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Association between the p53 arginine/arginine homozygous genotype at codon 72 and human papillomavirus E6/E7 mRNA expression

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ABSTRACT

Objective: To evaluate the association between p53 polymorphisms and human papillomavirus (HPV) E6/E7 mRNA expression.

Methods: We analyzed 175 cervical samples from women aged 16–69 years old who were tested for HPV E6/E7 mRNA expression (NucliSENS® EasyQ® HPV). The samples were divided into three groups: positive ($n = 75$) those with positive HPV E6/E7 mRNA expression and positive high-risk HPV Hybrid Capture (HR-HC) test; negative ($n = 52$) those with negative HPV E6/E7 mRNA expression and positive HR-HC; and control ($n = 48$) those with negative HPV E6/E7 mRNA expression and negative HR-HC. The p53 polymorphisms at codons 11, 72, and 248 were evaluated through *polymerase chain reaction-restriction fragment length polymorphism* (PCR-RFLP).

Results: The frequency of the arginine/arginine homozygous genotype at codon 72 was significantly higher in the positive (49.3%) than in the negative (32.7%) and control groups (20.8%, $p = 0.002*$). The frequency of the arginine allele was also significantly higher in the positive (67.3%) than in the negative (53.8%) and control groups (38.5%, $p < 0.001*$). The arginine/arginine homozygous genotype was significantly associated with positive HPV E6/E7 mRNA expression (positive group) compared with negative and control groups (odds ratio: 2.633; 95% CI, 1.399–4.954, $p = 0.003$). The frequency of arginine/arginine homozygous genotype at codon 72 remained significantly more frequent in the positive group of women aged ≥30 years than in the other two groups.

Conclusion: The presence of the p53 arginine/arginine homozygous genotype at codon 72 was significantly associated with the positive HPV E6/E7 mRNA expression.

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Introduction

The human papillomavirus (HPV) E6 and E7 viral proteins play an important role in malignant transformation in the cervical carcinogenesis mechanism induced by high-risk types of HPV. The E6 protein binds and inactivates the p53 tumor-suppressor protein and induces its degradation via ubiquitin, while E7 binds and inactivates the pRb tumor-suppressor protein, leading to cell cycle dysregulation.[1]

The expression of these proteins can be detected by the HPV E6/E7 mRNA expression test, which when positive indicates the virus-transforming capacity that results in immortalized proliferation and mutation of the host's squamous epithelial cells. Thus, some authors suggest that persistent expression of E6/E7 can serve as an indicator of progression to cervical intraepithelial neoplasia and invasive cancer.[2] Studies have shown that the detection of HPV E6/E7 mRNA has a positive predictive value higher than techniques based on the detection of viral DNA; moreover, it can identify women with high-risk HPV-persistent infection who are at increased risk of developing high-grade precursor lesions.[3,4]

The p53 protein is related to cell cycle regulation and apoptosis; cell growth instability and cell cycle progression are observed when the p53 gene is mutated. The p53 protein, when not mutated, activates other tumor-suppressor genes that are key to cell cycle regulation and progression, and apoptosis in cases of DNA damage.[5,6]

Studies have shown that p53 polymorphisms at codons 11, 72, and 248 are associated with different tumors.[7–10] The polymorphism at codon 72 is the most common, and different authors have suggested that the presence of arginine/arginine homozygous (Arg/Arg) genotype is related to high-grade cervical intraepithelial neoplasia and cervical cancer.[11–15] This association is not observed with heterozygous genotypes containing one arginine allele and one proline allele (Arg/Pro), or with proline/proline homozygous (Pro/Pro) genotype.[16] However, there are little data on the p53 polymorphism at codons 11 and 248 associated with lower genital tract diseases.

This study evaluated the association between p53 polymorphisms at codons 11, 72, and 248 and positive HPV E6/E7 mRNA expression. The secondary objective was to determine whether this association was different between the age groups <30 and ≥30 years old.

Methods

Sample description

We evaluated a total of 175 endocervical secretion samples from women tested for HPV E6/E7 mRNA expression using the NucliSENS EasyQ® kit (Biomerieux), which detects the 16, 18, 31, 33, and 45 HPV mRNAs. Samples were collected and provided by the Salomão Zoppi Diagnostics Laboratory (São Paulo, SP, Brazil) from February 2009 to September 2011. The collecting tubes were identified, cataloged, and stored after collection. All participants had previously signed an informed consent and standard laboratory responsibility forms; the

Codon	Primer
Codon 11 – Glu/Gln or Lys (GAG → CAG or AAG)	Sense: 5′-CTTGGGTTGTGGTGAAACATTG-3′ Antisense: 5′-GTCAGTCCCATGAATTTTCGCT-3′
Codon 72 – Arg/Pro (CGC → CCC)	Sense: 5′-TCCCCCTTGCCGTCCCAA-3′ Antisense: 5′-CGTGCAAGTCACAGACTT-3′
Codon 248 – Arg/Trp or Gln (CGG → TGG or CAG)	Sense: 5′-TAGGTTGGCTCTGACTGTACCA-3′ Antisense: 5′-TGTGATGAGAGGTGGATGGGTA-3′

Table 1 – Sequences of *primers* used in the study.

study had protocol approval by the Ethics Committee of Unifesp.

The samples included in the study were originated from patients who had been submitted to both high-risk HPV Hybrid Capture test (HR-HC) and HPV E6/E7 mRNA expression test. The samples were divided into three groups based on the results of these two tests: positive ($n = 75$, 42.8%), positive HR-HC and positive E6/E7 mRNA expression; negative ($n = 52$, 29.7%), positive HR-HC and negative E6/E7 mRNA expression; and control ($n = 48$, 27.4%), negative HR-HC and negative E6/E7 mRNA expression. The age of the women providing samples ranged from 16 to 69 years (mean 34 ± 9.97 years).

DNA extraction

The genomic DNA was extracted from scraped mucosal cells according to the GenElute™ Mammalian Genomic DNA (Sigma) kit protocol. The extracted and purified genomic DNA was stored at $-80\,°C$ until use. The amount of DNA in each sample was measured by spectrophotometry in the NanoDrop® 2000 (full-spectrum spectrophotometer Spectronic® Genesy 5 model).

Evaluation of p53 polymorphisms

Three p53 polymorphisms were selected for analysis in the study through *polymerase chain reaction-restriction fragment length polymorphism* (PCR-RFLP). Table 1 shows the sequences of primers used.

The PCR reactions included: 10 μL of the Master Mix (PCR Master Mix, 2.0x, Promega), 1 μL of each primer pair (10 pmol/μL), 100 ng of genomic DNA (×μL), and ×μL of nuclease-free water (Nuclease-Free Water, Promega) to a final reaction volume of 25 μL.

PCR products were analyzed on a 2.0% agarose gel horizontal electrophoresis for 30 min at 110 V in 1× TAE (0.04 M Tris–acetate, 1 mM EDTA pH8), and stained with ethidium bromide (1 mg/ml). The amplified fragments were visualized on a UV transilluminator and documented with the "Kodak Digital Science 1D" photo system.

Following the PCR-RFLP, all right-sized PCR products were digested with the restriction enzymes appropriate to identify each polymorphism (*Taq I* for codon 11, *BstU I* for codon 72, and *Hpa II* for codon 248) according to the manufacturer's

Table 2 – Fragment patterns after restriction digestion.			
Enzyme	Wild-type homozygous genotype	Heterozygous genotype	Mutant homozygous genotype
Taq I	Codon GAG (Glu/Glu) 239 bp + 140 bp	Glu/Gln or Lys - 379 bp + 239 bp + 140 bp	Codon CAG (Gln/Gln) or AAG (Lys/Lys) 379 bp
BstU I	Codon CGC (Arg/Arg) 160 bp + 119 bp	Arg/Pro 279 bp + 160 bp + 119 bp	Codon CCC (Pro/Pro) 279 bp
Hpa II	Codon CGG (Arg/Arg) 164 bp + 69 bp	Arg/Trp or Gln 233 bp + 164 bp + 69 bp	Codon TGG (Trp/Trp) or CAG (Gln/Gln) 233 bp

instructions. Table 2 shows the patterns of fragments after digestion corresponding to the three studied genotypes.

Statistical analysis

The genotype distribution and allelic frequencies were analyzed using the Pearson's chi-square test and Fisher's exact test, or its extension, considering the alpha significance level of 5%.[17] The odds ratio (OR) and the confidence interval (CI) of 95% by the Mantel–Haenszel method were calculated to estimate the risk of the studied polymorphisms to be associated with positive HPV E6/E7 mRNA expression.

Results

The vast majority of the total sample showed wild-type homozygous genotype at codon 11 (Glu/Glu, 91.4%) and codon 248 (Arg/Arg, 98.8%). The distribution of genotypes was more homogeneous for codon 72; the heterozygous (Arg/Pro, 37.7%) genotype was the most frequent, followed by the wild-type homozygous genotype (Arg/Arg, 36.6%) and mutant homozygous (Pro/Pro, 25.7%) genotypes (Table 3).

The analysis of genotype distribution showed that the frequency of wild-type homozygous genotypes was similar between the positive, negative, and control groups for codons 11 and 248. The Arg/Arg genotype was significantly ($p = 0.002^*$) more frequent in the positive group (49.3%) compared to the negative (32.7%) and control (20.8%) groups for codon 72 (Table 3).

The allele frequency analysis showed that the majority of samples presented wild-type allele (Glu) at codon 11. The same was observed at codon 248. At codon 72, 55.4% presented the Arg allele and 44.6% the Pro allele. The distribution of the wild-type allele at codons 11 and 248 was similar between the positive, negative, and control groups. The frequency of the Arg allele at codon 72 was 67.3% in the positive group, 53.8% in the negative group, and 38.5% in the control group ($p < 0.001^*$) (Table 3).

The OR of the Arg/Arg genotype at codon 72 versus the Arg/Pro + Pro/Pro genotype to be associated with a positive HPV E6/E7 mRNA expression test was 2.633 (95% CI: 1.399–4.954, $p = 0.003^*$) compared to the other groups. The OR was even higher, 3.7 (95% CI: 1.612–8.492, $p = 0.002^*$), when compared to the control group alone. Considering only the positive samples for high-risk HPV (positive and negative groups), the OR of the Arg/Arg genotype at codon 72 to be

associated with the HPV E6/E7 mRNA expression test was 2.005 (95% CI: 0.961–4.182, $p = 0.064$).

The OR of the Arg allele at codon 72 to be associated with the HPV E6/E7 mRNA expression test was 1.767 (95% CI: 1.056–2.956, $p = 0.030^*$) when the positive group was compared to the negative group; this OR was 2.372 (95% CI: 1.527–3.682, $p < 0.001^*$) when the positive group was compared to the other two groups (negative and control), and 3.287 (95% CI: 1.927–5.608, $p < 0.001^*$) when compared to the control group only.

No significant differences were observed in the genotype distribution for the presence of polymorphisms at codons 11, 72, and 248 in women younger than 30 years old ($n = 62$). Regarding allelic frequency, the Glu allele at codon 11 was significantly more frequent in the negative group (100%) than in the control group (84.4%, $p = 0.025^*$). No statistically significant differences were observed in the allelic frequencies at codons 72 and 248.

In women aged ≥30 years ($n = 113$), the homozygous Arg/Arg genotype and presence of the Arg allele at codon 72 was significantly more frequent in the positive group. No statistically significant differences were observed in the distribution of genotypes or allelic frequencies at codons 11 and 248 between the studied groups (Table 4).

Discussion

The analysis of p53 polymorphism at codon 72 shows that the Arg/Arg genotype was significantly more frequent in positive samples for HPV E6/E7 mRNA expression compared to samples in the negative and control groups. The presence of homozygous Arg/Arg genotype or the Arg allele significantly increased the chance of HPV E6/E7 mRNA expression, especially when compared to the control group.

Our results are partially in agreement with those reported by Bertuccio et al.[18] in Italy, who analyzed 80 positive cervical samples for high-risk HPV DNA, of which 16 (20%) were positive and 64 (80%) were negative for the HPV E6/E7 mRNA expression. The researchers also observed a higher frequency of the Arg/Arg genotype in the positive E6/E7 group compared to the negative group (62.5% vs. 50%, respectively), although not statistically significant. The Arg/Pro genotype was observed in 18.75% and 39% of samples in the positive and negative groups, respectively, and the Pro/Pro genotype was observed in 18.75% and 11% of samples, also respectively. In our study, we also observed a higher frequency of the Arg/Pro genotype in the negative group compared to the positive.

Table 3 – Genotype distribution and allelic frequencies of codons 11, 72, and 248 according to the studied groups.

	Codon 11						
	Genotype distribution (n = 175)[a,c]				Allelic frequencies (n = 350)[b,d]		
Groups	Glu/Glu N (%)	Glu/Gln or Lys N (%)	Gln/Gln or Lys/Lys N (%)	Total N (%)	Glu N (%)	Gln or Lys N (%)	Total N (%)
Positive	67 (89.3%)	5 (6.7%)	3 (4.0%)	75 (100%)	139 (92.7%)	11 (7.3%)	150 (100%)
Negative	49 (94.2%)	2 (3.8%)	1 (1.9%)	52 (100%)	100 (96.2%)	4 (3.8%)	104 (100%)
Control	44 (91.7%)	2 (4.2%)	2 (4.2%)	48 (100%)	90 (93.8%)	6 (6.3%)	96 (100%)
Total	160 (91.4%)	9 (5.1%)	6 (3.4%)	175 (100%)	329 (94.0%)	21 (6.0)%	350 (100%)

	Codon 72						
	Genotype distribution (n = 175)[b,e]				Allelic frequencies (n = 350)[b,f]		
Groups	Arg/Arg N (%)	Arg/Pro N (%)	Pro/Pro N (%)	Total N (%)	Arg N (%)	Pro N (%)	Total N (%)
Positive	37 (49.3%)	27 (36.0%)	11 (14.7%)	75 (100%)	101 (67.3%)	49 (32.7%)	150 (100%)
Negative	17 (32.7%)	22 (42.3%)	13 (25.0%)	52 (100%)	56 (53.8%)	48 (46.2%)	104 (100%)
Control	10 (20.8%)	17 (35.4%)	21 (43.8%)	48 (100%)	37 (38.5%)	59 (61.5%)	96 (100%)
Total	64 (36.6%)	66 (37.7%)	45 (25.7%)	175 (100%)	194 (55.4%)	156 (44.6%)	350 (100%)

	Codon 248						
	Genotype distribution (n = 175)[a,g]				Allelic frequencies (n = 350)[a,h]		
Groups	Arg/Arg N (%)	Arg/Trp or Gln N (%)	Trp/Trp or Gln/Gln N (%)	Total N (%)	Arg N (%)	Trp or Gln N (%)	Total N (%)
Positive	74 (98.7%)	1 (1.3%)	–	75 (100%)	149 (99.3%)	1 (0.7%)	150 (100%)
Negative	52 (100%)	–	–	52 (100%)	104 (100%)	–	104 (100%)
Control	47 (97.9%)	–	1 (2.1%)	48 (100%)	94 (97.9%)	2 (2.1%)	96 (100%)
Total	173 (98.8%)	1 (0.6%)	1 (0.6%)	175 (100%)	347 (99.1%)	3 (0.9%)	350 (100%)

[a] Extension of the Exact Fisher's test.
[b] Pearson's Chi-square test.
[c] $p = 0.900$.
[d] $p = 0.512$.
[e] $p = 0.002^*$.
[f] $p < 0.001^*$.
[g] $p = 0.562$.
[h] $p = 0.360$.

However, the frequency of the Pro/Pro genotype was higher in the negative compared to the positive group. This was the only study found in the literature on the association of p53 polymorphism with the expression of HPV E6/E7 proteins.

This polymorphism has been well studied regarding its association with high-grade lesions or cervical cancer, with varying results between different geographical regions. In Italy, two studies showed that the Arg/Arg genotype at codon 72 was associated with increased risk of high-grade lesions,[14,15] HPV infection,[14] and high-risk HPV infection[14] compared to the control group.

Other studies showed that homozygous Arg/Arg genotype was associated with an increased risk of cervical cancer.[16,19] Conversely, authors from different regions of the world such as China, Thailand, Korea, the United States, and Portugal found no association between Arg/Arg homozygosity and increased risk of high-grade lesions and/or cervical cancer.[20–25]

Meta-analyses conducted to establish the role of the Arg/Arg homozygous genotype also found divergent results,

some showing association only with invasive cervical cancer, but not with pre-invasive lesions[12,13] while others found no association.[26,27]

It is postulated that the Pro and Arg polymorphic forms have different biochemical and biological properties that can influence the interaction with the HPV E6 protein.[11,28] This indicates that the degradation of p53 by E6 is different according to the polymorphism at codon 72; Arg is more efficiently inactivated by E6 from high-risk viruses, in particular HPV 16 and 18, than Pro. Thus, the Arg/Arg homozygous genotype is more susceptible to degradation than the heterozygous form (Arg/Pro) or the Pro/Pro homozygous genotype.[11,14,24] A study by Storey et al.,[11] which gave rise to the possible role of Arg in carcinogenesis related to HPV, points out that individuals who are carriers of the Arg/Arg homozygous genotype are seven times more susceptible to tumorigenesis associated with HPV compared to those with the Arg/Pro heterozygous genotype.

Moreover, some authors point out that results showing no association between the Arg/Arg homozygosity and increased

Table 4 – Genotype distribution and allelic frequencies of codons 11, 72, and 248 in the age group ≥30 years.

	Codon 11						
	Genotype distribution ($n = 113$)[a,c]				Allelic frequencies ($n = 226$)[a,d]		
Groups	Glu/Glu N (%)	Glu/Gln or Lys N (%)	Gln/Gln or Lys/Lys N (%)	Total N (%)	Glu N (%)	Gln or Lys N (%)	Total N (%)
Positive	45 (90.0%)	3 (6.0%)	2 (4.0%)	50 (100%)	93 (93.0%)	7 (7.0%)	100 (100%)
Negative	28 (90.3%)	2 (6.5%)	1 (3.2%)	31 (100%)	58 (93.6%)	4 (6.4%)	62 (100%)
Control	31 (96.9%)	1 (3.1%)	–	32 (100%)	63 (98.4%)	1 (1.6%)	64 (100%)
Total	104 (92.0%)	6 (5.3%)	3 (2.7%)	113 (100%)	214 (94.7%)	12 (5.3%)	226 (100%)

	Codon 72						
	Genotype distribution ($n = 113$)[b,e]				Allelic frequencies ($n = 226$)[b,f]		
Groups	Arg/Arg N (%)	Arg/Pro N (%)	Pro/Pro N (%)	Total N (%)	Arg N (%)	Pro N (%)	Total N (%)
Positive	23 (46.0%)	19 (38.0%)	8 (16.0%)	50 (100%)	65 (65.0%)	35 (35.0%)	100 (100%)
Negative	9 (29.0%)	12 (38.7%)	10 (32.3%)	31 (100%)	30 (48.4%)	32 (51.6%)	62 (100%)
Control	5 (15.6%)	12 (37.5%)	15 (46.9%)	32 (100%)	22 (34.4%)	42 (65.6%)	64 (100%)
Total	37 (32.7%)	43 (38.1%)	33 (29.2%)	113 (100%)	117 (51.8%)	109 (48.2%)	226 (100%)

	Codon 248						
	Genotype distribution ($n = 113$)[a,g]				Allelic frequencies ($n = 226$)[a,h]		
Groups	Arg/Arg N (%)	Arg/Trp or Gln N (%)	Trp/Trp or Gln/Gln N (%)	Total N (%)	Arg N (%)	Trp or Gln N (%)	Total N (%)
Positive	49 (98.0%)	1 (2.0%)	–	50 (100%)	99 (99.0%)	1 (1.0%)	100 (100%)
Negative	31 (100%)	–	–	31 (100%)	62 (100%)	–	62 (100%)
Control	31 (96.9%)	–	1 (3.1%)	32 (100%)	62 (96.9%)	2 (3.1%)	64 (100%)
Total	111 (98.2%)	1 (0.9%)	1 (0.9%)	113 (100%)	223 (98.7%)	3 (1.3%)	226 (100%)

[a] Extension of the Exact Fisher's test.
[b] Pearson's Chi-square test.
[c] $p = 0.781$.
[d] $p = 0.309$.
[e] $p = 0.016$*.
[f] $p < 0.001$*.
[g] $p = 0.432$.
[h] $p = 0.462$.

risk of high-grade lesions or cervical cancer may be related to various factors such as the methodology used in different laboratories, misclassification resulting from inter-laboratory variation in protocols, study design, sample selection errors (e.g., choice of control group), ethnic differences that can display changes in allelic frequencies, source of DNA samples, sample size, HPV infection status, and genotypic geographical variation.[11,12,14,19,24,27,29]

Regarding geographical variation, populations living closer to the equator seem to have a higher percentage of Pro alleles compared to those living in countries further North.[25] Beckman et al.[30] commented further that ethnic and climate variations suggest that the polymorphism of codon 72 is balanced and maintained by natural selection. The allele encoding Arg in the p53 gene is found most commonly in people of Caucasians in African American ethnicity.[22] In a family association study, the Arg allele was significantly overly transmitted in people of the Caucasian ethnicity with

high-grade lesions and invasive cervical cancer, especially in cases of infection by HPV 16 and/or 18.[24]

The meta-analysis conducted by Koushik et al.[13] highlighted that some studies on p53 polymorphism at codon 72 were not in Hardy–Weinberg equilibrium, which could explain discordant results. This is a principle based on both gene and genotype frequencies that remain constant between generations in a population with infinitely broad interbreeding in which mating occurs randomly, and there is no selection, migration, or mutation.[22] In our study, the distribution of genotypes at codon 72 was in Hardy–Weinberg equilibrium in the three analyzed groups (data no shown), which favors the obtained results.

Considering age, we observed that the association between Arg/Arg homozygosity at codon 72 and HPV E6/E7 mRNA expression was detected only in samples from women aged ≥30 years. In these women HPV infection may result from new HPV infections in sexually active women or reactivation

of a latent or previously acquired infection. This may correspond to persistent infection by oncogenic viral types, and consequently, it may be associated with increased risk of pre-neoplastic and neoplastic lesions in the cervix. In association, the decreased immune function that sets in with age leads to a reduced capacity of the innate and adaptive immune system to respond to previous and new infections.[31] These factors may explain the findings of our study and suggest that the Arg/Arg homozygous genotype is another characteristic that may, in conjunction with other risk factors, facilitate the acquisition of new infections or persistence of high-risk HPV infection in women over 30 years of age.

Some studies on gynecologic and non-gynecologic malignant tumors and benign diseases evaluated p53 polymorphisms at codons 11 and 248; codon 248 is the site where most p53 cancer-related mutations occur.[32] Other studies report polymorphisms at codons 11 and 248 in gastric cancer,[33] lung tumors,[8] ovarian cancer,[9] and bladder cancer.[7]

The lack of data on p53 polymorphisms at codons 11 and 248 in lower genital tract diseases motivated the inclusion of these analyses in our study. However, we found no statistically significant difference in genotype distribution or allelic frequencies in polymorphisms at codons 11 and 248 between the three studied groups.

Our study has some limitations, such as a control group with fewer samples than the experimental groups, and the impossibility to evaluate the presence of other risk factors for high-risk oncogenic HPV infection in order to verify if they could interfere with the results. In addition, because this was a cross-sectional study, a long-term follow-up, to evaluate if the association between the Arg/Arg homozygosity and positive HPV E6/E7 mRNA expression is maintained over time or associated with an increased chance of developing pre-neoplastic or neoplastic lesions, was not possible. In fact, cytological/histological results of our study were normal in 35.1%, low-grade lesion in 52.1%, and high-grade lesion in 12.8%. No statistically significant difference in genotype distribution of p53 polymorphisms was found among the three studied groups (data not shown). It is also important to highlight that, as hybrid capture detects more types of HPV than NucliSENS®, the negative group can be E6/E7 mRNA positive for other HPV genotypes other than 16, 18, 31, 33, and 45.

This is the first study to evaluate the association between p53 polymorphisms at three codons and HPV E6/E7 mRNA expression providing new information about factors that can interfere with high-risk HPV infection. Because the role of p53 polymorphism at codon 72 may be different depending on geographical regions, our data are the first to suggest that the Arg/Arg homozygous genotype can increase the chance of positive HPV E6/E7 mRNA expression. Considering that this study was conducted in Brazil, we believe that the polymorphism is associated with this population.

In conclusion, p53 Arg/Arg homozygous genotype at codon 72 was associated with positive HPV E6/E7 mRNA expression; the chance for a positive expression in the studied group with this genotype was significantly increased by a factor of 2.6 times compared to the other two studied groups, and by a factor of 3.7 times when compared to the control group alone. This association was stronger in samples from women in the age group ≥30 years. The p53 polymorphisms at codons 11

and 248 were not associated with positive HPV E6/E7 mRNA expression.

Conflicts of interest

The authors declare no conflicts of interest.

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