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# Variant Toll-Like Receptor4 (Asp299Gly and Thr399Ile Alleles) and Toll-Like Receptor2 (Arg753Gln and Arg677Trp Alleles) in Colorectal Cancer

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## ABSTRACT

The innate immune system recognizes the presence of bacterial products through the expression of a family of membrane receptors known as Toll-like receptors (TLR**s**). Polymorphisms in TLRs have been shown to be associated with increased susceptibility to diseases such as inflammatory bowel disease.

The aim of this study was to determine whether there was a correlation between polymorphisms of TLR4 (Asp299Gly; Thr399Ile) and TLR2 (Arg677Trp; Arg753Gln) genes and risk of colorectal cancer. DNA from 60 colorectal carcinoma patients from 3 major races in Malaysia (22 Malays, 20 Chinese and 18 Indians) and blood from 50 apparently healthy individuals were evaluated. Control group were matched to study group by race and age. The polymorphisms were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Genotyping results showed two out of sixty tumor specimens (3.3%) harbored both variant TLR4 Asp299Gly and Thr399Ile alleles. In contrast, DNA isolated from blood cells of 50 apparently healthy individuals harbored wild type TLR4. In the case of TLR2 Arg753Gln genotyping, all of the fifty normal and 60 tumors were of the wild type genotype. TLR2 Arg677Trp genotyping showed a heterozygous pattern in all samples. However, this may not be a true polymorphism of the TLR2 gene as it is likely due to a variation of a duplicated (pseudogene) region. There was only a low incidence (2/60; 3.3%) of TLR4 polymorphism at the Asp299Gly and Thr399Ile alleles in colorectal cancer patients. All normal and tumor samples harbored the wild type TLR2 Arg753 allele.

Our study suggests that variant TLR4 (Asp299Gly and Thr399Ile alleles) as well as TLR2 (Arg753Gln allele) are not associated with risk of colorectal cancer.

**Keywords:** Colorectal Cancer; Polymorphisms; Toll-like Receptors

## INTRODUCTION

Colorectal malignancies remain one of the leading causes of cancer deaths in the world.[1]

Chronic infection and inflammation are the most important epigenetic factors contributing to tumorigenesis and tumor progression.[2-7] The risk of developing colorectal cancer was found to be 10-fold greater in the presence of inflammatory bowel diseases (e.g. ulcerative colitis and Crohn's disease) dna certain

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anti-inflammatory agents used in the control of colitis can reduce the incidence of colon cancer.[8,9] Several studies showed that bacterial- and viral-induced inflammatory process can mediate tumorigenesis.[10] The human large intestine (colon) contains an enormous (~$10^{14}$) population of microorganisms (Our bodies consist of only ~$10^{13}$ cells).

Toll-like receptors (TLRs) are important pattern recognition receptors expressed by immune cells, through which host recognizes microbial conserved components such as lipopolysaccharide (LPS), lipopeptides, dsRNA, and bacterial DNA.[11,12] The key TLRs that bind bacterial cell wall components are TLR2 and TLR4. Mutations in these genes decrease the response to bacterial components, impact gut homeostasis, results in impairment of TLRs activation and this may influence cancer development and progression.[13-15]

The important role of TLR polymorphisms in cancer, are shown in gastric and prostate cancer, the two malignancies in which chronic inflammation is the basic pathological incident.[16-20] In one study, eight TLR4 variants were investigated in a Swedish population and the 11381G/C variant was associated with prostate cancer.[16] In another report, 16 variants of TLR4 were evaluated and 8 variants showed an inverse association with prostate cancer, although the 11381G/C variant was not associated with disease.[17] TLRs have also been concerned in several diseases, including systemic inflammation ,[20] sepsis,[21] asthma, atopic dermatitis,[22] and cardiovascular disease.[23]

Two co-segregating single nucleotide polymorphisms (SNPs) within TLR4 gene, A896G (rs4986790) and C1196T (rs4986791) lead to Asp299Gly and Thr399Ile amino acid substitutions, respectively.[24-26] Achyut et al. (2007) found a significant association of the TLR-4 Thr399Ile allele with gastritis; the frequencies of Ile399Ile allele and Asp299-Ile399 haplotype were higher in patients as compared to control subjects.[18] The two most common TLR2 SNPs are Arg677Trp and Arg753Gln.[27] Jelavic et al. (2006) reported that TLR2 microsatellite GT polymorphisms and TLR4 at the Asp299Gly allele was associated with sporadic colorectal cancer among Croatians.[28]

Several studies showed significant differences in allele frequency between ethnic groups in TLR2 (Arg677Try andArg753Gln) and TLR4 (Asp299Gly and Thr399Ile). Two common TLR4 polymorphisms have been detected in Caucasians at a prevalence rate of 10% while these were not detected in Chinese and jupons ethnic groups.[29]

There are several types of Gram-negative and Gram- positive bacteria in intestine.TLR2 has been shown to binds to bacterial peptidoglycan and TLR4 is the predominant receptor for LPS from Gram-negative organisms. The function of these two TLRs is critical in immune response and inflammation toward various bacteria in the intestine. Evidence on the association of polymorphisms in TLR2 and TLR4 genes with ulcerative colitis and Crohn's disease, as well as other inflammatory conditions[30-32] provided the rationale for our study. We hypothesized that the presence of TLR2 and TLR4 variants affect gut homeostasis resulting in impairment of TLRs activation may lead to inflammation and colorectal cancer.

## MATERIALS AND METHODS

Fifty normal blood samples and sixty human colorectal cancer paraffin-embedded blocks were used for detection of TLR2 and TLR4 variants. The blocks were randomly selected from the archived colorectal carcinoma tissues from a local tertiary hospital in Kuala Lumpur (patients between 30-50 years old) and 50 blood samples as a control group from apparently healthy individuals. Control group were matched to study group by race, age and a 1:1 sex ratio. Permission for this study was obtained from the Human Ethics Committee, University Putra Malaysia. Statistical analyses were carried out using chi-square tests. Data with $p<0.05$ were considered to be significant.

The following cell lines were also used in this study, namely, human colorectal carcinomas, HT-29 (ATCC, HTB-38) and HCT116 (ATCC, CCL-247), human nasopharyngeal carcinoma epitheloid cell lines (CNE1 SUNE, TWOI, TWO4, TWO6) from Professor George Tsao of Hong Kong University, liver cancer cell line [HepG2 (CRL-11997)], Chang liver cell line (CCL 13) and breast cancer cell line (MDA) from ATCC.

### DNA Extraction

DNA was extracted from cell lines by a kit from Gene all. Sections of 10µm thickness were obtained from paraffin-embedded blocks using a standard microtome. DNA was extracted from tumor tissues by conventional proteinase K digestion method.

In the conventional method, the paraffin was removed using octane extraction and the octane was removed by performing repeated ethanol extractions.[33] Spectrophotometric determination of the yield and purity of DNA was performed.

**Genotyping of the TLR2 and TLR4**

Detection of the *TLR*2 and *TLR4* gene polymorphisms was performed with polymerase chain reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP).

**PCR Amplification of the Extracted DNA**

PCR reaction conditions were modified using temperature gradient PCR (Eppendorf Mastercycler gradient). Reactions were performed in a 25 µl volume containing 200 µM of each dNTP (0.5 µl of dNTP mix, 10mM each), 0.2 µM of each of the forward and reverse primers (0.5 µl of each 10µM primers), 2 mM $MgCl_2$ (1µl of $MgCl_2$, 50mM) and 1 U of Taq DNA polymerase (1µl of 1U/ µl enzyme), 1x PCR buffer (2.5µl of 10X PCR buffer). PCR cycling conditions were as follows: an initial denaturation step (95°C for 3min), 35 cycles (94°C for 30 s, 52°C (Asp299Gly), 60°C (Thr399Ile), 47°C (Arg677Trp) and 59°C ( Arg753Gln) for 30 s, 72°C for 30 s), and a final extension step (72°C for 10 min).

The primer sequences are presented in table 1.[34] PCR products were analyzed by 2 % agarose gel electrophoresis.

**Restriction Fragment Length Polymorphism (RFLP)**

PCR products were subjected to restriction enzyme digestion as indicated in table 2. The expected size of the fragments for *TLR2* and *TLR4* polymorphism are as listed in table 2. Lambda DNA was digested by each enzyme to show the efficiency of the enzymes. The restriction assay contained 1x restriction buffer, 20 units of the restriction enzyme (New England Biolabs, USA) for 10 µl of PCR product. It was incubated overnight at 37°C (Or 60°C) and analyzed by electrophoresis on a 2% agarose gel.

**Table 1. Sequences of primers as used for PCR**

| Gene | Polymorphism | Primers |
|---|---|---|
| *TLR4* | Asp299Gly | F: 5'- AGCATACTTAGACTACTACCTCCATG-3' |
| | | R: 5'- GAGAGATTTGAGTTTCAATGTGGG-3' |
| *TLR4* | Thr399Ile | F: 5'GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3' |
| | | R:5'-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3' |
| *TLR2* | Arg677Trp | F:5'-CCCCTTCAAGTTGTGGCTTCATAAG-3' |
| | | R:5'-AGTCCAGTTCATACTTGCACCAC-3' |
| *TLR2* | Arg753Gln | F;5'-CATTCCCCAGCGCTTCTGCAAGCTCC-3' |
| | | R:5'-GGAACCTAGGACTTTATCGCAGCTC-3' |

**Table 2. Restriction enzymes and length of the restriction fragments**

| Gene | Polymorphism | Restriction enzyme | Restriction temp °C | Size of the Restriction fragments |
|---|---|---|---|---|
| *TLR4* | Asp299Gly | *Nco I* | 37°C | Wild type (allele A): 188 bp<br>Asp299Gly (allele G): 168 bp + 20 bp |
| *TLR4* | Thr399Ile | *Hinf I* | 37°C | Wild type (allele C): 124 bp<br>Thr399Ile (allele T): 98 bp + 26 bp |
| *TLR2* | Arg677Trp | *Mwo I* | 60°C | Wild type (allele C): 130 bp + 22 bp<br>Arg677Trp (allele T): 152 bp |
| *TLR2* | Arg753Gln | *Msp I* | 37°C | Wild type (allele G): 104 bp + 25 bp<br>Arg753Trp (allele A): 129 bp |

**Table 3. TLR2 and TLR4 Polymorphisms in individuals with colorectal cancer and the healthy control subjects**

| Samples | *TLR2* | *TLR4* | |
|---|---|---|---|
| | Arg753Gln | Asp299Gly | Thr399Ile |
| Patients (60) (colorectal cancer paraffin-embedded blocks) | 60 - (100 %) | 2 + (3.3 %) | 2 + (3.3 %) |
| Control (50) (Blood samples) | 0 – (100 %) | 0 – (100 %) | 0 – (100 %) |

(-) Indicates wild type genotype

(+) Indicates the presence of variant allele

**Table 4. *TLR2* and *TLR4* Polymorphisms in cell lines**

| Cell lines | TLR2 | TLR4 | |
|---|---|---|---|
| | Arg753Gln | Asp299Gly | Thr399Ile |
| CNE1 SUNE (Human nasopharyngeal carcinoma) | - | - | - |
| TWOI (Human nasopharyngeal carcinoma) | - | - | - |
| TWO4 (Human nasopharyngeal carcinoma) | - | - | - |
| TWO6 (Human nasopharyngeal carcinoma) | - | - | - |
| HT-29 (Colorectal carcinoma) | + | - | - |
| HCT116 (Colorectal carcinoma) | - | - | - |
| HepG2 (Hepatocellular carcinoma) | - | + | + |
| Chang liver cell line (Normal liver tissue) | - | + | + |
| MDA-MB-231 (Breast cancer cell line) | - | - | - |

- Indicates wild type genotype

+ Indicates the presence of variant allele

Since the smaller fragments of DNA were not visible in standard agarose gel, the high-resolution metaphor agarose gel was used to separate 98bp and 124 bp. Restriction fragments were electrophoresed in 3% MetaPhorTM agarose gel then visualized by staining with ethidium bromide. The presence of *TLR4* wild type and variant forms from tumour tissues and blood samples were confirmed by sequencing. Sequencing was performed using an automated capillary ABIPRISM 3100 Genetic analyzer (Applied Biosystems, USA). Before DNA sequencing, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

## RESULTS

A screen of 60 paraffin-embedded tissues showed that two specimens were heterozygous for both *TLR4* Asp299Gly and Thr399Ile alleles in the *TLR4* gene (Figure 1a; Lanes 2, 3 and 4). These two individuals carried the *TLR4* variant at both alleles, confirming that these mutations are rarely seen to segregate individually.[35] Wild type *TLR4* was detected in the DNA from blood of 50 apparently healthy individuals.

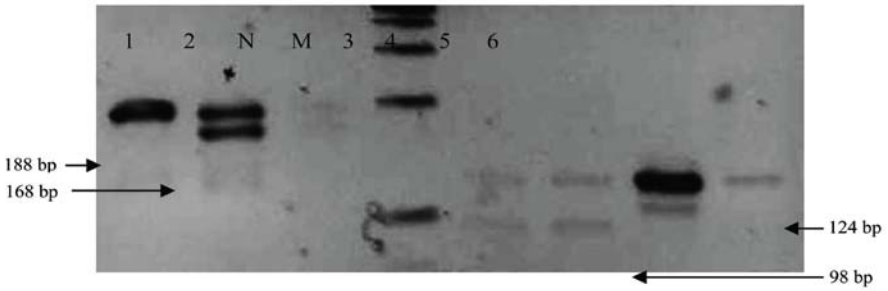


**Figure 1a. Agarose gel electrophoresis of restriction fragments to determine the TLR4 Asp299Gly and Thr399Ile polymorphisms by *NcoI* and *HinfI* restriction digest analysis of amplicons from CRC samples. M: 100bp DNA Ladder Marker, N: No template control, 1: Undigested 188bp product, 2: CRC sample with *NcoI* restriction site (Heterozygous for Asp299Gly; 188 & 168 bp), 3, & 4: CRC samples with *HinfI* restriction site (Heterozygous for Thr399Ile; 124&98 bp), 5: Undigested 124bp product, 6: CRC sample without *HinfI* restriction site (wild-type; 124bp). The 20bp and 26bp products from *Nco*I and *Hinf*I restriction digest in lanes 2, 3 and 4 were not resolved in this gel composition.**

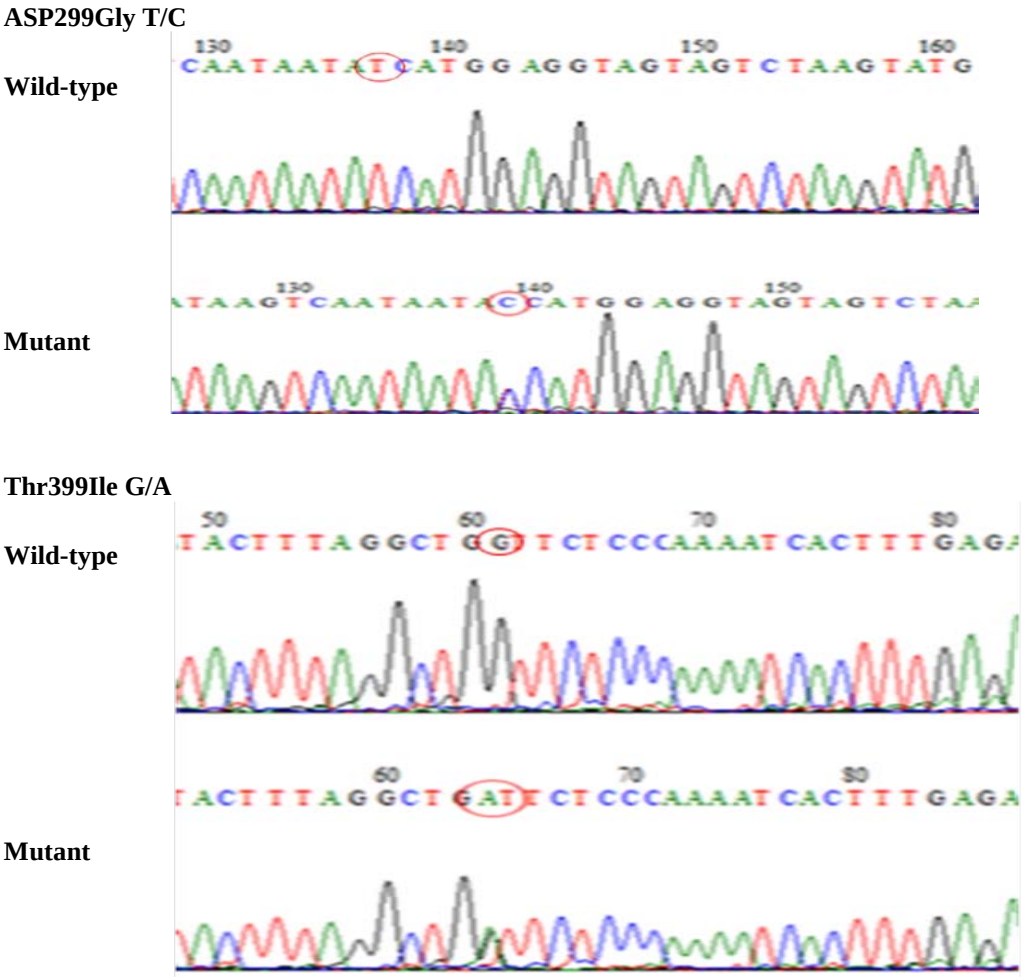


**Figure 1b. Sequence analysis of the *TLR4* Asp299Gly and Thr399Ile polymorphisms in colon cancer patients. The circled area demonstrates the nucleotide base changes; T nucleotide in Wild-type was substituted with doublet of T and C nucleotide in Asp299Gly polymorphism and G nucleotide in the wild-type was replaced with a doublet peak of G and A nucleotide in Thr399Ile polymorphism.**

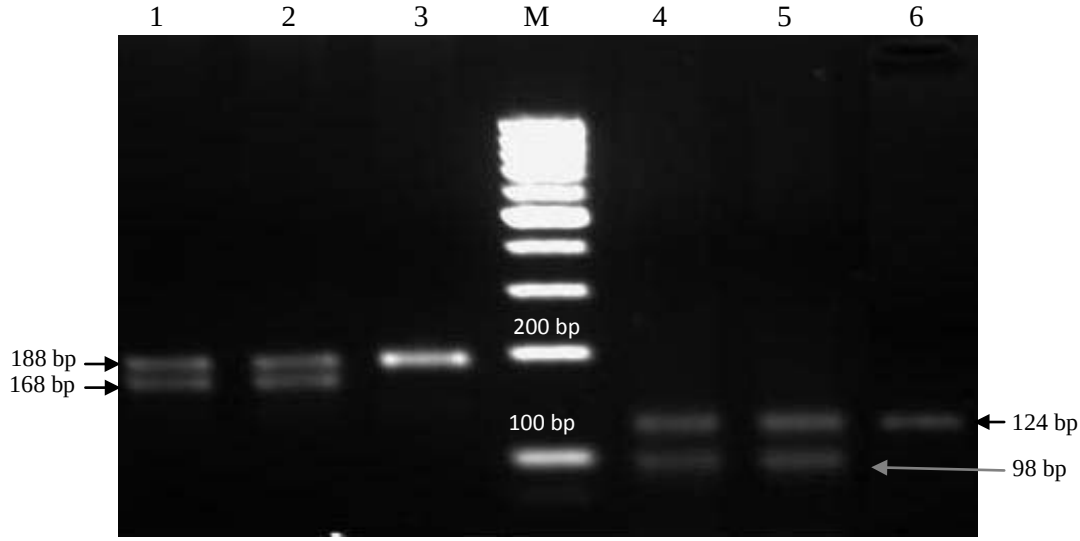


**Figure 2. Agarose gel electrophoresis of restriction fragments to determine the TLR4 Asp299Gly and Thr399Ile polymorphisms from cell lines: *NcoI* and *HinfI* restriction digest analysis of HepG2 and Chang liver cell lines. M: 100bp DNA Ladder Marker, 1: HepG2 cell line with *NcoI* restriction site (Heterozygous for Asp299Gly; 188 & 168 bp), 2: Chang liver cell line with *NcoI* restriction site (Heterozygous for Asp299Gly; 188 & 168 bp), 3: Undigested 188bp product, 4: HepG2 with *HinfI* restriction site (Heterozygous for Thr399Ile; 124 & 98 bp), 5: Chang liver cell line with *Hinfi* restriction site (Heterozygous for Thr399Ile; 124 & 98 bp) dna 6: Undigested 124bp product.**

The PCR products harboring the *TLR4* variants from RFLP analysis were confirmed via DNAsequencing These include two samples harboring the variant and ten of the wild type samples (five from CRC, and five from blood control group). DNA sequencing data confirmed that a heterozygous allele at Asp299Gly and Thr399Ileu (Figure 1b). The T nucleotide in wild type was replaced with a double peak showing T and C nucleotide in the Asp299Gly allele. The G nucleotide in wild-type was replaced with a double peak of G and A nucleotide in the Thr399Ileu allele.

RFLP analysis of fifty normal blood samples and sixty human colorectal carcinoma tissues showed a heterozygous pattern for *TLR2* Arg677Trp for all samples (Figure 3; Lane 1-15). This was an unexpected result. For *TLR2* Arg753Gln, we found that all of the fifty normal blood samples and sixty human colorectal cancer specimens were of the wild-type form. As summarized in table 4, no TLR4 polymorphism was found in all cell lines tested except for HepG2 and Chang liver cell lines, which were heterozygous for TLR4 Asp299Gly and Thr399Ile alleles (Figure 2; Lane 1, 2, 4 and 5).

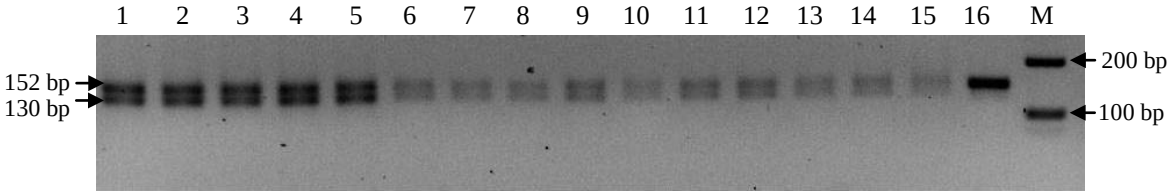


**Figure 3. Representative agarose gel electrophoresis of restriction fragments to determine TLR2 Arg677Trp polymorphism: *Mwo*I restriction digest analysis of CRC samples. M: 100bp DNA Ladder Marker, 1-15: CRC samples with *MwoI* restriction site (152 & 130bp), 16: Undigested 152 bp PCR product.**

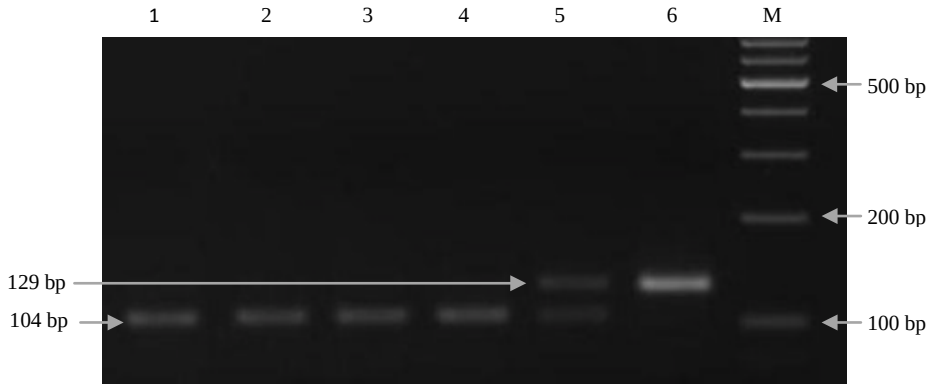


**Figure 4. Agarose gel electrophoresis of restriction fragments to determine the *TLR2* Arg753Gln polymorphism: *MSPI* restriction digest analysis of amplicons from CRC samples and HT29 cell line. M: 100bp DNA Ladder Marker, 1-4: CRC samples with *MSPI* restriction site (wild-type; 104bp), 5: HT29 cell line with *MSPI* restriction site (Heterozygous for *TLR2* Arg753Gln polymorphism; 129 & 104bp) and 6: Undigested 129 bp PCR product.**

The cell lines as listed in Table 4 were genotyped for *TLR4* Asp299Gly, Thr399Ile, and *TLR2* Arg753Gln. All cell lines were of wild type except HT-29, a colon cancer cell line (Figure 4; Lane 5) whereby a heterozygous pattern for *TLR2* Arg753Gln allele was observed.

## DISCUSSION

Recently host genetic factors are considered to be involved in tumor progression. The Toll-like receptors genes have been shown to be polymorphic. The frequency of many of these polymorphisms varies significantly across different races. Genetic association studies could be as a powerful tool to find etiologic agents of human diseases. The present study was designed to detect the *TLR2* and *4* polymorphisms in colorectal cancer. Several studies on Chinese and Korean people showed no homozygous or heterozygous variant genotypes of the *TLR4* at Asp299Gly or Thr339Ile.[36-38] The 60 paraffin embedded tomour tissues, which were used in this study, were from three major races in Malaysia (Malay 22, Chinese 20, and 18 Indian). Since our positive rates were too low (%3.3), we were unable to find an association of these polymorphisms with susceptibility to colorectal cancer. Our results showed that only two out of sixty colorectal tumour samples showed TLR4 polymorphism at Asp299Gly and Thr399Ile; no Arg753Gln *TLR2* polymorphism was found. Interestingly, both of the mutant *TLR4* individuals were from Indian patients. In a study by Achyuta on a northern Indian control population, 2007, the frequency of Gly and Ile alleles in *TLR4* gene was reported to be 8 and 3.3 percent, respectively.[18,39]

One unexpected finding was that, in fifty normal blood samples and sixty human colorectal cancers paraffin-embedded blocks, genotyping of the *TLR2* Arg677Trp showed a heterozygous pattern in all samples. This finding is consistent with the study by Malhotra on 286 leprosy patients and 183 healthy controls. Genotyping of the *TLR2* showed heterozygosity at nucleotide position 2029 leading to amino-acid substitutions Arg677Trp in all 286 leprosy patients and 183 healthy controls. They designed another primer pair to specifically amplify the *TLR2* intracellular signaling region. Genotyping results with these primers showed an absence of any variation at the same nucleotide.[40] Thus, TLR2 polymorphisms need to be studied with caution, because of the presence of variations in the duplicated (pseudogene) region representing exon 3 of the TLR2 gene. It would be interesting to determine the prevalence of TLR2 Arg677Trp with a different set of primers in a larger scale study. Genotyping of cell lines showed the heterozygous pattern of *TLR4* at Asp299Gly and Thr399Ile alleles, in HepG2 (hepatoma) and Chang liver cell lines. Nishimura and Naito reported that, HepG2 cells express *TLR*-2, *TLR*-3, *TLR*-6, and *TLR*-9, however *TLR*-1, *TLR*-4, *TLR*-5, *TLR*-7, *TLR*-8, and *TLR*-10 showed very weak or no expression. [41] Down regulated expression of the *TLR4* in HepG2 cell line

may be due to the mutations in this gene. It is recommended that further research be undertaken to investigate the association between the liver cancer diseases specially hepatoma and *TLR4* mutations.

In conclusion, *TLR4* polymorphism at Asp299Gly and Thr399Ile were found in two out of 60 colorectal samples (3.3%). All 50 blood samples from apparently healthy individuals exhibited wild-type *TLR4*. It is likely that the TLR4 polymorphism detected in our study is linked to ethnicity but large-scale studies are needed to clearly define the incidence of *TLR4* polymorphisms in the Malaysian population according to ethnic groups and the probability of the association of these polymorphisms with CRC. In the case of cell lines, nasopharyngeal, colon and breast cancer cell lines harbored wild-type *TLR4* and the two liver cell lines, namely HepG2 and Chang showed a heterozygous pattern for *TLR4* Asp299Gly and Thr399Ile. *TLR2* polymorphism at Arg753Gln was absent in 50 normal blood samples and 60 colorectal tumor specimens. *TLR2* Arg677Trp genotyping need to be repeated with new primers since the primers used in this study spanned a pseudogene region in exon 3. Wild-type *TLR2* was found in all cell lines except for HT299, a colon cancer cell line exhibited heterozygous pattern for TLR2 Arg753Gln.

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