Association of the p53 codon 72 polymorphism with clinicopathological characteristics of colorectal cancer through mRNA analysis

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Abstract. *TP53* represents a suitable candidate for a colorectal cancer susceptibility locus. The polymorphism in the p53 72nd codon involves a proline to arginine substitution, leading to changes in gene transcription activity, interaction with other proteins and modulation of apoptosis. Studies evaluating the association between this polymorphism and colorectal cancer (CRC) have shown inconsistent results, and none have evaluated the mRNA status of *TP53*. The aim of the present study was to evaluate the association between this SNP expression at the mRNA level in CRC samples and patient clinicopathological variables and prognosis, p53 protein expression and *TP53* mutation. This is the first report to describe the mRNA expression of p53 codon 72 alleles in CRC. We evaluated 101 non-related patients with CRC treated at the A.C. Camargo Cancer Center in Brazil. RNA was isolated from frozen tumor tissues using a TRIzol-based protocol. The polymorphism was detected using RT-PCR followed by Sanger sequencing. Associations were analyzed using Pearson's Chi-square or Fisher's exact tests, logistic regression and Cox. This polymorphism was significantly associated with clinicopathological variables related to increased tumor aggressiveness. The expression of Arg72 (OR, 3.83; CI 1.02-14.35; P=0.046) and the TNM stage (OR, 7.15; CI 1.45-35.29; P=0.016) were found to be independent predictors for recurrence. These data suggest that the mRNA expression of the Pro72 allele is associated with less favorable tumor features. The allele frequency of the p53 Pro72 was 0.26. The analysis of mRNA is important to determine the specific contribution of the allele expressed. These results suggest that this polymorphism may play a role in CRC.

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Introduction

Colorectal cancer (CRC) is the third most common cancer type in men and women worldwide. CRC is thought to result from an interaction between environmental and genetic factors (1).

Currently, functional variation of DNA repair and cell cycle control-related genes in the presence of carcinogen-mediated cell damage is believed to be a mechanism for explaining inter-individual variation in CRC susceptibility (1).

Analysis of phenotype concordance in monozygotic twin CRC cases suggest that inherited susceptibility underlies 35% of all CRCs. However, only 6% of CRCs occur in the context of a known high-penetrance cancer predisposition syndrome, such as familial adenomatous polyposis or Lynch syndrome (2,3). Therefore, most of the genetic risks for CRC remain unknown (4).

Fearon and Vogelstein (5) proposed a model for the development of CRC whereby colorectal carcinoma arises and progresses through histological stages due to an accumulation of genetic and epigenetic changes. A particular stage of progression of late adenoma to adenocarcinoma involves mutations in *TP53*. p53 regulates many cellular functions including cell cycle progression, DNA repair, senescence, apoptosis and cellular metabolism (6). In normal cells, the expression level of p53 is extremely low. However, p53 protein levels increase in response to various stress signals, such as DNA damaging agents, oxidative stress, amino acid depletion and temperature change (7).

In addition to the gene mutation, which represent the most common *TP53* genetic alteration, multiple single nucleotide polymorphisms (SNPs) have been identified in this gene. However, the relevance of the majority of the SNPs remains

unclear. The p53 codon 72 SNP (rs1042522), which is located in exon 4 within the p53 transactivation domain in a proline-rich region, results in the expression of either a proline or an arginine due to a nucleotide substitution of the second base in the codon (CCC>CGC), thus, changing from an amino acid with a non-polar aliphatic side chain to an amino acid with a positively charged basic side chain (1).

Several lines of evidence suggest that the resulting two alleles confer different properties to the p53 protein. These p53 variants are not biochemically equivalent, since they show different transcriptional regulation activities, interactions with p73 (a homologue of p53) and degradation rates mediated by the proteasome (8). The allele with proline (Pro72) is considered the wild-type one (9), and it appears less efficient than the allele with arginine (Arg72) at suppression of cell transformation and induction of apoptosis (10). Structural and functional features of p53 might be useful as a molecular prognostic marker (11).

An association between the genotyped p53 codon 72 SNP and human cancer risk has been reported in breast (12,13), gastric (14,15), thyroid (16,17), lung (18,19), vulval (20) and bladder cancers (21,22). However, this SNP does not appear to affect the risk of cervical (23,24), prostate (25,26) and endometrial cancers (27,28) and head and neck squamous cell carcinomas (29,30).

Previous studies have shown that the p53 codon 72 SNP is associated with the risk of CRC or its precursor lesion adenoma (8,31-36), while others have reported discordant results (37,38). The p53 codon 72 SNP was not found to be associated with the alteration of colorectal cancer risk in a meta-analysis with 20 case-control studies (1).

Mammano *et al* (8) demonstrated that the genotyped p53 codon 72 SNP is associated with a higher risk of CRC and with more advanced and undifferentiated tumors, suggesting that this SNP may play a role in the progression of CRC.

Most epidemiological studies have evaluated the genotypes of polymorphic genes, searching for alterations in cancer risk. However, it is imperative to evaluate which allele is expressed in the tumor as there may be preferential expression of a specific allele in heterozygotes, which may explain the discordance data related to association of p53 codon 72 SNP with CRC. Moreover, evaluation of the association of the expression of this SNP with patient clinicopathological variables and prognosis can provide relevant data not previously identified.

In order to shed light on the role of this SNP in CRC, we conducted a p53 codon 72 SNP expression analysis searching for associations with p53 protein expression, *TP53* mutations, patient clinicopathological variables and prognosis.

Materials and methods

Study population. We examined mRNA from 101 patients with sporadic origin colorectal tumors who were treated at the Hospital A.C. Camargo (São Paulo, Brazil) and who underwent surgical resection for colorectal adenocarcinoma between 1992 and 2006. Individuals fulfilling any familial syndrome clinical criteria or with inflammatory bowel diseases and those treated with preoperative chemoradiotherapy were excluded from the present study which was reviewed and approved by a duly appointed ethics committee (1042/08).

Clinicopathological data. All clinical data were collected from patient reports, and pathological data of the CRC cases were systematically evaluated by an experienced gastrointestinal pathologist (R.A.C.). The data collected include gender, age at diagnosis, smoking habit (yes or no), CRC location, histological grade, TNM stage (UICC/AJCC), dirty necrosis, desmoplasia, Crohn's-like lymphocytes, infiltrating lymphocytes, vascular and lymphatic invasion, budding, tumor border pattern of growth (expanding or infiltrating), tumor recurrence, use of post-chemoradiotherapy (radiotherapy only for rectal tumors) and follow-up time. Tumor budding was defined as an isolated single cancer cell or a cluster composed of fewer than 5 cancer cells observed in the stroma of the actively invasive area (39).

Immunohistochemistry. The expression status of p53 was evaluated using immunohistochemistry (IHC) technique. IHC staining was performed on 3-μm formalin paraffin-embedded (FFPE) tissues. The reactions were performed using a p53 monoclonal antibody (DO7 clone, 1:100 dilution; Dako, Glostrup, Denmark) and a polymer-based detection system (Advance HRP Link Polymer amplification system; Dako). Positive staining was defined as an unequivocal nuclear staining of neoplastic cells. The percentage of positively stained neoplastic cells was quantified. A tumor was considered positive when >20% of its cells were stained.

RNA extraction. Fresh samples were matched with FFPE tissues used for IHC. Total RNA was extracted from manually microdissected frozen tissues with at least 70% of tumor cells (10-100 mg) by homogenizing each tissue sample using Precellys equipment (Bertin Technologies, Villeurbanne, France) in 1 ml of TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA), and RNA was stored at -80˚C prior to use.

RT-PCR. Total mRNA was employed to synthesize cDNA for *TP53* allele expression and mutation analyses using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The *TP53* transcript was amplified as two overlapping fragments, from exon 2 to 6 and from exon 6 to 11, covering the entire coding region. PCR was performed in 25-μl reactions containing 20 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM of forward and reverse primers and 1.5 U of Platinum® Taq Polymerase (Invitrogen). PCR products were analyzed on an agarose gel containing SYBR Safe (Invitrogen). PCR primers are described in Table I.

Sequencing analysis. Samples were screened for mutations over the entire coding region of *TP53* and for the presence of polymorphic variants at codon 72. ExoSAP-IT (1 μl, USB; Affymetrix, Cleveland, OH, USA) was used to purify 7 μl of the PCR product. Sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) with specific primers that overlapped the region amplified (Table I) and an ABI PRISM 3730xl Automatic Genetic analyzer (Applied Biosystems), according to the manufacturer's recommendations.

Table I. Amplification and sequencing primers.

Use	Primer	Sequence	Amplicon (bp)
PCR fragment 1	E2ForE RNAm	GACGGTGACACGCTTCCCTG	708
	E6RevE RNAm	CACCACCACACTATGTCG	
PCR fragment 2	E6ForE RNAm	CCTCAGCATCTTATCCGAG	645
	E11RevE RNAm	AGGCTGTCAGTGGGGAAC	
Sequencing fragment 1	E2ForI RNAm	CAGCCAGACTGCCTTCCGGGTC	654
	E6RevI RNAm	CTGTCATCCAAATACTCCACACG	
Sequencing fragment 2	E6ForI RNAm	GGAAATTTGCGTGTGGAG	604
	E11RevI RNAm	CAAGAAGTGGAGAATGTC	

The *TP53* polymorphic and mutation status was analyzed using CLC Main Workbench software (CLCbio version 4.6) with p53 NM_000546 reference sequence. Allele expression was determined using electropherogram data from the sequencing analysis. Double peaks of C and G nucleotides were considered to indicate heterozygosity.

Statistical analysis. To test the distribution of genotypes and the relationship between the p53 codon 72 SNP and clinical variables, data were analyzed using the 2-sided Pearson's Chi-square or Fisher's exact tests in the SPSS v17.0 program (SPSS, Inc., Chicago, IL, USA). A $P<0.05$ was considered to indicate a statistically significant result.

To identify the variables associated with recurrence, univariate analysis was performed. Variables with $P<0.20$ were selected for multiple logistic regression model. In this model we considered variables with $P<0.05$ and present the OR and the 95% CI. To determine the variables associated with survival, univariate analysis was performed using the Kaplan-Meier and log rank test. Variables with $P<0.20$ were selected for the Cox proportional hazards regression model and the OR and 95% CI are presented. An α error of 5% was considered.

Results

Clinicopathological characteristics. The present study was performed using samples from 101 patients consisting of 49 men and 52 women with a mean age of 62.4 years (median age 63 years; age range 27-88 years). There were 52 (51.5%) tumors with negative and 49 (48.5%) with positive p53-protein nuclear accumulation. The tumor was located in the proximal colon in 36 cases (35.6%), in the distal colon in 42 cases (41.6%) and in the rectum in 23 cases (22.8%). Regarding the histological grade, 9 (8.9%) were well differentiated, 80 (79.2%) were moderately differentiated and 12 (11.9%) were poorly differentiated. Based on TNM staging criteria, 20 (19.8%) tumors were stage I, 34 (33.7%) were stage II, 26 (25.7%) were stage III and 21 (20.8%) were stage IV; 58 (57.4%) tumors were N0, 21 (20.8%) were N1 and 22 (21.8%) were N2; and 80 (79.2%) tumors were M0 and 21 (20.8%) were M1 (Table II).

Allelic expression associations. All significant associations are shown in Fig. 1. The expression allelic frequencies were 0.26 for Pro72 and 0.74 for Arg72. With regard to allele expression, 66.4% (n=67) of individuals were homozygote for Arg72, 15.8% (n=16) were heterozygote and 17.8% (n=18) were homozygote for Pro72. The analysis also considered the presence of expression of the Arg72 allele: 83 samples (82.2%) expressed Arg72 and 18 (17.8%) did not express it. When Arg72 allele expression was correlated with gender, we found that among the CRC tissues from women, 90.4% expressed Arg72 (at least one allele) and 72.2% of the CRC tissues from men expressed Pro72 exclusively (P=0.037). Of the declared smokers, 64.3% were men.

Correlations between the alleles and the IHC-derived p53 monoclonal antibody staining revealed that the presence of either the Pro72 or Agr72 allele did not affect protein expression. However, the presence of both alleles was associated with normal cellular conditions since 81.3% of the heterozygotes showed the normal absence of expression of the p53 protein (P=0.034).

The association between the SNP and tumor stage revealed that 94.4% of those patients not expressing the Arg72 allele presented tumors with T3 and T4 stages, whereas 95.8% of the tumors in T1 and T2 stages were Arg72 expressers (P=0.064).

Correlations between the polymorphism and the tumor characteristics showed that the pattern border of tumor growth varied with the allele expressed. An infiltrating border of tumor growth was present in 88.9% of those tumors not expressing the Arg72 allele, and among all expanding-border tumors, 93.8% expressed Arg72 (P=0.05).

Another finding from the present study was the relationship between tumor recurrence and this SNP since 93.8% of the Arg72-exclusive expresser tumors did not show tumor recurrence (P=0.008). Similarly, 91.3% of those tumors with Arg72 expression did not show recurrence (P=0.013). The average time for tumor recurrence was 17 months post surgery.

Further analysis showed a statistically significant relationship between the p53 codon 72 SNP and the use of postoperative adjuvant chemoradiotherapy. Of the tumors expressing Pro72 exclusively, 82.4% of individuals underwent some type of post-chemoradiotherapy (P=0.002). Of those who did not receive post-chemoradiotherapy, 78.8% of the tumors expressed Arg72 exclusively (P=0.005).

The present study did not evidence significant difference between this SNP and the relative CRC-free survival rates.

Table II. Association between p53 codon 72 polymorphism and clinicopathological variables.

Variables	N (%)	Allele expression			P-value	Arginine allele expression		P-value
		Arginine (%)	Arg/Pro (%)	Proline (%)		Expressers (%)	No expressers (%)[a]	
CRC cases	101 (100)	67 (66.4)	16 (15.8)	18 (17.8)	-	83 (82.2)	18 (17.8)	-
Gender								
Males	49 (48.5)	29 (43.3)	7 (43.8)	13 (72.2)	NS	36 (43.4)	13 (72.2)	0.037
Females	52 (51.5)	38 (56.7)	9 (56.3)	5 (27.8)		47 (56.6)	5 (27.8)	
Age at onset (years)								
<50	19 (18.8)	10 (14.9)	5 (31.3)	4 (22.2)	NS	15 (18.1)	4 (22.2)	NS
≥50	82 (81.2)	57 (85.1)	11 (68.8)	14 (77.8)		68 (81.9)	14 (77.8)	
Smoking habit								
No	82 (85.4)	52 (82.5)	13 (86.7)	17 (94.4)	NS	65 (83.3)	17 (94.4)	NS
Yes	14 (14.6)	11 (17.5)	2 (13.3)	1 (5.6)		13 (16.7)	1 (5.6)	
Tumor location								
Proximal	36 (35.6)	26 (38.8)	5 (31.3)	5 (27.8)	NS	31 (37.3)	5 (27.8)	NS
Distal	42 (41.6)	26 (38.8)	9 (56.3)	7 (38.9)		35 (42.2)	7 (38.9)	
Rectum	23 (22.8)	15 (22.4)	2 (12.5)	6 (33.3)		17 (20.5)	6 (33.3)	
Histological grade								
Well differentiated	9 (8.9)	8 (11.9)	1 (6.3)	0	NS	9 (10.8)	0	NS
Moderately differentiated	80 (79.2)	52 (77.6)	14 (87.5)	14 (77.8)		66 (79.5)	14 (77.8)	
Poorly differentiated	12 (11.9)	7 (10.4)	1 (6.3)	4 (22.2)		8 (9.6)	4 (22.2)	
Tumor infiltration (T)								
T1+T2	24 (23.8)	19 (28.4)	4 (25)	1 (5.6)	NS	23 (27.7)	1 (5.6)	NS
T3+T4	77 (76.2)	48 (71.6)	12 (75)	17 (94.4)		60 (72.3)	17 (94.4)	
Nodal status (N)								
N0	58 (57.4)	41 (61.2)	9 (56.3)	8 (44.4)	NS	50 (60.2)	8 (44.4)	NS
N1+N2	43 (42.6)	26 (38.8)	7 (43.8)	10 (55.6)		33 (39.8)	10 (55.6)	
Metastasis (M)								
M0	80 (79.2)	55 (82.1)	12 (75)	13 (72.2)	NS	67 (80.7)	13 (72.2)	NS
M1	21 (20.8)	12 (17.9)	4 (25)	5 (27.8)		16 (19.3)	5 (27.8)	
TNM stage								
I-II	54 (53.5)	39 (58.2)	9 (56.3)	6 (33.3)	NS	48 (57.8)	6 (33.3)	NS
III-IV	47 (46.5)	28 (41.8)	7 (43.8)	12 (66.7)		35 (46.5)	12 (66.7)	

Table II. Continued.

Variables	N (%)	Allele expression			P-value	Arginine allele expression		P-value
		Arginine (%)	Arg/Pro (%)	Proline (%)		Expressers (%)	No expressers (%)[a]	
Dirty necrosis								
Not observed	51 (51)	36 (54.5)	9 (53.6)	6 (33.3)	NS	45 (54.9)	6 (33.3)	NS
Present	49 (49)	30 (45.5)	7 (43.8)	12 (66.7)		37 (45.1)	12 (66.7)	
Desmoplasia								
Not observed	29 (28.7)	20 (29.9)	5 (31.3)	4 (22.2)	NS	25 (30.1)	4 (22.2)	NS
Present	72 (71.3)	47 (70.1)	11 (68.8)	14 (77.8)		58 (69.9)	14 (77.8)	
Crohn's-like lymphocytes								
Not observed	100 (99)	66 (98.5)	16 (100)	18 (100)	NS	82 (98.8)	18 (100)	NS
Present	1 (1)	1 (1.5)	0	0		1 (1.2)	0	
Infiltrating lymphocytes								
Not observed	3 (3)	3 (4.5)	0	0	NS	3 (3.6)	0	NS
Present	98 (97)	64 (95.5)	16 (100)	18 (100)		80 (96.4)	18 (100)	
Vascular invasion								
Not observed	91 (90.1)	62 (92.5)	14 (87.5)	15 (83.3)	NS	76 (91.6)	15 (83.3)	NS
Present	10 (9.9)	5 (7.5)	2 (12.5)	3 (16.7)		7 (8.4)	3 (16.7)	
Lymphatic invasion								
Not observed	88 (87.1)	57 (85.1)	14 (87.5)	17 (94.4)	NS	71 (85.5)	17 (94.4)	NS
Present	13 (12.9)	10 (14.9)	2 (12.5)	1 (5.6)		12 (14.5)	1 (5.6)	
Budding								
Not observed	70 (69.3)	48 (71.6)	11 (68.8)	11 (61.1)	NS	59 (71.1)	11 (61.1)	NS
Present	31 (30.7)	19 (28.4)	5 (31.3)	7 (38.9)		24 (28.9)	7 (38.9)	
Tumor border								
Infiltrating	69 (68.3)	42 (62.7)	11 (68.8)	16 (88.9)	NS	53 (63.9)	16 (88.9)	0.05
Expanding	32 (31.7)	25 (37.3)	5 (31.3)	2 (11.1)		30 (36.1)	2 (11.1)	
Recurrence								
Negative	85 (86.7)	61 (93.8)	12 (80)	12 (66.7)	0.008	73 (91.3)	12 (66.7)	0.013
Positive	13 (13.3)	4 (6.2)	3 (20)	6 (33.3)		7 (8.8)	6 (33.3)	
Chemoradiotherapy								
No	52 (53.1)	41 (62.1)	8 (53.3)	3 (17.6)	0.005	49 (60.5)	3 (17.6)	0.002
Yes	46 (46.9)	25 (37.9)	7 (46.7)	14 (82.4)		32 (39.5)	14 (82.4)	

Table II. Continued.

Variables	N (%)	Allele expression			P-value	Arginine allele expression		P-value
		Arginine (%)	Arg/Pro (%)	Proline (%)		Expressers (%)	No expressers (%)[a]	
p53 IHC expression								
Negative	52 (51.5)	31 (46.3)	13 (81.3)	8 (44.4)	0.034	45 (54.2)	8 (44.4)	NS
Positive	49 (48.5)	36 (53.7)	3 (18.8)	10 (55.6)		38 (45.8)	10 (55.6)	
p53 mutation								
Not observed	46 (45.5)	28 (41.8)	13 (81.3)	5 (27.8)	0.004	41 (49.4)	5 (27.8)	NS
Positive	55 (54.5)	39 (58.2)	3 (18.8)	13 (72.2)		42 (50.6)	13 (72.2)	

[a]Proline exclusive expressers. NS, not significant. Sample size is presented for each variable when data were not available for all subjects.

Table III. Multivariate analysis.

Variables	OR	CI 95%	P-value
Survival			
TNM stages			
I-II	1	1.84-10.43	0.001
III-IV	4.38		
Perineural invasion			
Not observed	1	1.26-8.19	0.014
Observed	3.21		
Recurrence			
Arg 72			
Expressers	1	1.02-14.35	0.046
No expressers	3.83		
TNM stages			
I-II	1	1.45-35.29	0.016
III-IV	7.15		

In the Cox proportional survival model, the independent variables were TNM stage (TNM I-II and III-IV; OR, 4.38; CI 1.84-10.43; P=0.001) and perineural invasion (OR, 3.21; CI 1.26-8.19; P=0.014) (Table III).

In the multiple logistic regression model, the variables that were independent predictors for recurrence were the expression of Arg72 allele (OR 3.83; CI 1.02-14.35; P=0.046) and the TNM stage (TNM I-II and III-IV; OR, 7.15; CI 1.45-35.29; P=0.016) (Table III). This model explained 24.5% of the variation of recurrence.

Mutation analysis. We also evaluated the mutation status of the *TP53* gene in the CRC samples, published in detail (40). Relative to the alleles expressed, pathogenic mutations were detected in 72.2% (n=13) of the Pro72-exclusive expressers, in 18.8% (n=3) of the heterozygotes and in 58.2% (n=39) of the Arg72-exclusive expressers (P=0.004). Detailed analyses revealed that among the tumors showing no mutations, 89.1% were Arg72 expressers.

Discussion

mRNA analysis. Genome-wide association studies (GWAS) assaying hundreds of thousands of SNPs have successfully identified a large number of genetic variants associated with complex traits, but each of those variant often confers only a modest increase in risk. One consequence of these small effects is that even combined, these discoveries only explain a small proportion of the entire genetic contribution to risk of disease, thus, leading to the 'missing heritability' question (41).

Many reasons for the missing heritability have been discussed (42,43). Complex patterns of inheritance (44), epigenetic modifications of the genome, common copy-number variants (CNVs) (45), analysis of gene-environment and gene-gene interactions (epistasis) (46) and the recently proposed 'synthetic association' signals created by rare variants in

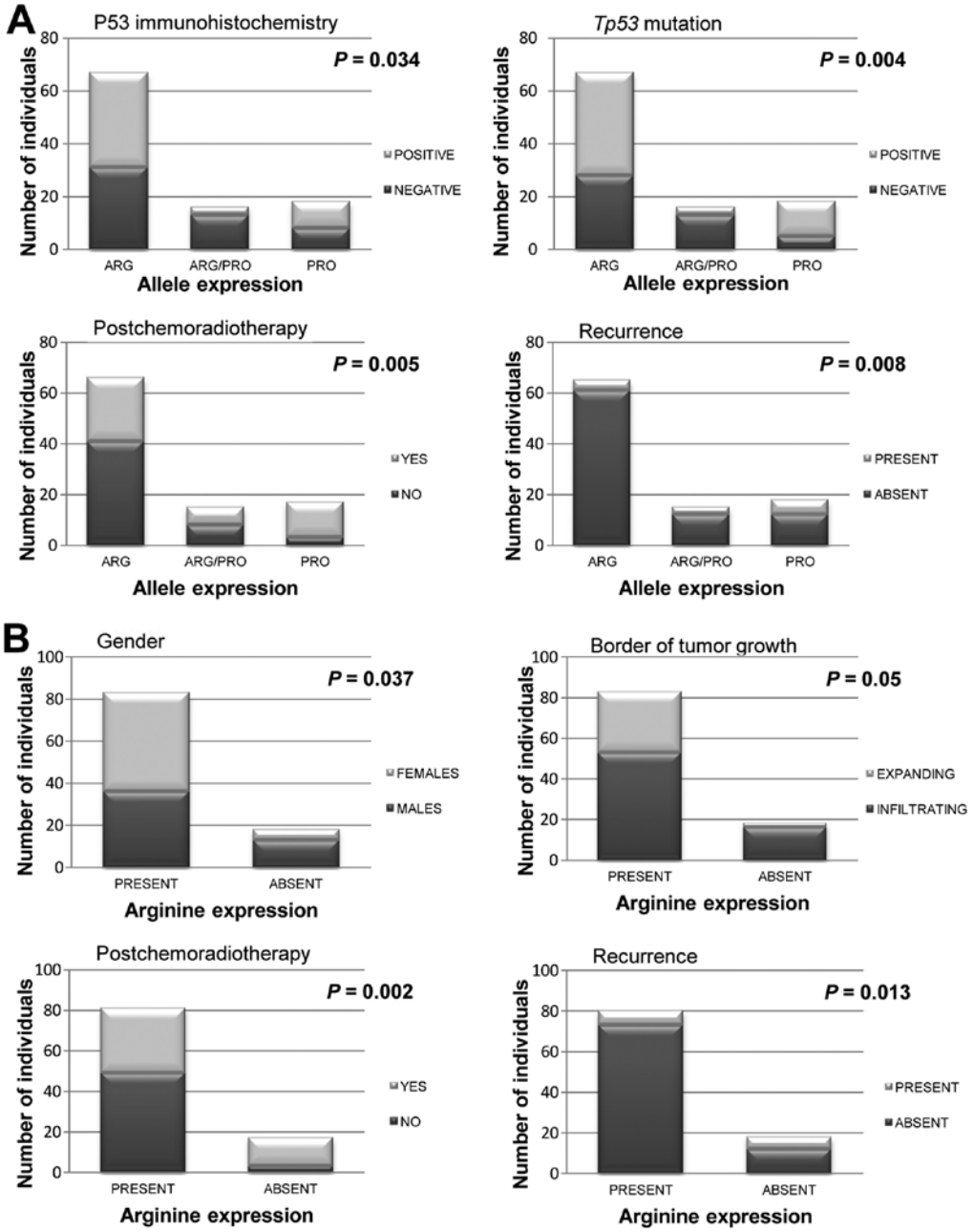

Figure 1. Significant associations between p53 codon 72 SNP and clinicopathological variables. (A) Associations related to genotype expressed by the tumor. (B) Associations relative to the presence of Arg72 allele expression.

GWAS (47) can contribute to the missing heritability. However, we propose that there may be differences in the expression of different polymorphic alleles in large heterozygote populations that may explain cancer behavior and that the real associations can be missed looking only for genomic alterations.

Studies have shown that variations exist in the relative allelic expression levels of specific genes in heterozygotes that contribute to phenotypic variation between individuals. (48-53). Monoallelic expression with random choice between paternal and maternal alleles has also been shown to affect hundreds of autosomal genes and, thus, contribute to individual cell variability (54).

The results presented in the present study highlight a major limitation in comparing our results with other reports since the majority of studies looking for relationships between polymorphisms and CRC examine the genotype of the individuals rather than the allele expressed in tumors. Siddique *et al* (13) showed that breast tumors from heterozygous Chinese women preferentially expressed the Pro72 allele compared to health ones. Thus, the expression status of the p53 alleles in tumors, rather than the genotype, may be a determining factor to better understand the tumor process.

Pro72 vs. Arg72. The p53 codon 72 SNP occurs in the proline-rich domain of p53, which is necessary for the protein to fully induce apoptosis. The polymorphic forms of the p53 protein result in marked alterations in the protein primary structure (55). Data from Marin *et al* (10) suggest that the Pro72 allele displays decreased efficiency in binding p73 and consequently inhibits p73-dependent apoptosis in p53 mutants.

Dumont *et al* (56) found that in cell lines containing inducible versions of alleles encoding the Pro72 and Arg72 variants, and in cells with endogenous p53, the Arg72 variant induced much greater levels of apoptosis than the Pro72 variant. The higher induction of apoptosis by the Arg72 allele results from the increased localization of p53 Arg72 to the mitochondria, which is accompanied by the release of cytochrome *c* into the cytosol (56). Although further studies are required, differences in the mitochondrial localization of the isoforms may also indicate that the p53 codon 72 SNP affects the ability of p53 to regulate mitochondrial respiration and other metabolic factors (7). Oseki *et al* (57) showed that these two polymorphic variants differed particularly within the N-terminal region and consequently, they differ in post-translational modifications at this portion. The Arg72 variant shows significantly enhanced phosphorylation at Ser-6 and Ser-20 compared with the Pro72 variant.

Allelic expression associations. We investigated whether the expression of the Pro/Arg alleles of p53 in CRC correlates with cancer behavior and progression. In studies evaluating genetic polymorphisms and cancer, typically only the association with cancer risk is investigated. However, the relationship between polymorphisms and clinicopathological characteristics of the tumor must be elucidated to enable understanding of the tumor pathogenesis and the tumor course.

Our data revealed that there was a high number of tumors expressing Arg72 in the CRC cohort although our results also showed that the expression of the Arg72 allele may exert a protective effect in this population. The expression of the Pro72 allele in CRC tissues may confer a poorer prognosis since expression of this allele was associated with tumor recurrence. Individuals who were Arg72 allele expressers presented a low number of cancer recurrences, lower grade tumors, expanding tumor borders in the majority of the cases and less frequent *TP53* mutations.

The relationship between gender and the SNP showed an apparent advantage for women as fewer tumors expressed the Pro72 allele in women. A common environmental source of DNA damage is cigarette smoke, which contains many mutagenic compounds. If p53 protects cells from DNA damage caused by exposure to these mutagens, the degree of protection should vary with the strength and nature of the p53 response. Thus, individuals with a weaker p53 response may be less capable of responding appropriately to cigarette smoke, which in turn will affect the ability to promote an apoptotic function (58). Indeed, epidemiological evidence reveals an association between this SNP and cigarette smoking in lung and bladder cancer patients (58,59). Thus, one possible explanation for our finding is that men typically initiate smoking earlier and smoke more frequently than women (60,61). These findings were replicated in the present study (64.3% of smokers were men) although we did not find a statistically significant correlation between smoking, gender and the expression of a specific allele. Furthermore, it is important to note that data of this type of habit are derived from self-reported 'yes or no' questionnaires, which may underestimate the true extent of smoking (62).

Several studies have indicated that individual susceptibility factors, including DNA repair capacity, metabolic capacity and variation in genes involved in these processes, may modulate the genotoxicity of xenobiotics (63,64). Hanova *et al* (65) showed a possible relationship between styrene exposure, DNA damage and the transcript levels of *TP53*.

Tumor-host interaction at the invasive front of colorectal cancer represents a critical interface where tumor progression and tumor cell dissemination arise. The expanding tumor border, identified as presenting margins reasonably well-circumscribed, is often associated with a well-developed inflammatory infiltrate (66,67). In contrast, the infiltrative tumor border is characterized by widespread dissection of normal tissue structures with a loss in the clear boundary between tumor and host tissues. The infiltrating tumor border configuration promotes progression and dissemination of tumor cells by penetrating the vascular and lymphatic vessels (66,67). Studies have revealed that the infiltrative pattern of growth is an adverse prognostic factor and may predict local recurrence (68), whereas the expanding pattern was related with improved survival (67), which is consistent with our results.

The p53 pathway is critical in mediating the response of commonly used cancer therapies. There is evidence that the *TP53* gene has functional SNPs that affect p53 signaling, thus, possibly altering cancer risk and clinical outcome (69). How the functional p53 SNPs interact with known cancer risk factors and therapeutics remains to be answered. The present study provides evidence for the protective effect of Arg72 expression on the requirement for postoperative adjuvant chemoradiotherapy.

Adjuvant therapy for colorectal cancer forms an essential component of an effective treatment strategy. Initially, adjuvant chemotherapy for colorectal cancer was delivered in the post-operative setting following 'curative' surgery to destroy any residual or micrometastatic disease. Today, the effects of chemotherapy for colorectal cancer include delaying and possibly preventing recurrences following 'curative' surgery, downsizing incurable disease in the pre-operative setting and significantly expanding the median survival in the advanced metastatic setting (70). Despite the large number of factors involved in predicting clinical outcome in patients with colorectal cancer, the histologic stage at surgical diagnosis remains the most important prognostic variable (71). Thus, the selection of appropriate patients to receive adjuvant therapy has been based on their risk of recurrence after surgery only and on disease variables known to adversely affect prognosis, and the selection of systemic agents has been typically based on antitumor activity in patients with advanced disease of similar histology (72).

In particular, 5-fluorouracil (5-FU) is widely used in the treatment of a range of cancers and has demonstrated the largest impact on CRC. *TP53* can be activated by 5-FU through more than one mechanism including incorporation of fluorouridine triphosphate into RNA, fluorodeoxyuridine triphosphate into DNA and inhibition of thymidylate synthase with resultant DNA damage (73). *TP53* status expectedly appears to have predictive value for the survival of CRC patients receiving 5-FU chemotherapy (74).

One study suggest that cells from individuals that carry the Pro72 allele may undergo less apoptosis in response to DNA

damage-inducing therapies when compared with individuals carrying the Arg72 allele. This effect has been suggested to be caused by reduced transcriptional activation of apoptotic effectors (75). In this study, Arg72 expression in presence of chemotherapeutic treatment was shown to induce up to 8-fold more apoptosis than the Pro72 with chemotherapeutics. Studies using p53-inducible isogenic cell lines also noted the greater apoptotic potential of the Arg72 both in the presence (75) and absence (76,77) of chemotherapeutics. Patients, homozygote for the Arg72 allele, with breast or lung cancers have been shown to survive and respond more favorably to chemotherapy and radiotherapy (78-80). Further studies of p53 variants could help to define patient populations by their abilities to respond to stress, suppress tumor formation and respond to DNA damaging therapies (69).

In the present study, we showed that 54.5% of individuals harbor a pathogenic *TP53* mutation. The total number of mutations found in this population is consistent with the literature. Petitjean *et al* (81) stated that *TP53* appeared to be mutated in ~50% of cases in the majority of human tumors. The simultaneous presence of Arg72 allele in the mutated form of *TP53* may serve as a predictor of enhanced tumor development due to inactivation of p73. On the other hand, Arg72 allele over wild-type background may potentially increase apoptotic ability (74). A modifier effect of this SNP has been also reported in germline *TP53* mutation carriers, where Arg72 was found to be associated with an earlier age at the initial cancer diagnosis (82).

In most studies, this SNP has been identified by amplifying the exon 4 followed by digestion using the *Acc*II restriction enzyme. However, partial digestion of the Arg72 homozygote leads to the same pattern as that derived from a heterozygote, causing erroneous conclusions. In the present study, analyses were conducted using direct sequencing, considered the gold standard for mutation/SNP detection. The method used here is appropriate for determining the quantity of C or G nucleotides in RNA samples, as described by Siddique *et al* (13).

Although we have not used more robust techniques for quantifying allelic expression, the mRNA sequencing of a gene allows for the direct verification of which relation exists between the alleles being expressed and tumor characteristics.

The discrepancies between the present study and others are most likely due to differences in population stratification and the methods used to ascertain the polymorphism. Further studies using larger samples and a more detailed analysis of genetic variations within *TP53* are required to examine the role of Pro/Arg alleles in carcinogenesis and to determine whether the proposed association is in linkage disequilibrium with other alleles.

In summary, the data presented here demonstrated that there is a strong correlation between expression of the p53 Pro allele and the aggressiveness of CRC. Thus, we propose that the expression status, rather than the conventionally analyzed genomic status, of p53 variants should be used in studies searching for associations between exonic SNPs and cancers.

Allelic variation of gene expression is of particular interest due to its potential contribution to variation in heritable traits. Therefore, understanding the degree of, structure of, and patterns of variations in gene expression is of central importance to determine its role in the pathogenesis of CRC.

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