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Analysis of *CYP1A1* and *COMT* polymorphisms in women with cervical cancer

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ABSTRACT. The aim of this case-control study was to obtain a comprehensive panel of genetic polymorphisms present only in genes (cytochrome P-450 1A1 - *CYP1A1* and catechol-O-methyl transferase - *COMT*) within the metabolic pathway of sex steroids and determine their possible associations with the presence or absence of cervical cancer. Genotypes of 222 women were analyzed: a) 81 with cancer of the cervix treated at the Cancer Hospital Alfredo Abram, between June 2012 and May 2013, with diagnosis confirmed surgically and/or through histomorphological examination; and b) 141 healthy women who assisted at the Endocrine Gynecology and Climacteric Ambulatory, Department of Gynecology, UNIFESP-EPM. These polymorphisms were detected by polymerase chain reaction amplification-restriction fragment length

polymorphism analysis and visualized on 3% agarose gels stained with ethidium bromide. We found a significant association between the frequency of the *CYP1A1* polymorphism and the development of cervical cancer. A statistical difference was observed between patient and control groups for *CYP1A1* polymorphism genotype distributions ($P < 0.05$). However, no significant differences were found in the *COMT* gene polymorphism genotype distributions between the patient and control groups ($P > 0.05$) or between other risk variables analyzed. The *CYP1A1* gene involved in the metabolic pathway of sex steroids might influence the emergence of pathological conditions such as cervical cancer in women who carry a mutated allele, and result in 1.80 and 13.46 times increased risk for women with heterozygous or homozygous mutated genotypes, respectively.

Key words: Gene polymorphism; *CYP1A1*; *COMT*; Cervical cancer

INTRODUCTION

Cervical cancer is one of the main causes of death in women worldwide, ranking together with breast cancer in developing countries (Parkin and Bray, 2006). The high-risk types of human papillomavirus are responsible for almost 90% of anogenital and oropharyngel cancers, including cervical cancer. Despite consistent evidence of association between HPV and cervical cancer, other factors are still necessary for the development and progression of this disease. These include the number of presumably infected sexual partners, which increases the chances of HPV infection, and an increased viral load, tabagism, nutritional deficiencies, hormone therapies, and the use of immunosuppression drugs and the natural immunity state of the individual (Pereira et al., 2007; Öslem et al, 2015). Additional factors, critical to the establishment of the disease, as well as death prevention are available for population in general on web sites, for example on INCA (www suppl. inca.gov.br).

However, with the understanding that cancer development is dependent upon a number of factors such as the time and intensity of exposure to carcinogens and individual genetic susceptibilities, we note that most genotoxic carcinogens that contribute to the development of tumors in humans are considered to be pro-carcinogens and are unable to react with DNA directly as they are stable compounds under physiological pH conditions. These, however, undergo biotransformation by enzymes such as cytochrome P450 (phase I) and glutathione *S*-transferases (phase II) into water-soluble compounds, which, therefore, are liable to be excreted. On the other hand, these products are also electrophilic and might react with regions of DNA leading to the formation of adducts that must be repaired prior to cell division, otherwise they might result in permanent DNA lesions including point mutations, or chromosome breaks or rearrangements. Lesions that occur in functional regions of genes that inhibit and/or stimulate cellular proliferation, such as in tumor-suppressing genes and proto-oncogenes, respectively, might lead to the loss or gain of function of these genes, and consequently might promote tumor onset. This type of DNA damage is irreversible and cumulative and, after as many as 5 to 7 alterations to relevant genes, can result in the acquisition of a neoplastic phenotype (Ribeiro et al., 2003).

Furthermore, it has been demonstrated that genetic alterations relating to genes involved in estrogen synthesis and metabolism, such as the cytochrome P450 polymorphisms 1A1 (*CYP*

1A1), 1B1 (*CYP 1B1*), 3A4 (*CYP 3A4*), 17 (*CYP 17*), and 19 (*CYP 19*), and polymorphisms in the catechol-O-methyltransferase (*COMT*) and glutathione *S*-transferase M1 (*GST*) genes and in estrogen and progesterone receptors might lead to an increase in estrogen and/or circulating catechol estrogens in response to sexual steroids. These phenomena might contribute in particular to the genesis of gynecologic neoplasias (Kitawaki et al., 2001).

The two main types of estrogen, estradiol and estrone (E2 and E1, respectively), are ligands of the estrogen receptor and substrates for oxidative enzymes such as *CYP1A1* (Dawling et al., 2004). Thus, one of the actuation mechanisms of estrogen (mainly E2) in cervical carcinogenesis involves proliferative stimulation of epithelial cells, which facilitates a higher occurrence of spontaneous mutations (Yager, 2000). The risk of endometrial cancer appears to be elevated for women whose levels of estrogens are relatively high, regardless of whether the estrogen originates exogenously (e.g., by means of hormone medication) or endogenously (e.g., as a result of obesity) (Amankwah et al., 2013). The carcinogenic potential of estrogen has been related not only to its mitotic activity but also to the role of estrogen catechols as carcinogenic metabolites, which can bind to DNA and lead to its damage and, in addition, upon their metabolism, can produce reactive oxygen species able to promote oxidative damage to the DNA as well (Liehr, 2000).

Several enzymes, therefore, are seen to be involved in estrogen metabolism both along phase I or phase II, some of them appear in the official literature as being associated with neoplastic processes. Of these, CYP1A1 (Nakachi et al., 1993; Sivaraman et al., 1994; Kawajiri et al., 1996; Vadlamuri et al., 1998; Taioli et al., 2013), COMT (Dawling et al., 2001; Mitrunen and Hirvonen, 2003; Lachman, 2008), and GST. In this study, we evaluated genes that codifying two of these enzymes: *CYP1A1* and *COMT*.

MATERIAL AND METHODS

A total of 222 women were recruited for this study, including 81 (patients) who had cervical cancer and were examined at Alfredo Abrão Cancer Hospital from June 2012 to May 2013, with confirmed diagnosis of cervical cancer based on histopathological findings, and 141 (control) healthy women assisted at the Endocrine Gynecology and Climacteric Ambulatory, Department of Gynecology, UNIFESP-EPM.

For both groups (patients and controls), genomic material was collected through peripheral venous puncture. Blood collection was performed using vacuum tubes containing an anticlotting agent, ethylenediaminetetraacetic acid (EDTA). The tubes were centrifuged for 15 min at 4000 cps for separation into plasma, leukocytes (pellet), and red blood cells. Nucleated cells (leukocytes) were deposited in 1.5-µL Eppendorf-type tubes for subsequent DNA extraction.

The study was approved by the Research Ethics Committee of the Federal University of São Paulo/São Paulo Hospital under protocol number 0118/08; all participants agreed with and signed an informed consent form.

DNA extraction

DNA extraction was performed by Kit Illustra™ Blood Genomic Prep protocol (GE, Little Chalfont, UK) for extraction of the material from the blood and epithelial cells scrapings. The purified DNA was stored at -80°C until its utilization. The amount of DNA in each sample was measured by spectrophotometry using a NanoDrop® 2000 full spectrum spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Gene polymorphisms were analyzed by means of a PCR-RFLP assay. The pairs of primers used were as follows: *CYP1A1* (*3801T>C* in the 3'-untranslated region), S5'-CAG TGA AGA GGT GTA GCC GCT-3' and AS 5'-TAG GAG TCT TGT CTC ATG CCT-3'; and *COMT* (*Val158Met*), S5'-TAC TGT GGC TAC TCA GCT GTG C-3' and AS5'-GTG AAC GTG GTG TGA ACA CC-3'.

For the PCR, the following items were used: 12 µL Master Mix (PCR Master Mix, 2.0X, Promega, Madison, WI, USA), 1 µL each primer (10 pmol/µL), 50-100 ng genomic DNA, and nuclease-free water, to obtain a 25-µL total reaction volume.

For PCR amplification to detect the *CYP1A1* polymorphism, the following temperature conditions and times were used: 94°C for 5 min for initial denaturation; 40 cycles at 94°C (denaturation), 62°C (annealing), and 72°C (extension) for 60 s at each stage; and 72°C for 7 min. For detection of the *COMT* polymorphism, the same times and temperature conditions as above were utilized, except that the annealing temperature was adjusted to 59°C.

The RFLP were carried out by appropriate endonucleases restrictions for the respective polymorphism: *CYP1A1*; *Msp*I (New England Bio Labs, Ipswich, MA, USA) and, *COMT; Nla*III (New England Bio Labs, Ipswich, MA, USA), complying with each manufacturer protocol.

The PCR products were loaded on a 3.0% agarose gel and the amplified fragments were visualized using an ultraviolet light transilluminator and photo-documented with the Kodak Digital Science 1D system (Eastman Kodak Company, Rochester, NY, USA). The migration of the PCR products presented a qualitative profile that enabled the differentiation of three genotypes: wild type, heterozygote, and mutant homozygote (Figures 1 and 2).

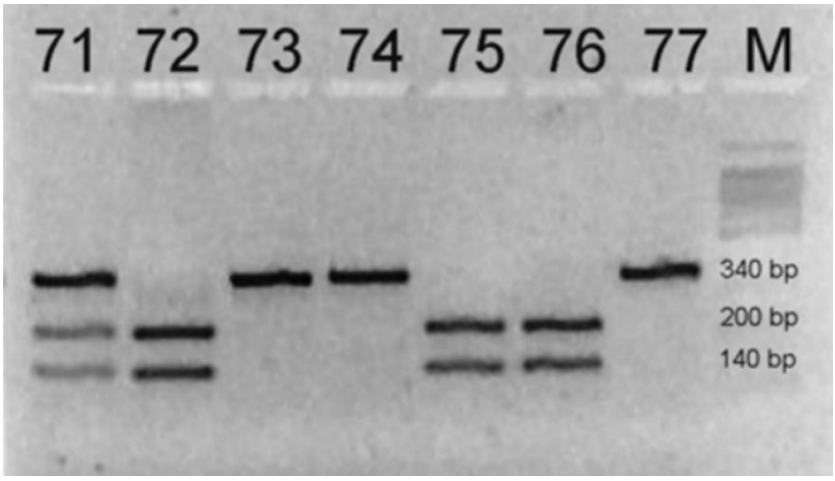

Figure 1. Electrophoretic analysis of the CYP1A1-*Msp*I polymorphism. Agarose gel (3.0%) stained with ethidium bromide, showing the results of restriction length polymorphism-polymerase chain reaction analysis of the CYP1A1 polymorphism. "M" represents the 100-bp molecular weight standard (Invitrogen, Carlsbad, CA, USA). *Lanes 73*, *74*, and *77* show the results for a wild-type homozygote patient with the CYP1A1-*Msp*I TT genotype; *lanes 72*, *75*, and *76* show the mutated homozygote (CC) genotype; *lane 71* shows the heterozygote standard (TC) genotype.

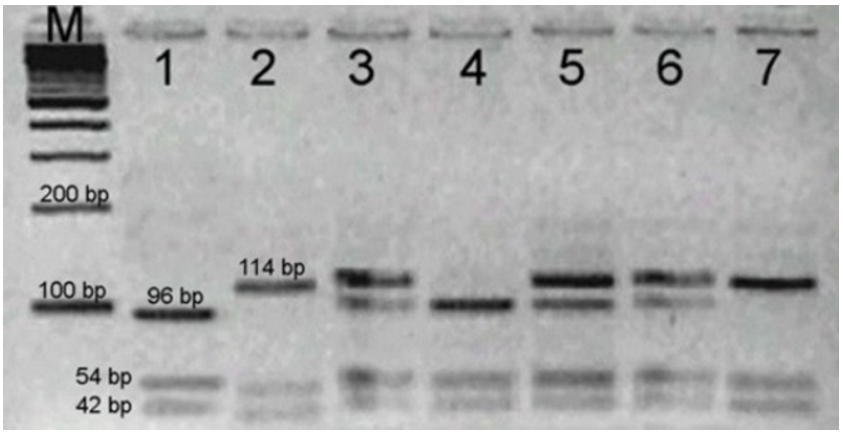

Figure 2. Electrophoretic analysis of the COMT polymorphism after digestion with *Nla*III. Agarose gel (3.0%) stained with ethidium bromide, showing results of restriction length polymorphism-polymerase chain reaction analysis of the COMT polymorphism. "M" represents the 100-bp molecular weight standard (Invitrogen, Carlsbad, CA, USA). *Lanes 2* and *7* illustrate the presence of the wild-type GG homozygote genotype for the COMT polymorphism; *lanes 3*, *5*, and *6* show the heterozygote GA genotype; *lanes 1* and *4* show the mutated homozygote standard genotype (AA).

RESULTS

Data analyses were performed using the SPSS Statistical Package for Social Sciences program (v16.0) (SPSS, Chicago, IL, USA). For a comparison of qualitative variables, i.e., frequencies and ratios, the exact Fisher test was used and the expected genotypic frequencies were estimated based on the allelic frequencies noted; deviations in the Hardy-Weinberg equilibrium (HWE) were calculated through the χ^2 test. The OR and CI estimates were performed through binary logistic regression. The amount of statistical significance was established at 5%, or $P < 0.05$.

Table 1 demonstrates the observed and expected numbers in the patient and control groups for the polymorphisms being studied. χ^2 values are presented for shifts from HWE. The genotypic distribution of polymorphisms fit HWE in the patient and control groups ($P > 0.05$).

Table 1. Hardy-Weinberg equilibrium analysis of the polymorphisms tested.

	Wild-type	Heterozygote	Mutant	χ^2	P value
CYP1A1					
Patients					
Observed	29.0	33.0	19.0	2.66	0.10
Expected	26.4	40.3	15.4		
Controls					
Observed	85.0	52.0	4.0	1.44	0.23
Expected	87.4	47.2	6.4		
COMT					
Patients					
Observed	32.0	38.0	11.0	0.00	0.98
Expected	32.0	38.0	11.0		
Controls					
Observed	57.0	71.0	13.0	1.89	0.17
Expected	60.7	63.6	16.7		

P value with 1 degree of freedom. χ^2 (chi-square test).

Table 2 separates the genotypic distributions of the polymorphisms being studied, both in the patient and control groups, as well as the P values corresponding to the exact Fisher test. A statistical difference between the patient and control groups was noted for the genotypic distributions of the *CYP1A1* (P < 0.05) polymorphism. No statistical difference was noticed between the patient and control groups for the genotypic distributions of the *COMT* polymorphism (P > 0.05).

Table 2. Genotype analysis of the polymorphisms studied.

	Wild-type	Heterozygote	Mutant	Total	Fisher exact test
CYP1A1					
Patients					
N	29.0	33.0	19.0	81.0	
%	35.8	40.7	23.5	100.0	<0.001*
Controls					
N	85.0	52.0	4.0	141.0	
%	60.3	36.9	2.8	100.0	
COMT					
Patients					
N	32.0	38.0	11.0	81.0	
%	39.5	46.9	13.6	100.0	0.58
Controls					
N	57.0	71.0	13.0	141.0	
%	40.4	50.4	9.2	100.0	

Comparison between wild-type *vs* heterozygote *vs* mutant (P < 0.001)*.

Table 3 illustrates a statistical difference between the patient and control groups regarding the allelic distributions of the polymorphism in the *CYP1A1* gene (P < 0.05). For the allelic distributions of *COMT*, however, no statistical difference was noted between the patient and control groups (P > 0.05).

Table 4 exhibits the OR analysis and the respective CIs. For the *CYP1A1* polymorphism, according to the distributions of genotypes shown in Table 2, the heterozygote + genotype data were combined, since both exhibited an increased frequency in the patient group.

Table 3. Allele analysis of the polymorphisms studied.

	Wild-type	Mutant	Total	Fisher exact test
CYP1A1				
Patients				
N	91.0	71.0	162.0	
%	56.2	43.8	100.0	<0.0001*
Controls				
N	222.0	60.0	282.0	
%	78.7	21.3	100.0	
COMT				
Patients				
N	102.0	60.0	162.0	
%	63.0	37.0	100.0	0.67
Controls				
N	185.0	97.0	282.0	
%	65.6	34.4	100.0	

Allelic comparison between wild-type *vs* mutant (P < 0.0001)*.

Table 4. Analysis of odds ratios without adjustment for the polymorphisms studied.

	Odds ratio	95%CI	P
CYP1A1			
Wild-type	1.0	-	-
Heterozygote	1.80	0.98-3.29	0.06
Mutant	13.46	4.24-42.75	<0.0001*
CYP1A1*			
Wild-type	1.0	-	-
Heterozygote + mutant	2.63	1.50-4.61	0.001**
COMT			
Wild-type	1.0	-	-
Heterozygote	0.92	0.52-1.65	0.79
Mutant	1.46	0.59-3.63	0.41
COMTb			
Wild-type	1.0	-	-
Heterozygote + mutant	1.53	0.65-3.58	0.33

Homozygous mutant genotype OR = 13.46 (*P < 0.0001). Presence at least one mutant allele OR = 2.63 (**P < 0.001).

DISCUSSION

It is believed that estrogen-induced cancers can be attributed at least in part to certain estrogen metabolites, as estrogen effects have profound consequences on the biologic as well as pathologic processes. In fact, evidence shows that the *in situ* formation of estrogen metabolites can exhibit biological properties, even at very low concentrations (Bradlow et al., 1985; Mueck and Seeger, 2007). It has been suggested that many of the estrogen effects cannot be caused by estradiol itself, but might result from the formation of active estrogen metabolites that act as local mediators or activate their own original receptors or effectors (Scandlyn et al., 2008). 17β-estradiol (E2) is metabolized into a variety of different compounds that occasionally act with oncogenic hormonal potential. It is already understood that E2 and 4-hydroxyestradiol (4-OHE2) induce oxidative stress, instabilities in microsatellites, and the neoplastic transformation of glandular epithelium cells in the human endometrium, whereas 2-hydroxyestradiol (2-OHE2) does not cause such cellular transformation or genomic instabilities (Salama et al., 2008). Thus, the unbalanced expression of genes involved in the metabolism of estrogens, the profile of estrogen metabolites from each individual might be crucial for regulating or influencing many of the physiological and pathological processes in cervical cancer.

This study demonstrated that the gene polymorphism present in the *CYP1A1* that is involved in the metabolic pathway of sexual steroids seem to have an influence on the onset of pathological conditions such as cervical cancer. Our analysis showed a significant association between the *CYP1A1* polymorphism and cervical cancer development.

After verifying that the genotypic distribution of polymorphisms was under HWE (Table 1) in the patient and control groups (P > 0.05) a statistical difference in the genotype distributions between the patients and controls was noted (Table 2) for the *CYP1A1* polymorphism (P < 0.05). However, no significant difference was noted between the patient and control groups (P > 0.05) for the *COMT* polymorphism, suggesting that this gene does not play a major role in the development of cervical carcinoma in our studied sample population, which in turn suggests that other biological mechanisms or other etiologies might be responsible for disease development in this cohort. In addition, the co-factors associated with cervical cancer development, not only with respect to environmental risk factors but also in relation with genetic susceptibility, might be different for each gene (Altekruse et al., 2003).

The development of cervical cancer is a process that occurs in a somewhat complex manner, from the onset of precursor lesions of the cervix of the intraepithelial neoplastic type (NIC) to the cancer itself (Altekruse et al., 2003). Our study demonstrated that the *CYP1A1* polymorphism might be related to the risk of development of cervical intraepithelial neoplasia and suggests that

such genes could be involved in the carcinogenesis process, from the onset of NIC through to the development of the carcinoma.

Upon analysis, a statistical difference was also noted between the patient and control groups regarding the allelic distribution (Table 3) of the *CYP1A1* gene polymorphism ($P < 0.05$). In contrast, for the allelic distribution of the *COMT* polymorphism, no statistical difference was identified between the patients and controls ($P > 0.05$).

The information obtained shows that women with at least one mutated allele present with a nearly 1.8-times greater chance to develop cervical cancer than do women having two wild-type alleles for the *CYP1A1* polymorphism (OR = 1.80; 95%CI = 0.98-3.29; P = 0.06). This can be even more exaggerated when both alleles are mutated, resulting in a nearly 13.5-times greater chance of developing cervical cancer in comparison with women carrying two wild-type alleles (OR = 13.46; 95%CI = 4.24-42.75; P = < 0.0001).

For the *CYP1A1* polymorphism, in order to correct the discrepancies in the cancer-risk due to the low "N" of the samples, and based on the distribution of genotypes shown in Table 2, instead of joining the heterozygote genotypes with the wild-type, as is customary, its more appropriate to combine the mutated and heterozygote genotypes, since both have an elevated frequency in the patient group, and compare them against the wild-type genotype, which represents the majority within the control group. Thus, one might predict a high risk of nearly 2.6-times greater chance of cervical cancer development in women having one or both mutated alleles (OR = 2.63; 95%CI = 1.50-4.61; P = 0.001). For the *COMT* polymorphism, after calculating the genotypic distributions in all possible allelic groups, no significant result was found associated with the a risk of predisposition to cervical cancer (OR = 1.53; 95%CI = 0.65-3.58; P = 0.33).

Thus, in view of our results, we believe that the presence of polymorphisms, in combination with a wide range of other factors already might play a role in cervical carcinogenesis. Finally, this study discusses the relevance of polymorphisms of genes involved in the metabolic path of sexual steroids and indicates the importance of studies that have demonstrated genetic alterations related to genes responsible for the synthesis and metabolism of estrogens, such as the polymorphisms in *CYP1A1*, *CYP 17*, *COMT*, GST, and estrogen and progesterone receptors. This variation might lead to a rise in the estrogens and/or circulating catechol estrogen levels through changes in the response to sexual steroids, which in turn might be liable in the onset of gynecologic neoplasms as well as the modulation of patient response to chemotherapeutic treatment and in survival (Dirven et al., 1994; Kuligina et al., 2000; Berstein et al., 2001; Lacey et al., 2001; Huber et al., 2002; Worda et al., 2003; Yamamoto et al., 2012).

Conflicts of interest

The authors declare no conflict of interest.

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