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TP53 Intronic Polymorphisms and Risk of Esophageal Cancer in Southern Thai Population

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ABSTRACT

Objective: The study aimed to determine the frequency of intron 3 16 base pair (bp) duplication polymorphism and intron 6 G to C substitution (G>C) of the TP53 gene and to evaluate the association these two intronic variants with the risk of esophageal cancer (EC).

Material and Methods: A case-control study was conducted to evaluate the frequency and association of cancer. Cases were patients with squamous cell carcinoma of esophagus and controls were age and sex-matched non-cancer patients. Blood samples were also obtained from healthy blood donors. Polymerase chain reaction (PCR) was used to detect intron 3 16 bp duplication and PCR-restriction fragment length polymorphism was applied to detect intron 6 G>C. Logistic regression was used for the analysis.

Results: Heterozygous intron 3 16 bp duplication (Del/Ins) was found in 10.1% (31/308) of blood donors, 9.3% (28/302) of controls and 8.6% (26/301) of EC cases. Intron 6 G>C was found in 0.3% (1/308) of blood donors, in 2.6% (8/310) of controls and 3.9% (12/307) of EC cases. Both variants displayed no significant association with risk of esophageal cancer (odd ratio (OR) = 1.16 [95% confidence interval (CI) = 0.64–2.11] for intron 3 16 bp duplication and OR = 0.81 [95% CI = 0.47-4.61] for intron 6 G>C.

Conclusion: Southern Thai population have low frequency of intron 3 16 bp duplication polymorphism and intron 6 G>C variant, both of which are not likely to be associated with esophageal cancer susceptibility.

Keywords: esophageal cancer; intronic variation; risk TP53; polymorphism

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INTRODUCTION

Esophageal cancer (EC) is among the ten leading cancers worldwide. It ranked seventh in terms of incidence (572,000 new cases) and the sixth in mortality (509,000 deaths) in 2018.[1] The age standardized rate (ASR) is 9.3 per 100,000 males and 3.5 per 100,000 in females. The incidence rates vary by nearly 16-fold, with the highest rates found in southern and eastern Africa and eastern Asia and lowest rates observed in Western and Middle Africa and Central America in both males and females.[1] In Thailand, the incidence in male is highest in Songkhla province, southern Thailand (ASR 9.1 per 100,000 in 2013-2015) compared to other regions of the country.[2] Likewise, the incidence of EC in female is also higher (ASR 0.8 per 100,000) in Songkhla than the national average value (ASR 0.6 per 100,000).

The TP53 gene is located on chromosome 17. It encodes p53 protein, a nuclear phosphoprotein key regulator, functioned on cell cycle arrest and induction of apoptosis in response to DNA damage. Mutations of TP53 have been found to be the most common genetic alteration in cancer and have been considered to be the primary event in the development of various cancers including EC.[3-5] Apart from somatic mutations, genomic variation in the DNA sequence, mainly in the form of single nucleotide polymorphisms, may also affect p53 function which leads to cancer susceptibility. Most TP53 polymorphisms locate in introns, outside consensus splicing sites.[6] Although the role of intronic variation on the gene function has not been clarified, studies have demonstrated that intron variation affects the expression or function of the TP53 gene.[7,8]

One of the most common intronic polymorphisms in TP53 gene is 16 base pair (bp) duplications in intron 3 (rs17878362). The wild type allele (no duplication) has one copy while the polymorphic allele has two copies of the sequence ACCTGGAGGGCTGGGG (rs17878362).[6,9] This polymorphism has been found to be associated with risk of various cancers including breast, colorectal and lung cancer.[10-14] The evidence is strengthened by the result of two meta-analyses that have demonstrated significant association of this polymorphism with increased risk of cancers, in particular breast cancer.[15,16] However, the evidence in EC is limited and conflicting. It has been reported in northern Indian population by two groups of authors. Umar et al. evaluated the association in 255 patients and 255 healthy controls and found no significant association with the risk of EC (odd ratio (OR) 2.2, 95% confidence interval (CI) = 0.85-1.74) while Malik et al. (135 patients and 195 healthy controls) reported significant association (OR = 2.31, 95% CI=1.08-4.97).[17, 18]

According to a previous study investigating TP53 mutations in esophageal squamous cell carcinoma (ESCC) from southern Thailand, a high frequency (8 of 42 mutations from 165 cases) of G to C substitution (G>C) in intron 6 at the 18th base after the end of exon 6 (c.672+18 G>C, rs199578278) were found.[5] In addition, the study confirmed that the variant was germline alteration. This intronic variant has been recorded as a validated genomic variant in IARC database release version R20, July 2020 (https://p53.iarc.fr/).[6] Currently, there is limited information on clinical significance of this variant in terms of cancer risk association. Therefore, it is worth to explore whether this intron 6 G>C is associated with risk of EC.

In the present study, we aimed to determine the frequency of two intronic variations of TP53 gene including intron 3 16 bp duplication polymorphism and intron 6 G>C in healthy donors to estimate minor allele frequency (MAF) in southern Thai population. In addition, we evaluated the association of these two intronic variations with the risk of EC through a hospital-based case-control study.

MATERIAL AND METHODS

Subjects and blood samples

We used DNA samples from our previous hospital-based case-control study investigating the association of genetic polymorphism of xenobiotic-metabolizing enzymes

with the risk of EC. The study protocol was approved by the Human Research Ethics Committee, Faculty of Medicine, Prince of Songkla University (REC. 55-067-04-1-2). The case subjects were patients who were diagnosed with-ESCC at Songklanagarind Hospital, a university hospital in southern Thailand, during 2004-2006. The controls were non-cancer patients from various out-patient clinics who underwent blood taking at the Department of Pathology during the same period. They were frequency matched to cases by age (+5 years) and sex. The controls who had alcohol or smoking related disease such as alcoholic cirrhosis, alcoholic pancreatitis, chronic obstructive pumonary disease were excluded. We also obtained blood samples from healthy donors during 2012 as a represen-tative of general population. Five ml of blood samples from all subjects were collected after written informed consents were obtained.

Detection of intron 3 16 bp duplication polymor-phism

Intron 3 16 bp duplication polymorphism was detected by PCR technique as described previously.[19] Briefly, genomic DNA was amplified with the sense primer 5'-CTGAAA ACAACGTTCTGGTA-3' and the antisense primer 5'-AAGGGGGACTGTAGATGGGTG-3'. The PCR conditions are 95°C for 10 min, followed by 32 cycles of 94°C denaturation for 30 sec, 60°C annealing for 30 sec, and 72°C extension for 30 sec, with a final extension of 72°C for 10 min. The PCR product was detected by electrophoresis on 4% agarose gel and visualized by ethidium bromide staining. Wild type allele (no duplication, designated as Del) resulted in 119 bp fragment and the variant allele (with 16 bp duplication, designated as Ins) resulted in 135 bp fragment.

Detection of intron 6 G>C

Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method was used to detect intron 6 G>C (rs199578278). The detail of procedure was previously described.[5] Briefly, PCR was performed in a total volume of 25 µL reaction mixture using primers, forward 5'-GCCTCTGATTCCTCACTGAT-3'; and reverse 5'-TTAACCCCTCCTCCCAGAGA-3'. The cycling condi-tions were 95°C for 10 min, 35 cycles of 94°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 1 min, with a final extension of 72°C for 10 min. The resultant 180 bp PCR product was digested with BsaHI restriction enzyme and separated on 10% polyacrylamide gel. A complete digestion (denoted base change or mutation) gave 158 bp and 23 bp DNA fragments.

Statistical analysis

Descriptive statistics were presented as mean (+standard deviation (S.D.)) or as percentage as appro-priate. Chi-square test or Fisher's exact test was used for the comparison of categorical variables between cases and controls. Adjusted odds ratio (adjOR) for the risk of EC was obtained using logistic regression analysis. Hardy-Weinberg equilibrium (HWE) of the genotype frequency in control and healthy donors was evaluated by goodness-of-fit chi-square test. Intercooled Stata version 6.0 (Stata Corp, College station, TX) was used for the analyses. P-value <0.050 was considered statistical significance.

RESULTS

Characteristics of esophageal cancer cases and hospital controls

The case-control study included 365 cases of EC and 344 non-cancer controls. **Table 1** hows charac-teristics of EC cases and non-cancer controls. The age and sex distribution among cases and controls were not different. Majority of both cases and controls had history of smoking and drinking. The cases had a higher percentage of smokers than the controls (72.6% versus 65.4%, p-value = 0.038).

Table 1 Characteristics of esophageal cancer cases and hospital controls in the case-control study for esophageal cancer in southern Thailand

Variables	Case No (%) N = 365	Control No (%) N = 344	p-value*
Age (years) (mean, S.D.)	61.44 (12.3)	62.73 (11.8)	0.158
Sex			0.896
Male	289 (79.2)	271 (78.8)	
Female	76 (20.8)	73 (21.2)	
Family history of cancer			
No	331 (90.9)	317(93.2)	0.259
Yes	33 (9.07)	23 (6.76)	
Smoking			0.038
No	100 (27.4)	119 (34.6)	
Yes	265 (72.6)	225 (65.4)	
Alcohol drinking			0.375
No	164 (44.9)	166 (48.3)	
Yes	201 (55.1)	178 (51.7)	
Betel chewin			0.802
No	267 (73.4)	248 (72.5)	
Yes	97 (26.8)	94 (27.5)	

*chi-square tese

Intron 3 16 bp duplication and intron 6 G>C in esophageal cancer cases and control

Successful laboratory identification was achieved in 603 samples for intron 3 16 bp duplication and 617 for intron 6 G>C. The genotype frequency of both Intron 3 16 bp duplication and intron 6 G>C in the controls were in HWE (chi-square = 0.097, p-value = 0.756 and chi-square = 0.059, p-value = 0.818, respectively). **Table 2** shows the genotypes and minor allele frequencies of both variants. The frequency of heterozygous intron 3 16 bp duplication (Del/Ins) was 8.6% (26/301) in cases and 9.3% (28/302) in controls, while homozygous genotype (Ins/Ins) was found in only one case in both cases and controls. For intron 6 G>C, G/C genotype was found in 3.9% (12/307) of cases and in 2.6% (8/310) of controls. No homozygous C/C was found. The genotype frequencies of both variants were not significantly different between cases and controls.

Table 2 Frequency of intron 3 16- bp duplication and intron 6 G>C of TP53 gene in esophageal cancer cases and hospital controls in the case-control study for esophageal cancer in southern Thailand

Genotypes	Case No (%)	Control No (%)	p-value*
Intron 3 16 bp duplication	N = 301	N = 302	
Del/Del	274 (91.3)	273 (90.4)	0.943
Del/Ins	26 (8.6)	28 (9.3)	
Ins/Ins	1 (0.3)	1 (0.3)	
MAF	0.0465	0.0497	
Intron 6 G>C	**N = 307**	**N = 310**	
G/G	295 (96.1)	302 (97.4)	0.373
G/C	12 (3.9)	8 (2.6)	
C/C	0	0	
MAF	0.0195	0.0129	

Del = deletion; Ins = insertion; G = guanine; C = cytosine; MAF = minor allele frequency *Fisher's exact test

Intron 3 16 bp duplication and intron 6 G>C in healthy donors

Three-hundred and eight healthy donors were examined. The mean age was 33.5 years (S.D. = 10.6 years) with a range of 17 to 68 years. The frequency of heterozygous intron 3 16 bp duplication (Del/Ins) was 10.1% (31/308) **(Table 3)**. Heterozygous intron 6 G>C was found in 0.3% (1/308). Minor allele frequency (MAF) of intron 3 16 bp duplication and intron 6 G>C were 0.0503 and 0016 respectively. The frequencies of both

Table 3 Frequency of intron 3 16– bp duplication and intron 6 G>C of TP53 gene in healthy donors (N = 308)

Genotypes	Total No (%)	Male No (%)	Female No (%)	p-value
Intron 3 16– bp duplication				
Del/Del	277 (89.9)	148 (90.8)	129 (89.0)	0.705[a]
Del/Ins	31 (10.1)	15 (9.2)	16 (11.0)	
Ins/Ins	0	0	0	
MAF	0.0503			
Intron 6 G>C				
G/G	307 (99.7)	163 (100)	144 (99.3)	0.471[b]
G/C	1 (0.3)	0 (0.0)	1 (0.7)	
G/G	0	0	0	
MAF	0.0016			

Del = deletion; Ins = insertion; G = guanine; C = cytosine; MAF = minor allele frequency;
[a]chi–square test; [b]Fisher's exact test

Table 4 Univariate and multivariate logistic regression for the risk of esophageal cancer

Variables	Crude OR (95% CI)	adjOR (95% CI)
Age	0.99 (0.98–1.00)	0.99 (0.98–1.01)
Sex		
Female versus Male	1.02 (0.71–1.47)	0.51 (0.27–0.92)
Family history of cancer		
Yes versus No	1.37 (0.79–2.39)	0.83 (0.41–1.65)
Smoking		
Yes versus No	1.40 (1.02–1.93)	2.21 (1.29–3.78)
Alcohol drinking		
Yes versus No	1.14 (0.85–1.54)	1.07 (0.69–1.65)
Betel chewing		
Yes versus No	0.96 (0.69–1.34)	0.89 (0.59–1.36)
Intron 3 16 bp duplication		
Del/Ins or Ins/Ins versus Del/Del	0.93 (0.54–1.61)	1.16 (0.64–2.11)
Intron 6 G>C		
G/C versus G/G	1.54 (0.62–3.81)	0.81 (0.47–4.61)

Del = deletion; Ins = insertion; G = guanine; C = cytosine; OR = odds ratio; adjOR = adjusted odds ratio; CI = confidence interval

variants were not different between males and females. The genotype frequency of intron 3 16 bp duplication and intron 6 G>C were both in HWE (chi-square = 0.864, p > 0.352 and chi-square = 0.0008, p = 0.977, respectively).

Association of intronic variants with risk of esophageal cancer

Table 4 shows the results of univariate and multivariate logistic regression for risk of EC. All clinical variables and both intronic variants were included for the analysis. Multivariate analysis showed that smoking and sex significantly associated with risk of EC. However, both intronic variants displayed no significant association with risk of EC (adjusted OR = 1.16 [95% CI = 0.64–2.11] for intron 3 16 bp duplication and adjusted OR = 0.81 [95% CI = 0.47–4.61] for intron 6 G>C. 0.81].

DISCUSSION

In addition to somatic mutation, intronic variation in TP53 gene has long been a focus of research for its potential association with risk of cancer. In the present study, duplication allele of intron 3 16 bp duplication polymorphism was found in 5.0% and the minor allele of intron 6 G>C was found in 0.2% of healthy donors. We did not find the association of either intron 3 16 bp duplication polymorphism or intro 6 G>C with the risk of EC.

Approximately 10–30% of MAF of intron 3 16 bp duplication polymorphism have been reported in Western and Indian population.[20] However, we found a much lower frequency. Heterozygous Del/Ins was found in 9.3% of non-cancer patients and in 10.1% of healthy donors with MAF of 5.0% in both groups. This frequency is considerably similar to Chinese population (5.0%) in one study.[21] This polymorphism is also rare in Japanese population, reported as 0% (0/189), and 1.4% in two studies.[22–23] This evidence reflects ethnic difference and indicates low frequency of intron 3 16 bp polymorphism in southeast Asian or east-Asian population.

In the present study, we did not find significant association of intron 3 16 bp duplication polymorphism with the risk of EC. Only two previous studies in EC have been published, both were from the northern region of India.[17–18] The two groups of authors, however, reported inconsistent results. Our study is consistent with those of Umar et al.[17] where no significant association was found. Regarding the result of previous meta-analysis, which included a total of 25 published studies with 10,786 cases and 11,760 controls, homozygote carriers of the duplicated allele (Ins/Ins) had a significantly increased cancer risk (various cancer sites combined) compared with those with Del/Del (aggregated OR=1.45, 95% CI 1.22–1.74).[20] However, there was no significant effect for the heterozygotes Del/Ins (aggregated OR 1.08, 95% CI 0.99–1.18). Notably, the study populations in this meta-analysis are mostly from Western countries, few from India and none from East-Asia or Southeast Asia. Therefore, intron 3 16-bp duplication still has an uncertain role in the risk of EC.

In our previous study, intron 6 G>C (c.672+18G>C) was found in 8 out of 42 mutations in 165 ESCC and was confirmed to be germline alteration.[5] In the present study, we intended to estimate the MAF of this variant in our population. We found considerably low frequency of intron 6 G>C with an MAF of 0.0129 in non-cancer patients and 0.0016 in healthy donors. This variant has rarely been reported in literature. By definition, a genomic variant will be considered as polymorphism if there is more than one allele at a specific locus and each allele must occur in the population at a rate of at least 1%. Therefore, this intron 6 G>C is likely to be rare genomic variant rather than common polymorphism.

Regarding the risk association, we did not find significant association between intron 6 G>C with the risk of EC. Only one study evaluating the association of this variant with risk of cancer has been published so far.[24] The authors found intron 6 G>C in 14/880 (1.6%) breast cancer cases and 2/270 (0.7%) controls. Consistent with

our study, the authors found no significant association of this variant with risk of breast cancer. However, as the frequency of this variant is enormously low, the power of study may be insufficient to detect a statistically significant association.

Certain limitation of our study should be addressed. Theoretically, suitable control for a case-control study should be representative of the population "at risk" of becoming cases and population control is favored than the hospital control in this respect. We reduced the potential bias from the imbalance of confounding among case and hospital controls by using matching technique (age and sex as matching variables). In addition, hospital controls are unhealthy patients which may have similar behavioral styles to cases which may eventually reduce the association of the certain risk factors. We reduced this potential bias by excluding patients who had alcohol or smoking related disease as a control. By all attempts, our study still found that smoking, a known strong risk factor, was significant factor in the analysis.

CONCLUSION

In conclusion, our study demonstrated low frequency of intron 3 16 bp duplication polymorphism in southern Thai population. Intron 6 G>C was found as a rare TP53 genetic variant. Both intronic variations are not likely to be associated with increased risk of EC.

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CONFLICT OF INTEREST

There are no potential conflicts of interest to declare.

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