Precancerous squamous intraepithelial lesions by human papillomavirus infection and *p53* R72P polymorphism in Mexican women

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Abstract

The aim was to determine the association between R72P polymorphism of p53 gene and the risk of developing squamous intraepithelial cervical lesions in HPV-16 and /or 18 infected women. Two groups of women were included in this study: 74 patients HPV-16 and /or 18 positive with a cytological and colposcopy diagnosis of squamous intraepithelial lesion and a group of unrelated 130 healthy blood-donors. The viral genotype, allele and genotype of the polymorphism frequencies were determined by PCR approached. The results were analyzed with the statistical programs DeFinetti and STAT intercooled v11.1. Patients with high-grade squamous intrahepitelial lesions (HG-SIL) were infected mainly by HPV-16 (60.72%) compared to low-grade lesions (LG-SIL) (39.28%) (OR 3.14; p= 0.037), with HPV-18 genotype 68.96% of LG-SIL and 31.04% were HG-SIL (OR=0.24, p=0.006). HG-SIL were more common in patients carrying both viral genotypes (70.59% *vs* 29.41%) (OR 2.8, p = 0.008). A statistically significant association was observed between the genotype R/R and HG-SIL (OR=11.25, IC 3.8-33.29, p= 0.000) compared to those with LG-SIL. The P/R genotype was significantly more frequent in patients LG-SIL, compared to HG-SIL (OR=0.27, p=0.00). In conclusion patients with the R/R genotype showed more susceptibility to HPV-16 infection and they have almost 12 times more risk probability of HG-SIL compared to women having the heterozygous genotype and HPV-18.

Keywords: Human papillomavirus, p53 gene, *R72P* polymorphism, cervical cancer

Introduction

Harald zur Hausen (2009) established the main oncogenic genotypes of human papillomavirus (HPV) were the causative agents in the development of cervical lesions and cervico-uterine cancer (CUC). In fact, the genotypes HPV-16 and 18 are responsible for over 75% of cases of CUC worldwide (Brown et al., 2005; Smith et al., 2007). However, it must be noted that most HPV infections no progress, albeit of its molecular search; which suggests that non-viral causes for develop of cancer could exist. This differential biological response can be explained by several polymorphisms within p53 gene, one of the most well studied. One specific single-nucleotide polymorphism of 53 gene is the G-to-C variation in exon 4 (rs1042522, Arg72Pro), which results in an arginine-to-proline substitution located in codon 72 (El tahir et al., 2012; Habbous et al., 2012). Its alleles can exert different biochemical properties: p53 72R is a more efficient inducing apoptosis than p53 72P, whereas p53 72P has been reported to be a more efficient activator of DNA-repair and cell cycle arrest (Proestling et al., 2012).

Since 1998 it was reported that individuals homozygous for arginine 72 are about seven times more susceptible to HPV-associated tumorigenesis than heterozygotes, at least in White and East Asian populations (El khair et al., 2010; Storey et al., 1998). This was attributed to the viral oncoprotein E6 being able to bind and suppress the p53 protein of the arginine form more easily (Richard et al., 2010), disabling it from fulfilling its tumor-suppressors role (Storey t al., 1998). Many studies have been carried out trying to look for a possible association between p53 R72P polymorphism and the risk of developing CUC (El tahir et al., 2012; Klug et al., 2009; Sousa et al., 2007; Storey et al., 1998) including Mexican women (Piña-Sanchez et al., 2011; Sifuentes-Alvarez and Reyes-Moreno, 2003; Suarez-Rincon et al., 2002) with mixed results. The main objective of the present study was to determine the association between p53 R72P polymorphism

and the risk of developing cervical lesions in Mexican patients infected with the HPV-16 and /or 18 genotypes.

Material and Methods

We analyzed 74 gynecological patients with high-grade (HG-SIL) and low-grade squamous intraepithelial lesions (LG-SIL) positive for HPV-16 and /or 18, with an average of 40.62 years old (range of 17-77) and were classified according to type of injury: 38 (51.35%) had HG-SIL and 36 (48.65%) had LG-SIL by vaginal cytology. All patients attended the dysplasia clinics at the Hospital General Regional "Dr Bernardo J. Gastelum" at Culiacan, state of Sinaloa, Mexico, and the Hospital Civil of Urban Health Center at Culiacan, Sinaloa, Mexico, during July-September, 2012 as a measure to prevent CUC. Each patient answered a standard questionnaire survey which included a background of pathological and non-pathological past medical history. Also a vaginal swab sample was taken for DNA extraction. Other group consisted of 130 women unrelated healthy blood donors from the Blood Bank, Hospital General Regional "Dr Bernardo J. Gastélum" was used as a control, and with an average age of 27 years old (range 18-51). The Mexican blood banks perform a questionnaire, based in the Official Mexican Standard (NOM-003-SSA2-1993), about risk factor of several diseases and discard to those women with moderate or severe cervical dysplasia and the blood-donors should be serologically negative for HIV, hepatitis C or B viruses. After written informed consent was obtained, bloods samples were collected from participants. Patients and blood-donors were recruited considering their origin from the northwest state of Sinaloa, Mexican mestizo's ethnicity. The study was approved by the Public Health Ethics and Research committee of the Universidad Autonoma de Sinaloa.

Vaginal swab samples were used for DNA extraction and purification by phenol-chloroform and proteinase K method, following conditions previously described (Chan et al. 1994). DNA of blood samples was obtained by the DTAB-CTAB method according to the conditions described

by Gustincich et al. (1991). The DNA concentration and purity was determined by spectrophotometry at a wavelength of 260/280 nm. The amplification of HPV fragments was performed by polymerase chain reaction (PCR), which was carried out following the conditions previously used by Salazar, Mercado and Calzada (2005), with the nucleotide sequences: 5'-aaggccaactaaatgtca-3' and 5'-gcggatcctgtctgcttttatactaa-3' for HPV-16 which amplifies a fragment of 228 bp, and 5'-accttaatgaaaaacgacga-3' and 5'-cgtcgttggagtcgttcctg-3' for the HPV-18 amplifying a 100 bp fragment.

The p53 R72P polymorphism was determined using polymerase chain reaction technique with specific oligonucleotides (PCR-ALO) with the following primers of the reaction: for allele arginine (5'-tccccttgccgtcccaa-3 and 5'-ctggtgcaggggccacgc-3') which amplifies a fragment of 144 bp, and for the proline allele the primers (5'-gccagaggctgctccccc-3' and 5'-cgtgcaagtcacagactt-3') were used to amplify a fragment of 171 pb, whose amplification conditions were previously described (El tahir et al., 2012). The PCR products were separated by electrophoresis in 6% of polyacrylamide gels and subsequently stained with silver nitrate. The allele and genotype frequencies were established by direct counting. Genotype distribution deviations from Hardy-Weinberg expectations, and comparison between groups were evaluated by Fisher's exact tests. *De Finetti* program was employed for these analyses (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl).

Results

Atypical squamous cells of undetermined significance were reported in the 74/240 (30.83%) patients and classified to accord to type of cervical lesion. The females were evaluated trough vaginal cytology and HG-SIL 38 (51.35%) and LG-SIL in 36 (48.65%) women were observed (Table 1). Molecular identification showed 28 (37.84%) carry HPV-16 genotype, 29 (39.19%) HPV-18 and 17 (22.97%) had both viral genotypes. Patients with HG-SIL were infected mainly

by HPV-16 (60.72%) compared to LG-SIL (39.28%) (OR 3.14; p= 0.037). The HPV-18 genotype was found in 60.98% of women with LG-SIL and 31.04% HG-SIL (OR=0.24, IC= 0.09-0.67, p=0.006). However, HG-SIL was more common in patients carrying both viral genotypes (70.59%, OR 2.8, IC=0.89-9.1, p = 0.008) (Table 1).

The genotype R/R and the presence of HG-SIL (OR=11.25, IC 3.8-33.29, p=0.000) compared to those having LG-SIL are showed in Table 2. This statistical significance data show that patient with the arginine homozygous genotype has a probability of 11.25 more times the risk of developing HG-SIL, compared with those who have the heterozygous genotype. Additionally, the P/R genotype was significantly more frequent in patients having LG-SIL, compared to HG-SIL patients (OR=0.27, IC=0.15-0.47, p=0.00), showing also a statistical significance (Table 2). When hemo-donors volunteer women and HPV-infected patients results were analyzed, we found that in blood-donor group, 43.85% had the R/R risk genotype, while in HPV-infected group they also had a higher frequency of R/R genotype 39/74 (52.7%) mainly those who were HPV-16 positive 20 (71.42%). Even the sample number difference among HPV-infected patients (n=74) and hemo-donors (n=130) women, the heterozygous P/R genotype was (45.9 *vs* 41.12%); however, a statistical significance was observed (p=0.017). Respect to the homozygous P/P genotype, a strong statistical significance was observed between patients and hemo-donors (p=0.032) (data not show). A survey was applied to the 74 patients who were positive for HPV viral subtypes 16 and /or 18, about some activities and /or habits considered to be risk factors for the development of cervical cancer. This information was analyzed in relation to the polymorphism genotyping result R72P and the type of cervical lesions in the patients. Logistic regression analysis was used to determine a possible association between the probabilities of the event in relation to other variables. The results showed that HPV genotype and a family history

of tumors were associated with the type of cervical lesion (p=0.009), but no association was found for other factors included in the survey (data not show).

Discussion

This work represents the first study in population of Sinaloa, Mexico, focused on relating the p53 R72P polymorphism with the risk for the development of cervical cancer in patients with high and low grade squamous intraepithelial cervical lesions and also having a molecular diagnosis for HPV-16 and /or 18.

In this study 270 samples of cervical cells were analyzed by PCR of which, 74 (27.4%) were positive for HPV, (28 for HPV-16, 29 for HPV- 18 and 17 with both genotypes) (Table 1). Thirty eight (51.35%) of the 74 patients had HG-SIL and 36 (48.65%) LG-SIL. This can be related to previous data (Lopez-Saavedra and Lizano-Soberon, 2006; Salazar, Mercado and Calzada, 2005) which report the presence of HPV DNA in approximately 90% of cervical cancer tissue samples analyzed, and to almost 55% in samples of penile cancer. In these studies, HPV-16 is the most common with a positivity of more than 50%, followed by HPV-18 with about 15% and the rest spread over almost 10 viral subtypes also considered with oncogenic potential. In contrast to our study, the low percentage that showed positivity could be explained by the fact that we only analyzed for the genotypes HPV-16 and HPV-18 and not other HPV genotypes, and that our samples were from patients with a precancerous cervical lesions and not from documented cancerous tissue as previous reports (Klug et al., 2009). The association between the type of cervical lesion and viral genotype showed that high-grade lesions are strongly associated with HPV-16 with a probability of 3.14 times to develop this type of injury compared with HPV-18 with a value of p=0.006. Similarly the patients with co-infection with both genotypes are 2.8 times more likely to develop high-grade cervical lesions (p=0.008) compared to infected patients with HPV-18 alone.

Table 1. Precancerous lesions and HPV status in women with from Sinaloa, Mexico.

n= 74 (%)	LG-SIL	HG-SIL	OR	CI	*p*
	36 (48.65%)	38 (51.35 %)			
HPV-16 (n=28)	11 (39.28 %)	17 (60.72 %)	3.14	0.7-4.7	0.037*
HPV-18 (n=29)	20 (68.96 %)	9 (31.04 %)	0.24	0.09-0.67	0.006**
Co-infection (n=17)	5 (29.41 %)	12 (70.59 %)	2.8	0.89-9.1	0.008***

LG-SIL: Low-grade squamous intraepithelial lesion; HG-SIL: High-grade squamous intraepithelial lesion; HPV: Human papillomavirus; OR: Odds ratio, *p*=Pearson chi-square test. **p*-value <0.05 for patients with HG-SIL and HPV16-infected compared to LG-SIL group. ***p*-value <0.05 for patients with LG-SIL and HPV18-infected compared to HG-SIL group. ****p*-value < 0.05 for patients with HG-SIL and both genotype viral compared to LG-SIL.

The genotype and allele frequencies of the patients were obtained by direct counting and were organized according to the type of HPV infection; it was observed that in patients infected with HPV-16 genotype, the more prevalent genotype was the homozygous form for the arginine allele (R/R) with 71.42% compared to patients with the heterozygote form (R/P) representing 28.58 %, and only one patient with the homozygous genotype (P/P) for proline allele.

In HPV-18 infection patients, it was found that 31.03% had the R/R genotype, smaller percentage (40.39% less), than that of HPV-16 positive patients. In patients with the genotype P/R, this showed that HPV-18 has higher affinity to infect. The allelic frequencies of arginine showed the difference between HPV-16 infections with 84% against 63% of HPV-18. Co-infection of HPV

showed similarity between genotype frequencies of patients with HPV-16 infection (0.80 vs 0.84). In our study 52.7% of patients had the R/R genotype, this data is similar to other studies carried out in European populations, such as a study in the UK that showed 59% of patients R/R, in another study conducted in Germany was observed 56%, in the Netherlands with a 57%, but differs with another study in Greece, in which patients with CUC showed 31% of genotype R/R (Sousa et al., 2007). In a case-control population trial conducted in Jalisco, Mexico using cervical tissue samples embedded in paraffin, it concluded that there was no statistically significant evidence that the p53 R72P polymorphism was associated as a risk factor for developing cervical cancer (Suarez-Rincon et al., 2002). Our data differ from what was done in that study, since polymorphism genotype frequencies were frequently observed for the arginine allele in patients with precursor lesions of cervical cancer. In another study of a cohort of 102 patients with CUC in the state of Durango, Mexico, they analyzed the gene polymorphism R72R in P53 to determine the frequency and possible association with the risk of developing cervical cancer, and they found that a homozygous genotype for the arginine allele could be considered a factor that increases the likelihood to develop this cancer in the presence of an oncogenic HPV infection (Sifuentes-Alvarez et al., 2003). Although our study did not include samples of patients with cervical cancer, the fact that both studies showed similar results in terms of the frequencies of the polymorphism, may be possibly explained by a common genetic ancestry background, as both states (Sinaloa and Durango) are neighbors and there is a high rate of gene flow between populations of both states.

Table 2. HPV status, p53 Arg72Pro polymorphism and clinical stage in women from Sinaloa, Mexico.

	P/P(%)	P/R(%)	R/R (%)	OR	CI	*p*
HPV genoype						
HPV-16 (n=28)	…	8(28.58)	20(71.42)*	…	…	0.031*
HPV-18 (n=29)	1 (3.46)	19(65.51)	9(31.03)	…	…	…
Co-infection (n=17)	…	7(41.18)	10(58.82)	…	…	…
SIL stage						
HG-SIL (n=38)	1(2.64)	7(18.42)	30(78.94)	11.25	3.8-33.29	0.000**
LG-SIL (n=36)	…	27(75)	9(25)	0.27	0.15-0.47	0.000***
Blood donors				Pro(%)	Arg(%)	
Female (n=130)	12(9.23)	61(46.92)	57(43.85)	85(32.7)	175(67.3)	
Male (n=191)	25(13.09)	71(37.17)	95 (49.74)	12(31.68)	261(68.32)	

P/P: Proline homozygosity; P/R: Heterozygous; R/R: Arginine homozygosity; HPV: Human papillomavirus; SIL: Squamous intraepithelial lesion; HG: High grade; LG: Low grade; OR: Odds ratio; CI: Confidence interval. *p*: Pearson chi-square test. **p*-value <0.05 for patients infected by HPV-16 with arginine homozygosity compared to heterozygous genotype. ** *p*-value <0.05 for patients with HG-SIL and Arg/Arg genotype compared to heterozygous genotype. *** *p*-value < 0.05 for patients with LG-SIL and heterozygous genotype compared to arginine homozygous genotype.

The arginine allele is significantly more frequent than proline in ancestral european population as data observed in this study. There was no disequilibrium in the Hardy-Weinberg equation between the women healthy hemo-donors, which reinforces the hypothesis that polymorphism is associated with the risk of developing cervical lesions in patients with HPV-16 and /or 18. Using the Armitage's statistical test (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) in hemo-donors women, an equilibrium of Hardy-Weinberg in genotypes was observed, but a statistical significance was obtained between the P/P genotype of patients (p=0.032) where a minor frequency (1.35%) was observed respect the expected data according to the Hardy-Weinberg used model (4.38%). Additionally the heterozygous genotype P/R show statistical significance (p=0.017); while in patients having the R/R genotype no statistical significance was observed (p=0.053) (data not shown).

In this work we sought a possible association between the type of cervical lesion and the genotype polymorphisms. We observed a marked statistically significant association between the R/R genotype and the risk of developing HG-SIL with a p value= 0.000 and an odds ratio (11.25) showing that for patients with the genotype R/R, they are almost 12 times more likely to develop a HG-SIL compared with patients with at least one copy of the proline allele at codon 72 of p53. Regarding the possible relationship between polymorphism and HPV infection, we observed that patients with the R/R genotype have a higher likelihood of acquiring infection with HPV-16, followed by HPV-16 and 18 co-infection and less likely with infection with HPV-18 alone. Data show that patients with the R/R genotype were more susceptible to infection with HPV-16 genotype that HPV-18.

Because cancer is a multifactorial disease where genetic and environmental factors converge, this study analyzed some pertinent risk factors in the past medical history of our patients and applied logistic regression to evaluate whether there was any association between these factors and the

development of cervical lesions and R72P polymorphism. We did not find any statistically significant relationship between the polymorphism and tobacco consumption, whether active or passive. Patients with family history of cancer failed to demonstrate any statistically significant relationship; no statistically significant association was observed indicating a relationship between oral contraceptive use or not. Further, having one or more sexual partners in the group of patients showed no significant relationship between the polymorphism and cervical lesions. Finally initiation of sexual activity at early age also did not show a significant association, with a *p* value = >0.05.

According to logistic regression, none of the risk factors studied showed statistically significant relationship with the polymorphism, although drug use, family history of cancer and tumors showed a tendency of association. The types of HPV demonstrated an association with the type of injury and also the family history of tumors showed a statistically significant association. Our results are consistent with the study conducted by Piña-Sanchez et al. (2011) in a population of central Mexico which also showed a statistically significant association between the type of cervical lesion and HPV type. Data showed that patients with the genotype R/R have more susceptibility to HPV-16 and the development of high-grade cervical lesions and patients with the genotype P/R were more susceptible to HPV-18 and the development of low grade cervical lesions. Nested PCR regarding the presence and distribution of other HPV genotypes in our group of studies are currently underway in our laboratory.

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