p53Arg72 Homozygosity and its Increased Incidence in Left-sided Sporadic Colorectal Adenocarcinomas, in a Greek-Caucasian Population

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Abstract. *Background: The current case-control study was conducted in order to elucidate any possible association of the single nucleotide polymorphism (SNP) of codon 72 of the p53 gene (Arg72Pro) and sporadic colorectal adenocarcinoma development in a Caucasian population in Greece. The distribution of its alleles, in relation to many clinical parameters of the cancer group, was also investigated. Materials and Methods: Genomic DNA samples from 93 sporadic colorectal adenocarcinoma cases and 95 healthy controls (age and ethnicity matched) were used to genotype the p53 codon 72 polymorphism. Results: A strong association of the homozygous 72Arg allele with the development of colorectal cancer was observed (Chi-Square=11,212, p=0.001, O.R=2.902, 95% (CI)=1.540-5.469, for Arg/Arg vs. Arg/Pro and Pro/Pro). When tumor location was accounted for, the Arg/Arg carrier genotypes were associated with an increased incidence of left colon cancer (Chi-Square=5.256, p=0.026, OR=2.975, 95% (CI)=1.150-7.699). Conclusion: p53Arg homozygosity is associated with the development of sporadic colorectal adenocarcinoma, in the Greek-Caucasian population studied and this polymorphism may have a significant prognostic value, where tumor location is concerned.*

The *p53* tumor suppressor gene is one of the most well studied human genes, because of its great importance in many cellular processes such as cell cycle arrest, programmed cell death and DNA repair (1). It has been estimated that *p53* signaling pathways are dysfunctional or non-functional in at least 80% of all human carcinomas (2). Apart from gene mutations, there are at least 10 genetic polymorphisms in the *p53* gene described so far (3). The most intensively studied polymorphic site in the p53 gene is the one at codon 72 at exon 4, encoding either arginine (CGC, *p53Arg*) or proline (CCC, *p53Pro*) (4). The *p53* codon 72 polymorphism is located in a proline rich region (residues 64-92), in which the proline isoform constitutes one of the five P*xx*P motifs resembling a *Src* homology 3 (*SH3*) binding domain, which is important for p53 dependent apoptosis and growth suppression, but not for cell cycle arrest (5). These two polymorphic variants have many different activities including their kinetic profiles in SDS-PAGE (Sodium-dodecyl sulfate polyacrylamide gel electrophoresis) (5), their response in cancer chemotherapy (6, 7) and their apoptotic potential (8, 9).

Since the elucidation of the above differences between the two alleles, there have been many efforts to outline the association of this polymorphism with several neoplasias such as cervical neoplasia (10), lung cancer (11, 12), breast cancer (13-15), gastric cancer (16, 17), hepatocellular carcinoma (18) and many other neoplastic and non-neoplastic diseases, however most of the results were contradictory and inconclusive. More specifically, some of these studies suggested that the *p53Arg* isoform was associated with increased risk of developing cancer, whilst others pointed to the *p53Pro* form as possibly being associated with cancer development and others that have not shown any association.

Colorectal cancer is the second most common type of neoplasia in the U.S and Europe, with a constantly increasing incidence (19). In Greece, although the incidence is lower than in the rest of the European countries,

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colorectal cancer remains a very important cause of death, since it is the fourth most common neoplasia, in the general population. Colorectal carcinogenesis is characterised by several alterations in DNA sequences involved in numerous molecular pathways, such as tumor suppressors, oncogenes and genes involved in the DNA repair process, such as *APC* (Adenomatous Polyposis Coli), *K-ras* (Kirsten-ras), *DCC* (Deleted in Colorectal Cancer), *p53* and mismatch repair (*MMR*) (20). The above information suggests that any functional change between the two polymorphic variants of the *Arg72Pro* polymorphism may affect the association of the protein with colorectal adenocarcinoma development. So far, there are a few studies that have examined the possible association of this polymorphism with colorectal carcinogenesis (21-27). However, the results between these studies do not all agree. In addition to that, it is known that the *p53Arg* isoform of the protein is more susceptible to inactivation through the ubiquitin-dependent proteolytic pathway by the *E6* protein encoded by the human papilloma virus (HPV), than the *p53Pro* isoform (10). Due to this fact, most of the studies of the *p53 Arg72Pro* polymorphism also include the possible association of HPV. It is also known that the *Arg72Pro* polymorphism shows significant heterogeneity, with geographical location and ethnicity accounting for many fluctuations and changes in the distribution of its alleles (22, 28, 29).

The association of the polymorphism of codon 72 of the *p53* tumor suppressor gene and the development of colorectal adenocarcinoma was evaluated in a Caucasian population in Greece. The distribution of the polymorphic variants in the population of sporadic colorectal adenocarcinoma was also evaluated, in terms of its association with various clinicopathological characteristics, such as colorectal tumor site, age at diagnosis, survival, Duke's stage, differentiation and gender.

Materials and Methods

Study populations. Ninety-three consecutive Greek Caucasian patients with sporadic colorectal adenocarcinoma (all Duke's stages), who received surgical treatment in the First Propaedeutic and Fourth Department of Surgery of the Medical School of the University of Athens, Greece, during the period of 2000-2003 were included. The mean age at diagnosis was 66.8±10.4 years with men accounting for 49.5% and women 50.5% of the cases. The cases were all sporadic adenocarcinomas of the colon and rectum and were classified according to Duke's staging system. Small pieces of the solid tumors were obtained during surgery and served as the genomic DNA source. The control group consisted of 95 Greek healthy individuals (matched for age, ethnicity and origin), who had no clinical evidence of any neoplastic disorder, at the time of the study. The mean age of the control group was 62.74±9.62 years (50.5% males, 49.5% females). Peripheral blood was obtained and served as the genomic DNA source.

Genomic DNA extraction. Genomic DNA was extracted from the solid tumors and peripheral blood samples using a Jetquick DNA extraction kit® (Genomed GmbH, Löhne, Germany), which is a modification of the original extraction method described by Bowtel in 1987 (30), according to the manufacturer's instructions. All the extracted DNAs were used for the genotyping process of the allele-specific PCR and the presence of DNA capable of amplification was tested in a PCR reaction using a set of primers for the β2-microglobulin gene.

p53 Arg72Pro genotyping process. For the determination of the p53 Arg72Pro genotype status of each patient and healthy individual the original allelic-specific PCR was used, as described by Storey *et al.*, 1998 (10), with a few modifications. This specific PCR reaction has the advantage of selectively detecting either the arginine allele or the proline allele. The PCR primers used for the amplification of the polymorphic site were: forward, 5'-TCC CCC TTG CCG TCC CAA-3', reverse, 5'-CTG GTG CAG GGG CCA CGC-3' for the *p53Arg* allele, and forward, 5'- GCC AGA GGC TGC TCC CCC-3', reverse, 5'-CGT GCA AGT CAC AGA CTT-3' for the *p53Pro* allele (10). Approximately 100 ng of genomic DNA from each sample of patients and controls was amplified in a PCR reaction containing 1 x buffer (100 mmol/L Tris-HCl pH 8.8, 25˚C, 500 mmol/L KCl, 8 mL/L Nonidet P40, Fermentas Life Sciences, USA), 2.5 mmol/L $MgCl_2$, 10 pmol of each primer, 10 mmol/L dNTP's (deoxyribose nucleotide triphosphates) and 0.5 U/μL of *Taq* polymerase (Fermentas Life Sciences, USA), in a final volume of 20 μL. The PCR conditions were as follows: denaturation at 94˚C for 3 min, followed by amplification for 40 cycles at 94˚C for 30 sec, 60˚C for the *p53Arg* allele and 54˚C for the *p53Pro* allele, both for 30 sec, with a final extension at 72˚C for 5 min. The products were visualised using agarose gel electrophoresis (15 mg/L gels, 1.5%), stained with ethidium bromide and photographed under UV illumination. The PCR product of the *p53Arg* pair of primers was a band of 141bp and for the *p53Pro* primer pair a band of 177bp (Figure 1). Samples showing only one product were assigned as homozygous, whereas those exhibiting both products as heterozygous.

Statistical analysis. Odds ratios and 95% confidence intervals (CI) were used in order to estimate the magnitude of the association between *p53 Arg72Pro* genotypes and sporadic colorectal adenocarcinoma. Pearson's Chi-square, Fisher's exact test (where necessary) and Kruskal-Wallis non-parametric test were also performed, using the statistical package SPSS 11.5 (SPSS Chicago, IL, USA). The statistical significance level was set at $p<0.05$.

Results

The tumors were of various differentiation levels (high: 7.5%, moderate: 79.6%, low: 12.9%) and of all Duke's stages, 29 (31.2%) were Duke's A, 17 (18.3%) Duke's B, 45 (48.4%) Duke's C and 2 (2.2%) were Duke's D. The descriptive characteristics and the allele frequencies observed in the case and control populations are shown in Table I.

The distribution of the alleles of the *Arg72Pro* polymorphism, in the 93 adenocarcinoma patients and the 95 controls is shown in Table I. The genotypic distributions showed an overall significant difference between cases and

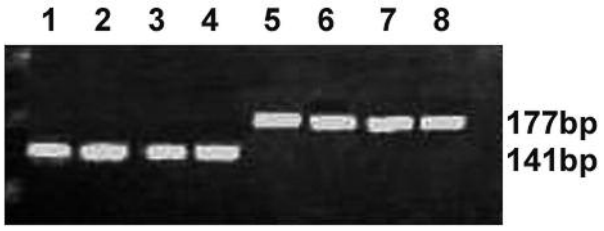

Figure 1. *Image from 15 mg/L agarose gel electrophoresis. 1-4 homozygous samples for p53Arg (141bp), 5-8 homozygous samples for p53Pro (177bp).*

controls as determined by Fisher's exact test=11.475, p=0.003. The relative frequencies of the two alleles *p53Arg* and *p53Pro* according to the Hardy-Weinberg equilibrium were 0.67 for *p53Arg* and 0.33 for the *p53Pro* in the cancer population and for the population of controls 0.55 and 0.45, respectively.

The statistical analysis using estimation of odds ratios and Pearson's Chi-square revealed a statistically significant difference of the homozygous *p53Arg* genotype compared with the combined *Arg*/*Pro* and *Pro*/*Pro* genotypes. The corresponding odds ratio for this comparison was OR=2.902 95% CI=1.540-5.469 Chi-square=11.212 p=0.001, showing that the homozygotes for *p53Arg* in the population studied were strongly associated with the presence of colorectal adenocarcinoma. The calculation of the odds ratios also gave a significant result, when the heterozygous genotype *Arg*/*Pro* was compared with the two homozygous genotypes (OR for *Arg*/*Pro vs. Arg*/*Arg* and *Pro*/*Pro*) with an estimated OR=0.420 95% CI=0.233-0.756 Chi-square=8.508 p=0.04. From the total 42 homozygous adenocarcinomas for the *p53Arg* allele, 34 (81.0%) of them were from the left colon (descending, sigmoid and rectum), whereas the remaining 8 (19.0%) were from the right colon (ascending and transverse colon). The estimated odds ratio of this difference between the left and right colon was equal to OR=2.975 95% CI=1.150-7.699 Chi-square=5.256 p=0.026, meaning that the adenocarcinomas homozygous for the *p53Arg* allele had an almost three times greater possibility of having arisen from the left colon than from the right. This difference was significantly associated with the status (alive/dead) of the *p53Arg* adenocarcinoma patients. Of the total 42 *p53Arg* homozygotes, from the 22 who were alive, 21 (95.5%) were from the left colon, whereas only 1 (4.5%) was from the right and out of the 20 who had died (at the time of survey) 13 (65.0%) were from the left and the remaining 7 (35.0%) were from the right colon, with an estimated odds ratio of OR=11.308 95% CI=1.245-102.718 Chi-square=6.301 p=0.018. There was no statistically significant difference in the distribution of the allelic genotypes (p>0.05 for Kruskal-Wallis significance), when the parameters of mean age at diagnosis (years), gender, survival, histological growth pattern, tumor differentiation or Duke's staging were taken into account.

Table I. *Descriptive characteristics, allelic distribution and population clinicopathological parameters.*

Descriptive characteristics	Adenocarcinomas	Normal controls
Study Populations, n	93	95
Gender, n (%)		
Male	46 (49.5%)	48 (50.5%)
Female	47 (50.5%)	47 (49.5%)
Age, mean±s.d, years	66.8±10.4	62.74±9.62
Allelic Distribution		
Arginine homozygotes (*Arg*/*Arg*)	42 (45.16%)	21 (22.10%)
Heterozygotes (*Arg*/*Pro*)	41 (44.08%)	62 (65.26%)
Proline homozygotes (*Pro*/*Pro*)	10 (10.75%)	12 (12.63%)

(*Arg*/*Arg vs*. *Arg*/*Pro* and *Pro*/*Pro*) OR=2.902, 95% CI=1.540-5.469, Chi-square=11.212, p=0.001.

Allelic distribution by tumor location	Left colon	Right colon	Total
n (%)	64 (68.8%)	29 (31.2%)	93 (100%)
Arg/*Arg*	34 (81.0%)	8 (19.0%)	42 (45.16%)
Arg/*Pro*	25 (61.0%)	16 (39.0%)	41 (44.08%)
Pro/*Pro*	5 (50.0%)	5 (50.0%)	10 (10.75%)

(For *Arg*/*Arg* left *vs*. right colon) OR=2.975, 95% CI=1.150-7.699 Chi-square=5.256, p=0.026; OR: odds ratio, CI: confidence intervals.

Discussion

In this case-control study the *p53 Arg72Pro* polymorphism was positively associated with the presence of sporadic colorectal cancer in the Greek-Caucasian population studied. The results suggested that homozygosity of *p53Arg* was strongly associated with colorectal cancer. In fact the homozygous *p53Arg* patients were almost three times as likely to develop sporadic colorectal adenocarcinoma (OR=2.902, p=0.001). These results were in agreement with the original report of the association of this specific polymorphism and malignancy (cervical neoplasia), by Storey *et al.* (10). In addition, our results agree with the recent report, of Perez *et al.*, (27), who observed a positive correlation of the homozygous *p53Arg* genotype with colorectal cancer, in a case-control study including 126 individuals with sporadic adenocarcinoma of the colon and rectum, in Argentina. On the other hand, many previous studies of the p53 *Arg72Pro* polymorphism and colorectal cancer had different results (24, 25), or failed to establish a significant correlation (23). In cervical cancer, an increased risk associated with the homozygous *p53Arg* genotype has been reported (31), but in contrast, for lung cancer, the

p53Pro carrier genotype was associated with increased risk (32). Data from many studies, including other types of neoplasias or disease associations have drawn opposite conclusions. This could be due to different experimental protocols or poor selection methods of the control group. The most important factor is ethnic background since different genotypic frequencies due to natural selection through ecological adaptation to ultra-violet radiation have been found (33), with the populations living closer to the equator having increased *p53Pro* allele frequencies. The frequency of the two alleles in the control population in the present study was similar to that obtained by other association studies that included controls and cases from the geographical area of Greece, for lung cancer (11), breast cancer (14), bladder cancer (34) and cervical neoplasia (35).

To our knowledge, the present study was the first to establish a positive correlation of the homozygous *p53Arg* genotype and sporadic colorectal adenocarcinoma in a population from Greece. In addition to that, the two populations of the current case – control study were selected from the same underlying population, thus minimizing the possibility of selection bias (26). As far as methodology is concerned, the PCR reaction used had the advantage of using separate PCR reactions for each allele, so that low copies of one allele were not affected by the presence of several copies of the other allele (27) and all genotyping results were 100% reproducible. The use of fresh tissue and blood specimens is advantageous when compared to archival or fixed tissues, since it has been shown that artificial mutation detection is less frequent, suggesting a much lower level of misclassification when using such specimens (32, 36). On the other hand, this technique has the tendency to under-estimate heterozygotes, whereas the method that uses analysis with restriction enzymes has the tendency to over-estimate heterozygotes (11). Additional studies confirming frequencies by more than one method and establishing a genetic "map" of ethnicities and geographical locations of the *p53 Arg72Pro* polymorphism could clarify the association between the polymorphism and malignancy.

The current study provides additional knowledge regarding the association of the *p53 Arg72Pro* polymorphism and colorectal cancer, in relation to the colorectal tumor site. Recent evidence from embryological and physiological studies has shown that proximal and distal parts of the colon (in relation to the splenic flexure) represent distinct anatomical and functional entities and that colorectal cancer is differentiated into two phenotypes on the basis of underlying genetic mutational pathways (37, 38). So far, there are only two studies that have analysed the distribution of the polymorphism according to the colorectal tumor site (26, 21), with the first showing a small difference between proximal and distal colorectal cancer when sex was taken into account and the second showing no significant variation of the genotypic distribution according to the tumor site. In our study, the distribution of the *p53 Arg72Pro* genotypes was found to be greatly dependent on the site of the tumor, with the homozygous *p53Arg* adenocarcinomas having an almost three times greater possibility of having arisen from the left colon than the right colon (OR=2.975, $p=0.026$), for both men and women. This difference also had a significant prognostic value, when stratified for the status (alive-dead) of the homozygous *p53Arg* patients.

In conclusion, the *p53Arg72Pro* polymorphism is positively associated with the presence of sporadic colorectal cancer, in Greek Caucasians and could have a significant prognostic value, when stratified for tumor location. More studies are needed including much larger population groups, with attention to ethnical and geographical origin to provide a clearer view of the status of this association.

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