GENETICS

Polymorphism in the Tumor Necrosis Factor Alpha Promoter Region and Its Influence on Colorectal Cancer Predisposition Risk in Malaysian Population

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ABSTRACT

Objective: **A case control study was designed to investigate the TNF-α -308 G>A polymorphism allele frequencies and to determine the influence of the polymorphic genotype on sporadic CRC susceptibility risk in Malaysian population.**

Materials and Methods: **Peripheral blood samples of 164 normal controls and 161 clinically and histopathologically confirmed CRC patients were genotyped for TNF-α -308 G>A polymorphism employing allele specific PCR. The relative associations of various genotypes with CRC susceptibility risk was determined by calculating Odds Ratios. Corresponding chi-square tests on the CRC patients and controls were carried out and 95% confidence interval (95% CI) were determined using Fisher exacts tests.**

Results: **On comparing the frequencies of genotypes of patients and controls, the homozygous variant AA was significantly higher in CRC patients (p = 0.030) compared to controls. On investigating the association of the polymorphic genotypes with CRC susceptibility risk, the homozygous variant TNF-α -308 AA showed significantly increased risk with OR 2.5842.**

Conclusion: **Our results suggest that, polymorphic genotype of inflammation response gene TNF-α is significantly associated with CRC susceptibility risk and could be considered as a high risk variant for CRC predisposition.**

KEY WORDS

colorectal cancer, inflammation response gene, TNF- α , plymorphism

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in developed countries and getting more and more attention in developing countries for its morbidity and mortality. Even though the definite mechanism of development is still unknown, both environmental factors and genetic susceptibility are believed to contribute to the onset of CRC. Excluding inherited types of CRC, the susceptibility of a certain individual to development of sporadic CRC remains largely undetermined. A recent leading theory is that the oxidative stress that accompanies chronic inflammation contributes to neoplastic transformation[1,2] Epidemiological observations, animal and clinical studies have established an association between continuous inflammatory condition and CRC[1,3,4] Patients with inflammatory bowel disease (IBD), including Crohn's disease (CD) and Ulcerative colitis (UC), are at increased risk of developing colorectal cancer[5]. The associations between inflammatory response genes and IBD make them attractive candidate susceptibility genes for colorectal cancer since approximately 1:6 individuals with IBD will develop malignancy[6]. Despite these evidences strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors which mediate inflammatory response and favors CRC development.

Genetic polymorphisms have emerged in recent years as important determinants of disease susceptibility and severity. Polymorphic variants of several genes are thought to play a key role in determining how individuals respond at the cellular level to various environment conditions including inflammation. If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes which mediate inflammatory response might be a group of candidate

Received on January 13, 2011 and accepted on April 19, 2011

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Table 1. Frequencies of TNF-α -308 G > A genotypes in CRC cases and controls

Genotype	Patients (n = 161)	Controls (n = 164)	P Value
Wild Type (GG)	78 (48.4%)	83 (50.61%)	0.697
Hetero (GA)	66 (41%)	74 (45.12%)	0.452
Variant (AA)	17 (10.6%)	7 (4.27%)	0.030*

*P < 0.05, statistically significant

Table 2. Association risk of TNF-α -308 G > A genotypes with CRC susceptibility

Genotype	Patients (n = 161)	Controls (n=164)	OR (95% CI)	P Value
Wildtype (GG)	78	83	Reference	
Hetero (GA)	66	74	0.9491 (0.603 -1.436)	0.8230
Variant (AA)	17	7	2.5842 (1.0167-6.5689)	0.04

* P < 0.05, statistically significant

genes for CRC predisposition. Few genes are known to be important for inflammation of colorectum and their allelic variants have been shown to have biological effect[7,8].

Tumor Necrosis Factor-alpha (TNF- α) is a pro-inflammatory cytokine that exist in both in membrane bound and in soluble forms, its action occurring as a result of binding to TNF receptor p55 and p75[9]. A role for TNF in the pathogenesis of inflammatory bowel disease (IBD) has been established. TNF- α is present at high level in intestinal mucosa of patients with IBD[10-12]. The -308 G>A polymorphism in the promoter region of TNF- α has been reported to be associated with various inflammatory and autoimmune diseases in western countries[13-15]. But no reports are available from Malaysia. So a study was designed to investigate the TNF- α -308 G>A polymorphic allele frequencies in healthy controls and CRC patients in Malaysian population and to determine the associated risk of polymorphic genotype of TNF- α -308 G>A on sporadic CRC susceptibility.

MATERIALS AND METHODS

The study has obtained approval from Research Review Board and Ethics Committee of University Sains Malaysia and Ministry of Health (MOH) Malaysia. For this case control study, 161 clinically and histopathologically confirmed, sporadic colorectal cancer (CRC) patients (90 males and 71 females) were recruited from Hospital University Sains Malaysia (HUSM), and also from few hospitals under Ministry of Health (MOH) Malaysia like Hospital Sultanah Bahiyah, Alor Setar, Kedah and Hospital Raja Perempuan Zainab II, Kota Bharu, Kelantan. The ages of patients ranged from 27 to 77 years with mean age of 57.26 years. One hundred and sixty four (164) age and sex matched normal healthy controls comprising of 84 males and 80 females were also recruited. For the controls, the age ranged from 33 to 78 years with mean age of 48.91 years. Peripheral blood samples of study subjects were collected in EDTA tubes, after getting written informed consent. The collected samples were stored at -20℃ till use. Genomic DNA was extracted using commercial DNA extraction kit (QIAGEN) and the gene of interest was amplified using appropriate primers.

Single nucleotide polymorphism -308 G>A in the TNF- α gene was determined using PCR-RFLP using primers Forward 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and reverse 5'-TCCTCC-

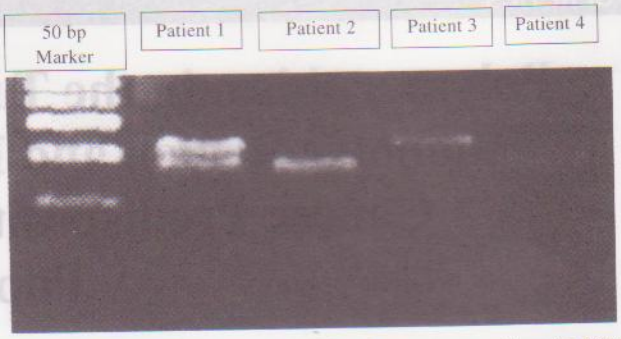

Figure 1. Gel showing TNF-α -251T > A genotype pattern in CRC patients
Patient 1: Heterozygous variant
Patient 2 & Patient 4: Homozygous wildtype
Patient 3: Homozygous variant

CTGCTCCGATTCCG-3' which amplified a 107 bp sequence. For PCR, the composition master mix composed of 100 ng DNA template, primer (0.2 μM), 2.0 mM MgCl2, 10x buffer, 10 mM dNTP (0.2) and 5 U *taq* DNA polymerase (Applied Biosystems) with total volume of 25 μl for PCR. The annealing temperature was 60℃, and 35 PCR cycles were carried out. The PCR products were isolated on 2% agarose gels and visualized with SYBR Green. PCR products with a G position- 308 digested by Nco1 to give two fragments of 87bp and 20 bp. Those with an A allele at position-308 was not digested by Nco1. TNF-α -308 G>A polymorphic genotypes were categorized into homozygous wild, heterozygous and homozygous variant. The difference in various genotypes frequencies of TNF-α among the cases and controls was calculated. The relative associations of various genotypes with CRC susceptibility risk was determined by calculating Odds Ratios. Corresponding chi-square tests on the CRC patients and controls were carried out and 95% confidence interval (95% CI) were determined using Fisher exacts tests. Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 18.

RESULTS

Frequencies of TNF-α -308 G>A genotypes in CRC cases and controls

The frequencies of TNF-α -308 G>A genotypes in cases and controls are shown in Table 1. Among the 164 controls, the homozygous wild type GG genotype was observed in 83 (50.61%), the heterozygous variant GA genotype was observed in 74 (45.12%) and the homozygous variant genotype AA was detected in 7(4.27%). In the case of 161 CRC patients, 78 (48.4%) showed homozygous wild type GG genotype, 66 (41%) showed heterozygous variant GA genotype and 17 (10.6%) showed homozygous variant AA genotype. On comparing the frequencies of the polymorphic genotypes among the cases and controls, the homozygous variant genotype frequency was significantly higher among CRC patients (p = 0.030).

Association risk of TNF-α -308 G>A genotypes with CRC susceptibility.

Table 2 shows the associated risk of TNF-α -308 G > A genotypes with CRC susceptibility in this population. When the association of the polymorphic genotypes with CRC susceptibility risk was investigated, the TNF-α homozygous variant genotype (AA) showed significantly increased risk with OR 2.5842 (CI 1.0167-6.5689, P = 0.04).

DISCUSSION

Inflammation, which is part of the immune response, may also induce or exaggerate some diseases through production of proinflam-

matory cytokines. Evidences have shown that the individual level of cytokine production is affected by single nucleotide polymorphisms (SNPs) in cytokine genes, and the observed differences in cytokine production among individuals can be at least partially explained by gene polymorphisms. Genetic polymorphisms might directly influence interindividual in the magnitude of inflammatory response and this might contribute to an individual's ultimate clinical outcome. Genetic polymorphisms of cytokine genes have been identified to play a role in susceptibility to various diseases including cancer[16]. Tumor necrosis factor is a pleiotropic cytokine that plays several roles such as in immune homeostasis, inflammation, host defenses and pivotal role in regulation of the immune response system. TNF-α is a pro-inflammatory cytokine that act as central mediator of the immune response and it also involved in a wide range of immuno-inflammatory and infectious diseases[17]. For example, at the site of inflammation in Inflammatory Bowel Disease (IBD) patients showed increase of expression of pro-inflammatory cytokines such as TNF-α [18]. Usually TNF-α has beneficial role against diseases, but if its production is uncontrolled, it can contribute to several disease pathogenesis[19,20].

Researchers had found that, TNF-α expression was mostly regulated at the transcriptional level and the polymorphisms within TNF-α promoter was related to the TNF-α production level[21,22]. According to Westendorp *et al* (1997), approximately 60% of the variation of TNF-α production could be under genetic control, which may be related to TNF-α polymorphism[23]. The polymorphism of TNF-α at position -308, which is substitution of Guanine to Adenine has been reported to be associated with increased of TNF-α production level[13,24]. This polymorphism play important role in functional implication for transcriptional activation and subsequent increased downstream inflammation. At present, relatively limited information exists on the relationship between colorectal cancer and TNF-α polymorphism.

We investigated the frequencies and potential risk modification of TNF-α -308 G>A polymorphic genotype on colorectal cancer susceptibility. Compared to controls, the prevalence of homozygous variant AA genotype was significantly higher in CRC patients, (4.27% vs 10.6%, P = 0.030) while for the homozygous wildtype genotypes (GG) and heterozygous variant genotypes (GA), there was no significant difference in frequencies between the two groups. Earlier, Sashio *et al* (2002) had reported a higher frequency of the TNF-α -308 AA genotype among Japanase patients with Ulcerative Colitis and Crohn's Disease[25]. In a study on US population, Garrity-Park *et al* (2008) had reported that the frequency of TNF-α -308 G>A homozygous variant and heterozygous variant genotypes were higher in UC-CRC cases compared to controls[26].

On examining the risk, our results showed that, the polymorphism in TNF-α gene was significantly associated with the development of CRC. The -308AA variant genotype was associated with a significantly increased risk of CRC as compared with -308GG genotype (OR 2.5842, CI 1.0167-6.5689, P = 0.04). The strong association that we observed in CRC patients prompts us to suggest that TNF-α gene -308AA polymorphism could be contributing significantly for CRC susceptibility. Polymorphism of TNF-α -308 G>A has been associated with high risk of several other cancer including Hodgkin's lymphoma, breast carcinoma, uterine endometrial cancer and prostate cancer[27,28]. In a study by Garrity-Park *et al* (2008), the heterozygous and homozygous variant at TNF-α -308 G>A was demonstrated to be associated with CRC susceptibility. Moreover they also reported that these polymorphism were associated with severity of CRC[26]. The TNF-α SNP has been associated with a high risk for gastric cancer[29,30]. It has been postulated that the pro-inflammatory and acid inhibitory properties of TNF-α could be enhancing *H. pylori* oncogenic or other effects on gastric mucosa[29,30]. A similar extrapolation regarding certain colorectal colonizing bacteria could be made based on our results.

However a study by Wu *et al* (2008) reported that TNF-α -308G/A gene polymorphism was not associated with the development of colorectal cancer, but TNF-α -308 A/A genotype and A allele were related to the progression of colorectal cancer in Chinese population[31]. There are a few other previous reports supporting that there is no significant association between TNF-α -308 A allele with crohn's disease, ulcerative colitis and inflammatory bowel disease in other populations[32-34]. This could be explained based on the difference in the polymorphic allele frequencies of TNF-α -308G>A in different population ethnic groups.

Yan *et al* (2006) has demonstrated that, high doses of TNF induced direct damage in Trp53$^{-/-}$ malignant cells[35]. Another study by Babbar and Casero in human lung bronchial epithelial cells showed that TNF-α exposure resulted in increased production of reactive oxygen species (ROS) and Hydrogen peroxide (H_2O_2) by the concomitant increase in the production of 8-oxo-deoxyguanosin a marker for oxidative DNA damage[36]. ROS play important role in DNA damage resulting in increased mutation rates and promote oncogenes transformation[37]. So these studies suggest that inflammation results in deregulated and sustained production of TNF which in turn produce ROS and thus could contribute to carcinogenesis and even in some cases be an initiating event. These data suggest a common pathway by which inflammation from multiple sources can lead to the mutagenic changes necessary for the development and progression of multiple epithelial cancers.

CONCLUSION

In conclusion, to best of our knowledge this is the first study on the association of the TNF-α gene 308G>A polymorphism and CRC risk in Malaysian population. Our results showed that the genetic diversity of TNF-α gene influences susceptibility to colorectal cancer in Malaysian population and could be considered as a potential genetic predisposition factor of CRC. Results are also in favor of inflammation mediated colorectal carcinogenesis pathway.

ACKNOWLEDGEMENT

This work was supported by University Sains Malaysia Research University (RU) Grants (1001/PPSP/812032). We would like to express our sincere thanks to Dr Nurul Ain Bin Abdullah Bayanuddin, Penang Health Center, for her help in normal control sample collection.

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