chi-square test.

Table III. *Distribution of alleles for interleukin-6 polymorphism.*

	Group		
Allele	Case n(%)	Control n(%)	*p*-Value*
G	229 (75.3)	379 (79.6)	0.186
C	75 (24.7)	97 (20.4)	
Total	304 (100)	476 (100)	

*Chi-square test.

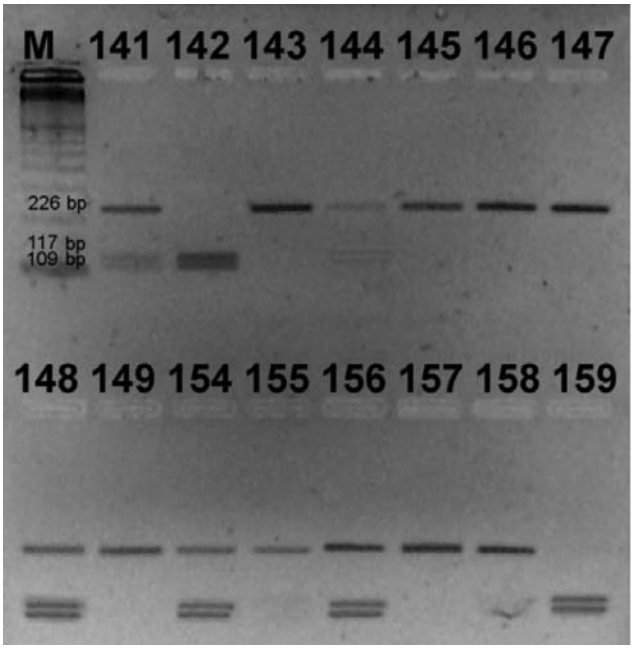

Figure 2. *Agarose gel after incubation of the DNA fragment (226 bp) target interleukin-6 (IL6) with endonuclease NLAIII. The 3.0% agarose gel was stained with ethidium bromide in the study of IL6 (174 G/C) polymorphism. The numbers 141-159 represent a small portion from our sample group and M represents a molecular weight standard of 100 bp. In the columns marked 143, 145, 146, 147, 149, 155, 157 and 158, note the presence of fragments reflecting the homozygous wild-type for the polymorphism (G/G); columns 142 and 159 reveal the mutated homozygous pattern of polymorphism (C/C); 141, 144, 148, 154 and 156 have the heterozygous pattern (G/C) for the IL6 polymorphism.*

Statistical analysis. Analyses were performed using the Statistical Package for Social Science (v14.0) (IBM softwares, USA). To compare qualitative variables between case and control groups, *i.e.* frequencies and genotypic and allelic proportions, the Chi-square test was used, when required. Statistical significance was set at 5%.

Results

Our study included 390 women, 152 in the case group (HPV-positive, E6/E7-positive) and 238 in the control group (HPV-negative, E6/E7-negative). There was no statistically significant differences between the groups according to genotype or allele, as shown in Tables I, II and III.

Discussion

Genotypic analysis showed a striking similarity of the distribution of *IL6* polymorphisms in both cases and controls. This is extremely interesting because it calls into question the actual role of *IL6* in carcinogenesis of the uterine cervix. The allelic distribution in cases and controls for G and C was very similar, with no significant (p=0.186) difference, which could indicate that IL6 functionality is similar for both groups.

It is well-known that *IL6* encodes a cytokine that acts in inflammatory processes and which has an important role in cervical carcinogenesis. The polymorphisms of *IL6* are associated with variations in immune response that contribute to an increased risk of cancer (22). Recently, *IL6* polymorphism was shown to be a marker for susceptibility to cervical carcinoma in 1,584 Chinese women with cervical carcinoma. However, these data need further evaluation regarding the relationship between polymorphism and HPV-induced cervical lesions (23). Our study was based on 152 women positive for HPV E6/E7, but with different degrees of cervical lesion. The lack of significant differences between the HPV-positive cases and the group of 238 HPV-negative women shows the variability of polymorphisms to be discriminatory only if analyzed in large population.

The genotype distribution between cases and controls had homogeneous proportions (p=0.280) for those with wild-type (GG: 58.6% of cases and 66.4% of controls), heterozygous (GC: 33.6% of cases and 26.5% of controls) and homozygous (CC: 7.9% of cases and 7.1% of controls) genotypes. Even dichotomously separating wild genotype *versus* heterozygous or homozygous genotypes, did not show any significant differences (p=0.144), which reinforces, in part, our assumption of no significant influence of *IL6* polymorphism in women with positive and negative reactions for HPV E6/E7 but without invasive carcinomas. In 131 women with cervical intra-epithelial neoplasias 2-3 and 209 controls, Grimm and co-workers observed that the polymorphism of *IL1*, *IL1A889*, but not *IL6*, was independently associated with an increased risk for high-grade intraepithelial lesions (24). Conversely, Gangwar and colleagues evaluated the association of the polymorphism of *IL6* (174G/C) for predisposition to cervical carcinoma in 160 cases of women with cervical cancer and 200 controls. They found a significant association between *IL6* 174CC genotype and increased risk of cervical cancer (odds ratio=3.16, p=0.014), in addition to the increased risk of developing carcinoma in women with stage I genotype (CG) (OR=3.63, p=0.003) (25).

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Received March 18, 2014
Revised June 3, 2014
Accepted June 5, 2014