Acta Scientiarum. Health Sciences
ISSN: 1679-9291
eduem@uem.br
Universidade Estadual de Maringá
Brasil

Suzuki, Patrícia Sayuri; Brajão de Oliveira, Karen; Nóbrega Aoki, Mateus; Hideko Tatakihara, Vera Lúcia; Donizete Borelli, Sueli; Ehara Watanabe, Maria Angelica
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Acta Scientiarum. Health Sciences, vol. 30, núm. 2, 2008, pp. 95-100
Universidade Estadual de Maringá
Maringá, Brasil

DOI: 10.4025/actascihealthsci.v30i2.944

Investigating the association of chemokine receptor 5 (CCR5) polymorphism with cervical cancer in human papillomavirus (HPV) positive patients

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ABSTRACT. HPV is one of the most frequent causes for the development of cervical cancer. It is known that chemokines are important determinants of early inflammatory responses. The CC chemokine receptor 5 (CCR5) gene is involved in the chemotaxis of leukocytes toward inflammation sites. In the present study, polymerase chain reactions (PCR) in genomic DNA samples, using specific CCR5 oligonucleotide primers surrounding the breakpoint deletion, detected a 225 bp product from the normal CCR5 allele and a 193 bp product from the 32 bp deletion allele. The wild type genotype was prevalent in both group, but it was not statistically significant, with $\chi^2 = 1.519$ (2 degrees of freedom; $p > 0.05$). As there are a small number of Δ32 allele carriers, further studies are needed to clarify the role of CCR5 in the cervical cancer.

Key words: cervical cancer, CCR5, HPV.

RESUMO. Investigação da associação do polimorfismo do receptor de quimiocinas CCR5 com câncer cervical em pacientes HPV positivos. O HPV é um dos maiores responsáveis pelo desenvolvimento do câncer cervical. É conhecido que as quimiocinas são importantes determinantes da resposta inflamatória precoce. O produto do gene do receptor de quimiocinas CCR5 está envolvido na quimiotaxia de leucócitos para sítios inflamatórios. No presente estudo, reações em cadeia da polimerase (PCR) de amostras de DNA genômico, utilizando iniciadores específicos para CCR5 que flanqueam a região de deleção, foram utilizadas para detectar produto de 225 bp para o alelo normal e 193 bp para o alelo que apresenta a deleção de 32 bp. O genótipo selvagem foi o mais prevalente em ambos os grupos e não houve diferença estatisticamente significativa, com $\chi^2 = 1{,}519$ (2 graus de liberdade; $p > 0{,}05$). Uma vez que a prevalência de indivíduos portadores do alelo Δ32 é pequena, são necessários mais estudos a fim de elucidar o papel do CCR5 no cancer cervical.

Palavras-chave: câncer cervical, CCR5, HPV.

Introduction

There have been about 500,000 incident cases of and 275,000 deaths due to cervical cancer worldwide in recent years, equivalent to about a tenth of all deaths in women due to cancer (Parkin *et al*., 2005). The burden of cervical cancer is disproportionately high (> 80%) in the developing world (Parkin and Bray, 2006).

Cervical cancer development is a multi-step process. The major steps are HPV infection and HPV persistence for more than one year, followed by slow progression to pre-cancerous lesions and to invasive cancer. The risk factors for persistence and pre-cancer have not been disentangled (Shiffman *et al.*, 2007) and the relative importance of genetic and environment factors in the development of cervical tumors are not yet known (Zheng *et al*., 2006).

In addition to the loss of cellular control mechanisms, the role of antigen-specific tumor-infiltrating leukocytes and other immunocompetent cells have been considered as decisive factors in cervical pathogenesis (O'Brien *et al*., 2001; Evans *et al*., 1997). Microbial persistence in or around epithelial cells may lead to a chronic inflammatory state, resulting in increased epithelial cell turnover, and provide a stimulus for recruitment and activation of inflammatory cells from the blood stream (Moss and Blaser, 2005).

Chemokines and their receptors have shown a potential function in immunity against cervical tumors (Ghaderi *et al*., 2000; Kleine-Lowinski *et al*., 1999; Ohta *et al*., 2002). They are small chemotactic cytokines that direct the migration of leukocytes during inflammation and organize the homing of lymphocytes and macrophages into secondary lymphoid organs. Chemokines also promote the adhesiveness of target cells, regulate angiogenesis and may consequently control tumor growth (Rossi and Zlotnik, 2000). The receptors for chemokines are mainly expressed on immune and inflammatory cells, such as B- and T-lymphocytes and professional antigen-presenting cells in which ligand-receptor interactions on these cells lead to cell migration (Zheng *et al*., 2006).

Since cytokines, chemokines and free radicals initiate and perpetuate inflammatory responses, gene polymorphisms or genetic variations in immune related genes might be related to HPV persistence and progression to cancer (Moss and Blaser, 2005).

CC chemokine receptor 5 (CCR5) is involved in the chemotaxis of leukocytes towards inflammatory sites (Baggiolini, 1998), and is present only in certain cell types, such as lymphocytes, dendritic cells and macrophages (Loetscher *et al.*, 1998).

The CCR5Δ32 deletion may alter the expression or the function of the protein product (Sidoti *et al.*, 2005), resulting in a non-functional form of the chemokine receptor. This variant allele has been characterized and causes significant defects in the chemotaxis mediated by CCR5 ligands (Yang *et al.*, 2004; Smith *et al*., 1997). CCR5Δ32 has been implicated in a variety of immune-mediated diseases, could be associated with resistance to HIV infection and confers protection against asthma, rheumatoid arthritis (Zúñiga *et al.*, 2003), multiple sclerosis (Kaimen-Maciel *et al.*, 2007), and in a less severe spectrum of clinical manifestations in cutaneous leishmaniasis (Oliveira *et al.*, 2007).

More recently, CCR5 variant allele has been reported to confer significant risk for gallbladder cancer (Srivastava *et al*., 2008), and CCR5 heterozygous genotype may significantly influence the early stage of cervical cancer development (Singh *et al.,* 2008).

Since inflammation plays a major role in the pathogenesis of cancers and CCR5 plays an important role in inflammation, the aim of this study was to investigate the association of human papillomavirus (HPV) infection and cancer progression to chemokine receptor 5 (CCR5) polymorphism.

Material and methods

Patients: Following the approval from the Human Ethics Committee of State University of Londrina and State University of Maringá, peripheral blood cells were collected from 117 Brazilian women. Molecular analyses were performed at State University of Londrina, Paraná, Brazil. They were divided into two groups: HPV - positive and HPV - negative. The samples were collected from women under Pap smears routine proceedings for cervical cancer screening.

DNA extraction: Genomic DNA was isolated from peripheral blood cells using the technique described by Miller *et al.* (1988). DNA was extracted from whole blood in the presence of 0.2 M NaCl and 0.25% SDS, for 4h at 37°C. After precipitation with ethanol, the pellet was dried and resuspended in 50 μL of *milli Q* water.

CCR5 Genotyping: DNA was analyzed using polymerase chain reaction (PCR) with specific primers for CCR5, which were, Primer sense: 5' ACC AGA TCT CAA AAA GAA 3' and Primer anti-sense: 5' CAT GAT GGT GAA GAT AAG CCT CA 3', GenBank Accession number: AF009962. Reaction conditions for the PCR rounds were the same (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP and 1.25 units of Taq polymerase). PCR procedure was 5 min. denaturation at 94°C, 35 cycles of 1 min. at 94°C, 1 min. at 58°C and 1 min. at 72°C, and 10 min. elongation at 72°C, carried out in a thermocycler (PCR sprint, ThermoHybaid, Ashford, Middlesex, UK). All DNA amplification reactions were performed with appropriate negative controls in parallel to detect contamination at each step of the procedure. PCR products of 225 and 193 base pairs were analyzed by electrophoresis in a 10% acrylamide gel and visualized using the silver staining method.

Statistical analysis: Data were analyzed through the chi-square (χ_2) test with the level of significance set at $p < 0.05$. Demographic characteristics were evaluated by Anova, Microcal Origin™ 4.1 (Northampton, MA).

Results

During the gynecological examination, specimens for the Papanicolaou test were obtained from the endocervical canal with a cytobrush and from the exocervix using an Ayre spatula. Cytological changes HPV - related collected from the Pap smears which showed evidence of koilocytotic cells, described by Koss and Durfee (1956), were considered as enlarged epithelial cells with irregular, hyperchromatic nuclei, encircled by transparent and clear space (Figure 1).

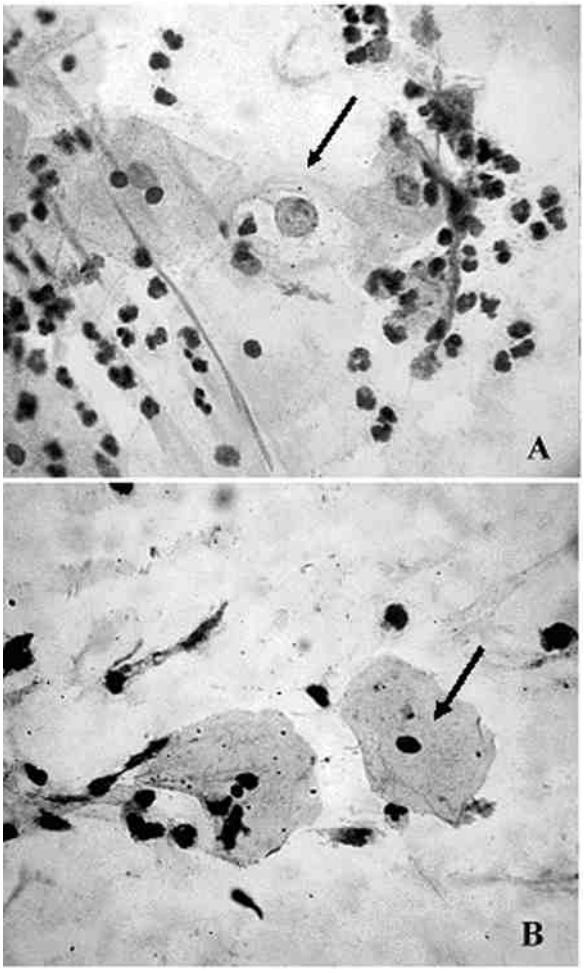

Figure 1. Pap smears: A - Koilocytosis (Papanicolaou stain, x 400); B - A normal Pap Smear at the screening power of 400X. Benign squamous cells

In this study, DNA samples from 117 Brazilian women, aged 13-81 were analyzed, including 43 HPV - negative women and 74 HPV - positive patients after Papanicolaou examinations (Table 1).

Table 1. Distribution of HPV - negative patients and HPV - positive patients according to age.

		HPV- (n = 43)	HPV+ (n = 74)
Age (years)	13-28	3	12
	29-44	8	35
	45-60	18	19
	61-76	10	7
	77-92	4	1

$X^2 = 17.742$; 4 degrees of freedom; $p = 0.0014$.

HPV infection was more prevalent in patients aged 29-44 years old, when patients and control groups were compared by chi square ($p = 0.0014$).

In order to verify the role of CCR5Δ32 polymorphism in the susceptibility of HPV infection, after DNA extraction, the deletion was assessed using Standard PCR Fragment Length Analysis. Figure 2 shows a breakdown of genotypes, wild-type (CCR5/CCR5) and Δ32 allele. It was possible to observe that genotypic distribution is similar between HPV - negative individuals and HPV - positive patients (Table 2).

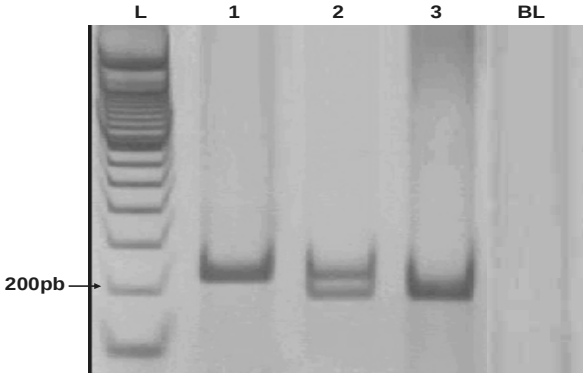

Figure 2. CCR5 Genotyping. The PCR products were detected using silver staining method in polyacrylamide gel. L: DNA Ladder 100 bp; 1, CCR5 wild type homozygous genotype (225 bp); 2 heterozygous genotype (193 bp and 225 bp); and 3 variant allele homozygous genotype (193 bp) and BL: represents Blank reaction.

Table 2. Genotypic and allelic frequencies for the Δ32 and wt alleles of CCR5.

Study subjects	Number of samples	Genotype (117)[a]			Allelic frequency	
		CCR5/CCR5 n (%)	CCR5/Δ32 n (%)	Δ32/Δ32 n (%)	wt	Δ32
HPV - negative	43⋆	38 (88.37)	04 (9.3)	01 (2.33)	0.93	0.07
HPV - positive	74⋆⋆	70 (94.59)	03 (4.05)	01 (1.35)	0.97	0.03

⋆HWE = 3.429; ⋆⋆HWE = 13.48; [a]HPV - negative patients X HPV - positive patients; $\chi^2 = 1.47$ (2 degrees of freedom; $p > 0.05$).

Regarding genotype distribution, the HPV - negative patient group is in accordance with the Hardy Weinberg equilibrium; however, the HPV - positive patient group showed a significant deviation from the Hardy Weinberg equilibrium, which may be supported by the small sample analyzed.

In both groups, the wild type genotype was more prevalent, and there was no significant difference in genotype and allele distribution between analyzed groups ($p > 0.05$). In spite of the wt/wt genotype predominance, 11.6% of HPV - negative individuals were Δ32 allele carriers compared to 5.4% from HPV - positive patients.

Among the 74 HPV - positive women, 60 (81%) were negative and 14 (19%) presented a positive diagnostic for cancer, of which 10 patients developed invasive epidermoid cancer. No difference in age range between HPV - positive women without cancer and the ones that developed cervical cancer, 13-81 and 29-74 years old, respectively, was observed.

Among cervical cancer patients (n = 14) 13 patients presented the wild type genotype and only one patient presented the heterozygous genotype. However, there was no significant difference in genotype frequency distribution when HPV - positive patients with and without cancer were compared ($p > 0.05$) (Table 3).

Table 3. Genotypic frequencies for the Δ32 and wt alleles of CCR5 for HPV - positive patients with or without cancer.

	CCR5/CCR5 n = 70	CCR5/Δ32 n = 3	Δ32/Δ32 n = 1
Cancer	13	1	0
Without cancer	57	2	1

HPV - positive with cancer X HPV - positive without cancer; $\chi^2 = 0.645$ (2 degrees of freedom; $p > 0.05$).

Discussion

Human papillomavirus (HPV) is common throughout the world. Although most infections with HPV cause no symptoms and are self-limiting, persistent genital HPV infection can cause cervical cancer in women (Walboomers *et al.*, 1999).

DNA samples from 117 Brazilian women, including 43 individuals negative for HPV and 74 HPV - positive patients after routine Papanicolaou examinations were analyzed.

In the present work it was not possible to determine which HPV type was responsible for cervical lesions once HPV - positive patients were diagnosed by cytopathological analysis. Koilocytotic atypia defined by Koss and Durfee (1956) and associated with HPV infection by Meisels and Fortin (1976) has been the most reliable indicative of cytomorphological lesions related to HPV (Yamamoto *et al.,* 2004). However, it is well established that, in the Brazilian population, the most prevalent types of high-risk HPVs that infect the uterine cervix are HPV 16 and HPV 18 (Bosch *et al.*, 1995; Eluf-Neto *et al.*, 1994).

In a study performed on cervical biopsy, in the samples of patients from 22 countries, HPV DNA was detected by polymerase chain reaction (PCR) in 99.7% of the cases of cervical cancer, indicating that HPV is in fact the main cause of this type of cancer (Muñoz, 2000).

Since only a small proportion of HPV infections will eventually lead to cervical cancer, other cofactors are needed for cervical cancer development (Schiffman and Kjaer, 2003). The relative importance of genetic and environment factors in the development of cervical tumors are not yet known (Zheng *et al.*, 2006).

Chemokines are important determinants of the early inflammatory response. It is known that chemokines have the potential to stimulate T-cell activation, although the pattern of activation may differ for different chemokine-chemokine receptor interactions (Nanki and Lipsky, 2001). Chemokines are reported to be produced by most of the cancer types (Dewin *et al.*, 2005). Strong evidence supports that chemokines are major determinants of macrophage and lymphocyte infiltrates found in melanomas; in carcinomas of the ovary, breast and cervix; and in sarcomas and gliomas (Luboshits *et al.*, 1999; Zheng *et al.,* 2006).

CCR5 plays an important role in the recruitment of macrophages, monocytes and T cells in inflammation (Panzer *et al.*, 2005; Spagnolo *et al.*, 2005) driving an immune response involving a Th1 cytokine pattern (Loetscher *et al.*, 1998). Host immunity, particularly T cell immunity (Th1/Th2 balance), plays an important role in clinicopathological features of malignant disease (Fujii *et al.*, 2004). Vaday *et al.* (2006) suggested that the pathogenesis of prostate cancer is influenced by inflammation, especially those caused by inflammatory chemokines, such as CCL5 (RANTES).

The CC chemokine receptor 5 (CCR5) delta 32 variant results in a non-functional form of the chemokine receptor, which is incapable of binding beta chemokines (RANTES, MIP-1alpha, MIP-1beta). CCR5Δ32 causes significant defects in the chemotaxis mediated by these ligands, once CCR5Δ32 deletion may alter expression or the function of the protein product (Smith *et al.*, 1997; Yang *et al.*, 2004). Recent evidence has also demonstrated the role of CCR5 in a variety of human diseases, ranging from infectious and inflammatory diseases to cancer (Balistreri *et al.*, 2007).

In the present study the wild type genotype was more prevalent in both groups, and no significant difference in genotype and allele distribution was observed between analyzed groups ($p > 0.05$). Although wild type genotype was predominant in both groups, Δ32 allele was observed in 11.6% of HPV - negative individuals and in 5.4% of HPV - positive patients; nonetheless, Zheng *et al.* (2006) reported that CCR5Δ32 deletion is imperceptibly associated with an increased risk of HPV infection ($p = 0.045$).

Cancer progression was verified in 19% (14/74) of HPV - positive women; however, no correlation was observed in genotype frequency distribution and cervical cancer development when HPV - positive patients with and without cancer were compared ($p > 0.05$).

Degerli *et al.* (2005) have also reported that

heterozygous CCR5/Δ32 genotype plays an important role in the progression of breast tumorigenesis. However, no statistically significant relationship was found between the Δ32 allele and laryngeal, breast carcinoma, thyroid carcinoma or brain carcinoma development. Although a discrete correlation between CCR5Δ32 allele and increased risk of HPV infection has been observed, cervical neoplasia was not associated with genetic polymorphism of CCR5 and CCR2 (Zheng *et al.,* 2006).

Singh *et al.* (2008) reported that CCR5 heterozygous genotype may significantly influence the early stage of cervical cancer development. Prior studies have also implicated the role of CCR5Δ32 in pancreatitis inflammation and its progression to pancreatic adenocarcinoma (Duell *et al*., 2006). However, no association was observed in CCR5 genotypes and allele frequencies in the susceptibility of patients to squamous cell carcinoma of head and neck cancer (Khademi *et al.*, 2008).

Any defect in the inflammatory cell recruitment because of defect in the chemokine receptors may lead to suppressed immune response (Azenshtein *et al.,* 2002). Oliveira *et al.* (2007) has proposed that leishmaniasis patients with mucocutaneous lesions present a stronger T-cell response and that delta32 carriers could have a weaker inflammatory immune response and migratory capacity due to the non-functional chemokine receptor.

Although there was no difference in CCR5Δ32 distribution between assessed groups in the present work, it was observed that 92.8% (13/14) of cancer patients presented the wild type genotype, and only one patient presented the variant allele in heterozygous state, which may be explained by a stronger inflammatory response, due HPV infection in wild type patients.

Conclusion

No correlation was observed between human papillomavirus (HPV) infection and chemokine receptor 5 (CCR5) polymorphism. However, due to the small number of Δ32 allele carriers examined and since CCR5 is an inflammatory chemokine, further studies comprising larger numbers of individuals carrying non-wild-type haplotypes are needed to elucidate the role of CCR5Δ32 in cervical cancer pathogenesis.

Acknowledgements

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, and Patricia Sayuri Suzuki received a fellowship from Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Pro-Reitoria de Pós-Graduação - State University of Londrina - PROPG-UEL. We acknowledge Prof. Philip Sidney Pacheco Badiz for English correction.

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Received on February 27, 2008.
Accepted on August 25, 2008.