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BRIEF ARTICLES

Lack of correlation between *p53* codon 72 polymorphism and anal cancer risk

Simone S Contu, Grasiela Agnes, Andrea P Damin, Paulo C Contu, Mário A Rosito, Claudio O Alexandre, Daniel C Damin

Simone S Contu, Paulo C Contu, Mário A Rosito, Daniel C Damin, Department of Surgery, Division of Coloproctology, Clinicas Hospital of Porto Alegre and Federal University of Rio Grande do Sul, 90.035-930 Porto Alegre, Brazil
Grasiela Agnes, Andrea P Damin, Claudio O Alexandre, Daniel C Damin, Laboratory of Molecular Biology and Department of Pathology, Federal University of Health Science of Porto Alegre, 90.035-930 Porto Alegre, Brazil
Author contributions: Contu SS supervised the collection of research material and patient data and prepared the manuscript; Agnes G performed laboratory analysis of the biological material; Damin AP designed the research and performed laboratory analysis; Contu PC collected the biological material and wrote the manuscript; Rosito MA recruited patients and prepared clinical data; Alexandre CO coordinated the laboratory activities and analyzed the laboratory results; Damin DC designed the research, coordinated collection of clinical data and biological material and wrote the manuscript.
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Correspondence to: Dr. Daniel C Damin, Division of Coloproctology, Clinicas Hospital of Porto Alegre and Federal University of Rio Grande do Sul, 90.035-930 Porto Alegre, Brazil. damin@terra.com.br
Telephone: +55-51-33598232 Fax: +55-51-33598001
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Abstract

AIM: To investigate the potential role of *p53* codon 72 polymorphism as a risk factor for development of anal cancer.

METHODS: Thirty-two patients with invasive anal carcinoma and 103 healthy blood donors were included in the study. *p53* codon 72 polymorphism was analyzed in blood samples through polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing.

RESULTS: The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls. No significant differences in distribution of the codon 72 genotypes between patients and controls were found.

CONCLUSION: These results do not support a role for the *p53* codon 72 polymorphism in anal carcinogenesis.

Peer reviewers: Atsushi Mizoguchi, Assistant Professor, Experimental Pathology, Massachusetts General Hospital, Simches 8234, 185 Cambridge Street, Boston, MA 02114, United States; Fritz von Weizsacker, Professor, Department of Medicine, Schlosspark Klinik, Humboldt University, Berlin 14059, Germany

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INTRODUCTION

Squamous cell carcinoma (SCC) of the anus is a relatively uncommon malignancy, affecting approximately 4600 patients per year in the United States[1]. Globally, annual incidence rates of invasive anal cancer range from 0.1 to 2.8 cases per 100 000 among men and 0.0-2.2 cases per 100 000 among women[2]. In particular, anal cancer rates among men who have sex with men are notably higher[3]. Invasive anal cancer, like invasive cervical cancer, has been causally linked to high-risk human papillomavirus (HPV) infection[4,5]. According to a recent review, HPV is detected in 71% of invasive anal cancers, with approximately 72% of the HPV-positive cases being associated with HPV 16 and/or 18 infection[6]. This estimate of HPV 16 and 18 prevalence is similar to that found in invasive cervical cancer[7].

Although many risk factors for the development of anal cancer have been identified, such as the practice of receptive anal intercourse and immunodeficiency, the molecular mechanisms related to anal carcinogenesis remain unclear. Mutations in the *p53* gene are the most common genetic alterations in human cancer and they can be found in up to 80% of anal carcinomas[8]. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies[9-11]. Recent studies have focused on a common single-base-pair polymorphism at codon

72, which results in a Pro (CCC) or Arg (CGC) residue at this position. The two polymorphic variants have been shown to have not only structural differences, as reflected by distinct electrophoresis patterns of migration, but also different biological properties[12-14]. The Arg variant has been demonstrated to be more susceptible to degradation by the HPV E6 protein than the Pro variant, with individuals who are homozygous for Arg having a higher risk of being affected by HPV-associated malignant tumors[15].

In this article, we present the results of what is believed to be the first study to investigate the potential association of *p53* codon 72 polymorphism with invasive carcinoma of the anal canal.

MATERIALS AND METHODS

Cases and controls

Thirty-two patients with histologically confirmed primary SCC of the anal canal (mean age 60.3 years, range 30-81 years) were enrolled prospectively in the study. As a non-malignant control group, we studied 103 consecutive healthy blood donors with no previous history of cancer (mean age 47.7 years, range 40-72 years). Demographic characteristics of cases and controls are shown in Table 1. After pretreatment assessment, including a complete medical history and physical examination, colonoscopic examination, computed tomography of the abdomen and pelvis, and chest radiography, the patient's AJCC (American Joint Committee on Cancer) tumor stage was determined[16]. The distribution was as follows: 6.2% stage Ⅰ (n = 2), 53.1% stage Ⅱ (n = 17), 34.3% stage Ⅲ (n = 11) and 6.2% stage Ⅳ (n = 2).

The study was approved by the Ethics and Scientific Committee of the Santa Casa Hospital Complex and Hospital de Clínicas de Porto Alegre. Informed consent was obtained from all patients and controls before being enrolled in the study.

DNA extraction and genotyping

p53 codon 72 polymorphism was studied in blood samples collected by venous puncture. Genomic DNA was extracted from peripheral lymphocytes using Ultra Clean DNA Bloodstain Kit (MoBiolabs, Solana Beach, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of codon 72, modified from the technique described by Ara *et al*[17], was used to identify *p53* genotypes. The forward primer used was 5'-TTGCCGTCCCAAAGCAATGGATGA-3', and the reverse primer was 5'-TCTGGGAAGGGACA GAAGATGAC-3'. Each PCR reaction mixture (50 mL) contained 10 pmol each primer, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 1 U Platinum®*Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil), and 100-300 ng genomic DNA. Reaction mixtures were preincubated for 5 min at 94℃. PCR conditions were 94℃ for 1 min and 55℃ for 1 min, followed by 72℃ for 1 min for 35 cycles. The final extension was at 72℃ for 10 min.

Table 1 Demographic characteristics of cancer patients and controls *n* (%)

Characteristics	Cases	Controls	*P*
Gender			
Male	7 (22)	22 (21)	NS
Female	25 (78)	81 (79)	
Race			
White	28 (87)	96 (93)	NS
Non-white	4 (13)	7 (7)	
Age (yr); mean (range)	60.3 (30-81)	47.7 (40-72)	< 0.001
Total	32 (100)	103 (100)	

NS: Not significant.

After confirmation of an amplified fragment of the expected size (199 bp) on an agarose gel, 10 μL PCR product was digested with 6 U restriction enzyme *Bst*UI (New England Biolabs, Ipswich, MA, USA) at 60℃ for at least 4 h. DNA fragments were electrophoresed through a 2.5% agarose gel and stained with ethidium bromide. RFLP results were confirmed by sequencing the PCR fragments from nine randomly selected samples (three of each genotype) using an automated sequencing system (ABI Prism 310 Genetic Analyzer; Applied BioSystems, Foster City, CA, USA). Sequencing reactions were performed using the BigDye® Terminator V3.1 cycle sequencing reaction kit (Applied BioSystems) according to the manufacturer's instructions. Forward and reverse primers were utilized as sequencing primers.

Statistical analysis

Univariate statistics were used first to compare cases and controls for demographic variables and genotype prevalence. The χ^2 test was used to analyze categorical variables and ANOVA was used to compare the continuous variable age. The association between the *p53* polymorphism and anal cancer was determined using the logistic regression method to assess ORs and 95% CI. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of *p53* codon 72 polymorphism by PCR-RFLP was performed in all cases and controls. The Arg allele was cleaved by *Bst*UI, which yielded two small fragments (113 and 86 bp). The Pro allele was not cleaved by *Bst*UI, which had a single 199-bp band. Heterozygotes contained three bands, which corresponded to 199, 113 and 86 bp. The PCR results were confirmed by DNA sequencing.

The distribution of the codon 72 genotypes in patients and controls did not deviate from the Hardy-Weinberg equilibrium. The genotype frequencies in cases and controls are presented in Table 2, with no association with anal cancer risk being observed. The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls.

We also analyzed the codon 72 polymorphism of the healthy controls according to their age. The genotype

Table 2 Distribution of *p53* codon 72 polymorphism in cancer patients and controls *n* (%)

	Total	Arg/Arg	Arg/Pro	Pro/Pro	OR[1]	CI	*P*
Anal cancer	32	10 (31.2)	19 (59.4)	3 (9.4)	1.6	0.6-4.9	0.325
Controls	103	31 (30.1)	62 (60.2)	10 (9.7)			

[1]Adjusted for age. Arg/Arg *vs* Arg/Pro and Pro/Pro.

distribution in the 78 controls under 50 years old was as follows: 25 Arg/Arg (32.1%), 6 Pro/Pro (7.7%), and 47 Arg/Pro (60.3%). The distribution in the 25 controls over 50 years old was: 6 Arg/Arg (24.0%), 4 Pro/Pro (16.0%), and 15 Arg/Pro (60.0%). No significant difference in the genotype distribution was found between these two age groups ($P = 0.407$).

DISCUSSION

High-risk HPV infection has been implicated in the pathogenesis of different malignancies[17-21]. Several biochemical and genetic studies have shown that HPV E6 and E7 proteins exert a cooperative effect on cellular transformation and immortality by interfering with the function of cellular tumor suppressor proteins[22-24].

A common polymorphism has been known in codon 72 of the *p53* gene, with two alleles encoding either Arg (*p53*Arg) or Pro (*p53*Pro)[13,14]. Storey *et al*[15] have investigated the effect of this polymorphism on the susceptibility to E6-mediated degradation and found that individuals homozygous for Arg are seven times more susceptible to HPV-associated cervical carcinogenesis than heterozygotes are. Since then, the effect of codon-72 polymorphism of *p53* on cervical cancer has been studied, with contradictory results being reported. Overall, as demonstrated in a recent meta-analysis, compared with the heterozygous genotype (Pro/Arg), the homozygous genotype (Arg/Arg) of codon-72 of *p53* is associated with an approximately 20% increased risk of cervical cancer[25].

Invasive anal cancer, like invasive cervical cancer, has well-documented precursors, known as anal intraepithelial neoplasia 2-3 (histology) or high-grade squamous intraepithelial lesions (cytology)[6]. Anal cancer also has been causally linked to high-risk HPV infection, therefore, we decided to evaluate, perhaps for the first time, the potential role of codon 72 polymorphism as a risk factor for development of this type cancer.

In order to minimize sources of bias and avoid misinterpretation of the results, standard safeguards were adopted. Patients and controls were matched ethnically and derived from a population living in the same geographic region (Southern Brazil), and were enrolled consecutively in a single institution. All PCR results were confirmed by DNA sequencing.

We investigated the allele and genotype frequencies at *p53* codon 72 in 32 patients with anal cancer and 103 healthy individuals from southern Brazil. No significant differences in the relative allele frequency and in the distribution of genotypes were found between patients and controls. These results are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral SCCs[26,27].

The association between codon 72 polymorphism and risk of cancer has been reported in different populations[28]. Studies have been conducted to evaluate this polymorphism as a risk factor for different types of cancer, such as gastric[29], lung[9] and breast carcinomas[11]. So far, the published data have been inconclusive. The conflicting results found in the literature might be attributed to variations in protocols among different laboratories, or to poor selection of control groups[29]. They also might have been caused by the inherent characteristics of the population being analyzed, as there are considerable variations in the distribution of the codon 72 genotypes in various populations. This polymorphism seems to be maintained by natural selection influenced by environmental factors, such as the degree of exposure to the UV-B component of sunlight[30]. The resulting North-South Arg/Pro gradient has been reported in different geographical regions. Population-based studies have indicated that the Arg allele is most prevalent in individuals with light complexion and least prevalent in those with darker complexion, with a clear and consistent decline in the prevalence of the Pro allele, with increasing northern latitude[30-32].

The population from Southern Brazil, in contrast with other regions of the country, is composed mainly of Caucasian individuals who are descended from European immigrants[33]. Although most of these immigrants came from Portugal, Germany and Italy[34], the genotype distribution found in our healthy controls was notably different from the genotype distribution observed in those countries[35-37]. This can be explained partially by the process of miscegenation among different ethnic groups (Caucasians, Amerindians and Afro-Brazilians) that took place during Brazilian colonization[38]. Each specific population seems to have its own characteristic genotype distribution that can differ markedly from the polymorphic frequencies found in other populations, even when neighboring countries are compared.

We believe, however, that the lack of correlation between the codon 72 genotype distribution and anal cancer risk observed in our study cannot be interpreted solely as a result of population ethnicity. In a previous study of cancer patients and normal individuals from Southern Brazil, we were able to detect a significant

association of *p53* codon 72 polymorphism with breast cancer risk[39]. We analyzed blood samples collected from 118 women with primary breast carcinoma and from 202 female blood donors (healthy controls) through PCR-RFLP and DNA sequencing. The Arg/Arg genotype was significantly associated with an increased risk for breast cancer (OR 2.9; 95% CI: 1.43-3.6; $P < 0.002$). The relative frequency of each allele was 0.75 for Arg and 0.25 for Pro in patients with cancer, and 0.62 for Arg and 0.38 for Pro in normal controls ($P < 0.001$). In the present study, the relative frequency of each allele observed within the control group (0.61 for Arg and 0.39 for Pro) was therefore very similar to our previous observation in normal controls derived from the same population.

In summary, we did not detect significant differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. Our results do not support the hypothesis that *p53* codon 72 polymorphism is associated with anal cancer susceptibility. The role of the genetic susceptibility to high-risk HPV infection and anal cancer, however, merits further investigation.

COMMENTS

Background

A common Arg/Pro polymorphism at codon 72 of the *p53* gene has been studied as a risk factor for human papilloma virus (HPV)-associated malignancies. Although anal cancer has been associated repeatedly with high-risk HPV infection, this polymorphism has not been investigated in this type of cancer up until now.

Research frontiers

Although several risk factors for the development of anal cancer have been determined, the molecular mechanisms involved in anal carcinogenesis remain unclear. In this context, the identification of new factors involved in progression of the anal carcinoma represents a critical step towards development of new anticancer strategies in this malignancy.

Innovations and breakthroughs

This is believed to be the first study to investigate *p53* codon 72 polymorphism in patients with anal cancer. The authors did not detect differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. In contrast to previous observations with cervical cancer, this polymorphism does not seem to be associated with anal cancer susceptibility. The results, however, are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral squamous cell carcinomas.

Applications

In the process of identifying genetic causes of cancer, it is important to determine precisely which elements of a biological pathway are responsible for affecting tumor suppression or development. Then, treatments and preventive measures can be directed to those individuals who would benefit most. Previous studies have identified *p53* codon 72 polymorphism as a potential contributing factor in HPV-associated carcinogenesis. This study found that *p53* codon 72 polymorphism is not a likely risk factor for anal cancer, and future research should focus on other parts of the *p53* gene pathway to understand its role in development of this type of cancer.

Terminology

Mutations in the *p53* gene are the most common genetic alterations in human cancer. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies. A polymorphism is known in codon 72 of the *p53* gene, which results in a Pro or Arg residue at this position. These two polymorphic variants have been shown to have different biological properties, including differences in cancer susceptibility.

Peer review

Contu *et al* Have described the lack of a functional role of a specific *p53* polymorphism in the pathogenesis of anal cancer. The major point of this paper and the spectrum of methods used are rather confined, but the point is clear and the paper is well written. Although this paper presents negative data, I feel that the attempt to perform genetic analysis on a relatively uncommon malignancy (anal cancer) may be viewed favorably clinically.

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