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ORIGINAL ARTICLE

Case Control Study

Polymorphism of genes encoding drug-metabolizing and inflammation-related enzymes for susceptibility to cholangiocarcinoma in Thailand

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Abstract

BACKGROUND

Cholangiocarcinoma (CCA) is an intractable cancer, and its incidence in north-

eastern Thailand is the highest worldwide. Infection with the liver fluke *Opisthorchis viverrini* (OV) has been associated with CCA risk. However, animal experiments have suggested that OV alone does not induce CCA, but its combination with a chemical carcinogen like nitrosamine can cause experimentally induced CCA in hamsters. Therefore, in humans, other environmental and genetic factors may also be involved.

AIM

To examine relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes.

METHODS

This hospital-based case-control study enrolled 95 case-control pairs matched by age (± 5 years) and sex. We examined relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes, serum anti-OV, alcohol consumption, and smoking. Polymorphisms of *CYP2E1*, *IL-6* (-174 and -634), *IL-10* (-819), and *NF-κB* (-94) and their co-occurrence with polymorphisms in the drug-metabolizing enzyme gene *GSTT1* or *GSTM1* were also analyzed.

RESULTS

Although CCA risk was not significantly associated with any single polymorphism, persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had an increased risk (OR = 3.33, 95%CI: 1.23-9.00) as compared with persons having the *GSTT1* wild-type and *CYP2E1* c1/c1 wild genotype. The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk, and smoking level was related to an OR of 1.5-1.8 in multivariable analyses adjusted for each of the seven genetic polymorphisms.

CONCLUSION

In addition to infection with OV, gene-gene interactions may be considered as one of the risk factors for CCA development.

Key Words: *Opisthorchis*; Glutathione transferase; Cytochrome P-450 *CYP2E1*; Case-control study

Core Tip: Cholangiocarcinoma (CCA) is an intractable cancer, and its prevalence in northeastern Thailand is the highest worldwide. An inflammatory condition produced by infection with the liver fluke *Opisthorchis viverrini* (OV) has been associated with CCA risk, but the susceptibility of individuals has not been fully examined. Our study revealed that persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had an increased risk for developing CCA (OR = 3.33, 95%CI: 1.23-9.00). Therefore, both gene-gene interactions and OV infection should be considered as risk factors for cholangiocarcinogenesis.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a cancer of the hepatobiliary tract, and its incidence is extremely high in northeastern Thailand[1]. This is related to the lifestyle of the people of this area who often consume raw fish, which carries the risk of ingesting fish-borne parasites[2]. Infection with the liver fluke, *Opisthorchis viverrini* (OV), is a known CCA risk factor for inhabitants of northeastern Thailand[3-5], where approximately one-third of the population has been infected with OV[6,7]. Although infection with OV has been listed as a carcinogen to humans by The International Agency for Research on Cancer (IARC)[8], infection with OV alone is not sufficient for CCA development. Indeed, experiments with hamsters have suggested that parasitic infection alone is not sufficient to develop CCA. In fact, co-administration of a chemical carcinogen such as *N*-nitrosodimethylamine is necessary to induce CCA in hamsters[9]. In addition, genetic background related to the activation or detoxification of chemical

carcinogens is reported to be involved in CCA risk[10]. Also, elevated plasma IL-6 was associated with increased risk of CCA in patients infected with OV[11]. Thus, maintenance of chronic infection, exposure to a chemical carcinogen(s), and genetic background may explain CCA risk in northeastern Thailand. We previously reported that infection with OV and genetic polymorphism of a drug-metabolizing enzyme gene, namely *GSTM1*, is related to CCA risk[5] and that the combined effect of polymorphisms of the genes *8-oxoguanine glycosylase 1* and *GSTM1* is also relevant CCA risk[12] in northeastern Thailand. Here, we report our analysis of the association between polymorphisms of inflammation-related genes *(IL-6*, *IL-10* and *NF-kB*), and *CYP2E1*, *GSTT1* and *GSTM1* with risk of developing CCA. We also analyzed potential interactions among genetic polymorphisms of these genes.

MATERIALS AND METHODS

This work was conducted after receiving the approval from the ethics committees of the Nagahama Institute of Bio-Science and Technology, Shiga, Japan, and the National Cancer Institute, Bangkok, Thailand.

Study subjects

All cases with CCA were identified between 1999 and 2005 upon a visit to the Ubon Ratchathani Cancer Centre in the northeastern province of Thailand, one of the cancer centers administered by the National Cancer Institute of Thailand. Diagnosis was based on abdominal ultrasonography by a single radiologist at Ubon Ratchathani Cancer Centre with serological supportive evidence including an elevated carbohydrate antigen 19-9 level (≥ 40 μ/mL). Each case was matched by sex and age (within 5 years) with each control subject who lived in the same Ubon Ratchathani area and visited Ubon Ratchathani Cancer Centre for health check-up. All control subjects were without any clinical, ultrasonographical, or serological abnormalities. Finally, the 95 case-control pairs having data for an antibody against infection with OV were employed although the number of subjects for each of the analyses involving genetic polymorphisms was not equal because the amount of blood samples was limited. Still, there was a narrow range (91-95) for the number of case-control pairs for each of the genetic polymorphisms examined (Table 1).

OV infection

Infection with OV was determined with an enzyme-linked immunosorbent assay using an antibody ("anti-OV") raised against an OV antigen[13].

DNA extraction

Blood samples were frozen and stored at -80°C. DNA was extracted from 2 mL blood with the QIAGEN DNA Blood Midi kit[14].

Analysis of GSTM1 and GSTT1 polymorphisms

Polymorphisms in each of *GSTM1* and *GSTT1* were determined with polymerase chain reaction (PCR) [5] using the following primers: *GSTM1*, 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and 5′-GTT GGG CTC AAA TAT ACG GTG G-3′; *GSTT1*, 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′; *CYP1A1* (internal control), 5′-GAA CTG CCA CTT CAG CTG TCT-3′ and 5′-CAG CTG CAT TTG GAA GTG CTC-3′. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM deoxyribonucleoside triphosphates (dNTPs), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase (AmpliTaqGold; Cetus PerkinElmer, Norwalk, CT). The PCR cycling parameters were 3 min at 95°C (initial denaturation) followed by 45 cycles of 1 min at 95°C, 30 sec at 59°C, and 1 min at 72°C, with a final 10-min extension step. The PCR products (299 bp for *GSTM1*, 507 bp for *GSTT1*) were separated on a 2% agarose gel.

Analysis of CYP2E1 polymorphisms

The 5′-flanking *Rsa*I site polymorphism of *CYP2E1* was detected by PCR combined with restriction-fragment-length polymorphism analysis (PCR-RFLP). The forward and reverse primers were 5′-CCA GTC GAG TCT ACA TTG TCA-3′ and 5′-TTC ATT CTG TCT TCT AAC TGG-3′, respectively. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 12 min at 95°C (initial denaturation) followed by 40 cycles of 1 min at 95°C, 30 sec at 60°C, and 1 min at 72°C, with a final 10-min extension step at 72°C. A 10-μl aliquot of the PCR products was digested with 0.2 U of the restriction enzyme *Rsa*I (GTaU) (TOYOBO Co., Ltd., Japan) for 16 h at 37°C. The resulting fragments were separated by 3% agarose gel electrophoresis and stained with ethidium bromide. The expected PCR product was 410 bp. Upon digestion with *Rsa*I, the c1/c1 wild-type homozygotes had fragments of 360 and 50 bp, the c1/c2 heterozygotes had fragments of 410, 360 and 50 bp, and the c2/c2 variant homozygotes had a fragment of 410 bp[15].

Table 1 Number of subjects with non-missing and with missing values for serum anti-*Opisthorchis viverrini*, alcohol consumption, and smoking dependent on each matched-pair analysis

Principle independent variable	Number of matched pairs	Anti-OV		Alcohol consumption		Smoking	
		Missing	Non-missing	Missing	Non-missing	Missing	Non-missing
Anti-OV	95	Not applicable		3	187	2	188
GSTM1	95	10	180	2	188	2	188
GSTT1	95	10	180	2	188	2	188
CYP2E1	93	9	177	2	184	2	184
IL6, -634 G/C	91	10	172	2	180	2	180
IL6, -175 G/C	92	10	174	2	182	2	182
IL10, -819 T/C	91	10	172	2	180	2	180
NF-kB, -94 ins/del ATTG	92	10	174	2	182	2	182

OV: *Opisthorchis viverrini*.

Analysis of IL-6 (rs1800795) polymorphisms

Polymorphism -174 (G/C) (rs1800795) of *IL-6* was detected by PCR-RFLP with forward primer 5′-ATG CCA AGT GCT GAG TCA CTA-3′ and reverse primer 5′-TCG AGG GCA GAA TGA GCC TC-3′. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 30 sec at 54°C, and 40 sec at 72°C, with a final 10-min extension step at 72°C. A 10-μl aliquot of the PCR products was digested with 0.2 U of the restriction enzyme *Nla*III (CATG↓) (New England Biolabs, Japan) at 37°C for 16 h and separated by PAGE (10% polyacrylamide gel). The expected PCR product was 308 bp. Upon digestion with *Nla*III, the G/G wild-type homozygotes had fragments of 233 and 75 bp, the G/C heterozygotes had fragments of 233, 121, 112 and 75 bp, and the C/C variant homozygotes had fragments of 121, 112 and 75 bp[16].

Analysis of IL-6 (rs1800796) polymorphisms

Polymorphism -634 G/C (rs1800796) of *IL-6* was analyzed by PCR with confronting two-pair primers [17]. The four primers used were *IL-6*-634 F1 5′-CCT CTA AGT TGG GCT GAA GCA GG-3' and *IL-6*-634 R1 5′-GTT CTG GCT CTC CCT GTG AGG-3′ for amplifying the variant type, and *IL-6*-634 F2 5′-CCA GGC AGT TCT ACA ACA GCC G-3′ and *IL-6*-634 R2 5′-TGA GTT TCC TCT GAC TCC ATC GC-3′ for amplifying wild type. Each 25-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 36 cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C, with a final 5-min extension step at 72°C. The PCR products were separated by PAGE. The G/G wild-type homozygotes had fragments of 157 and 240 bp, G/C heterozygotes had fragments of 125, 157, 240 and 75 bp, and the C/C variant homozygotes had fragments of 125 and 240 bp.

Analysis of IL-10 (rs1800871) polymorphisms

Polymorphism -819 T/C (rs1800871) of *IL-10* was analyzed by PCR-RFLP. The forward and reverse primers were *IL-10*-819 F 5′-TCA TTC TAT GTG CTG GAG ATG G-3′ and *IL-10*-819 R 5′-TGG GGG AAG TGG GTA AGA GT-3′, respectively. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 5 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 45 sec at 59°C, and 1 min at 72°C, with a final 10-min extension step at 72°C. A 10-μl aliquot of the PCR products was digested with 0.03 U of the restriction enzyme *Mae* III (↓GTNAC) (Roche Diagnostics GmbH Germany) for 16 h at 55°C and separated by PAGE. The expected PCR product was 209 bp. Following digestion with *Mae*III, the T/T wild-type homozygotes had a fragment of 209 bp, the T/C heterozygotes had fragments of 209, 125 and 84 bp, and the C/C variant homozygotes had fragments of 209 and 84 bp[18].

Analysis of NF-κB polymorphisms

Polymorphism -94 ins/del ATTG of the *NF-κB* promoter was determined by PCR-RFLP. The forward and reverse primers were 5′-TTT AAT CTG TGA AGA GAT GTG AAT-3′ and 5′-CTA GCA GGG CGC TCC CGA AT-3′, respectively. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50

Table 2 Relationship between serum anti-*Opisthorchis viverrini* and cholangiocarcinoma risk, Ubon Ratchathani, Thailand

Number of case-control pairs according to serum anti-OV status: positive (+) *vs* negative (-)				Crude OR	95%CI		*P* value	Adjusted[1] OR	95%CI		*P* value	Adjusted[2] OR	95%CI		*P* value
Case (+) / Control (+)	Case (+) / Control (-)	Case (-) / Control (+)	Case (-) / Control (-)		LL	UL			LL	UL			LL	UL	
67	23	2	3	11.50	2.71	48.78	< 0.001	8.96	2.06	38.99	0.001	9.40	2.16	40.85	0.003

[1]Adjusted for alcohol consumption and smoking.
[2]Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.
OV: *Opisthorchis viverrini;* CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 1 min at 56°C, and 1 min at 72°C, with a final 10-min extension at 72°C. A 10-µl aliquot of the PCR products was digested with 2 U of the restriction enzyme *PflMI* (New England Biolabs Inc., Japan) at 37°C for 16 h and separated by PAGE. The expected PCR product was 254 bp. Upon digestion with *PflMI*, the del/del ATTG wild-type homozygotes had a fragment of 254 bp, the ins/del heterozygotes had fragments of 254 and 206 bp, and the ins/ins variant homozygotes had a fragment of 206 bp[19].

Data collection for lifestyle-related factors

Smoking and alcohol consumption status were ascertained alongside dietary habits at the hospital by local health personnel using a structured questionnaire used in previous studies[5,12]. Smoking status was classified into four categories: Never, occasional, former, and current. A current smoker was defined as smoking at least 1 cigarette *per* day, whereas the former smoker was defined as having stopped smoking regularly at least 1 year prior to our study. Alcohol consumption status was classified the same as for smoking status into four categories: Never, occasional, former, and current. "Current" drinker was defined as drinking more than once a week, whereas "former" drinker was defined as having stopped regular drinking at least 1 year prior to the study.

Statistical analysis

CCA risk attributable to infection with OV was defined as positivity for anti-OV, and the potential contributions of genetic polymorphisms of genes encoding inflammation-related enzymes were examined by calculating an OR. Each OR was calculated using a conditional logistic regression model, keeping matched case-control pairs. Interactions between each of the genetic polymorphisms of the inflammation-related genes and each of the metabolic enzyme genes were first examined using a log-likelihood ratio test. The tests compared the main effect with no interaction terms with a full model that included the interaction term for the variables concerned. When the p value for the log-likelihood ratio statistic was < 0.1, we calculated the OR owing to the co-occurrence of two genetic polymorphisms. Three subjects lacked data for alcohol consumption, and two lacked data for smoking status (Table 1). Most of the multivariable statistical analyses were performed with imputation for the missing data for these five subjects and produced comparable results (Tables 2-4). All statistical analyses were performed

Table 3 Relationship between serum anti-*Opisthorchis viverrini*, alcohol consumption, and smoking and cholangiocarcinoma risk, Ubon Ratchathani, Thailand, shown by analysis for each of the genetic polymorphisms

Independent variable	Gene adjusted for	Adjusted[1] OR	95%CI		*P* value	Adjusted[2] OR	95%CI		*P* value
			LL	UL			LL	UL	
Positive serum anti-OV	*GSTM1*	9.16	2.00	41.90	0.004	7.12	1.76	28.81	0.006
	GSTT1	8.16	1.85	35.96	0.006	7.33	1.74	30.93	0.007
	CYP2E1	7.75	1.73	34.69	0.007	4.90	1.41	17.05	0.013
	IL-6, -174 G/C	8.07	1.80	36.07	0.006	5.39	1.51	19.29	0.010
	IL-6, -634 G/C	7.21	1.57	32.98	0.011	6.13	1.54	24.32	0.010
	IL-10, -819 T/C	9.04	1.97	41.39	0.005	6.03	1.65	22.04	0.007
	NF-kB	11.81	2.31	60.44	0.003	8.14	1.90	34.90	0.005
Alcohol drinking	*GSTM1*	1.06	0.70	1.60	0.779	1.12	0.76	1.67	0.560
	GSTT1	1.05	0.70	1.58	0.817	1.10	0.74	1.63	0.643
	CYP2E1	1.05	0.70	1.58	0.801	1.10	0.75	1.63	0.614
	IL-6, -174 G/C	1.03	0.68	1.56	0.907	1.09	0.73	1.62	0.674
	IL-6, -634 G/C	1.00	0.65	1.52	0.989	1.06	0.70	1.59	0.784
	IL-10, -819 T/C	0.98	0.63	1.53	0.933	1.06	0.70	1.61	0.785
	NF-kB	0.99	0.63	1.54	0.956	1.05	0.68	1.60	0.836
Smoking	*GSTM1*	1.56	1.01	2.40	0.044	1.49	0.99	2.23	0.053
	GSTT1	1.56	1.01	2.42	0.044	1.51	1.00	2.27	0.049
	CYP2E1	1.55	1.00	2.41	0.052	1.46	0.98	2.18	0.060
	IL-6, -174 G/C	1.73	1.05	2.85	0.030	1.62	1.04	2.54	0.035
	IL-6, -634 G/C	1.79	1.10	2.91	0.018	1.73	1.08	2.76	0.022
	IL-10, -819 T/C	1.80	1.09	2.98	0.022	1.65	1.05	2.59	0.030
	NF-kB	1.78	1.08	2.94	0.023	1.80	1.11	2.91	0.016

[1]Adjusted for alcohol consumption and smoking.
[2]Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.
OV: *Opisthorchis viverrini;* CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

with the statistical package STATA 16.1 (College Station, TX, USA), and statistical significance was defined as $P < 0.05$ unless indicated otherwise.

Although the CCA-related risk of infection with OV among the current study subjects was reported in the multivariable analyses that included the interaction terms between the *GSTM1* and *hOGG1* polymorphisms in our previous study[12], a crude or adjusted OR for each polymorphism has not been reported. Data for CCA risk owing to polymorphism of *GSTM1* or *GSTT1* alone also has not been reported[12]. Findings for those factors are, thus, reported in the present study.

RESULTS

Concerning environmental factors, we confirmed the strong association between anti-OV-positive individuals and CCA risk adjusted for smoking status and alcohol consumption (Table 2). The OR values (95%CI) for a one-category change in smoking category (never, occasional, former, current) were approximately 1.5 regardless of the particular genetic polymorphism(s) examined or whether imputed data were included (Table 3). Alcohol consumption was not materially related to CCA risk (Table 3).

There was no significant association between polymorphisms for any of the inflammation-related genes or drug-metabolizing genes and CCA risk. When an inflammation-related gene (*IL-6*, *IL-10*, *NF-kB*) wild-type genotype was used as the reference, CCA risk was not significantly associated with any homozygous or heterozygous genotype (Table 4). Likewise, when the *GSTT1* wild-type genotype was used as the reference, CCA risk was not significantly associated with the null genotype. Similarly, when

Table 4 Relationship between polymorphisms of drug-metabolizing and inflammation-related enzyme genes and cholangiocarcinoma risk, Ubon Ratchathani, Thailand

Gene	Number of pairs according to gene polymorphism of case and control					Polymorphism	Crude OR	95%CI		*P* value	Adjusted[1] OR	95%CI		*P* value	Adjusted[2] OR	95%CI		*P* value
								LL	UL			LL	UL			LL	UL	
GSTM1																		
			Case															
			Wild	Null														
	Control	Wild	8	18		Wild	1.00	Reference			1.00	Reference			1.00	Reference		
		Null	18	51		Null	1.00	0.52	1.92	1.000	0.67	0.29	1.56	0.357	0.81	0.37	1.75	0.594
GSTT1																		
			Case															
			Wild	Null														
	Control	Wild	44	23		Wild	1.00	Reference			1.00	Reference			1.00	Reference		
		Null	21	7		Null	1.10	0.61	1.98	0.763	0.94	0.44	1.98	0.865	1.02	0.52	2.02	0.957
CYP2E1																		
			Case															
			c1/c1	c1/c2	c2/c2													
	Control	c1/c1	56	14	4	c1/c1	1.00	Reference			1.00	Reference			1.00	Reference		
		c1/c2	12	4	2	c1/c2	1.11	0.52	2.38	0.782	1.02	0.39	2.70	0.966	1.14	0.47	2.77	0.773
		c2/c2	1	0	0	c2/c2	6.19	0.74	52.08	0.093	2.02	0.20	20.67	0.552	4.17	0.45	38.24	0.207
						c1/c2 or c2/c2	1.38	0.68	2.83	0.371	1.14	0.47	2.77	0.767	1.35	0.58	3.11	0.484
IL-6																		
			Case			-174												
			G/G	G/C	C/C													
	Control	G/G	90	0	0	G/G	1.00	Reference			1.00	Reference			1.00	Reference		
		G/C	1	0	0	G/C	0.00	-	-	-	0.003	0.00	-	0.995	0.0037	0.00	-	0.999
		C/C	0	0	0	C/C	-	-	-	-	-	-	-	-	-	-	-	-
IL-6						-634												

			Case													
			G/G	G/C	C/C											
	Control	G/G	0	4	5	G/G	1.00	Reference		1.00	Reference		1.00	Reference		
		G/C	2	13	12	G/C	1.93	0.63	5.93	0.252	1.90	0.49	7.30	0.351	1.69	0.46	6.25	0.432
		C/C	4	18	34	C/C	1.28	0.44	3.73	0.654	1.38	0.40	4.71	0.607	1.45	0.43	4.87	0.551
						G/C or C/C	1.50	0.53	4.21	0.442	1.53	0.46	5.05	0.488	1.52	0.46	4.98	0.489
IL-10						-819 T/C												
			Case													
			T/T	T/C	C/C											
	Control	T/T	23	15	6	T/T	1.00	Reference		1.00	Reference		1.00	Reference		
		T/C	15	17	4	T/C	0.81	0.41	1.60	0.537	0.66	0.27	1.63	0.371	0.75	0.34	1.70	0.497
		C/C	8	0	3	C/C	1.19	0.46	3.06	0.715	1.59	0.43	5.93	0.487	1.63	0.51	5.17	0.405
						T/C or C/C	0.91	0.51	1.65	0.763	0.85	0.39	1.87	0.686	0.95	0.47	1.93	0.891
NF-kB						-94 ins/del ATTG												
			Case													
			del/del	ins/del	ins/ins											
	Control	del/del	3	5	3	del/del	1.00	Reference		1.00	Reference		1.00	Reference		
		ins/del	4	16	13	ins/del	0.83	0.32	2.17	0.711	1.11	0.27	4.61	0.884	0.79	0.24	2.67	0.708
		ins/ins	9	20	19	ins/ins	0.48	0.19	1.23	0.128	0.32	0.08	1.20	0.090	0.33	0.10	1.03	0.057
						ins/del or ins/ins	0.91	0.51	1.65	0.763	0.49	0.14	1.69	0.258	0.47	0.16	1.38	0.167

[1]Adjusted for alcohol consumption and smoking.
[2]Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.
CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

the *GSTM1* wild-type genotype was used as the reference, CCA risk was not significantly associated with the null genotype. When the *CYP2E1* c1/c1 wild-type genotype was used as the reference, CCA risk was not significantly associated with c1/c2, c2/c2, or the combination of the c1/c2 + c2/c2 genotypes (Table 4).

Gene-gene interactions between GSTT1 and CYP2E1

Interaction of the *CYP2E1* polymorphism and the *GSTT1* polymorphisms was suggested by a p value of

Table 5 Risk of cholangiocarcinoma due to the combination of polymorphisms of *GSTT1* and *CYP2E1*: Matched case-control study, Ubon Ratchathani, Thailand

CYP2E1	*GSTT1* wild		*GSTT1* null		*CYP2E1*	*GSTT1* wild				*GSTT1* null				
	Number		Number			Odds ratio[1]	95%CI		*P* value	Odds ratio[1]	95%CI		*P* value	*P* for interaction
	Case	Control	Case	Control			LL	UL			LL	UL		
c1/c1	49	59	25	18	c1/c1	1.00	Reference			2.04	0.95	4.39	0.07	< 0.01
c1/c2 + c2/c2	24	9	5	11	c1/c2 + c2/c2	3.33	1.23	9.00	0.02	0.54	0.14	2.13	0.38	

[1]Based on the conditional logistic regression model.

CCA: Cholangiocarcinoma; CI: Confidence interval; LL: Lower limit; UL: Upper limit.

0.003, whereas other interactions between drug-metabolizing enzyme genes (*GSTM1* or *GSTT1*) and other genetic polymorphisms were not substantiated ($P > 0.10$). Persons with the *GSTT1* wild-type plus *CYP2E1* c1/c2 + c2/c2 genotype had an increased risk for CCA (OR = 3.33, 95%CI: 1.23-9.00) as compared with persons having the *GSTT1* wild-type plus *CYP2E1* c1/c1 wild-type genotype that was used as the reference (Table 5).

DISCUSSION

Early diagnosis of CCA is difficult, and most patients die within a year after diagnosis[20]. Identification of risk factors and means of preventing cholangiocarcinogenesis is thus highly desirable. We confirmed that positivity for anti-OV constitutes a significant risk factor for CCA, as reported previously[3,5,21]. Smoking was not a significant risk factor for CCA, whereas alcohol consumption was in fact related to increased risk as we reported previously for subjects (and matched controls) recruited from another part of northern Thailand[5] In the present study, control subjects were persons seeking a health check-up and thus possibly may have led a relatively healthier lifestyle compared with our experimental subjects. However, the discordance between the findings on smoking and alcohol drinking of the two studies are not yet explained. Although we did not find any significant association between CCA risk and polymorphisms in inflammation-related genes (*IL-6*, *IL-10*, *NF-kB*) or in drug metabolism-related genes (*CYP2E1, GSTT1* and *GSTM1*), persons with the *GSTT1* wild-type plus *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold greater risk than persons having the *GSTT1* wild-type plus *CYP2E1* c1/c1 wild-type genotype that was used as the reference. Hayashi *et al*[22] reported that the c2/c2 homozygous sequence placed upstream of the SV40 promoter and *chloramphenicol acetyltransferase* gene enhanced the expression of that gene, and the enhancement of expression by the c2/c2 sequence was about 10-fold that by the c1/c1 sequence. Thus, it is possible that CYP2E1 is expressed at a higher level in the presence of the c2/c2 sequence than in the presence of the c1/c1 sequence, which is consistent with the known function of CYP2E1 as a carcinogen-activating enzyme[23,24]. Because the numbers of cases and controls were not large, the conclusions from this work should be confirmed in a future study with

more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions.

CONCLUSION

The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk for CCA independently of the genetic polymorphisms of carcinogen-metabolizing and inflammation-related genes. While any single polymorphism was not significantly associated with CCA risk, persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk as compared with persons having the *GSTT1* wild-type and *CYP2E1* c1/c1 wild genotype. In addition to infection with OV, gene-gene interactions may be considered as one of the risk factors for CCA development.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma (CCA) is a cancer of the hepatobiliary tract, and its incidence is extremely high in northeastern Thailand. This is related to the lifestyle of the inhabitants of this area consuming often raw fish, which carries the risk of ingesting fish-borne parasites, *Opisthorchis viverrini* (OV), a known CCA risk factor. While infection with OV has been listed as a carcinogen to humans by The International Agency for Research on Cancer, the parasitic infection alone is not sufficient to develop CCA; in fact, co-administration of a chemical carcinogen such as *N*-nitrosodimethylamine is necessary to induce CCA in animal model. In addition, genetic background related to the activation or detoxification of chemical carcinogens is reported to be involved in CCA risk. Also, elevated plasma IL-6 was associated with increased risk of CCA in patients infected with OV.

Research motivation

We already reported that infection with OV and genetic polymorphism of a drug-metabolizing enzyme gene, namely *GSTM1*, is related to CCA risk and that the combined effect of polymorphisms of the genes *8-oxoguanine glycosylase 1* and *GSTM1* is also relevant CCA risk in northeastern Thailand. In the present study, we further investigated possible associations of maintenance of chronic infection, exposure to a chemical carcinogen(s) and genetic background with CCA risk in northeastern Thailand.

Research objectives

To examine relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing (*CYP2E1*, *GSTT1* and *GSTM1*) and inflammation-related genes (*IL-6, IL-10* and *NF-kB*), and potential interactions among genetic polymorphisms of these genes on the CCA risk.

Research methods

All cases with CCA were identified between 1999 and 2005 upon a visit to the Ubon Ratchathani Cancer Centre in the northeastern province of Thailand. This hospital-based case-control study enrolled 95 case-control pairs matched by age (± 5 years) and sex. We examined relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes, serum anti-OV, alcohol consumption, and smoking. Smoking and alcohol consumption status were ascertained using a structured questionnaire used in previous studies. Conditional logistic regression was employed to estimate CCA risk as OR due to each of genetic polymorphisms and possible interactions of those polymorphisms.

Research results

Although any single polymorphism was not significantly associated with CCA risk, persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk as compared with persons having the *GSTT1* wild-type and *CYP2E1* c1/c1 wild genotype. The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk for CCA independently of the genetic polymorphisms of carcinogen-metabolizing and inflammation-related genes.

Research conclusions

An inflammatory condition produced by infection with OV indicated as raised anti-OV in serum has been associated with CCA risk. Our study added the finding that persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk for developing CCA. Therefore, both gene-gene interactions and OV infection should be considered as risk factors for cholangiocarcinogenesis in northeastern Thailand.

Research perspectives

CCA is still an intractable cancer. While our study revealed the interaction of polymorphisms of *GSTT1* and *CYP2E1* possibly contributes to development of CCA, the numbers of cases and controls were not large. The conclusions from this work should be confirmed in a future study with more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions.

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FOOTNOTES

Author contributions: Miwa M secured funds and started the collaborative study in 1998 with the late Srivatanakul P who had been Thai side's active organizer based on the National Cancer Institute, Bangkok, until her death in 2020; Viwatthanasittiphong C, Muangphot M, Chenvidhya D, Jedpiyawongse A, Sripa B, Honjo S, and Miwa M designed and conducted the epidemiological study; Sripa B measured anti-OV and providing microbiological advice; You G, Zeng L, Tanaka H, Ohta E, Fujii T, Ohshima K, Tanaka M, Hamajima N performed analyses concerning genetic polymorphisms; You G, Zeng L, Miwa M and Honjo S conducted statistical analyses and prepared the manuscript; All authors have read and approved the final manuscript.

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