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RsaI Polymorphism in CYP2E1 Gene Increases the Risk to Gastrointestinal Cancer in Malaysian.

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ABSTRACT

Gastrointestinal cancer (GIC) has high mortality rate of 4.71 per 100,000 populations in Malaysia and hence early screening using panels of biomarker for GIC is crucial. *CYP2E1* C-1019T *Rsa*I and T7678A *Dra*I polymorphisms revealed conflicting results to risk of GIC in Asian and non-Asian populations but remain unclear in Malaysian.

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 GIC in Malaysian.

Design: Case-control study.

Setting: Malaysia.

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

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 SNP significantly increased the risk to 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 towards GIC compared to the Malays, Indians and KadazanDusun. An increased risk of GIC was observed in individuals who aged >40 years old and females had a 2.22- and 1.58-fold increased risk to stomach and colorectal cancers, respectively, when compared with males.

Limitations: Future research should be conducted with 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 to GIC and may be used as a genetic biomarker for early screening of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers than males.

ARTICLE SUMMARY

Article focus

- Malaysia has a high mortality rate of 4.71 per 100 000 populations for gastrointestinal cancer (GIC). Therefore, a screening system for the detection of GIC is needed.
- *CYP2E1* C-1019T and T7678A SNPs were associated to increase the risk of GIC in Asian and non-Asian populations and other factors such as age, gender, and ethnicity may influence the risk of GIC development. We wished to determine how these SNPs and factors affect the risk of GIC in Malaysian population.

Key messages

- This study reveals the prevalence of *c2* allele and carrier with at least one *c2* allele of C-1019T SNP in the 5′-flanking region of *CYP2E1* gene can be used as a potential biomarker for GIC in Malaysian.
- Risk of GIC has been shifted to early age of 40s in Malaysian population and females have a greater risk towards stomach and colorectal cancers when compared with males in this population.

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 subjects, gene-gene and gene-environment interactions were not included in this study.

INTRODUCTION

Gastrointestinal cancer (GIC) remains a major challenge worldwide because it is among the top cause of cancer-related mortality in the world.[1] In Malaysia, carcinomas of digestive system ranked top of the cancer-related mortality in recent statistics from Ministry of Health Malaysia with mortality rate of 4.71 per 100,000 populations,[2] indicates that a screening system for early detection of GIC is extensively needed. *CYP2E1* gene receives great attention due to previous studies demonstrated the correlation of this gene to cancer carcinomas development such as esophageal, hepatocellular, stomach, colorectal, and lungs in both Asian and non-Asian populations.[3-7]

CYP2E1 is a member of the cytochrome *P450* superfamily, mapped at the 10q24.3 region of chromosome 10.[8] *CYP2E1* enzymes are involved in the metabolism of more than eighty low molecular weight substrates and are found to be highly expressed in the hepatic tissue,[9-10] and sufficient amount of these enzymes are also localized in extrahepatic tissues including nasal mucosa, stomach, small intestine, and colon.[11-12] More than 10 single nucleotide polymorphism (SNP) sites have been identified along the *CYP2E1* gene.[13] Of these, *Rsa*I polymorphism in the 5'-flanking region and *Dra*I polymorphism in the intron 6 of the gene are well-characterized with C to T replacement in position -1019 and T to A replacement in position 7678, respectively.[14] The *Rsa*I and *Dra*I polymorphisms of *CYP2E1* gene revealed conflicting findings to GIC development among different populations in the past studies, but it is not apparent how these polymorphisms affect Malaysian population.[4,6,15-17]

In addition, Malaysia is a multiethnic country with a majority in Malays, Chinese, and Indians whereas KadazanDusun is the dominant native ethnic in East Malaysia, or known as North Borneo. Previous study reported that Malaysian Chinese and Indians ethnics had a greater risk towards GIC than the Malays in Malaysian.[18] Nevertheless, GIC was predominant in elderly who aged >50 years old in Asian and non-Asian populations but to the best of our knowledge, no significantly different on gender to risk of GIC was reported previously.[6,19-20] 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 Malaysian.

METHODS

Samples Collection and DNA Extraction

The design and analysis for this study were according to STREGE guidelines.[21] A total of 520 consented healthy blood donors with no previous GIC record and 175 GIC patients who admitted to Queen Elizabeth Hospital in Sabah, East Malaysia and University Malaya Medical Centre (UMMC), West Malaysia were recruited for this preliminary case-control study to represent the whole Malaysians. Data including age, gender, and ethnicity of the subjects were collected, regardless of data-matching for controls and cases. DNA was extracted from 2 ml of peripheral blood collected in a BD Vacutainer® EDTA-coated tube using alkaline lysis method.

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

Genotyping analysis for both SNPs was carried out at Biotechnology Research Laboratory, School of Science and Technology, Universiti Malaysia Sabah. Polymerase chain reaction (PCR) was performed for both polymorphisms in a 20 µl mixture containing 100 ng extracted DNA as template, 1X PCR buffer (Promega, USA), 1.0 mM of $MgCl_2$, 0.2mM of dNTPs and 1 U *Go Taq*® Flexi DNA polymerase (Promega, USA). 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 94ºC, 35 cycles at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min, followed by a final extension for 7 min at 72ºC. The PCR products were subjected to overnight 2.5 U *Rsa*I and 5.0 U *Dra*I (New England BioLabs® Inc., Ipswich, MA) restriction enzymes digestion for C-1019T and T7678A SNPs, respectively. The digested fragments were electrophoresed and analyzed on 2% agarose gel stained with ethidium bromide in 1X TAE buffer.

Direct Sequencing

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

Statistical Analysis

Hardy-Weinberg proportion for both cases and controls were calculated using Online Encyclopedia for Genetic Epidemiology (OEGE) software.[22] The odds ratio (OR) and 95% confidence intervals (95% CI) of the allele and genotype risk associations to GIC were performed using SPSS Software V17.0 (SPSS Inc., Chicago, Illinois) 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 Chi-square test to examine the genotype differences between cases and controls in this study.

RESULTS

Out of 695 subjects, 62.2% was males and 37.8% was females, with mean age ± S.D. of 42.13 ± 0.140 and 45.96 ± 0.271, respectively. Cases and controls were not deviate from Hardy-Weinberg equilibrium in this study (χ^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 subjects carried the wild-type (*c1/c1*) genotype, followed by 29.2% heterozygous (*c1/c2*) and 3.9% variant (*c2/c2*). No significant different of 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- and 1.45-fold greater risk to GIC significantly ($p < 0.05$).

The frequencies of *D* and *C* allele in T7678A *Dra*I polymorphism were 77.9% and 22.1%, respectively. Frequency of 62.0% was found for subjects who carried the wild-type (*D/D*) genotype, followed by 31.8% for heterozygous (*D/C*) and 6.2% for variant (*C/C*). The distribution of genotype was found to be very similar between cases and controls. The allelic and genotypic risk associations of this polymorphism suggested the T7678A SNP was not likely to be involved in 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 (Table 2), we found that the Malaysian Chinese had a greater risk towards GIC than the Malays, Indians, and KadazanDusun in Malaysian. Besides, elderly who aged >40 years old and females had a higher risk of GIC significantly ($p < 0.05$). Further investigation revealed that females had a 2.22- and 1.58-fold higher risk to gastric and colorectal cancers, respectively, when compared to males in this study (Table 3).

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 to GIC in Malaysian. Previous investigations reported conflicting findings of this polymorphism to risk of GIC in different populations. A study in Fujian province of China revealed that the presence of *c2* allele elevated the risk to gastric cancer, but was found as a protective factor for esophageal squamous cell cancer in another study in China.[3,5] Besides, Ladero *et al*. (1996) reported that carrier with one copy of *c2* allele increased the risk of hepatocellular carcinoma with addition of regular alcohol intake in Spanish, supported the findings by Lee *et al*. (1997) in Korean and Japanese populations.[4,16]

Although the reason that contribute 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 to *c1* allele and the variant *c2/c2* genotype is correlated with about 10-fold greater of gene transcription of *CYP2E1* gene.[23-25] 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.[24] 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, results to intensified lipid and protein peroxidation, DNA damage and carcinogenesis which may leads to the development of GIC.[10]

We found no significant association between T7678A *Dra*I polymorphism and GIC in this study. Similarly, Darazy *et al*. (2011) reported that *D/C* genotype had a non-significant reduced risk to GIC in Lebanese.[26] It might be due to the T7678A SNP was 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.[27] In any case, a study revealed that the *C/C* genotype significantly associated to increase the risk of colorectal cancer although Lin *et al*. (1998) claimed that this genotype might be a protective factor for esophagus cancer.[15,20] Furthermore, combined analysis on both polymorphisms in this study showed no significant association to increase the risk to GIC, which similar to previous published data.[28-29] Malaysia

is a multiracial country but we report here as overall Malaysians in genotyping analysis, regardless the ethnic of the subjects as it is for early GIC screening program development.

As the past decade, elderly who aged >50 years old was predominant in cancer diseases including GIC.[6,19-20] Our study suggests that this pattern has been shifted to younger age in which patients who aged >40 years old had a highly significant increased risk towards GIC in Malaysian. Recently, an annual report by Clinical Research Centre, Ministry of Health Malaysia revealed that 12.7% of GIC was contributed by patients ranging from 40 to 49 years old.[30] Taken together, this suggests that screening programs for early detection of GIC should start at age 40 instead of 50 as practised in many countries. In addition, we also found that the Malaysian Chinese had a higher risk towards GIC than the Malays, Indians and KadazanDusun. Daily diet and different cultural of each ethnicity might play an important role to the development of GIC in Malaysian, yet to be further investigate.

It is worth to note that we unexpectedly found that females had a significant 1.71-fold increased risk towards GIC when compared to males. The further analysis also suggested that females were predominant to gastric and colorectal cancers than males in Malaysian. One possible explanation is the unstable of hormone during menopausal as about 75% of our female patients were more than 50 years old. Caucasian women in postmenopausal were reported to have significantly higher risk to esophageal and stomach cancers than in pre- or peri-menopausal women.[31] Previous study also showed that females who older than 50 years old had 7% greater activity of *CYP2E1* enzyme when compared to males,[32] suggesting that elderly females had a higher risk towards GIC than males.

In conclusion, 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 to GIC and may be used as a genetic biomarker for early screening of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers when compared to males in Malaysian. However, future research should be conducted with larger sample population and including the gene-gene and gene-environmental interactions.

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Competing interests

None.

Ethics approval

University Malaya Medical Centre (UMMC) Ethical Board with Ref. 654.1.

Contributors

PCL, KHC and JAC were responsible for design of the study. PCL, KHC, JAC and ETJC were involved in samples collection. ETJC and CCL performed the laboratory analysis. ETJC and PCL were responsible for statistical analysis and writing of the manuscript. All authors read and approved the final version of the manuscript.

Provenance and peer review

None.

Data sharing statement

None.

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LIST OF TABLES

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

* Statistically significant ($p < 0.05$)

▲ In Hardy-Weinberg equilibrium ($\chi^2 < 5.991$, $df = 2$)

Table 2 Association of other factors to risk of GIC.

	Cases, *N*	Controls, *N*	OR (95% CI)	*p*-value
Age				
≤ 40	19	451	1.00 (Reference)	-
> 40	156	69	53.67 (31.29 – 92.04)	**<0.001***
Gender				
Male	92	340	1.00 (Reference)	-
Female	83	180	1.71 (1.20 – 2.41)	**0.003***
Ethnics				
Chinese	53	137	1.00 (Reference)	-
Malays	11	51	0.56 (0.27 – 1.15)	0.131
Indians	4	30	0.35 (0.12 – 1.03)	0.054
KadazanDusun	33	121	0.71 (0.43 – 1.16)	0.210
Others	74	181	1.06 (0.70 – 1.60)	0.832

* Statistically significant ($p < 0.05$)

Table 3 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 – 4.35)	**0.021***
Colorectal	62	52	1.58 (1.05 – 2.39)	**0.032***
Others[▲]	13	11	1.60 (0.70 – 3.64)	0.279

[#] ORs were calculated by taking males as the reference group

[▲] Including esophagus ($N = 5$), liver ($N = 3$), pancreas ($N = 9$), gallbladder ($N = 4$) and uncommon GI cancers ($N = 3$)

* Statistically significant ($p < 0.05$)

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

Section/Topic	Item #	Recommendation	Reported on #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1 & 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5
		(*b*) For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	5
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	6
		(*c*) Explain how missing data were addressed	-
		(*d*) If applicable, explain how matching of cases and controls was addressed	-
		(*e*) Describe any sensitivity analyses	6
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	7
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	7
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted	7

		estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	7
Discussion			
Key results	18	Summarise key results with reference to study objectives	8 & 9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	8 & 9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	8 & 9
Generalisability	21	Discuss the generalisability (external validity) of the study results	8 & 9
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	10

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

RsaI but not DraI Polymorphism in CYP2E1 Gene Increases Risk of Gastrointestinal Cancer in a Malaysian Population: A Case-Control Study

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*Rsa*I but not *Dra*I Polymorphism in *CYP2E1* Gene Increases the Risk to Gastrointestinal Cancer in Malaysian: A Case-Control Study

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ABSTRACT

Background: Gastrointestinal cancer (GIC) has high mortality rate of 4.71 per 100,000 populations in Malaysia and hence early screening using panels of biomarker for GIC is crucial. *CYP2E1* C-1019T *Rsa*I and T7678A *Dra*I polymorphisms revealed conflicting results to risk of GIC in Asian and non-Asian populations but remain unclear in Malaysian.

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 GIC in Malaysian.

Design: Case-control study.

Setting: Malaysia.

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

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 SNP significantly increased the risk to 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 towards GIC compared to the Malays, Indians and KadazanDusun. An increased risk of GIC was observed in individuals who aged >40 years old and females had a 2.22- and 1.58-fold increased risk to stomach and colorectal cancers, respectively, when compared with males.

Limitations: Future research should be conducted with 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 to GIC and may be used as a genetic biomarker for early screening of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers than males.

ARTICLE SUMMARY

Article focus

- Malaysia has a high mortality rate of 4.71 per 100 000 populations for gastrointestinal cancer (GIC). Therefore, panel of biomarkers for the early detection of GIC is necessary.
- *CYP2E1* C-1019T and T7678A SNPs were associated to increase the risk of GIC in Asian and non-Asian populations and other factors such as age, gender, and ethnicity may influence the risk of GIC development. We wished to determine how these SNPs and factors affect the risk of GIC in Malaysian population.

Key messages

- This study reveals the prevalence of *c2* allele and carrier with at least one *c2* allele of C-1019T SNP in the 5′-flanking region of *CYP2E1* gene can be used as a potential biomarker for GIC in Malaysian.
- Risk of GIC has been shifted to early age of 40s in Malaysian population and females have a greater risk towards stomach and colorectal cancers when compared with males in this population.

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 subjects, gene-gene and gene-environment interactions were not included in this study.

INTRODUCTION

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

CYP2E1 is a member of the cytochrome *P450* superfamily, mapped at the 10q24.3 region of chromosome 10.[9] *CYP2E1* enzymes are involved in the metabolism of more than eighty low molecular weight substrates and are found to be highly expressed in the hepatic tissue,[10-11] and sufficient amount of these enzymes are also localized in 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-characterized 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 to 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 in Malays, Chinese, and Indians whereas KadazanDusun is the dominant native ethnic in East Malaysia, or known as North Borneo. Previous study reported that Malaysian Chinese and Indians ethnics had a greater risk towards GIC than the Malays in Malaysian.[19] Nevertheless, GIC was predominant in elderly who aged >50 years old in Asian and non-Asian populations but to the best of our knowledge, no significantly different 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 Malaysian.

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 GIC patients that were not available.[22] A total of 520 consented healthy blood donors with no previous GIC record and 175 GIC patients who 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 the whole Malaysians. Data including age, gender, and ethnicity of the subjects were collected, regardless of data-matching for controls and cases. Two ml of peripheral blood was 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 via a Nanophotometer (Implen, Germany).

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

Genotyping analysis for both SNPs was carried out at Biotechnology Research Laboratory, School of Science and Technology, Universiti Malaysia Sabah. Polymerase chain reaction (PCR) was performed for both polymorphisms in a 20 µl mixture containing 100 ng extracted DNA as template, 1X PCR buffer (Promega, USA), 1.0 mM of $MgCl_2$, 0.2mM of dNTPs and 1 U *Go Taq*® Flexi DNA polymerase (Promega, USA). 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 94ºC, 35 cycles at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min, followed by a final extension for 7 min at 72ºC. The PCR products were subjected to overnight 2.5 U *Rsa*I and 5.0 U *Dra*I (New England BioLabs® Inc., Ipswich, MA) restriction enzymes digestion for C-1019T and T7678A SNPs, respectively. The digested fragments were electrophoresed and analyzed on 2% agarose gel stained with ethidium bromide in 1X TAE buffer.

Direct Sequencing

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

Statistical Analysis

Hardy-Weinberg proportion for both cases and controls were calculated using Online Encyclopedia for Genetic Epidemiology (OEGE) software.[23] The odds ratio (OR) and 95% confidence intervals (95% CI) of the allele and genotype risk associations to GIC were performed using SPSS Software V17.0 (SPSS Inc., Chicago, Illinois) 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 Chi-square test to examine the genotype differences between cases and controls in this study.

RESULTS

Out of 695 subjects, 62.2% was males and 37.8% was females, with mean age ± S.D. of 42.13 ± 0.140 and 45.96 ± 0.271, respectively. Cases and controls were not deviate from Hardy-Weinberg equilibrium in this study (χ^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 subjects carried the wild-type (*c1/c1*) genotype, followed by 29.2% heterozygous (*c1/c2*) and 3.9% variant (*c2/c2*). No significant different of 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- and 1.45-fold greater risk to GIC significantly (p < 0.05).

The frequencies of *D* and *C* allele in T7678A *Dra*I polymorphism were 77.9% and 22.1%, respectively. Frequency of 62.0% was found for subjects who carried the wild-type (*D/D*) genotype, followed by 31.8% for heterozygous (*D/C*) and 6.2% for variant (*C/C*). The distribution of genotype was found to be very similar between cases and controls. The allelic and genotypic risk associations of this polymorphism suggested the T7678A SNP was not likely to be involved in 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 towards GIC than the Malays, Indians, and KadazanDusun in Malaysian (Table 2). Overall, elderly who aged >40 years old and females had a higher risk of GIC significantly (p < 0.05) (Table 3 and Table 4). Further investigation revealed that females had a 2.22- and 1.58-fold higher risk to gastric and colorectal cancers, respectively, when compared to males 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 to GIC in Malaysian. Previous investigations reported conflicting findings of this polymorphism to 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 Fujian province of China revealed that the presence of *c2* allele elevated the risk to gastric cancer, but was found as a protective factor for esophageal squamous cell cancer in another study in China.[3,5] Besides, Ladero *et al*. (1996) reported that carrier with one copy of *c2* allele increased the risk of hepatocellular carcinoma with addition of regular alcohol intake in Spanish, supported the findings by Lee *et al*. (1997) in Korean and Japanese populations and by Deka *et al*. (2010) in Indian population.[4,8,17]

Although the reason that contribute 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 to *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, results to intensified lipid and protein peroxidation, DNA damage and carcinogenesis which may leads 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*. (2011) reported that *D/C* genotype had a non-significant reduced risk to GIC in Lebanese.[28] It might be due to the T7678A SNP was 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*. (1998) claimed that this genotype might be a protective factor for esophagus cancer.[16,21] Furthermore, combined analysis on both polymorphisms in this study showed no significant

association to increase the risk to GIC, which similar to previous published data.[30-31] Malaysia is a multiracial country but we report here as overall Malaysians in genotyping analysis, regardless the ethnic of the subjects 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 study,[14,31-33] further analysis on the association of other SNPs in the gene to GIC in Malaysians is necessary.

As the past decade, elderly who aged >50 years old was predominant in cancer diseases including GIC.[6,20-21] Our study suggests that this pattern has been shifted to younger age in which patients who aged >40 years old had a highly significant increased risk towards GIC in Malaysian. Recently, an annual report by Clinical Research Centre, Ministry of Health Malaysia revealed that 12.7% of GIC was contributed by patients ranging from 40 to 49 years old.[34] Taken together, this suggests that screening of GIC should start earlier at age 40 instead of 50 as practised in many countries. In addition, we also found that the Malaysian Chinese had a higher risk towards GIC than the Malays, Indians and KadazanDusun. Daily diet and different cultural of each ethnicity might play an important role to the development of GIC in Malaysian, yet to be further investigate.

It is worth to note that we unexpectedly found that females had a significant 1.71-fold increased risk towards GIC when compared to males. The further analysis also suggested that females were predominant to gastric and colorectal cancers than males in Malaysian. One possible explanation is the unstable of hormone during menopausal as about 75% of our female patients were more than 50 years old. Caucasian women in postmenopausal were reported to have significantly higher risk to esophageal and stomach cancers than in pre- or peri-menopausal women.[35] Previous study also showed that females who older than 50 years old had 7% greater activity of *CYP2E1* enzyme when compared to males,[36] suggesting that elderly females had a higher risk towards GIC than males.

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 to GIC and may be used as a genetic biomarker for early detection of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers when compared to males in Malaysian. However, future research should be conducted with larger sample population and including the gene-gene and gene-environmental interactions.

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Competing interests

None declared.

Ethics approval

University Malaya Medical Centre (UMMC) Ethical Board with Ref. 654.1.

Contributors

PCL, KHC and JAC were responsible for design of the study. PCL, KHC, JAC and ETJC were involved in samples collection. ETJC and CCL performed the laboratory analysis. ETJC and PCL were responsible for statistical analysis and writing of the manuscript. All authors read and approved the final version of the manuscript.

Provenance and peer review

None.

Data sharing statement

Sequencing data are available upon request to corresponding author.

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LIST OF TABLES

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

* Statistically significant ($p < 0.05$)

▲ In Hardy-Weinberg equilibrium ($\chi^2 < 5.991$, $df = 2$)

Table 2 Association of ethnic groups to risk of GIC.

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

[#] ORs were calculated by taking Chinese as the reference group

Table 3 Association of age to risk of GIC.

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

[#] ORs were calculated by taking age ≤40 as the reference group

* Statistically significant ($p < 0.05$)

Table 4 Association of gender to risk of GIC.

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

[#] ORs were calculated by taking males 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 – 4.35)	0.021*
Colorectal	62	52	1.58 (1.05 – 2.39)	0.032*
Others[▲]	13	11	1.60 (0.70 – 3.64)	0.279

[#] ORs were calculated by taking males as the reference group

[▲] Including esophagus (*N* = 5), liver (*N* = 3), pancreas (*N* = 9), gallbladder (*N* = 4) and uncommon GI cancers (*N* = 3)

* Statistically significant ($p < 0.05$)

*Rsa*I but not *Dra*I Polymorphism in *CYP2E1* Gene Increases the Risk to Gastrointestinal Cancer in Malaysian: A Case-Control Study

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ABSTRACT

Background: Gastrointestinal cancer (GIC) has high mortality rate of 4.71 per 100,000 populations in Malaysia and hence early screening using panels of biomarker for GIC is crucial. *CYP2E1* C-1019T *Rsa*I and T7678A *Dra*I polymorphisms revealed conflicting results to risk of GIC in Asian and non-Asian populations but remain unclear in Malaysian.

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 GIC in Malaysian.

Design: Case-control study.

Setting: Malaysia.

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

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 SNP significantly increased the risk to 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 towards GIC compared to the Malays, Indians and KadazanDusun. An increased risk of GIC was observed in individuals who aged >40 years old and females had a 2.22- and 1.58-fold increased risk to stomach and colorectal cancers, respectively, when compared with males.

Limitations: Future research should be conducted with 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 to GIC and may be used as a genetic biomarker for early screening of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers than males.

ARTICLE SUMMARY

Article focus

- Malaysia has a high mortality rate of 4.71 per 100 000 populations for gastrointestinal cancer (GIC). Therefore, panel of biomarkers for the early detection of GIC is necessary.
- *CYP2E1* C-1019T and T7678A SNPs were associated to increase the risk of GIC in Asian and non-Asian populations and other factors such as age, gender, and ethnicity may influence the risk of GIC development. We wished to determine how these SNPs and factors affect the risk of GIC in Malaysian population.

Key messages

- This study reveals the prevalence of *c2* allele and carrier with at least one *c2* allele of C-1019T SNP in the 5′-flanking region of *CYP2E1* gene can be used as a potential biomarker for GIC in Malaysian.
- Risk of GIC has been shifted to early age of 40s in Malaysian population and females have a greater risk towards stomach and colorectal cancers when compared with males in this population.

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 subjects, gene-gene and gene-environment interactions were not included in this study.

INTRODUCTION

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

CYP2E1 is a member of the cytochrome *P450* superfamily, mapped at the 10q24.3 region of chromosome 10.[9] *CYP2E1* enzymes are involved in the metabolism of more than eighty low molecular weight substrates and are found to be highly expressed in the hepatic tissue,[10-11] and sufficient amount of these enzymes are also localized in 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-characterized 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 to 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 in Malays, Chinese, and Indians whereas KadazanDusun is the dominant native ethnic in East Malaysia, or known as North Borneo. Previous study reported that Malaysian Chinese and Indians ethnics had a greater risk towards GIC than the Malays in Malaysian.[19] Nevertheless, GIC was predominant in elderly who aged >50 years old in Asian and non-Asian populations but to the best of our knowledge, no significantly different 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 Malaysian.

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 GIC patients that were not available.[22] A total of 520 consented healthy blood donors with no previous GIC record and 175 GIC patients who 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 the whole Malaysians. Data including age, gender, and ethnicity of the subjects were collected, regardless of data-matching for controls and cases. Two ml of peripheral blood was 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 via a Nanophotometer (Implen, Germany).

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

Genotyping analysis for both SNPs was carried out at Biotechnology Research Laboratory, School of Science and Technology, Universiti Malaysia Sabah. Polymerase chain reaction (PCR) was performed for both polymorphisms in a 20 µl mixture containing 100 ng extracted DNA as template, 1X PCR buffer (Promega, USA), 1.0 mM of $MgCl_2$, 0.2mM of dNTPs and 1 U *Go Taq*® Flexi DNA polymerase (Promega, USA). 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 94ºC, 35 cycles at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min, followed by a final extension for 7 min at 72ºC. The PCR products were subjected to overnight 2.5 U *Rsa*I and 5.0 U *Dra*I (New England BioLabs® Inc., Ipswich, MA) restriction enzymes digestion for C-1019T and T7678A SNPs, respectively. The digested fragments were electrophoresed and analyzed on 2% agarose gel stained with ethidium bromide in 1X TAE buffer.

Direct Sequencing

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

Statistical Analysis

Hardy-Weinberg proportion for both cases and controls were calculated using Online Encyclopedia for Genetic Epidemiology (OEGE) software.[23] The odds ratio (OR) and 95% confidence intervals (95% CI) of the allele and genotype risk associations to GIC were performed using SPSS Software V17.0 (SPSS Inc., Chicago, Illinois) 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 Chi-square test to examine the genotype differences between cases and controls in this study.

RESULTS

Out of 695 subjects, 62.2% was males and 37.8% was females, with mean age ± S.D. of 42.13 ± 0.140 and 45.96 ± 0.271, respectively. Cases and controls were not deviate from Hardy-Weinberg equilibrium in this study (χ^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 subjects carried the wild-type (*c1/c1*) genotype, followed by 29.2% heterozygous (*c1/c2*) and 3.9% variant (*c2/c2*). No significant different of 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- and 1.45-fold greater risk to GIC significantly (p < 0.05).

The frequencies of *D* and *C* allele in T7678A *Dra*I polymorphism were 77.9% and 22.1%, respectively. Frequency of 62.0% was found for subjects who carried the wild-type (*D/D*) genotype, followed by 31.8% for heterozygous (*D/C*) and 6.2% for variant (*C/C*). The distribution of genotype was found to be very similar between cases and controls. The allelic and genotypic risk associations of this polymorphism suggested the T7678A SNP was not likely to be involved in 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 towards GIC than the Malays, Indians, and KadazanDusun in Malaysian (Table 2). Overall, elderly who aged >40 years old and females had a higher risk of GIC significantly (p < 0.05) (Table 3 and Table 4). Further investigation revealed that females had a 2.22- and 1.58-fold higher risk to gastric and colorectal cancers, respectively, when compared to males 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 to GIC in Malaysian. Previous investigations reported conflicting findings of this polymorphism to 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 Fujian province of China revealed that the presence of *c2* allele elevated the risk to gastric cancer, but was found as a protective factor for esophageal squamous cell cancer in another study in China.[3,5] Besides, Ladero *et al*. (1996) reported that carrier with one copy of *c2* allele increased the risk of hepatocellular carcinoma with addition of regular alcohol intake in Spanish, supported the findings by Lee *et al*. (1997) in Korean and Japanese populations and by Deka *et al*. (2010) in Indian population.[4,8,17]

Although the reason that contribute 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 to *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, results to intensified lipid and protein peroxidation, DNA damage and carcinogenesis which may leads 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*. (2011) reported that *D/C* genotype had a non-significant reduced risk to GIC in Lebanese.[28] It might be due to the T7678A SNP was 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*. (1998) claimed that this genotype might be a protective factor for esophagus cancer.[16,21] Furthermore, combined analysis on both polymorphisms in this study showed no significant

association to increase the risk to GIC, which similar to previous published data.[30-31] Malaysia is a multiracial country but we report here as overall Malaysians in genotyping analysis, regardless the ethnic of the subjects 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 study,[14,31-33] further analysis on the association of other SNPs in the gene to GIC in Malaysians is necessary.

As the past decade, elderly who aged >50 years old was predominant in cancer diseases including GIC.[6,20-21] Our study suggests that this pattern has been shifted to younger age in which patients who aged >40 years old had a highly significant increased risk towards GIC in Malaysian. Recently, an annual report by Clinical Research Centre, Ministry of Health Malaysia revealed that 12.7% of GIC was contributed by patients ranging from 40 to 49 years old.[34] Taken together, this suggests that screening of GIC should start earlier at age 40 instead of 50 as practised in many countries. In addition, we also found that the Malaysian Chinese had a higher risk towards GIC than the Malays, Indians and KadazanDusun. Daily diet and different cultural of each ethnicity might play an important role to the development of GIC in Malaysian, yet to be further investigate.

It is worth to note that we unexpectedly found that females had a significant 1.71-fold increased risk towards GIC when compared to males. The further analysis also suggested that females were predominant to gastric and colorectal cancers than males in Malaysian. One possible explanation is the unstable of hormone during menopausal as about 75% of our female patients were more than 50 years old. Caucasian women in postmenopausal were reported to have significantly higher risk to esophageal and stomach cancers than in pre- or peri-menopausal women.[35] Previous study also showed that females who older than 50 years old had 7% greater activity of *CYP2E1* enzyme when compared to males,[36] suggesting that elderly females had a higher risk towards GIC than males.

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 to GIC and may be used as a genetic biomarker for early detection of GIC in Malaysian. The risk age-group has been shifted to a younger age at 40s and females showed a significant greater risk towards stomach and colorectal cancers when compared to males in Malaysian. However, future research should be conducted with larger sample population and including the gene-gene and gene-environmental interactions.

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Competing interests

None declared.

Ethics approval

University Malaya Medical Centre (UMMC) Ethical Board with Ref. 654.1.

Contributors

PCL, KHC and JAC were responsible for design of the study. PCL, KHC, JAC and ETJC were involved in samples collection. ETJC and CCL performed the laboratory analysis. ETJC and PCL were responsible for statistical analysis and writing of the manuscript. All authors read and approved the final version of the manuscript.

Provenance and peer review

None.

Data sharing statement

None.

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LIST OF TABLES

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

* Statistically significant ($p < 0.05$)

▲ In Hardy-Weinberg equilibrium ($\chi^2 < 5.991$, $df = 2$)

Table 2 Association of ethnic groups to risk of GIC.

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

[#] ORs were calculated by taking Chinese as the reference group

Table 3 Association of age to risk of GIC.

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

[#] ORs were calculated by taking age ≤40 as the reference group

* Statistically significant ($p < 0.05$)

Table 4 Association of gender to risk of GIC.

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

[#] ORs were calculated by taking males 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 – 4.35)	**0.021***
Colorectal	62	52	1.58 (1.05 – 2.39)	**0.032***
Others[▲]	13	11	1.60 (0.70 – 3.64)	0.279

[#] ORs were calculated by taking males as the reference group

[▲] Including esophagus ($N = 5$), liver ($N = 3$), pancreas ($N = 9$), gallbladder ($N = 4$) and uncommon GI cancers ($N = 3$)

* Statistically significant ($p < 0.05$)

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

Section/Topic	Item #	Recommendation	Reported on #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1 & 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5
		(*b*) For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	5
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	6
		(*c*) Explain how missing data were addressed	-
		(*d*) If applicable, explain how matching of cases and controls was addressed	-
		(*e*) Describe any sensitivity analyses	6
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	7
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	7
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted	7

		estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	7
Discussion			
Key results	18	Summarise key results with reference to study objectives	8 & 9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	8 & 9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	8 & 9
Generalisability	21	Discuss the generalisability (external validity) of the study results	8 & 9
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	10

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.