XRCC1 Arg399Gln polymorphism and risk for cervical cancer development in Argentine women

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Summary

Background: XRCC1 (X-ray repair cross-complementing group 1) plays a central role in the DNA base excision repair mechanism. Single nucleotide polymorphisms (SNPs) in the XRCC1 gene are thought to modulate DNA repair capacity and have been linked to cancer risk in several studies. *Materials and Methods:* We conducted a case-control study comprising 217 cervical samples, including 103 cervical carcinomas and 114 normal tissue samples. Cervical samples were genotyped for two XRCC1 SNPs (*Arg194Trp* and *Arg399Gln*) by PCR-RFLPs. *Results:* Subjects carrying heterozygous *Arg399Gln* or the combined *Gln399Gln* + *Arg399Gln* variant genotypes had a significantly reduced risk for cervical cancer development. In addition, the *194Arg-399Gln* haplotype was also found to be associated with a decreased risk for cervical carcinoma. *Conclusion:* Our findings suggest that XRCC1 genotypes and haplotypes contribute in reducing the risk for cervical cancer development. Furthermore, genetic susceptibility conferred by *Arg399Gln* polymorphism operates independently of human papillomavirus infection of cervical tissue.

Key words: Cervical Cancer; HPV; Single Nucleotide Polymorphisms; XRCC1.

Introduction

Cervical cancer is the second most common cancer among women worldwide, with an estimated global incidence of 493,000 new cases and 274,000 deaths in the year 2002. In developing countries, where widespread screening is still unavailable, cervical cancer accounts for 15% of female cancers. The highest incidence rates are observed in sub-Saharan Africa, Melanesia, the Caribbean, South central and Southeast Asia and Latin America [1]. In Argentina, where disparate distribution of cervical cancer prevalence is remarkable, mortality rates associated with cervical cancer are higher in provinces with a lower socioeconomic level [2, 3].

Infection with high-risk HPV has been considered to be the major etiological factor in the development of cervical cancer. However, most of HPV infections are transient and only 1% of women infected with high-risk HPV will develop cervical carcinoma. This indicates that HPV infection is a necessary event but not sufficient for cervical carcinogenesis. Therefore, other factors, including environmental agents and host genetic background, may play crucial roles in the development of cervical cancer [4].

Exposure to different endogenous and exogenous mutagens and carcinogens can result in various types of DNA damage. These alterations, if not repaired, can cause genetic instability, mutagenesis and cancer [5]. The damage is fixed by multiple DNA repair pathways including base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair. A defect or reduced efficiency in any of these DNA repair mechanisms plays a critical role in the development of various age-related diseases, including cancer [5].

Among the five main DNA maintenance mechanisms operating in humans, base excision repair (BER) is the primary adopted by cells against reactive oxygen species, methylation, deamination, and hydroxylation [5]. The *XRCC1* gene (X-ray repair crosscomplementing group 1) is located on chromosome 19q13.2 and encodes a scaffolding protein involved in DNA BER. This protein interacts with a complex of DNA repair proteins, including poly (ADP-ribose) polymerase, DNA ligase 3, and DNA polymerase-β, promoting efficiency of the BER pathway [6, 7]. Shen *et al.* have identified three coding polymorphisms in the *XRCC1* gene at codons *194* (Arg to Trp), *280* (Arg to His) and *399* (Arg to Gln). *Arg194Trp* polymorphism is located in a linker region connecting the domains that interact with poly (ADP-ribose) polymerase and DNA polymerase-β, while *Arg399Gln* polymorphism is located in the functionally poly (ADP-ribose) polymerase binding region, within an identified *BRCA1* COOH terminus domain [8]. These polymorphisms, involving an amino acid change at evolutionarily conserved regions, can cause subtle structural alterations in the repair protein and consequently change the host susceptibility to cancer.

To date, there are controversial results related to the contribution of *XRCC1* SNPs to cancer susceptibility. Several molecular epidemiologic studies revealed that *XRCC1* polymorphisms were significantly associated with increased risk of cervical cancer [9, 10], as well as breast cancer [11, 12], lung cancer [13, 14], bladder cancer [15] and melanoma [16]. However, a null association with *XRCC1* polymorphisms has been reported in other studies [17-19].

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The XRCC1 protein has no enzymatic activity and during repair interacts with DNA polyb, PARP and DNA ligase III [20]. Moreover, the XRCC1 protein may also upregulate the activity of many other DNA repair enzymes [21] and genetic polymorphisms in the same and other DNA repair genes may influence cancer risk [22]. Because of these reasons, we hypothesized that variations in the XRCC1 gene may contribute to cervical carcinoma susceptibility. Consequently, we carried out a population-based case-control study considering XRCC1 as a candidate susceptibility gene for cervical cancer. We assessed the association of two XRCC1 SNPs, *Arg194Trp* and *Arg399Gln*, with the risk for cervical cancer development and their interaction with HPV infection.

Materials and Methods

A total of 217 cervical samples were obtained from an anonymous cervical specimen data bank in La Plata, Argentina. The specimens comprise 114 normal cytologies (collected from women who were attending screening), and 103 squamous cervical cancers. The mean age of women comprising the control group was ~40 years old (± 10.26 SD), and ~44 years old (± 9.87 SD) for those in the case group. Cervical specimens comprise exfoliated cells from the ecto-endocervix, collected using a cytobrush or spatula and kept frozen at –80°C. Cervical-biopsy specimens were formalin-fixed and paraffin-embedded or freshly frozen.

DNA extraction

Paraffin-embedded samples were washed twice with xylol and finally with 100% ethanol, re-suspended in 350 µl of proteinase K digestion buffer (250 mg/ml), and incubated for two hours at 56°C. Cervical exfoliated cell pellets and frozen biopsies were suspended and washed twice with 1 ml of PBS, and incubated for 24 hours at 56°C in 400 µl of digestion buffer (50 mM Tris-ClH pH 8,5; 1 mM EDTA; 1% Triton *X*100 and 0,5% Tween 20) containing 250 mg/ml of proteinase K (Genbiotech, Buenos Aires, Argentina). After proteinase digestion, the samples were kept for 10 min at 100°C for proteinase inactivation. DNA purification was conducted by the *salting out* procedure (direct protein precipitation methodology) described by Miller [23]. Finally, the DNA was suspended in distillate water. The samples were stored at 20°C until used.

Human papillomavirus DNA detection

Sample preparation, polymerase chain reaction (PCR) setup and amplicon analysis were performed in separate rooms to prevent contamination. Human papillomavirus DNA was detected in cervical tissues using a nested PCR approach, using MY09/11 as external primers and GP5+/6+ as internal ones, according to the methods previously described [24, 25]. MY09/11 and GP5+/6+ primers are the most commonly used system in PCR methods for the detection of genital HPVs. This strategy has been demonstrated to be highly sensitive and specific. PCR products were analyzed in 2% agarose gels stained with Safer Green and visualized using a blue light transilluminator.

XRCC1 genotyping

Genotypes of *XRCC1 Arg194Trp* and *Arg399Gln* polymorphisms were detected using a specific PCR-RFLP assay. The primers were designed according to the human *XRCC1* gene sequence (GenBank accession N° L34079), using the software FastPCR version 5.4.6. (University of Helsinsky, Finland).

The PCR oligonucleotides for the *XRCC1 Arg194Trp* polymorphism were: 5'-caa gct tgg cca gtt ccg tg-3' (**X194F)** and 5'-acc cac gag tct agg tct caa cc-3' **(X194R)** for forward and reverse primers. Primers defined a region of 150 base pairs. The reaction mixture was performed in a final volume of 25 µl: 5 µl of genomic DNA; 0.85 pmol/µl of each primer; 20 mM of each deoxynucleoside triphosphate; PCR buffer 1X (50 mM KCl and 10 mM Tris-HCl-pH 8.3), and 0.1% Triton X-100); 1.2 mM of $MgCl_2$, and 1.5 units of Taq DNA polymerase (Invitrogen). The amplification reaction was carried out under the following conditions: an initial melting step of 92°C for 3 min followed by 35 cycles of 30'' at 92°C, 50'' at 60°C and 40'' at 72°C; with a final elongation step of 72°C for 5 min.

The primers for *Arg399Gln XRCC1* amplification were: 5'-gca tcg tgc gta agg agt g-3' (**X399F)** and 5'-cag gat aag gag cag ggt tgg cgt -3' (**X399R)** for forward and reverse primers. These primers define a 100 base pairs fragment. The PCR reaction mixture (25 l) consisted of 5 l of genomic DNA, 1 pmol/µl of each primer, 20 mM of deoxynucleotide triphosphate, 1X PCR buffer (50 mM KCl and 10 mM Tris-HCl; pH 8.3), 1.2 mM $MgCl_2$, and 1.5 units of Taq polymerase. The conditions for the amplification reaction included an initial melting step of 92°C for 3 min followed by 35 cycles of 30'' at 92°C, 40'' at 59°C and 40'' at 72°C; and a final extension step of 72°C for 5 min.

The PCR products for both polymorphisms were checked on 2% agarose gels, stained with Safer Green and visualized with Safe Imager (Invitrogen, USA).

Genotyping of both polymorphisms was assessed by restriction enzyme digestion. PCR products were digested overnight at 37°C with five units of *Msp*I (Genbiotech, Argentina) per sample. The obtained fragments were resolved on 10% polyacrylamide gels, stained with Sybr Safe and visualized with blue light. The homozygous *XRCC1* codon *194 Arg* allele yields bands of 80, 55 and 20 bp while the *194Trp* allele yields only two bands of 100 and 55 bp. The heterozygous *Arg194Trp* genotype is determined by the presence of 100, 80, 55 and 20 bp bands, although 20 bp is too small to be clearly appreciated in the gel. The 55 bp band, generated by a constant restriction site, was used as an internal control for complete digestion. On the other hand, *XRCC1* codon *399* digestion yields 65 and 35 bp bands for the homozygous *Arg399Arg* genotype, while the homozygous *399Gln* allele generates a unique 100 bp band. The heterozygous *Arg399Gln* genotype is determined by the presence of three bands at 100, 65, and 35 bp. To avoid misclassification, a randomly selected subset of 20% of the samples was redigested for genotype confirmation.

Statistical analysis

Descriptive statistics were analyzed using the SPSS™ (*Statistical Package for Social Science*s) software version 15.0. Pearson's Chi-square test was applied to examine the Hardy-Weinberg equilibrium and the independence of genotype frequencies between cases and controls. To determine the polymorphisms risk estimation unconditional logistic regression was used to calculate crude and age adjusted odds ratio (OR) and they relative 95% confidence interval (CI). Finally, haplotype analyses were performed using the CHAPLIN (Case-Control Haplotype Inference; Emory University School of Medicine; USA) software version 1.2.2. CHAPLIN is a program for identifying specific haplotypes or haplotype features that are associated with disease using genotype data from a case-control study. This program provides estimates of haplotype effects on disease and also tests whether such effects are significantly different from zero using likelihood-based statistics.

Table 1. — *Analysis of association between XRCC1 polymorphisms and risk for cervical carcinoma.*

XRCCI Genotypes	Control N=114	Carcinoma (SCC) N=103	Adjusted OR (95% CI)	*p*
Codon 194				
Arg/Arg (CC)	98 (86.0%)	79 (76.7%)	1 (reference)	
Arg/Trp (CT)	12 (10.5%)	20 (19.4%)	1.55 (CI = 0.62-3.86)	0.349
Trp/Trp (TT)	4 (3.5%)	4 (3.9%)	1.19 (CI = 0.248-5.71)	0.829
Arg/Trp +Trp/Trp (CT+TT)	16 (14%)	24 (23.3%)	1.45 (CI = 0.643-3.28)	0.369
Allele frequency				
Arg (C)	208 (91.2%)	178 (86.4%)	1 (reference)	
Trp (T)	20 (8.8%)	28 (13.6%)	1.63 (CI = 0.89-3.00)	0.148
Codon 399				
Arg/Arg (GG)	37 (32.5%)	54 (52.4%)	1 (reference)	
Gln/Arg (AG)	59 (51.8%)	31 (30.1%)	0.45 (CI = 0.24-0.85)*	0.014*
Gln/Gln (AA)	18 (15.8%)	18 (17.5%)	0.66 (CI = 0.30-1.46)	0.305
Gln/Gln+Gln/Arg (AA+AG)	77 (67.5%)	49 (47.6%)	0.47 (CI = 0.25-0.89)*	0.020*
Allele frequency				
Arg (G)	133 (58.4%)	139 (67.5%)	1 (reference)	
Gln (A)	95 (41.6%)	67 (32.5%)	0.67 (CI = 0.45-0.99)*	0.049*

Results

Population data

XRCC1 polymorphisms were successfully genotyped in all samples. The specimens were also tested for the presence of human papillomavirus (HPV) DNA. Among the 217 samples included in this study, the global age ranged from 26 to 69 years, with a median age of 39.6 years (SD ± 10.26) for the control group, and 43.9 years (SD ± 9.87) for the case group. The prevalence of HPV ranged from 31.6% among the control group to 85.3% among squamous cell carcinomas (OR = 12.57; CIs = 6.39-24.59; X^2 = 63.35; *p* = 0.01).

XRCC1 allele frequencies

Gene and genotype frequencies for XRCC1 codon *194* and *399* polymorphisms in controls and cases are shown in Table 1. While there was no significant differences between the XRCC1 *Arg194Trp* alleles between controls and carcinomas (91.2% vs 86.4%, respectively), the XRCC1 *Arg399Gln* polymorphism showed a higher prevalence of *399Arg* in controls (58.4% for Arg*399* and 41.6% for 399*G*ln) than cases (67.5% for Arg*399* and 32.5% for *399Gln*), this difference being statistically significant (OR = 0.67; CI = 0.45-0.99; *p* = 0.049). These findings suggest an inverse association between the *Gln399* allele and cervical cancer risk.

XRCC1 genotypes frequencies and risk for cervical cancer development

The distribution of genotypes for both polymorphisms fit well as expected by the Hardy-Weinberg model in the control group. Results for the XRCC1 codon *399* showed a statistically significant difference in genotypes distribution (X^2 = 11.03; *p* = 0.004). Age-adjusted logistic regression analysis revealed that the heterozygous condition *Arg399Gln* was inversely related with cervical cancer risk (OR = 0.45; CIs = 0.24-0.85; *p* = 0.014) compared with women homozygous for the *Arg399* allele. Besides, when we combined the *Gln399Gln* and *Arg399Gln* genotypes they also presented the same relationship with respect to cervical carcinoma (OR = 0.47; CIs = 0.25-0.89; *p* = 0.02).

On the other hand, we did not find significant differences in *Arg194Trp* genotype distribution. Conversely, when the analysis was carried out with the combination of *Arg194Trp+Trp194Trp* genotypes, they provided a modest risk of borderline statistical significance, with a crude OR of 1.86. However, no association was found when adjusting for age. Risk estimations for cervical cancer and statistical tests are presented in Table 1.

XRCC1 polymorphisms and risk for HPV infection

In spite of the association between codon *399* polymorphism and cervical cancer risk, *Arg399Gln* genotypes were uniformly distributed among HPV positive and HPV negative women. Similar to these findings, we did not observe any association of XRCC1 *Arg194Trp* genotypes and viral infection. In this sense, none of the studied polymorphisms were found to provide a higher risk of viral infection. Prevalences and distribution of HPV infection among XRCC1 polymorphisms are presented in Table 2.

Haplotypes and the risk of cervical cancer

For the analyses of haplotypes, all potential models were taken into consideration. We found that subjects carrying the *Arg194-Gln399* combination have a protective effect for cervical cancer development (β = –0.92; Wald = –2.04; *p* = 0.040; Akaike AIC = 682.20), and this haplotype reached the strongest association under the dominant model.

Table 2. — *Analysis of association between XRCC1 polymorphisms and risk for HPV infection in control group.*

XRCCI Genotypes	HPV Positive N=36	HPV Negative N=78	Adjusted OR (95% CI)	*p*
Codon 194				
Arg/Arg (CC)	31 (86.1%)	67 (85.9%)	1 (reference)	
Arg/Trp (CT)	4 (11.1%)	8 (10.3%)	1.09 (CI = 0.30-3.96)	0.89
Trp/Trp (TT)	1 (2.8%)	3 (3.8%)	0.67 (CI = 0.065-6.78)	0.73
Arg/Trp +Trp/Trp (CT+TT)	5 (13.8%)	11 (14.1%)	0.97 (CI = 0.31-3.07)	0.96
Allele frequency				
Arg (C)	66 (91.7%)	142 (91.1%)	1 (reference)	
Trp (T)	6 (8.3%)	14 (8.9%)	0.92 (CI = 0.33-2.50)	0.87
Codon 399				
Arg/Arg (GG)	14 (38.9%)	23 (29.5%)	1 (reference)	
Gln/Arg (AG)	15 (41.7%)	44 (56.4%)	0.56 (CI = 0.22-1.37)	0.21
Gln/Gln (AA)	7 (19.4%)	11 (14.1%)	0.74 (CI = 0.31 -1.78)	0.51
Gln/Gln+Gln/Arg (AA+AG)	22 (61.1%)	55 (70.5%)	0.65 (CI = 0.28-1.51)	0.32
Allele frequency				
Arg (G)	43 (59.7%)	90 (57.7%)	1 (reference)	
Gln (A)	29 (40.3%)	66 (42.3%)	0.92 (CI = 0.52-1.62)	0.77

Discussion

Recent studies have focused the attention on identifying the effect of single nucleotide polymorphisms (SNPs) in several DNA repair genes, suggesting that these polymorphisms could affect individual's susceptibility to cancer. The XRCC1 protein plays an important role in the base-pair excision repair (BER) pathway. Shen *et al*. reported three coding nonsynonymous SNPs, *Arg194Trp*, *Arg280His*, and *Arg399Gln*, in the XRCC1 gene. Polymorphism in codon *399* of this gene has been extensively investigated in many epidemiological studies in relation to various types of cancer. The variant *Gln* allele was thought to reduce DNA repair activity and hence lead to increased DNA damage [26]. Moreover, this genetic polymorphism has been linked to an increased risk of lung cancer [14, 27], stomach cancer [28], and head and neck cancer [29]. On the other hand, this allele was also reported to be associated with a reduced risk of bladder cancer [30], esophageal cancer [31], non-melanoma skin cancer [32] and head and neck cancer [33].

Our population-based case-control study of 103 cases and 114 controls, revealed a significant association between *Arg399Gln* polymorphism and cervical cancer risk. Our findings are consistent with those from previous studies that reported a reduced risk of cancer for this polymorphism [30-33]. A study by Nelson *et al*. reported a decreased risk of non-melanoma skin cancer for those individuals who carried the *Gln399Gln* genotype. In a hospital-based case-control study conducted by Olshan *et al*. [33], both the *Gln399Gln* and the combined *Arg399Gln/Gln399Gln* genotypes were found to be associated with a decreased risk of head and neck cancer among patients in North Carolina. Stern *et al*. [30] also reported a slight decrease in risk of bladder cancer for subjects who carried the homozygous *Gln399Gln* genotype compared with those homozygous for the *Arg399* allele, although this difference was not statistically significant. Accordingly to these works, we found that both *Arg399Gln* and the combined *Gln399Gln/Arg399Gln* variant genotypes provided a protective effect against cervical cancer development among women in La Plata, Argentina. Other epidemiological investigations reported contrary findings. Two population-based case-control studies conducted by Huang *et al*. [9] among Chinese women and Niwa *et al*. [34] among Japanese subjects revealed that individuals with the homozygous *Gln399Gln* genotype are at higher risk of developing cervical carcinoma.

It is worth noting that, in our study, genotypes frequencies among women are remarkably different to those observed among subjects in Chinese and Japanese studies. These significant differences in frequency distribution between Caucasian and Asian populations have already been reported [35]. Comparison of the obtained allele frequencies for XRCC1 codon *194* and codon *399* with those published for Caucasian, African and Asian populations [14] revealed that the Argentine population is highly similar to Caucasians and quite different from Asian populations. This situation is very important at the moment of searching associations and interpreting results, since different findings in different populations could be attributed to genetic and ethnic disparities.

Whether the *Arg399Gln* polymorphism is associated with increased or reduced cancer risk may be a function of type and location of tumor. Although the *Gln* allele has been shown to diminish XRCC1 protein efficiency, our results showed that *Arg399Gln* and *Gln399Gln/Arg399Gln* genotypes still provide a decreased risk for cervical cancer. An Interesting model was proposed by Stern *et al*. [30] who hypothesized that cells with excessive oxidative damage that carry such variants would have a decreased ability to repair DNA damage and might be more likely to undergo apoptosis or senescence. Such decreased efficiency could be an "advantage" if it prevents the transmission and clonal expansion of mutations that could arise during BER.

Controversial data was also found for the *Arg194Trp* polymorphism. For instance, *Trp194Trp* genotype was reported to be associated with increased risk of certain carcinomas, including colorectal carcinoma and esophageal SCC [36, 37]. Conversely, this genotype was regarded in other investigations as a protective factor against bladder [38] and gastric carcinoma [39]. Our findings revealed that the combined *Arg194Trp/Trp194Trp* variant genotype provided a modest risk of borderline statistical significance, with crude OR of 1, 86. However, no association was found when adjusting for age.

Although XRCC1 *Arg194Trp* polymorphism occurs at an evolutionary conserved site involving an amino acid substitution, we hypothesize that it would be less likely to cause a significant change in protein repair function, and affect cancer susceptibility, since it resides in the linker region of *XRCC1* N-terminal domain.

It has been widely demonstrated that HPV infection is a crucial factor in cervical cancer development. Consequently, we further investigated the association between the two SNPs of XRCC1 gene and the risk for HPV infection. For the 217 women analyzed in this study the prevalence of HPV ranged from 32.3% among the control group to 84.7% among the squamous cell carcinomas. The prevalence of HPV infection was similar to that previously reported by our group among control subjects but lower among women with SCC [40]. No significant differences were observed in genotype and allele frequencies of both SNPs between HPV positive and HPV negative women. Furthermore, none of all variant genotypes were found to confer higher risk of viral infection. We suggest that genetic susceptibility found for *Arg399Gln* polymorphism operates independently of the infection status of cervical tissue.

In addition to single polymorphism analyses, we have examined the effect of haplotype combinations for both XRCC1 loci. All potential models were takin into consideration. Interestingly, we found that subjects carrying the *Arg194-Gln399* haplotype have a decreased risk for cervical cancer development. We also observed that the protective haplotype reached the strongest association under a dominant model, denoting that a single copy is enough to show the inverse association between the *Gln399* allele and cervical cancer risk.

In summary, our data supply evidence for the association between the *XRCC1 Arg399Gln* polymorphism and the risk of cervical cancer. Our findings revealed that both *Arg399Gln* and the combined *Gln399Gln/Arg399Gln* variant genotypes provided a reduced risk for cervical cancer development among women in La Plata, Argentina. Furthermore, haplotypes analysis confirmed and strengthened the association of the XRCCI gene with disease susceptibility. In this way, haplotype *Arg194-Gln399*, acting with a dominant effect, was found to decrease risk of cervical cancer. On the other hand, no association between XRCCI *Arg194Trp* polymorphism and disease outcome has been demonstrated. Both studied SNPs and haplotypes did not confer more risk for HPV infection.

Our results allow for only preliminary conclusions due to the small sample size, and thus larger studies are needed to further test the effects of XRCCI genetic polymorphisms on the risk of cervical cancer.

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