RESEARCH COMMUNICATION

Association of Arg72Pro of *P53* Polymorphism with Colorectal Cancer Susceptibility Risk in Malaysian Population

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Abstract

Background: Colorectal cancer (CRC) results from the interaction between environmental exposures and genetic predisposition factors. Aims: A case control study was designed and to investigate the genotype frequencies of *P53* Arg72Pro polymorphism in Malaysian CRC patients and healthy controls and to determine the associated risk of this polymorphism with CRC predisposition. Methods: In this case-control study, peripheral blood samples of 202 sporadic CRC patients and 201 normal controls were collected, DNA extracted and genotyped using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique. Results: Genotype analysis showed the frequency of homozygous variant (Pro/Pro) genotype (21%) to be significantly higher in cases compared to controls (13%), (p=0.013). On examining the association between variant genotypes and CRC risk, the Pro/Pro homozygous variant genotype showed significantly higher risk association with CRC susceptibility (OR: 2.047, CI: 1.063-4.044, p=0.033). When stratified according to age, we observed that, individuals aged above 50 years and carriers of pro/pro genotype had significantly higher risk with OR: 3.642, CI: 1.166-11.378, p=0.026. Conclusions: Our results suggest that the codon 72 SNP which results in amino acid substitution of Arginine to Proline in cell cycle regulatory gene P53, is associated with sporadic CRC risk and carriers of Pro/Pro genotype and more than 50 years old may have high susceptibility.

Keywords: Colorectal cancer - TP53 codon 72 - Malaysia

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Introduction

Colorectal cancer (CRC) is the second to fourth most common cancer in developed countries. Worldwide, 875,000 or more people are diagnosed with CRC annually (de la Chapelle, 2004). The incidence of CRC is increasing in developing countries including Malaysia. In Malaysia, CRC has become the second commonest cause of cancer related mortality after breast cancer and has become the most common cancer in men and second in women (Malaysia Cancer Statistics, 2006). Being a complex and multifactorial disease, its etipathogenesis involve interaction between environmental and genetic factors. Age, gender environmental factors such as diet, tobacco smoke and alcohol consumption (Giovannucci, 2001; Terry et al., 2001; Neagoe et al., 2004; Yeh et al., 2005; Stern et al., 2007) in interaction with genetic factors have been shown to increase the risk of colorectal cancer (de la Chapelle, 2004)

Tumour suppressor genes play important role in mediating cellular responses to genotoxic insults through its effects on gene transcription, DNA synthesis and repair, genomic stability and apoptosis (Vogelstein and Kinzler, 1992). The most common mutated gene in various cancers is *P53* gene which is involved in 50% cancers. Being a guardian of the genome, p53 is involved in G1 arrest which facilitate DNA repair during replication or in induction of apoptosis and cell cycle regulation. In sporadic colorectal cancer, 75% of the tumours had been reported to have inactivation of p53 (Kressner et al., 1999). This inactivation could be single base substitution or loss of alleles with inactivation by viral or cellular proteins (Tommasino et al., 2003).

Genetic variation like Single Nucleotide Polymorphisms (SNPs) in candidate genes such as DNA damage and tumour suppressor genes are thought to play an important role in individual variation in colorectal cancer susceptibility. Genetic association studies have focused on SNPs as important tools for targeting the genes responsible for cancer susceptibility (Cao et al., 2009). A SNP at the codon 72, located at exon 4 of *P53* gene (Lima-Ramos et al., 2008) has been studied significantly for its association with various types of cancer such as colorectal, breast and other types of cancer (Tenesa et al., 2008;

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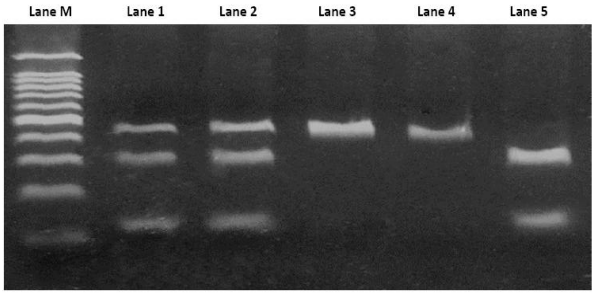

Figure 1. Genotype Patterns of *P53* Arg72Pro Polymorphism in Cases and Controls

Tomlinson et al., 2008; Zhu et al., 2007). Several studies showed positive association between this polymorphism with colorectal cancer susceptibility and the rest, failed to ascertain this relationship. Previous studies also have shown that, Arg72Pro polymorphism of *P53* varies in different ethnic and population groups. Since no reports are available from Malaysian population, we conducted a case control study to investigate the allele and genotype frequencies and the contribution of variant genotype of Arg72Pro of *P53* gene in modifying the susceptibility risk in Malaysian sporadic CRC patients.

Materials and Methods

Study subjects

The study was approved by Research Review Board and Ethics Committee of Universiti Sains Malaysia, Kelantan and Ministry of Health, Malaysia. For this Hospital based case control study, subjects were recruited from Hospital Universiti Sains Malaysia (HUSM), Hospital Raja Perempuan Zainab II and Hospital Sultanah Bahiyah, Kedah, Malaysia. Genotyping was carried out at the Human Genome Center, Universiti Sains Malaysia. Two hundred and two (202) hispathologically confirmed sporadic CRC patients and 201 healthy normal controls were recruited as study subjects. Cases with known (as indicated in the pathology reports) familial adenomatous polyposis, ulcerative colitis or Crohn's disease or any other previous malignancy were excluded. Controls were normal healthy individuals, volunteers who visit HUSM for other problems unrelated to colorectal cancer and selected by using the same eligibility criteria as those used for cases. Controls were biologically unrelated to the study patients and were cancer free participants. Epidemiological data was collected from patients using a pre-structured questionnaire which included socio-demographic status, physical status, dietary factors, occupation, tobacco/alcohol habits, previous illness, radiation exposure etc.

DNA extraction and P53 Arg72Pro genotyping

Three (3) ml of whole blood was collected from all study participants in sterile EDTA-coated vacutainer. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and stored at -200C until used for genotyping. Genotyping of SNP *P53* Arg72Pro was carried out employing Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Briefly, the region of interest was PCR amplified using appropriate primers (Foward: 5'-TCA AAC ATC CTG TCC CTA CT -3', Reverse: 5'-CTG CGA TTA AAG GCT GTG GA -3') which generated a 458 bp product containing the polymorphic site. The PCR reactions were carried out in a 25 μl volume of 1X PCR Buffer, 2.0mM of $MgCl_2$, 0.5mM dNTPs, 0.4mM of each primers and 1U of Ampli*taq* gold polymerase with a denaturation of 94˚C for 5 min, followed by 35 cycles at 94 ˚C for 30 s, 57˚C for 30 s and 72˚C for 30 s and finally 5 min at 72 ˚C. Following amplification, PCR products were digested using *BstUI* restriction enzyme (New England BioLab) for 1 hour at 600C and electrophoresed on 2% agarose gel. The Arg allele was cleaved by *BstUI* and yielded two small fragments (321 and 137 bp). The Pro allele was not cleaved by BstUI and had a single 458 bp band. The heterozygous contained three bands (458, 321 and 137 bp). The genotype categorized as wildtype (Arg/Arg), heterozygous (Arg/Pro) and homozygous variant (Pro/Pro) based on band sizes are shown in Figure 1.

Table 1. Distribution of Sex and Age Among Cases and Controls

	Cases n=202	Controls n=201	p-value
Sex			
Female	89 (44%)	106 (53%)	0.081
Male	113 (56%)	95 (47%)	
Age			
<50	37 (18%)	124 (62%)	<0.001*
≥50	165 (82%)	77 (38%)	

*p-value < 0.05, statistically significant

Table 2. Genotype and Allele Frequencies of Arg72Pro of P53 Polymorphism in Colorectal Cases and Controls

	Cases n=202	Controls n=201	p-value
Genotype			
Arg/Arg	70 (35%)	75 (37%)	0.578
Arg/Pro	88 (44%)	101 (50%)	0.179
Pro/Pro	44 (21%)	25 (13%)	0.013*
Allele			
Arg allele	0.564	0.624	
Pro allele	0.436	0.376	

*p-value < 0.05, statistically significant

Statistical Analysis

The difference in distribution of genotypes, gender and age between cases and controls were assessed using Chi Square, $\chi 2$ test. The Odds Ratios (ORs) and 95% Confidence Interval (CI) were calculated by using binary logistic regression (SPSS version 18) adjusted by sex and age to assess the risk association. All statistical tests were two sided, and statistical significance was determined as $p< 0.05$.

Results

In this case control study, cases involved 202 histopathologically confirmed sporadic CRC patients (113 males and 89 females) and controls comprised of 201 healthy normal individuals (95 males and 106 females).

Table 3. Association of Arg72Pro of P53 Polymorphism with Colorectal Susceptibility Risk

Genotype	Cases n=202	Controls n=201	Crude OR (95% CI)	AdjustedOR (95%CI) ‡	p-value
Arg/Arg	70	75	1 (Ref)	1 (Ref) †	-
Arg/Pro	88	101	0.934 (0.606-1.440)	0.827 (0.508-1.346)	0.444
Pro/Pro	44	25	1.886 (1.046-3.399)	2.047 (1.063-4.044)	0.033*

†The genotype served as reference category, ‡ Unconditional logistic regression adjusted to gender and age, *p-value < 0.05, statistically significant

Table 4. Association of Gender, Age and SNP P53 Arg72Pro Genotype with Colorectal Cancer Susceptibility Risk

Genotype	Cases	Controls	OR (95% CI)‡	p-value
Female				
Arg/Arg	30	40	1 (Reference)†	
Arg/Pro	40	51	0.861 (0.429-1.729)	0.674
Pro/Pro	19	15	1.912 (0.759-4.814)	0.169
Male				
Arg/Arg	40	35	1 (Reference)†	
Arg/Pro	48	50	0.840 (0.460-1.534)	0.750
Pro/Pro	25	10	2.187 (0.924-5.181)	0.075
Age <50 years				
Arg/Arg	10	52	1 (Reference)†	
Arg/Pro	21	51	2.141 (0.919-4.991)	0.078
Pro/Pro	6	21	1.486 (0.479-4.608)	0.493
Age ≥ 50 years				
Arg/Arg	60	23	1 (Reference)†	
Arg/Pro	67	50	0.519 (0.283-0.952)	0.034*
Pro/Pro	38	4	3.642 (1.166-11.378)	0.026*
Male & <50 years				
Arg/Arg	4	25	1 (Reference)†	
Arg/Pro	13	25	3.250 (0.831-11.347)	0.065
Pro/Pro	2	9	1.389 (0.219- 8.927)	0.729
Male & ≥ 50 years				
Arg/Arg	36	10	1 (Reference)†	
Arg/Pro	35	25	0.389 (0.163-0.927)	0.033*
Pro/Pro	23	1	6.387 (0.766-53.289)	0.087
Female & <50 years				
Arg/Arg	6	27	1 (Reference)†	
Arg/Pro	8	26	1.385 (0.422-4.541)	0.591
Pro/Pro	4	12	1.500 (0.357-6.308)	0.580
Female & ≥ 50 years				
Arg/Arg	24	13	1 (Reference)†	
Arg/Pro	32	25	0.693 (0.295-1.629)	0.401
Pro/Pro	15	3	2.708 (0.660-11.109)	0.166

† The genotype served as reference category, ‡ Unconditional logistic regression adjusted to gender and age, *p-value < 0.05, statistically significant

The mean age was 60.36 ± 12.32 years for the cases and 55.01 ± 12.17 years for the controls. The distribution of gender and age group among study subjects are shown in Table 1. There wcas significant difference in the incidence of CRC between age groups (p-value, <0.001). CRC incidence was higher among individuals more than 50 years compared to those less than 50 years. When the incidence of CRC was compared between males and females, a slight preponderance of CRC was observed among males, however difference was not statistically significant (p=0.081).

Table 2 shows the genotypes and allele frequencies of Arg72Pro of P53 polymorphism in colorectal cases and controls. In colorectal cancer cases, the frequencies of genotypes of Arg/Arg, Arg/Pro and Pro/Pro were 70 (35%), 88 (44%) and 44 (21%) respectively whereas in the controls, the genotype frequencies were 75(37%), 101(50%) and 25(13%) respectively. On comparing the genotype frequencies between cases and controls, the frequency of Pro/Pro genotype was significantly higher in cases (p-value: 0.013). The frequencies of minor allele (Pro allele) in cases and controls were 0.436 and 0.376 respectively.

The risk association of Arg72Pro of *P53* gene polymorphism with colorectal cancer susceptibility was examined using Binary Logistic Regression analysis and deriving Odds Ratios (ORs). All ORs were calculated relative to subjects with the major alelle Arg/Arg genotype as a reference. Table 3 shows the association of *P53* Arg72Pro genotypes with colorectal susceptibility risk. The homozygous variant (Pro/Pro) genotype showed significantly higher risk for colorectal cancer susceptibility with adjusted OR: 2.047, (95% CI: 1.063-4.044 and p-value = 0.033).

Additionally, we stratified our study subjects to investigate the relationship of the SNP studied with other confounding factors like gender and age with colorectal cancer susceptibility risk and the results are shown in Table 4. Age wise stratification showed that, carriers of Pro/Pro genotype with age 50 years and above had a significantly higher risk for colorectal cancer susceptibility (OR: 3.642, 95% CI 1.166-11.378). When stratified according to gender and age, males with age more than 50 years and carriers of Pro/Pro genotype also showed high risk for colorectal cancer susceptibility (OR: 6.387, 95% CI: 0.766-53.289), but was statistically insignificant. This could be due to the wide range of CI observed due to the presence of Pro/Pro genotype in only 1 normal control male aged more than 50 years. We also found that, Arg/Pro genotype displayed a protective effect against colorectal development for individuals with age more than 50 years old and especially for males with age more than 50 years old with OR: 0.519 and 0.389 respectively.

Discussion

Progression of sporadic colorectal cancer involve accumulation of multiple somatic mutations in cells, inactivation of DNA damage repair genes and tumour suppressor genes as well as activation of oncogenes. Interaction of endogenous and exogenous factors which lead to DNA damages will cause the abnormalities in the human genome, especially in genes that involved in DNA repair pathways. DNA damage should be repaired before the cells enter S-phase or mitosis. In this phase, check point mechanisms will ensure that DNA is intact before the cell proceeds for cell replication and division. Failure of DNA repair mechanism will lead to accumulation of DNA damage which turns to mutation. Defects in DNA

damage repair genes will also lead to involvement of *P53* tumour suppressor gene to overcome the damages by inducing apoptosis or programmed cell death. *P53* gene is involved in cell cycle regulation, controlling DNA repair and apoptosis. Single nucleotide polymorphism in *P53* may completely disrupt the function of the protein, resulting in high rate of uncontrolled cell growth or cancer and thus contribute to large number of tumours.

Amino acid substitution from arginine (CGC) to proline (CCC) at codon 72 has been identified and shown to alter the p53 protein structure (Matlashewski et al., 1987; Koushik et al., 2006). Studies have shown that, these two types of proteins are different in biology and biochemical pathways (Thomas et al., 1999). Study conducted by Pim and Banks, demonstrated that, these two proteins, either Arginine or Proline have different results in alteration of primary structure of protein. Their results showed that Arg72 allele is more efficient than the Pro72 allele at inducing apoptosis (Pim and Banks, 2004). Marin et al. showed that, p53 mutant inactivated the p73 protien and showed less effectiveness and low efficiency compared with Arg allele during apoptosis (Marin et al., 2000). It has been reported that, Arg72 is better than Pro72 in inducing apoptosis as Arg72 have a greater ability to localize to the mitochondria and enhance apoptosis whereas variant of p53 is more potent in binding with p73 and neutralizing p73 to induce apoptosis (Dumont et al., 2003). These studies have demonstrated that functional differences exist between these two alleles of the *P53* gene. Genetic variations in *P53* can result in the damage being left unrepaired and inefficient apoptosis which can lead to unregulated cell growth and cancer.

In this case control study, we investigated the genotype frequencies and associated causal role of the common SNP Arg72Pro of *P53* in sporadic colorectal predisposition in Malaysian population since no previous reports are available. We observed significant association between SNP Arg72Pro of *P53* gene and colorectal cancer susceptibility. *P53* codon 72 Pro/Pro genotype showed significantly higher risk for colorectal cancer susceptibility. This clearly indicated that, individuals who have Pro/Pro genotype have a twofold higher risk for colorectal cancer development compared to individuals who have Arg/Arg genotype.

Our results are concordant with few other studies that also showed significant risk association. Study conducted by Zhu et al. in Chinese population showed that, carriers of Arg/Pro and Pro/Pro genotype had a higher risk of colorectal cancer development with OR: 1.60 and 2.37 (Zhu et al., 2007). Significant association of variant Pro/Pro genotype with increased risk of colorectal cancer was reported by Cao et al.(2009) in a Korean population and Sameer et al. (2010) in a Kashmiri population. When analyzed based on the age-gender factors, no significant associations were found between these two confounding factors on CRC susceptibility (Zhu et al., 2007; Cao et al., 2009, Sameer et al., 2010). However, in the present study, we found a significant association on age and CRC susceptibility. Out of 202 CRC patients, 82% were above 50 years old and 18% were less than 50 years.

Study conducted by Joshi et al. (2010) found that men with Pro/Pro genotype and Pro allele showed significantly higher risk for colorectal cancer development compared to women. They combined the genotype of P53 polymorphism with *MDM2* polymorphism and found an association between these two polymorphisms with colorectal cancer susceptibility. Individuals who carried the Pro allele of *P53* and guanine allele of SNP309 showed significantly higher risk with OR: 1.67, CI: 1.11-2.51 (Joshi et al., 2010). Van Heemst et al., did a formal large meta-analysis of published results from the literature and studied the impact of P53 Pro/Pro and Arg/Arg polymorphisms upon the frequency of developing cancers and upon the longetivity of the population under study. From the analysis, they concluded that individuals with a Pro/Pro genotype had an increased risk of developing a cancer over their lifetimes compared to individuals with an Arg/Arg genotype. These researchers interpreted that the Arg/Arg genotype has a higher apoptotic rate in response to stress and so protects against cancer better (Van Heemst et al., 2005).

Kaushik et al. (2006) did not find significant overall association between *P53* Arg72Pro genotype and colorectal cancer but found significant association with colorectal adenoma risk. When stratified by gender, an increased risk of proximal colorectal cancer in women and distal colon cancer in men were observed (Koushik et al., 2006). Higher risk association between CRC and *P53* Arg72Pro polymorphisms were reported by Perez et al.(2006) in an Argentinean population and also by Mammano et al. (2009) in Italian population.

Few other studies have reported contradictory findings also. Tang et al. (2010) conducted a meta-analysis involving 17 case control studies and with a total of 3537 colorectal cancer cases and 5168 controls as study subjects. They did not find any significant association of Pro/Pro genotype of *P53* with colorectal cancer when compared with Arg/Arg genotype. In this study, risk for Pro/Pro genotype was OR: 1.02, (CI: 0.80-1.29) and for Arg/Pro, the OR was 1.00, CI: (CI: 0.86-1.16) . Similarly Economopoulos et al. also did not find any significant risk association when they conducted a Meta-analysis study involving 19 Caucasion , 6 Chinese and 2 mixed populations (Economopoulos et al., 2010).

The difference in results on risk association between the present study and other previous studies might be explained by difference in groups studied or populations, and also differences in environmental exposure and lifestyle factors. Smaller sample size and or inadequate controlling for certain confounder factors such as gender and age also might have contributed to differing results and lack of association. Further studies exploring the interaction of *P53* Arg72Pro with other genes involved in DNA repair pathways, either singly or in combination, and also correlating with environmental interactions such as smoking, alcohol consumption, and dietary habits as well as clinicopathological characteristics, would be beneficial in deriving more accurate risk predictive markers. In conclusion, our study provides evidence that P53 Arg72Pro polymorphism may contribute to the etiology of sporadic colorectal cancer in the Malaysian population and individuals who are above 50 years old

and carriers of Pro/Pro genotype especially have a higher risk for colorectal cancer susceptibility.

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