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*Rsa*I but not *Dra*I polymorphism in *CYP2E1* gene increases the risk of gastrointestinal cancer in Malaysians: a case–control study

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ABSTRACT

Objectives: Our study aimed to investigate the association of *CYP2E1* C-1019T *Rsa*I and T7678A *Dra*I polymorphisms and factors such as age, gender and ethnicity to the risk of gastrointestinal cancer (GIC) in Malaysians.

Design: Case–control study.

Setting: Malaysia.

Participants: 520 consented healthy blood donors with no previous GIC record and 175 patients with GIC.

Measurements: C-1019T *Rsa*I and T7678A *Dra*I genotyping of *CYP2E1* gene; direct sequencing.

Results: This study reveals that the variant *c2* allele and carrier with at least one *c2* allele of C-1019T single nucleotide polymorphism (SNP) significantly increased the risk of GIC but no significant association was found between T7678A SNP and combined analysis of C-1019T and T7678A SNPs to risk of GIC. The Malaysian Chinese had greater risk of GIC compared with the Malays, Indians and KadazanDusun. An increased risk of GIC was observed in individuals aged >40 years and women had a 2.22-fold and 1.58-fold increased risk of stomach and colorectal cancers, respectively, when compared with men.

Limitations: The future research should be conducted with a larger sample population and including the gene–gene and gene–environmental interactions.

Conclusions: Our study suggests that the rare *c2* allele and carrier with at least one *c2* allele of *CYP2E1* *Rsa*I polymorphism significantly elevated the risk of GIC and may be used as a genetic biomarker for early screening of GIC in Malaysians. The risk age-group has been shifted to a younger age at 40s and women showed a significant greater risk of stomach and colorectal cancers than men.

Strengths and limitations of this study

- Sequencing is performed to verify the homozygosity and heterozygosity of the samples.
- Each association test is provided with a significant value (p value).
- Data on the lifestyle of the participants, gene–gene and gene–environment interactions were not included in this study.

INTRODUCTION

Gastrointestinal cancer (GIC) remains as a major challenge worldwide because it is among the top cause of cancer-related mortality in the world.[1] In Malaysia, carcinomas of the digestive system ranked on top of the cancer-related mortality in recent statistics from the Ministry of Health Malaysia with a mortality rate of 4.71/100 000 populations,[2] indicates that finding appropriate biomarker(s) for early detection of GIC is extensively needed. The *CYP2E1* gene receives great attention owing to previous studies which demonstrated the correlation of this gene to cancer carcinomas development such as oesophageal, hepatocellular, stomach, colorectal and lungs in Asian and non-Asian populations.[3–8]

CYP2E1 is a member of the cytochrome *P450* superfamily, mapped in the 10q24.3 region of chromosome 10.[9] *CYP2E1* enzymes are involved in the metabolism of more than 80 low-molecular-weight substrates and are found to be highly expressed in the hepatic tissue,[10 11] and enough amount of these enzymes are also localised in the extrahepatic tissues including nasal mucosa, stomach, small intestine and colon.[12 13] More than 10 single nucleotide polymorphism (SNP) sites have been identified along the *CYP2E1* gene.[14] Of these, *Rsa*I polymorphism in the 5′-flanking region and *Dra*I polymorphism in the intron 6 of the gene are well-characterised with C to T replacement in position −1019 and T to A replacement in position 7678, respectively.[15] The *Rsa*I and *Dra*I polymorphisms of *CYP2E1* gene revealed conflicting findings in GIC development among

different populations in the past studies, but it is not apparent how these polymorphisms affect Malaysian population.[4 6 16–18]

In addition, Malaysia is a multiethnic country with a majority of Malays, Chinese and Indians, whereas KadazanDusun is the dominant native ethnic in East Malaysia, also known as North Borneo. A previous study reported that the Malaysian Chinese and Indians ethnics had a greater risk of GIC than the Malays in Malaysia.[19] Nevertheless, GIC was predominant in the elderly aged >50 years in Asian and non-Asian populations, but to the best of our knowledge, no significant difference on gender to risk of GIC was reported previously.[6 20–21] Therefore, we conducted this study to investigate the *CYP2E1 Rsa*I and *Dra*I polymorphisms, as well as other risk factors such as age, gender and ethnicity to the risk of GIC in Malaysians.

METHODS

Samples collection and DNA extraction

The design and analysis for this study were following the suggestions of STREGE guidelines, except the clinical characteristics of patients with GIC that were not available.[22] A total of 520 consented healthy blood donors with no previous GIC record and 175 patients with GIC who were admitted to Queen Elizabeth Hospital in Sabah, East Malaysia and University Malaya Medical Centre (UMMC), West Malaysia from year 2010 to 2013 were recruited for this preliminary case–control study to represent all Malaysians. Data including age, gender and ethnicity of the participants were collected, regardless of data-matching for controls and cases. Two millilitres of peripheral blood were collected in a BD Vacutainer EDTA-coated tube and genomic DNA was extracted using conventional alkaline lysis and phenol-chloroform extraction method. Both the quality and quantity of extracted DNA were determined by spectrophotometry using Nanophotometer (Implen, Germany).

Genotyping of C-1019T *Rsa*I and T7678A *Dra*I SNPs

Genotyping analysis for both SNPs was carried out in Biotechnology Research Laboratory, School of Science and Technology, Universiti Malaysia Sabah. PCR was performed for both polymorphisms in a 20 µL mixture containing 100 ng extracted DNA as template, 1×PCR buffer (Promega, USA), 1.0 mM of $MgCl_2$, 0.2 mM of dNTPs and 1 U *Go Taq* Flexi DNA polymerase (Promega). For C-1019T SNP, forward (5′-CCAGTCGAGTCTACATTGTCA-3′) and reverse (5′-TTCATTCTGTCTTCTAACTGG-3′) primers were used with PCR conditions set as followed: initial denaturation at 94°C for 5 min; amplification of 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min; final extension at 72°C for 7 min. In contrast, primers 5′-TCGTCAGTTCCTGAAAGCAGG-3′ (forward) and 5′-GAGCTCTGATGCAAGTATCGCA-3′ (reverse) were used to amplify the T7678A SNP and the PCR conditions were as followed: initial activation for 5 min at 94°C, 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, followed by a final extension for 7 min at 72°C. The PCR products were subjected to overnight 2.5 U *Rsa*I and 5.0 U *Dra*I (New England BioLabs Inc, Ipswich, Massachusetts, USA restriction enzymes digestion for C-1019T and T7678A SNPs, respectively. The digested fragments were electrophoresed and analysed on 2% agarose gel stained with ethidium bromide in 1×TAE buffer.

Direct sequencing

The PCR amplification for SNPs was highly repeatable and the SNPs were verified by direct sequencing. PCR products for both polymorphisms were purified using the QIAquick PCR purification kit following the instructions from the manufacturer (QIAGEN, USA) and were sequenced using ABI PRISM 3100 Genetic analyser (Applied Biosystems, USA).

Statistical analysis

Hardy-Weinberg proportion for cases and controls were calculated using the Online Encyclopedia for Genetic Epidemiology (OEGE) software.[23] The OR and 95% CIs of the allele and genotype risk associations to GIC were performed using SPSS Software V.17.0 (SPSS Inc, Chicago, Illinois, USA) and the associations were considered statistically significant at $p<0.05$ (Fisher's exact test). With respect to this, the common *c1* allele and *c1/c1* genotype for C-1019T SNP as well as the wild *D* allele and *D/D* genotype for T7678A SNP were taken as the reference group. The SPSS software was also used in χ^2 test to examine the genotype differences between cases and controls in this study.

RESULTS

Of 695 participants, 62.2% were men and 37.8% women, with a mean age±SD of 42.13±0.140 and 45.96 ±0.271, respectively. Cases and controls were not deviate from Hardy-Weinberg equilibrium in this study ($\chi^2<5.991$, df=2). Heterozygosity and homozygosity for both SNPs were sequenced and confirmed. Table 1 shows the risk association of *CYP2E1* polymorphisms to GIC. In C-1019T *Rsa*I polymorphism analysis, the frequencies of *c1* and *c2* allele were 81.5% and 18.5%, respectively. The genotype analysis showed that 66.9% of the participants carried the wild-type (*c1/c1*) genotype, followed by 29.2% heterozygous (*c1/c2*) and 3.9% variant (*c2/c2*). No significant difference in genotype distribution of this polymorphism was found between cases and controls. Our study revealed that *c2* allele and carrier with at least one *c2* allele (*c1/c2+c2/c2*) had a 1.38-fold and 1.45-fold greater risk of GIC significantly ($p<0.05$).

The frequencies of *D* and *C* allele in T7678A *Dra*I polymorphism were 77.9% and 22.1%, respectively. A frequency of 62.0% was found for participants who carried the wild-type (*D/D*) genotype, followed by 31.8% for heterozygous (*D/C*) and 6.2% for the variant (*C/C*). The

Table 1 Association of *CYP2E1* polymorphisms to risk of GIC

	Cases, N	Controls, N	OR (95% CI)	p Value
*C-1019T SNP**				
Allele				
c1	272	861	1.00 (Reference)	–
c2	78	179	1.38 (1.02 to 1.86)	0.038†
Genotype				
c1/c1	106	359	1.00 (Reference)	–
c1/c2	60	143	1.42 (0.98 to 2.06)	0.065
c2/c2	9	18	1.69 (0.74 to 3.88)	0.241
c1/c2+c2/c2	69	161	1.45 (1.02 to 2.07)	0.042†
*T7678A SNP**				
Allele				
D	277	806	1.00 (Reference)	–
C	73	234	0.91 (0.68 to 1.22)	0.552
Genotype				
D/D	111	320	1.00 (Reference)	–
D/C	55	166	0.96 (0.66 to 1.39)	0.850
C/C	9	34	0.76 (0.36 to 1.64)	0.583
D/C+C/C	64	200	0.92 (0.65 to 1.32)	0.719
C-1019T+T7678A SNPs				
Allele				
c1+D	549	1667	1.00 (Reference)	–
c2+C	151	413	1.11 (0.90 to 1.37)	0.329
Genotype				
c1/c1+D/D	217	679	1.00 (Reference)	–
c1/c2+D/C	115	309	1.17 (0.90 to 1.52)	0.277
c1/c2+C/C	69	177	1.22 (0.89 to 1.68)	0.245
c2/c2+D/C	64	184	1.09 (0.79 to 1.50)	0.617
c2/c2+C/C	18	52	1.08 (0.62 to 1.89)	0.773

*In Hardy-Weinberg equilibrium (χ^2<5.991, df=2).
†Statistically significant (p<0.05).
GIC, gastrointestinal cancer; SNP, single nucleotide polymorphism.

distribution of genotype was found to be very similar between cases and controls. The allelic and genotypic risk associations of this polymorphism suggested that T7678A SNP was not likely to be involved in the GIC development in Malaysian population (p>0.05). We did not find any significant associations to the risk of GIC when both polymorphisms were combined (table 1).

When other factors were examined, we found that the Malaysian Chinese had a greater risk of GIC than the Malays, Indians and KadazanDusun in Malaysia (table 2). Overall, the elderly aged >40 years and women had a higher risk of GIC significantly (p<0.05; tables 3 and 4). Further investigation revealed that women had a 2.22-fold and 1.58-fold higher risk of gastric and colorectal cancers, respectively, when compared with men in this study (table 5).

DISCUSSION

This study indicates that the rare *c2* allele and carrier with at least one *c2* allele (*c1/c2+c2/c2*) of C-1019T *Rsa*I polymorphism increased the risk of GIC in Malaysians. Previous investigations reported conflicting findings of this polymorphism to the risk of GIC in different populations. *Rsa*I polymorphism has been reported to increase susceptibility to colorectal cancer in Kashmir population in India.[24] A study in the Fujian province of China revealed that the presence of *c2* allele elevated

Table 2 Association of ethnic groups to risk of gastrointestinal cancer

Ethnics	Cases, N	Controls, N	OR (95% CI)*	p Value
Chinese	53	137	1.00 (reference)	–
Malays	11	51	0.56 (0.27 to 1.15)	0.131
Indians	4	30	0.35 (0.12 to 1.03)	0.054
KadazanDusun	33	121	0.71 (0.43 to 1.16)	0.210
Others	74	181	1.06 (0.70 to 1.60)	0.832

*ORs were calculated by taking Chinese as the reference group.

Table 3 Association of age to risk of gastrointestinal cancer

	Cases, N		Controls, N			
	≤40	>40	≤40	>40	OR (95% CI)*	p Value
Total	19	156	451	69	53.67 (31.29 to 92.04)	<0.001†
Chinese	1	52	117	20	304.20 (39.76 to 2327.25)	<0.001†
Malays	2	9	45	6	33.75 (5.85 to 194.81)	<0.001†
Indians	1	3	25	5	15.00 (1.28 to 175.30)	0.033†
KadazanDusun	2	31	110	11	155.00 (32.62 to 736.48)	<0.001†
Others	13	61	154	27	26.76 (12.96 to 55.26)	<0.001†

*ORs were calculated by taking age ≤40 as the reference group.
†Statistically significant (p<0.05).

the risk for gastric cancer, but was found as a protective factor for oesophageal squamous cell cancer in another study in China.[3 5] Besides, Ladero *et al*[4] who reported that a carrier with one copy of *c2* allele increased the risk of hepatocellular carcinoma with the addition of regular alcohol intake in the Spanish, supported the findings by Lee *et al*[17] in Korean and Japanese populations and by Deka *et al*[8] in Indian population.

Although the reason that contributes to inconsistent findings of C-1019T *Rsa*I polymorphism to GIC is unclear, previous in vitro studies reported that the *c2* allele express at a higher rate when compared with *c1* allele and the variant *c2/c2* genotype is correlated with about 10-fold greater of gene transcription of *CYP2E1* gene.[25–27] The *Rsa*I polymorphism has been reported to influence the binding of a transcriptional factor to the 5′-flanking region of *CYP2E1* and subsequently alters the expression of mRNA level.[26] Since the *CYP2E1* enzyme can reduce molecular oxygen to highly reactive oxygen forms (ROFs) even without the presence of substrate, overexpression of this enzyme will increase the synthesis of ROFs in the body, resulting in intensified lipid and protein peroxidation, DNA damage and carcinogenesis which may lead to the development of GIC.[11]

We found no significant association between T7678A *Dra*I polymorphism and GIC in this study. Similarly, Darazy *et al*[28] reported that *D/C* genotype had a non-significant reduced risk to GIC in the Lebanese. It might be due to T7678A SNP located in intron 6; the mechanisms of this SNP influencing the *CYP2E1* expression and catalytic activity of the enzyme were not well-demonstrated since no functional significance of this SNP appeared. In fact, published data suggested that this SNP did not influence the expression of *CYP2E1* gene and its catalytic activity.[29] In any case, a study revealed that the *C/C* genotype significantly associated to increase the risk of colorectal cancer although Lin *et al*[16] claimed that this genotype might be a protective factor for oesophagus cancer.[21] Furthermore, combined analysis on both polymorphisms in this study showed no significant association to increase the risk of GIC, which is similar to previous published data.[30 31] Malaysia is a multiracial country but we report here as overall Malaysians in genotype analysis, regardless of the ethnicity of the participants as it is for early GIC detection in all Malaysians. As most of the SNPs described in *CYP2E1* gene revealed inconsistent results in previous and current studies,[14 31–33] further analysis of the association of other SNPs in the gene to GIC in Malaysians is necessary.

In the past decade, the elderly aged >50 years were predominant in cancer diseases including GIC.[6 20 21] Our study suggests that this pattern has shifted to a younger age in which patients aged >40 years had a highly significant increased risk of GIC in Malaysians. Recently, an annual report by the Clinical Research Centre, Ministry of Health Malaysia revealed that 12.7% of GIC was contributed by patients ranging from 40 to 49 years.[34] Taken together, this suggests that screening of GIC should start earlier at age 40 instead of 50 as

Table 4 Association of gender to risk of gastrointestinal cancer

	Cases, N		Controls, N			
	Male	Female	Male	Female	OR (95% CI)*	p Value
Total	92	83	340	180	1.71 (1.20 to 2.41)	0.003†
Chinese	25	28	81	56	1.62 (0.86 to 3.07)	0.146
Malays	6	5	31	20	1.29 (0.35 to 4.80)	0.744
Indians	2	2	22	8	2.75 (0.33 to 22.92)	0.564
KadazanDusun	16	17	63	58	1.15 (0.53 to 2.49)	0.845
Others	43	31	143	38	2.71 (1.51 to 4.87)	0.001†

*ORs were calculated by taking men as the reference group.
†Statistically significant (p<0.05).

Table 5 Risk association of gender to type of GIC

Type of GIC	Male, N (mean age±S.D.=56.32±1.48)	Female, N (mean age±S.D.=59.25±1.54)	OR (95% CI)*	p Value
Gastric	17	20	2.22 (1.14 to 4.35)	0.021†
Colorectal	62	52	1.58 (1.05 to 2.39)	0.032†
Others‡	13	11	1.60 (0.70 to 3.64)	0.279

*ORs were calculated by taking men as the reference group.
†Including oesophagus (N=5), liver (N=3), pancreas (N=9), gallbladder (N=4) and uncommon GI cancers (N=3).
‡Statistically significant (p<0.05).
GIC, gastrointestinal cancer.

practised in many countries. In addition, we also found that the Malaysian Chinese had a higher risk of GIC than the Malays, Indians and KadazanDusun. Daily diet and different cultures of each ethnicity might play an important role in the development of GIC in Malaysians, yet to be further investigated.

It is worth noting that we unexpectedly found that women had a significant 1.71-fold increased risk of GIC when compared with men. Further analysis also suggested that women were predominant to gastric and colorectal cancers than men in Malaysia. One possible explanation is the unstable hormones during menopause as about 75% of our female patients were >50 years. Caucasian postmenopausal women were reported to have a significantly higher risk for oesophageal and stomach cancers than in premenopausal or perimenopausal women.[35] A previous study also showed that women older than 50 years had 7% greater activity of *CYP2E1* enzyme when compared with men,[36] suggesting that elderly women had a higher risk of GIC than men.

In conclusion, our study reveals that the rare *c2* allele and carrier with at least one *c2* allele of *CYP2E1 Rsa*I polymorphism significantly elevated the risk of GIC and may be used as a genetic biomarker for early detection of GIC in Malaysians. The risk age-group has been shifted to a younger age at 40s and women showed a significant greater risk for stomach and colorectal cancers when compared with men in Malaysia. However, the future research should be conducted with a larger sample population and should include the gene–gene and gene–environmental interactions.

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