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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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Keywords: Cholangiocarcinoma, Polymorphisms, DNA repair genes, Smoking, Alcohol drinking

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ABSTRACT

Objective: Cholangiocarcinoma (CCA) is the most common malignancy in a Northeast Thai population. Smoking and alcohol drinking are associated with production of free radical intermediates, which can cause several types of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. We therefore examined that whether polymorphisms in DNA base-excision repair (BER) genes, *XRCC1* G399A and *OGG1* C326G, were associated with CCA risk and whether they modified the effect of smoking and alcohol drinking in Thai population.

Design: A nested case-control study within cohort study was conducted: 219 subjects with primary CCA were each matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of *Opisthorchis viverrini* eggs in stools. Smoking and alcohol consumption were assessed on recruitment. Polymorphisms in BER genes were analyzed using a polymerase chain reaction with high resolution melting analysis. The associations were assessed using conditional logistic regression.

Results: Our results suggest that in Thai population polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, are associated with increased susceptibility to CCA, and that their role as modifiers of the effect of smoking and alcohol consumption influences the risk of CCA.

Conclusions: Better ways of reducing habitual smoking and alcohol consumption, targeted toward subgroups which are genetically susceptible, are recommended. CCA is multi-factorial disease, and a comprehensive approach is needed for its effective prevention. This approach would also have the additional advantage of reducing the onset of other cancers.

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Strengths and limitations of this study

- This was a nested case-control study within the cohort study. This design minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. It is advantage for examining the role of self-reported factors such as cigarette smoking and alcohol drinking, which are modified when cancer is present.
- Only some cholangiocarcinoma cases were histological confirmed diagnosis.
- This study did not include population aged 70 years and above.

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INTRODUCTION

The data from GLOBOCAN 2008 shows that liver cancer is the fourth most common cause of death from cancer worldwide.[1] It is the most common cancer in Northeast Thailand, where the great majority of cases are cholangiocarcinomas (CCA).[2] Data from the Khon Kaen Cancer Registry (KKCR) have shown that the estimated age-standardised incidence rates for men and women are 84.6 and 36.8 per 100 000, respectively.[3]

Among several types of DNA damage induced by carcinogens from tobacco and alcohol are oxidative base damage and single-stand breaks, which are repaired by the base-excision repair pathway (BER).[4] Genetic variants and polymorphisms in DNA repair pathways have been reported to affect the levels of DNA lesions, which accumulate in the epithelial tissue of the hepatobiliary tract system, thus influencing the susceptibility to biliary tract cancers and stones in a Chinese population[5, 6] and gallbladder cancer in an Indian population.[7] No studies on this topic in relation to CCA have been conducted in a Thai population, but there have been studies, which clarify the role of the accumulation of levels of oxidative DNA lesions in liver fluke-infected- and CCA patients[8, 9]; these studies concluded that some biomarkers from oxidative DNA lesions induced by liver fluke infection – a major risk factor – may play an important role in CCA development. However, the results from our previous study showed that not only liver fluke infection but also certain lifestyle and dietary co-factors were associated with increased risk of CCA.[10, 11] Long-term smoking[12, 13] and alcohol drinking[4, 14, 15] are associated with production of free radical intermediates, which can cause several kinds of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. With respect to genetic factors, a number of polymorphisms in various DNA repair genes have been discovered, and it is possible that these polymorphisms may affect DNA repair capacity and thus modulate susceptibility to cancer. Investigations are therefore needed into the different patterns of

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smoking and alcohol drinking in relation to susceptibility to CCA, along with DNA repair capacity induced by genetic variations or polymorphisms.

Although cigarette smoking was not associated with CCA development in the previous studies conducted in Thailand[11, 16-19] and elsewhere,[20] a modifying effect by DNA repair genes remains a possibility. With respect to the use of alcohol, this was related to a substantially increased risk of CCA in our previous study.[11] At the molecular level, it is known that single-nucleotide polymorphisms (SNPs) in genes participate biochemically in the base-excision repair pathway (X-ray repair cross-complementing group 1: *XRCC1* and 8-oxoguanine DNA glycosylase 1: *OGG1*), which repairs smoking- and alcohol-induced oxidative DNA base modifications.[4]

In this present study, we therefore investigated whether polymorphisms in DNA base-excision repair genes (*XRCC1* G399A and *OGG1* C326G) are associated with CCA risk and whether their effects are modified by cigarette smoking and alcohol drinking in a Northeastern Thai population residing in a high-incidence area of opisthorchiasis-related CCA. The selection of these SNPs was based on their putative effect on protein function and/or previous evidence of associations with the risk of hepatobiliary tract cancers.[5-7]

MATERIALS AND METHODS

Study subjects

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS). Details of the KKCS and the selection of CCA cases and controls used in the present study have been previously reported.[10, 11, 21] Briefly, 219 cohort members who had developed a primary CCA, diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology, six or more months after enrollment were identified. The vital

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status and date of death of potential cases were ascertained by linkage to the files of deaths in Thailand, which are held in the database of the National Health Security Office and the demographic database of Ministry of Interior. All cases had died within two years of diagnosis. Two non-cancer controls from the same cohort population were randomly selected for matching with each case on sex, age at recruitment (±3 years) and presence/absence of liver fluke eggs in the faeces at recruitment (detected by the formalin ethyl acetate concentration technique). This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Assessment of cigarette smoking and alcohol drinking

A smoker was defined as someone who had ever smoked any type of cigarette on a daily basis.

Ever drinkers were defined as those who consumed at least one type of alcoholic beverage (beer, *Sato*, white whisky, red whisky and other whiskies) at least once a month; those drinking less than this were defined as non-drinkers. The consumption of each subject was calculated in terms of units of alcohol, where a unit corresponds to 10 ml (approximately 8 gm) of ethanol. The number of units of alcohol in a drink was determined by multiplying volume of the drink (in milliliters) by its alcohol percentage (beer 5.0%, *Sato* 7.0%, white whisky 40%, red whisky 35%) and dividing by 1000. Drinkers were dichotomized according to units of alcohol consumed per month on the basis of the median monthly consumption for the controls.

Laboratory methods

Specimen collection and DNA extraction

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Blood samples (buffy coat) were available for 175 (80%) of the 219 eligible CCA cases; specimens were retrieved from the KKCS bio-bank for these cases and for the 350 matched controls. Genomic DNA was extracted from buffy coat fractions using the standard protocols of Genomic DNA Mini Kit with Proteinase K (Geneaid Biotech). Of those with DNA samples, genotyping was successfully carried out for 95% (499 out of 525) of all samples for *XRCC1* and for 90% (474 out of 525) of all samples for *OGG1*. Laboratory personnel were blinded to the case-control status of the available samples.

PCR amplification and genetic polymorphisms detection

The real-time polymerase chain reactions with high resolution melting analysis (Real-time PCR-HRM) technique of DNA amplification for the DNA base-excision repair genes (*XRCC1* and *OGG1*) polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System.

The amplification of *XRCC1* G399A used two primers, [F]:5′-AGT GGG TGC TGG ACT GTC-3′ and [R]:5′-TTG CCC AGC ACA GGA TAA-3′, and was performed in a LightCycler® 480 Real-Time PCR System with a final volume of 20 µl containing 10 µl of master mix, 4.4 µl of H_2O, 3 mM of $MgCl_2$, 0.3 µM of each primer and 200 ng of the DNA template. The amplification of the *OGG1* C326G was performed in the same way, but the primers were [F]:5′-CCT ACA GGT GCT GTT CAG T-3′ and [R]:5′-CCT TTG GAA CCC TTT CTG-3′.

HRM data were analyzed using the LightCycler 480® Gene Scanning Software version 1.5 (Roche). Normalized and temperature-shifted melting curves carrying a sequence variation were evaluated and compared with the wild-type sample. Sequence variations were distinguished by different shape of melting curves (Figure 1(A) for *XRCC1* G399A and (B) for *OGG1* C326G).

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Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plots were distinguished by different melting peaks for each genotype (Figure 1(C) for *XRCC1* G399A and (D) for *OGG1* C326G). To improve the genotyping quality and validation, genotyping of 10% of random samples was confirmed by the PCR with restriction fragment length polymorphism techniques (PCR-RFLP).

Statistical analysis

To assess the strength of the associations between polymorphisms in DNA base-excision repair genes (*XRCC1* and *OGG1*) and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. A univariate analysis using McNemar's chi-square test and conditional logistic regression was carried out to explore the associations between DNA-repair genes polymorphisms, smoking and alcohol drinking, and the risk of CCA. Factors found to have a strong association with CCA in the univariate analysis ($p < 0.05$), and factors without association in the univariate analysis, but found to play important roles as factors for CCA risk from literature reviews, were included in the multivariate analysis. Possible modifications of the effects of smoking and alcohol drinking by polymorphisms in *XRCC1* G399A and *OGG1* C326G were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

RESULTS

There were 219 cohort members who had developed a primary CCA six or more months after enrollment into the KKCS. They comprised 92 females and 127 males, with a median age of 57 years (minimum 31, maximum 69). There were two controls matched by age and sex for each

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case. Table 1 shows the distribution of *XRCC1* G399A and *OGG1* C326G polymorphisms in cases and controls, the joint distribution of those polymorphisms, and the associated odds ratios. While there were no associations between any of these individual polymorphisms and the risk of CCA, the combinations of *XRCC1* GA heterozygous and *OGG1* CC wild-type or CG heterozygous, and of *XRCC1* GG wild-type and *OGG1* CG heterozygous, were significantly related to a reduced risk of CCA (*P*-value <0.001). Conversely, the combinations of the *OGG1* GG mutant, homozygous with all three genotypes of *XRCC1* (wild-type, heterozygous and mutant homozygous), were significantly associated with an increased risk of CCA (*P*-value = 0.01, 0.03 and 0.004, respectively), but there were no interactions between the polymorphisms of *XRCC1* G399A and *OGG1* C326G on CCA risk.

In the univariate analysis (Table 2), there were significant associations between cigarette smoking and alcohol consumption and the risk of CCA, and their joint effect appeared to be multiplicative with no interactions between them. There was trend in risk with smoking frequency or number of years of habitual smoking (*P*-value for trend = 0.002).

In the multivariate analysis (Table 3), shows the adjusted ORs and 95% CIs from the multivariate analysis. There was a clear association with alcohol drinking: compared with non-drinkers, individuals who consumed <14 units of alcohol per month had an increased risk of CCA (OR=5.2, 95% CI 2.74 to 9.95), and the risk was higher still in those who consumed ≥14 units of alcohol per month (OR=7.6, 95% CI 3.67 to 15.72).

Table 4 shows the results of gene-environment interaction of the *XRCC1* and *OGG1* polymorphisms along with smoking and alcohol drinking on the risk of CCA. When compared with the reference subjects (nonsmokers with the *XRCC1* GG wild-type), smokers carrying the *XRCC1* GG wild-type had an increased susceptibility to CCA (OR=2.8, 95% CI 1.30 to 5.91),

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and this was higher still in smokers with GA heterozygous (OR=3.4, 95% CI 1.60 to 7.28). Moreover, smokers with the *OGG1* CC wild-type and CG heterozygous had an increased risk of CCA compared with nonsmokers with the wild-type. With respect to alcohol consumption, some combinations of alcohol drinking and mutant polymorphisms of both *XRCC1* and *OGG1* were statistically significant. For example, compared with the reference group (nondrinkers with the *XRCC1* GG wild-type), subjects with the *XRCC1* GA heterozygous who were nondrinkers or drinkers (<14 units of alcohol per month) had an increased susceptibility to CCA (4.4-fold increased to 8.8-fold). Similarly, subjects with the *XRCC1* AA variant genotype had an increased risk of CCA (9.2-fold in nondrinkers increased to 10.3-fold in drinkers of <14 units of alcohol per month). Again, there were no interactions between *XRCC1* or *OGG1* polymorphisms and smoking or alcohol drinking.

DISCUSSION

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS).[10, 11, 21] The design of this study minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. This design is very useful for examining the role of self-reported factors such as cigarette smoking and alcohol consumption, which are modified when cancer is present.

Smoking was not associated with CCA development in this present study (after adjustment for all other variables in the multivariate analysis) – polymorphisms in *XRCC1* and *OGG1*, cigarette smoking and units of alcohol per month of all alcohol consumption.

Alcohol consumption was significantly associated with an increased risk of CCA in previous studies in Thailand[17, 18] and elsewhere.[20] Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines[15, 18] and it is associated with production of free radical

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intermediate, which can cause several kinds of DNA lesions.[4, 14, 15] Reduced repair of these DNA lesions would therefore constitute a major risk factor for cancer development.[14, 15]

The genotype frequencies of *XRCC1* and *OGG1* polymorphisms found in the controls for the present study were consistent with those found in other studies in Thailand: the prevalence of the *XRCC1* codon 399 (A allele) was 56% in the present study compared with 42–59% in other studies[22-24] and that of the *OGG1* codon 326 (G allele) was 71% compared with 75% and 80%.[23, 25]

XRCC1 and *OGG1* are major DNA repair genes involved in BER. Mutations and polymorphisms in DNA repair genes, which affect the efficiency of DNA damage repair, may increase an individual's susceptibility to several cancers[22-25] including cancers of the hepatobiliary tract.[5-7] However, no studies on this topic have been conducted in relation to CCA susceptibility and especially not in Thailand where the incidence of CCA is highest in the world.[16] However, there are four studies in a Thai population, which investigated relationships between the BER gene polymorphisms and other common cancers. Whereas *XRCC1* G399A polymorphisms tend to be associated with a reduced risk of oral squamous cell carcinoma,[22] they are associated with increased risks of breast[23] and cervical cancers,[24] and *OGG1* C326G polymorphisms tend be related to increased risks of breast[23] and lung cancers.[25]

Several studies have explored the associations between polymorphisms of the DNA repair genes involved in BER (*XRCC1* G399A and *OGG1* C326G) and the risk of various cancers, but they have shown inconsistent results. This may be the consequence of the different modifying effects, which these polymorphisms have on the balance during the DNA repair process. Imbalance in the tightly regulated process can be caused by SNPs and will result in deficient DNA repair and an increase in DNA breaks.[26] The *XRCC1* codon 399 A allele and the *OGG1* 326 G allele have been associated with a reduced capacity of the DNA repair process as detected by

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the persistence of DNA bulky adducts[27-29] and γ-irradiation induced oxidative DNA damage.[30] In addition, the balance may be modified by smoking- and alcohol-induced oxidative DNA base modifications interacting with the repair inefficiency of BER polymorphisms[4]; for example, the *XRCC1* GA variant heterozygous carriers, who were nondrinkers or drinkers of <14 units of alcohol per month, had an increased susceptibility to CCA (4.4-fold in nondrinkers increase to 8.8-fold in drinkers) when compared with nondrinkers carrying the *XRCC1* GG wild-type (Table 4).

In conclusion, this study is the first report of possible associations between the main polymorphisms in BER genes (*XRCC1* G399A, *OGG1* C326G), the joint effects of both genes, and their modification of the effects of cigarette smoking and alcohol drinking on CCA risk in a Thai population. Our results suggest that polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, were associated with increased susceptibility to CCA. CCA is a multifaceted disease requiring a broad range of preventative actions. In the context of the present study, there is a need for more effective programmes for smoking cessation and reducing alcohol consumption, targeted especially at subgroups which are genetically at particular risk for CCA. Such an approach could also help in the prevention of other forms of cancer.

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Table 1 Univariate analysis of *XRCC1* and *OGG1* polymorphisms with the risk of cholangiocarcinoma in Khon Kaen, Thailand

Genetic polymorphisms		Cases		Controls		OR*	95% CI	*P*-value
		n	%	n	%			
***XRCC1* G399A†**								
GG		62	38.8	149	44.0	1.0		
GA		94	58.8	169	49.9	1.3	0.89 to 1.97	0.17
AA		4	2.5	21	6.2	0.5	0.16 to 1.42	0.18
GA or AA (any A allele)		98	61.3	190	56.1	1.2	0.82 to 1.78	0.33
***OGG1* C326G‡**								
CC		34	23.5	95	28.9	1.0		
CG		109	75.2	229	69.6	1.4	0.86 to 2.23	0.18
GG		2	1.4	5	1.5	1.2	0.22 to 7.03	0.80
CG or GG (any G allele)		111	76.6	234	71.1	1.4	0.86 to 2.22	0.18
Joint effects								
***XRCC1* G399A**	***OGG1* C326G**							
GG	CC	14	9.7	41	12.7	1.0		
GA	CC	19	13.1	47	14.5	0.3	0.15 to 0.55	<0.001
AA	CC	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
GG	CG	42	29.0	100	30.9	0.1	0.07 to 0.25	<0.001
GA	CG	56	38.6	108	33.3	0.1	0.07 to 0.22	<0.001
AA	CG	3	2.1	15	4.6	0.9	0.42 to 2.07	1.00
GG	GG	5	3.4	3	0.9	4.7	1.30 to 25.33	0.01
GA	GG	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
AA	GG	2	1.4	2	0.6	7.0	1.61 to 63.46	0.004
P-value for interaction = 0.21								

*Crude odds ratio from matched case-control analysis.

†DNA samples for XRCC1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

‡DNA samples for OGG1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 2 Univariate analysis of cigarette smoking and alcohol drinking on the risk of cholangiocarcinoma in Khon Kaen, Thailand

Variables	Cases		Controls		OR*	95% CI	*P*-value
	n	%	n	%			
Cigarette smoking							
Non-smoker	92	42.0	230	52.5	1.0		
Smoker	127	58.0	208	47.5	2.7	1.57 to 4.50	<0.001
Age at which smoking started of all cigarettes (years)							
Non-smoker	92	42.0	230	52.5	1.0		
<17	28	12.8	48	11.0	2.9	1.38 to 5.91	0.01
17–19	35	16.0	44	10.1	3.6	1.85 to 7.17	<0.001
20–21	40	18.3	70	16.0	2.5	1.33 to 4.62	0.004
22+	24	11.0	46	10.5	2.4	1.19 to 4.73	0.01
P-value for trend = 0.002							
Alcohol consumption							
No	57	26.0	254	58.0	1.0		
Yes	162	74.0	184	42.0	5.7	3.61 to 9.04	<0.001
Units of alcohol per month of all alcohol consumption							
Non-drinker	57	26.0	254	58.0	1.0		
<14	79	36.1	92	21.0	5.2	3.15 to 8.43	<0.001
≥14	83	37.9	92	21.0	6.7	3.90 to 11.38	<0.001
Alcohol consumption and smoking							
No alcohol, non-smoker	42	19.2	178	40.6	1.0		
No alcohol, smoker	15	6.9	76	17.4	2.2	0.93 to 5.00	0.07
Alcohol drinker, non-smoker	50	22.8	52	11.9	5.8	3.12 to 10.78	<0.001
Alcohol drinker, smoker	112	51.1	132	30.1	10.4	5.07 to 21.16	<0.001
P-value for trend <0.001							
P-value for interaction = 0.68							

*Crude odds ratio from matched case-control analysis.

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Table 3 Cases and controls, and odds ratios for cholangiocarcinoma associated with smoking, alcohol drinking, and *XRCC1* and *OGG1* polymorphisms

Variables	Cases		Controls		OR*	OR†	95% CI‡	*P*-value
	n	%	n	%				
***XRCC1* G399A polymorphisms§**								
GG	62	38.8	149	44.0	1.0	1.0		
GA	94	58.8	169	49.9	1.3	1.2	0.76 to 1.94	0.43
AA	4	2.5	21	6.2	0.5	0.4	0.10 to 1.62	0.20
***OGG1* C326G polymorphisms¶**								
CC	34	23.4	95	28.9	1.0	1.0		
CG	109	75.2	229	69.6	1.4	1.2	0.70 to 2.07	0.49
GG	2	1.4	5	1.5	1.2	0.7	0.10 to 5.11	0.75
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Smoker	127	58.0	208	47.5	2.7	1.9	0.94 to 4.01	0.07
Units of alcohol per month of all alcohol consumption								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.2	2.74 to 9.95	<0.001
≥14	83	37.9	92	21.0	6.7	7.6	3.67 to 15.72	<0.001

*Crude odds ratio from matched case–control analysis.

†Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).

‡95% confidence interval for OR†.

§DNA samples for *XRCC*1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

¶DNA samples for *OGG*1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 4 Gene-environment interactions of *XRCC1* and *OGG1* polymorphisms together with smoking and alcohol drinking on cholangiocarcinoma risk in Khon Kaen, Thailand

XRCC1	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value	*OGG1*	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value
G399A	**All cigarettes smoking**					*0.87†*	**C326G**	**All cigarettes smoking**					*0.54†*
GG	Non-smoker	22	71	1.0			CC	Non-smoker	14	55	1.0		
GA	Non-smoker	48	99	1.5	0.82 to 2.79	0.18	CG	Non-smoker	49	122	1.5	0.77 to 2.99	0.22
AA	Non-smoker	2	11	0.6	0.11 to 2.74	0.47	GG	Non-smoker	1	1	–	–	–
GG	Smoker	40	78	2.8	1.30 to 5.91	0.01	CC	Smoker	20	40	3.1	1.17 to 8.38	0.02
GA	Smoker	46	70	3.4	1.60 to 7.28	0.001	CG	Smoker	60	107	4.3	1.79 to 10.21	0.001
AA	Smoker	2	10	1.2	0.21 to 6.29	0.86	GG	Smoker	1	4	1.5	0.14 to 15.44	0.74
G399A	**Units of alcohol per month of all alcohol drinking**					*0.80†*	**C326G**	**Units of alcohol per month of all alcohol drinking**					*0.74†*
GG	Non-drinker	16	83	1.0			CC	Non-drinker	10	65	1.0		
GA	Non-drinker	20	33	4.4	1.81 to 10.83	0.001	CG	Non-drinker	9	9	8.1	2.30 to 28.36	0.001
AA	Non-drinker	26	33	9.2	3.65 to 23.21	<0.001	GG	Non-drinker	15	21	8.6	2.62 to 28.02	<0.001
GG	<14	22	102	1.1	0.49 to 2.28	0.89	CC	<14	24	130	1.3	0.55 to 3.20	0.52
GA	<14	39	35	8.8	3.76 to 20.71	<0.001	CG	<14	46	58	7.0	2.85 to 17.39	<0.001
AA	<14	33	32	10.3	4.14 to 25.80	<0.001	GG	<14	39	41	11.2	4.18 to 30.17	<0.001
GG	≥14	1	12	0.4	0.05 to 3.71	0.44	CC	≥14	0	1	–	–	–
GA	≥14	2	3	2.8	0.31 to 25.41	0.36	CG	≥14	1	1	7.7	0.29 to 26.73	0.22
AA	≥14	1	6	2.5	0.25 to 23.99	0.44	GG	≥14	1	3	6.4	0.48 to 86.72	0.16

*Crude odds ratio from matched case-control analysis.

†*P*-value for interaction.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 (Internet). Lyon, France: IARC; 2010. http://globocan.iarc.fr (accessed 27 Mar 2013).

2. Khuhaprema T, Attasara P, Sriplung H, *et al*. *Cancer in Thailand Volume VI, 2004-2006.* Bangkok: NCIB, 2012.

3. Parkin DM, Ohshima H, Srivatanakul P, *et al*. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev* 1993;2:537–44.

4. Stern MC, Conti DV, Siegmund KD, *et al.* DNA repair single-nucleotide polymorphisms in colorectal cancer and their role as modifiers of the effect of cigarette smoking and alcohol in the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2007;16:2363–72.

5. Huang WY, Gao YT, Rashid A, *et al.* Selected base excision repair gene polymorphisms and susceptibility to biliary tract cancer and biliary stones: a population-based case-control study in China. *Carcinogenesis* 2008;29:100–5.

6. Zhang M, Huang WY, Andreotti G, *et al.* Variants of DNA repair genes and the risk of biliary tract cancers and stones: a population-based study in China. *Cancer Epidemiol Biomarkers Prev* 2008;17:2123–7.

7. Srivastava A, Srivastava K, Pandey SN, *et al*. Single-nucleotide polymorphisms of DNA repair genes *OGG1* and *XRCC1*: association with gallbladder cancer in North Indian population. *Ann Surg Oncol* 2009;16:1695–703.

8. Thanan R, Murata M, Pinlaor S, *et al.* Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2008;17:518–24.

For peer review only

9. Dechakhamphu S, Pinlaor S, Sitthithaworn P, *et al*. Lipid peroxidation and etheno DNA adducts in white blood cells of liver fluke-infected patients: protection by plasma alpha-tocopherol and praziquantel. *Cancer Epidemiol Biomarkers Prev* 2010;19:310–8.

10. Songserm N, Promthet S, Sithithaworn P, *et al. MTHFR* Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand. *Asian Pac J Cancer Prev* 2011;12:1341–5.

11. Songserm N, Promthet S, Sithithaworn P, *et al.* Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms. *Cancer Epidemiol* 2012;36:e89–94.

12. Kosti O, Byrne C, Meeker KL, *et al.* Mutagen sensitivity, tobacco smoking and breast cancer risk: a case-control study. *Carcinogenesis* 2010;31:654–9.

13. Rouissi K, Ouerhani S, Hamrita B, *et al.* Smoking and polymorphisms in xenobiotic metabolism and DNA repair genes are additive risk factors affecting bladder cancer in Northern Tunisia. *Pathol Oncol Res* 2011;17:879–86.

14. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009;674:36–44.

15. Pelucchi C, Tramacere I, Boffetta P, *et al*. Alcohol consumption and cancer risk. *Nutr Cancer* 2011;63:983–90.

16. Parkin DM, Srivatanakul P, Khlat M, *et al.* Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991;48:323–8.

17. Chernrungroj G. Risk factors for cholangiocarcinoma: a case-control study (dissertation). Connecticut: Yale University, 2000.

For peer review only

18. Honjo S, Srivatanakul P, Sriplung H, *et al.* Genetic and environmental determinants of risk for cholangiocarcinoma via *Opisthorchis viverrini* in a densely infested area in Nakhon Phanom, northeast Thailand. *Int J Cancer* 2005;117:854–60.

19. Poomphakwaen K, Promthet S, Kamsa-Ard S, *et al.* Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. *Asian Pac J Cancer Prev* 2009;10:251–8.

20. Chalasani N, Baluyut A, Ismail A, *et al.* Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000;31:7–11.

21. Sriamporn S, Parkin DM, Pisani P, *et al.* A prospective study of diet, lifestyle, and genetic factors and the risk of cancer in Khon Kaen Province, northeast Thailand: description of the cohort. *Asian Pac J Cancer Prev* 2005;6:295–303.

22. Kietthubthew S, Sriplung H, Au WW, *et al*. Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand. *Int J Hyg Environ Health* 2006;209:21–9.

23. Sangrajrang S, Schmezer P, Burkholder I, *et al.* Polymorphisms in three base excision repair genes and breast cancer risk in Thai women. *Breast Cancer Res Treat* 2008;111:279–88.

24. Settheetham-Ishida W, Yuenyao P, Natphopsuk S, *et al*. Genetic Risk of DNA Repair Gene Polymorphisms (*XRCC1* and *XRCC3*) for High Risk Human Papillomavirus Negative Cervical Cancer in Northeast Thailand. *Asian Pac J Cancer Prev* 2011;12:963–6.

25. Klinchid J, Chewaskulyoung B, Saeteng S, *et al*. Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women. *Cancer Genet Cytogenet* 2009;195:143–9.

26. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutat Res* 2003;526:93–125.

27. Lunn RM, Langlois RG, Hsieh LL, *et al*. *XRCC1* polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999;59:2557–61.

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28. Duell EJ, Millikan RC, Pittman GS, *et al.* Polymorphisms in the DNA repair gene *XRCC1* and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:217–22.

29. Matullo G, Guarrera S, Carturan S, *et al.* DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer* 2001;92:562–7.

30. Vodicka P, Stetina R, Polakova V, *et al.* Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis* 2007;28:657–64.

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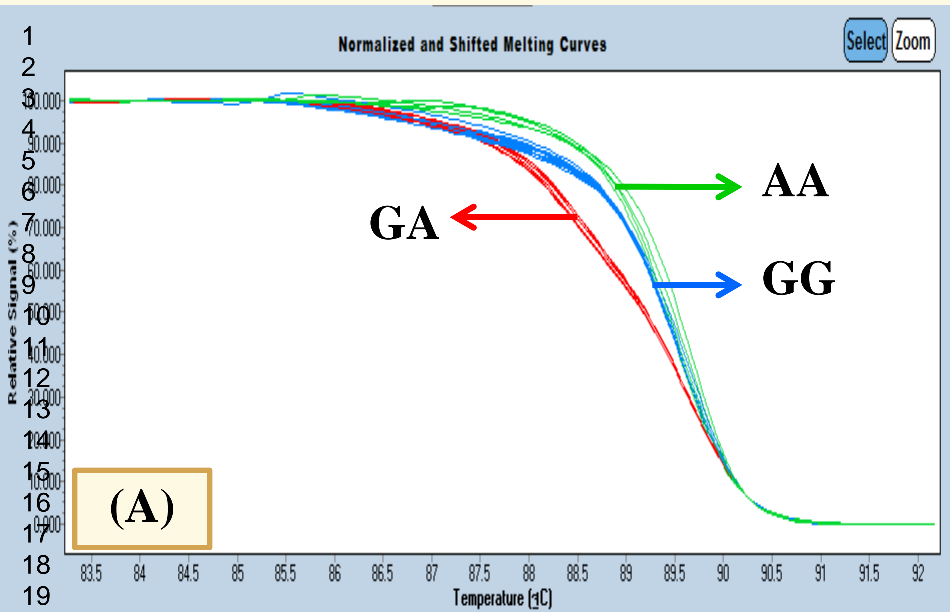
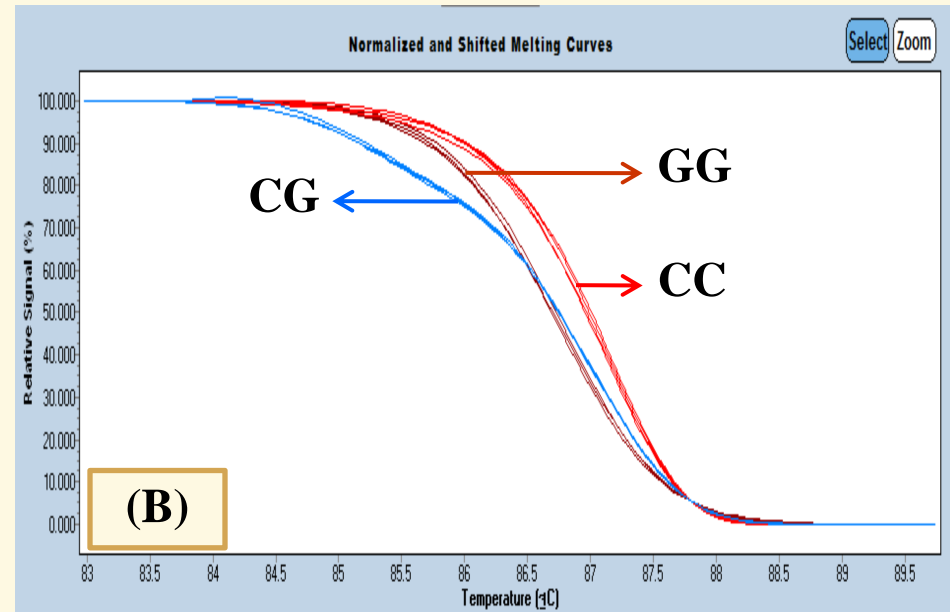
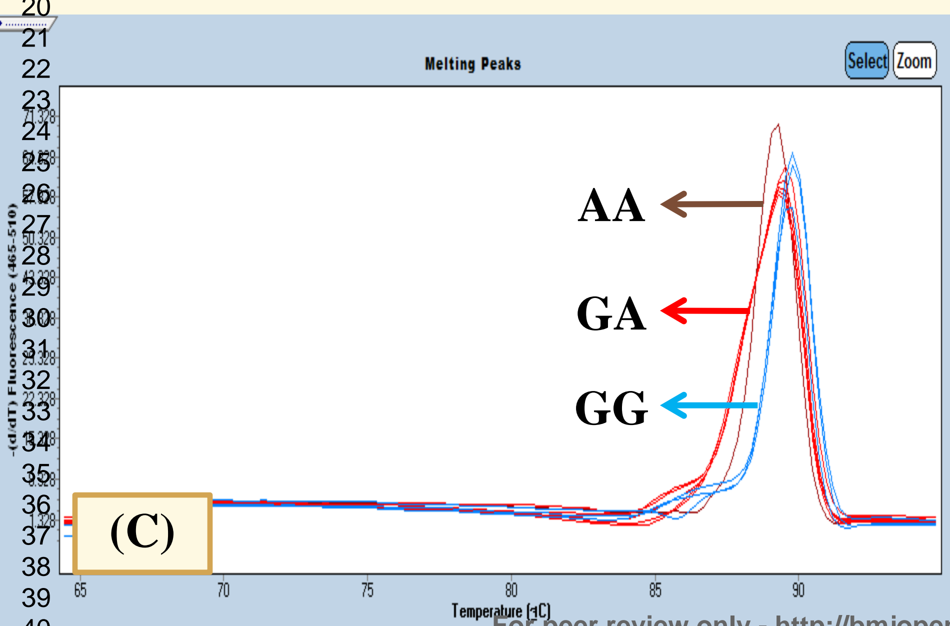
XRCC1 G399A

OGG1 C326G







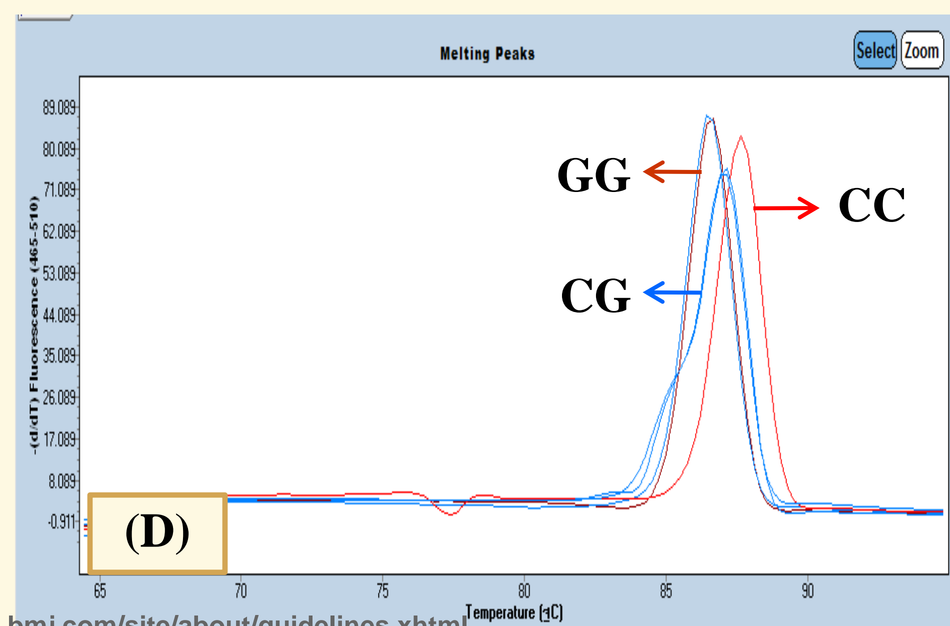


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Figure 1 Polymorphisms in *XRCC1* G399A and *OGG1* C326G were analyzed using PCR with high resolution melting analysis.

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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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ABSTRACT

Objective: Cholangiocarcinoma (CCA) is the most common malignancy in a Northeast Thai population. Smoking and alcohol drinking are associated with production of free radical intermediates, which can cause several types of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. We therefore examined that whether polymorphisms in DNA base-excision repair (BER) genes, *XRCC1* G399A and *OGG1* C326G, were associated with CCA risk and whether they modified the effect of smoking and alcohol drinking in Thai population.

Design: A nested case-control study within cohort study was conducted: 219 subjects with primary CCA were each matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of *Opisthorchis viverrini* eggs in stools. Smoking and alcohol consumption were assessed on recruitment. Polymorphisms in BER genes were analyzed using a polymerase chain reaction with high resolution melting analysis. The associations were assessed using conditional logistic regression.

Results: Our results suggest that in Thai population polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, are associated with increased susceptibility to CCA, and that their role as modifiers of the effect of smoking and alcohol consumption influences the risk of CCA.

Conclusions: Better ways of reducing habitual smoking and alcohol consumption, targeted toward subgroups which are genetically susceptible, are recommended. CCA is multi-factorial disease, and a comprehensive approach is needed for its effective prevention. This approach would also have the additional advantage of reducing the onset of other cancers.

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Strengths and limitations of this study

- This was a nested case-control study within the cohort study. This design has the advantage of minimising a systematic recall bias which can occur when the receipt of diagnosis of cancer leads to subjects modifying their self-reported personal histories, in this case, their recall of cigarette smoking and alcohol consumption. This was prevented in the present study because subjects were interviewed at the time of recruitment, before case or control status was defined.
- The diagnosis of cholangiocarcinoma was not histologically confirmed in all the cases.
- This study did not include the population aged 70 years and above.
- In future research, larger samples are needed to increase the power of the studies.

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INTRODUCTION

The data from GLOBOCAN 2008 shows that liver cancer is the fourth most common cause of death from cancer worldwide.[1] It is the most common cancer in Northeast Thailand, where the great majority of cases are cholangiocarcinomas (CCA).[2] Data from the Khon Kaen Cancer Registry (KKCR) have shown that the estimated age-standardised incidence rates for men and women are 84.6 and 36.8 per 100 000, respectively.[3]

Among several types of DNA damage induced by carcinogens from tobacco and alcohol are oxidative base damage and single-stand breaks, which are repaired by the base-excision repair pathway (BER).[4] Genetic variants and polymorphisms in DNA repair pathways have been reported to affect the levels of DNA lesions, which accumulate in the epithelial tissue of the hepatobiliary tract system, thus influencing the susceptibility to biliary tract cancers and stones in a Chinese population[5, 6] and gallbladder cancer in an Indian population.[7] There were very few studies on this topic in relation to CCA in Thai population, but there have been studies, which clarify the role of the accumulation of levels of oxidative DNA lesions in liver fluke-infected- and CCA patients[8-10]; these studies concluded that some biomarkers from oxidative DNA lesions induced by liver fluke infection – a major risk factor – may play an important role in CCA development. However, the results from our previous study showed that not only liver fluke infection but also certain lifestyle and dietary co-factors were associated with increased risk of CCA.[11, 12] Long-term smoking[13, 14] and alcohol drinking[4, 15, 16] are associated with production of free radical intermediates, which can cause several kinds of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. With respect to genetic factors, a number of polymorphisms in various DNA repair genes have been discovered, and it is possible that these polymorphisms may affect DNA repair capacity and thus modulate susceptibility to cancer. Investigations are therefore needed into the different patterns of

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smoking and alcohol drinking in relation to susceptibility to CCA, along with DNA repair capacity induced by genetic variations or polymorphisms.

Although cigarette smoking was not associated with CCA development in the previous studies conducted in Thailand[12, 17-20] and elsewhere,[21] a modifying effect by DNA repair genes remains a possibility. With respect to the use of alcohol, this was related to a substantially increased risk of CCA in our previous study.[12] At the molecular level, it is known that single-nucleotide polymorphisms (SNPs) in genes participate biochemically in the base-excision repair pathway (X-ray repair cross-complementing group 1: *XRCC1* and 8-oxoguanine DNA glycosylase 1: *OGG1*), which repairs smoking- and alcohol-induced oxidative DNA base modifications.[4]

In this present study, we therefore investigated whether polymorphisms in DNA base-excision repair genes (*XRCC1* G399A and *OGG1* C326G) are associated with CCA risk and whether their effects are modified by cigarette smoking and alcohol drinking in a Northeastern Thai population residing in a high-incidence area of opisthorchiasis-related CCA. The selection of these SNPs in DNA base-excision repair genes was based on their putative effect on protein function and/or previous evidence of associations with the risk of hepatobiliary tract cancers in Asian, Chinese and Indian populations[5-7]and the combined effects of a DNA-repair gene and metabolic gene polymorphisms on CCA risk in Thailand.[10] However, these studies involved only gene-gene interactions. There is need for studies of gene-environment interactions, especially between DNA-repair genes and exposure to the carcinogenic effects of tobacco/alcohol carcinogens in relation to CCA risk.

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MATERIALS AND METHODS

Study subjects

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS). Details of the KKCS and the selection of CCA cases and controls used in the present study have been previously reported.[11, 12, 22] Briefly, 219 cohort members who had developed a primary CCA, diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology, six or more months after enrollment were identified. The vital status and date of death of potential cases were ascertained by linkage to the files of deaths in Thailand, which are held in the database of the National Health Security Office and the demographic database of Ministry of Interior. All cases had died within two years of diagnosis. Two non-cancer controls from the same cohort population were randomly selected for matching with each case on sex, age at recruitment (±3 years) and presence/absence of liver fluke eggs in the faeces at recruitment (detected by the formalin ethyl acetate concentration technique). This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Assessment of cigarette smoking and alcohol drinking

Definitions of alcohol use, cigarette smoking and levels of consumption of alcohol were the same as those previously described.[12] A smoker was defined as someone who had ever smoked any type of cigarette on a daily basis. However, in the present study smokers were categorized into four groups according to age when they began smoking. The 1st, 2nd and 3rd quartiles obtained from the control group frequency distribution for age of starting to smoke were used as the cut-offs for the four groups.

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Ever drinkers were defined as those who consumed at least one type of alcoholic beverage (beer, *Sato*, white whisky, red whisky and other whiskies) at least once a month; those drinking less than this were defined as non-drinkers. The consumption of each subject was calculated in terms of units of alcohol, where a unit corresponds to 10 ml (approximately 8 gm) of ethanol. The number of units of alcohol in a drink was determined by multiplying volume of the drink (in milliliters) by its alcohol percentage (beer 5.0%, *Sato* 7.0%, white whisky 40%, red whisky 35%) and dividing by 1000. Drinkers were dichotomized according to units of alcohol consumed per month on the basis of the median monthly consumption for the controls.

Laboratory methods

Specimen collection and DNA extraction

Blood samples (buffy coat) were available for 175 (80%) of the 219 eligible CCA cases; specimens were retrieved from the KKCS bio-bank for these cases and for the 350 matched controls. Genomic DNA was extracted from buffy coat fractions using the standard protocols of Genomic DNA Mini Kit with Proteinase K (Geneaid Biotech). Of those with DNA samples, genotyping was successfully carried out for 95% (499 out of 525) of all samples for *XRCC1* and for 90% (474 out of 525) of all samples for *OGG1*. Laboratory personnel were blinded to the case-control status of the available samples.

PCR amplification and genetic polymorphisms detection

The real-time polymerase chain reactions with high resolution melting analysis (Real-time PCR-HRM) technique of DNA amplification for the DNA base-excision repair genes (*XRCC1* and *OGG1*) polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System.

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The amplification of *XRCC1* G399A used two primers, [F]:5′-AGT GGG TGC TGG ACT GTC-3′ and [R]:5′-TTG CCC AGC ACA GGA TAA-3′, and was performed in a LightCycler® 480 Real-Time PCR System with a final volume of 20 µl containing 10 µl of master mix, 4.4 µl of H_2O, 3 mM of $MgCl_2$, 0.3 µM of each primer and 200 ng of the DNA template. The amplification of the *OGG1* C326G was performed in the same way, but the primers were [F]:5′-CCT ACA GGT GCT GTT CAG T-3′ and [R]:5′-CCT TTG GAA CCC TTT CTG-3′.

HRM data were analyzed using the LightCycler 480® Gene Scanning Software version 1.5 (Roche). Normalized and temperature-shifted melting curves carrying a sequence variation were evaluated and compared with the wild-type sample. Sequence variations were distinguished by different shape of melting curves (Figure 1(A) for *XRCC1* G399A and (B) for *OGG1* C326G). Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plots were distinguished by different melting peaks for each genotype (Figure 1(C) for *XRCC1* G399A and (D) for *OGG1* C326G). To improve the genotyping quality and validation, genotyping of 10% of random samples was confirmed by the PCR with restriction fragment length polymorphism techniques (PCR-RFLP).

Statistical analysis

To assess the strength of the associations between polymorphisms in DNA base-excision repair genes (*XRCC1* and *OGG1*) and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. A univariate analysis using McNemar's chi-square test and conditional logistic regression was carried out to explore the associations between DNA-repair genes polymorphisms, smoking and alcohol drinking, and the risk of CCA. Factors found to have a strong association with CCA in the univariate analysis (p

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<0.05), and factors without association in the univariate analysis, but found to play important roles as factors for CCA risk from literature reviews, were included in the multivariate analysis. Possible modifications of the effects of smoking and alcohol drinking by polymorphisms in *XRCC1* G399A and *OGG1* C326G were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

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RESULTS

There were 219 cohort members who had developed a primary CCA six or more months after enrollment into the KKCS. They comprised 92 females and 127 males, with a median age of 57 years (minimum 31, maximum 69). There were two controls matched by age and sex for each case. Table 1 shows the distribution of *XRCC1* G399A and *OGG1* C326G polymorphisms in cases and controls, the joint distribution of those polymorphisms, and the associated odds ratios. While there were no associations between any of these individual polymorphisms and the risk of CCA, the combinations of *XRCC1* GA heterozygous and *OGG1* CC wild-type or CG heterozygous, and of *XRCC1* GG wild-type and *OGG1* CG heterozygous, were significantly related to a reduced risk of CCA (*P*-value <0.001). Conversely, the combinations of the *OGG1* GG mutant, homozygous with all three genotypes of *XRCC1* (wild-type, heterozygous and mutant homozygous), were significantly associated with an increased risk of CCA (*P*-value = 0.01, 0.03 and 0.004, respectively), but there were no interactions between the polymorphisms of *XRCC1* G399A and *OGG1* C326G on CCA risk.

In the univariate analysis (Table 2), there were significant associations between cigarette smoking and alcohol consumption and the risk of CCA, and their joint effect appeared to be

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multiplicative with no interactions between them. There was trend in risk with smoking frequency or number of years of habitual smoking (*P*-value for trend = 0.002).

In the multivariate analysis (Table 3), shows the adjusted ORs and 95% CIs from the multivariate analysis. There was a clear association with alcohol drinking: compared with non-drinkers, individuals who consumed <14 units of alcohol per month had an increased risk of CCA (OR=5.2, 95% CI 2.74 to 9.95), and the risk was higher still in those who consumed ≥14 units of alcohol per month (OR=7.6, 95% CI 3.67 to 15.72).

Table 4 shows the results of gene-environment interaction of the *XRCC1* and *OGG1* polymorphisms along with smoking and alcohol drinking on the risk of CCA. When compared with the reference subjects (nonsmokers with the *XRCC1* GG wild-type), smokers carrying the *XRCC1* GG wild-type had an increased susceptibility to CCA (OR=2.8, 95% CI 1.30 to 5.91), and this was higher still in smokers with GA heterozygous (OR=3.4, 95% CI 1.60 to 7.28). Moreover, smokers with the *OGG1* CC wild-type and CG heterozygous had an increased risk of CCA compared with nonsmokers with the wild-type. With respect to alcohol consumption, some combinations of alcohol drinking and mutant polymorphisms of both *XRCC1* and *OGG1* were statistically significant. For example, compared with the reference group (nondrinkers with the *XRCC1* GG wild-type), subjects with the *XRCC1* GA heterozygous who were nondrinkers or drinkers (<14 units of alcohol per month) had an increased susceptibility to CCA (4.4-fold increased to 8.8-fold). Similarly, subjects with the *XRCC1* AA variant genotype had an increased risk of CCA (9.2-fold in nondrinkers increased to 10.3-fold in drinkers of <14 units of alcohol per month). Again, there were no interactions between *XRCC1* or *OGG1* polymorphisms and smoking or alcohol drinking.

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DISCUSSION

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS).[11, 12, 22] The design of this study minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. This design is very useful for examining the role of self-reported factors such as cigarette smoking and alcohol consumption, which are modified when cancer is present.

Smoking was not associated with CCA development in this present study (after adjustment for all other variables in the multivariate analysis) – polymorphisms in *XRCC1* and *OGG1*, cigarette smoking and units of alcohol per month of all alcohol consumption.

Alcohol consumption was significantly associated with an increased risk of CCA in previous studies in Thailand[18, 19] and elsewhere.[21] Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines[16, 19] and it is associated with production of free radical intermediate, which can cause several kinds of DNA lesions.[4, 15, 16] Reduced repair of these DNA lesions would therefore constitute a major risk factor for cancer development.[15, 16]

The genotype frequencies of *XRCC1* and *OGG1* polymorphisms found in the controls for the present study were consistent with those found in other studies in Thailand: the prevalence of G allele of *XRCC1* codon 399 was 68.88% in the present study compared with 36.59%–73.20% in other studies[10, 23-25] and A allele of *XRCC1* codon 399 was 31.12% in controls (prevalence of A allele was 26.80%–52.56%). In our analysis of the *OGG1* codon 326, the genotype distribution in the controls deviated from the Hardy–Weinberg equilibrium. Genotype frequency of *OGG1* C326G in controls was slightly higher than rates noted in reports from other studies in Thailand.[10, 24, 26] The C allele of *OGG1* codon 326 was 63.68% in controls (C allele prevalence was between 50.12%–55.00%) and G allele of *OGG1* codon 326 was 36.32% in controls (prevalence of G allele in *OGG1* codon 326 was between 45.00%–49.88%).

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XRCC1 and *OGG1* are major DNA repair genes involved in BER. Mutations and polymorphisms in DNA repair genes, which affect the efficiency of DNA damage repair, may increase an individual's susceptibility to several cancers[23-26] including cancers of the hepatobiliary tract.[5-7] However, no studies on this topic have been conducted in relation to CCA susceptibility and especially not in Thailand where the incidence of CCA is highest in the world.[17] However, there are four studies in a Thai population, which investigated relationships between the BER gene polymorphisms and other common cancers. Whereas *XRCC1* G399A polymorphisms tend to be associated with a reduced risk of oral squamous cell carcinoma,[23] they are associated with increased risks of breast[24] and cervical cancers,[25] and *OGG1* C326G polymorphisms tend be related to increased risks of breast[24] and lung cancers.[26]

Several studies have explored the associations between polymorphisms of the DNA repair genes involved in BER (*XRCC1* G399A and *OGG1* C326G) and the risk of various cancers, but they have shown inconsistent results. This may be the consequence of the different modifying effects, which these polymorphisms have on the balance during the DNA repair process. Imbalance in the tightly regulated process can be caused by SNPs and will result in deficient DNA repair and an increase in DNA breaks.[27] The *XRCC1* codon 399 A allele and the *OGG1* 326 G allele have been associated with a reduced capacity of the DNA repair process as detected by the persistence of DNA bulky adducts[28-30] and γ-irradiation induced oxidative DNA damage.[31] In addition, the balance may be modified by smoking- and alcohol-induced oxidative DNA base modifications interacting with the repair inefficiency of BER polymorphisms[4]; for example, the *XRCC1* GA variant heterozygous carriers, who were nondrinkers or drinkers of <14 units of alcohol per month, had an increased susceptibility to CCA (4.4-fold in nondrinkers increase to

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8.8-fold in drinkers) when compared with nondrinkers carrying the *XRCC1* GG wild-type (Table 4).

In conclusion, this study is the first report of possible associations between the main polymorphisms in BER genes (*XRCC1* G399A, *OGG1* C326G), the joint effects of both genes, and their modification of the effects of cigarette smoking and alcohol drinking on CCA risk in a Thai population. Our results suggest that polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, were associated with increased susceptibility to CCA. CCA is a multifaceted disease requiring a broad range of preventative actions. In the context of the present study, there is a need for more effective programmes for smoking cessation and reducing alcohol consumption, targeted especially at subgroups which are genetically at particular risk for CCA. Such an approach could also help in the prevention of other forms of cancer.

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Table 1 Univariate analysis of *XRCC1* and *OGG1* polymorphisms with the risk of cholangiocarcinoma in Khon Kaen, Thailand

Genetic polymorphisms		Cases		Controls		OR*	95% CI	*P*-value
		n	%	n	%			
***XRCC1* G399A†**								
GG		62	38.8	149	44.0	1.0		
GA		94	58.8	169	49.9	1.3	0.89 to 1.97	0.17
AA		4	2.5	21	6.2	0.5	0.16 to 1.42	0.18
GA or AA (any A allele)		98	61.3	190	56.1	1.2	0.82 to 1.78	0.33
***OGG1* C326G‡**								
CC		34	23.5	95	28.9	1.0		
CG		109	75.2	229	69.6	1.4	0.86 to 2.23	0.18
GG		2	1.4	5	1.5	1.2	0.22 to 7.03	0.80
CG or GG (any G allele)		111	76.6	234	71.1	1.4	0.86 to 2.22	0.18
Joint effects								
***XRCC1* G399A**	***OGG1* C326G**							
GG	CC	14	9.7	41	12.7	1.0		
GA	CC	19	13.1	47	14.5	0.3	0.15 to 0.55	<0.001
AA	CC	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
GG	CG	42	29.0	100	30.9	0.1	0.07 to 0.25	<0.001
GA	CG	56	38.6	108	33.3	0.1	0.07 to 0.22	<0.001
AA	CG	3	2.1	15	4.6	0.9	0.42 to 2.07	1.00
GG	GG	5	3.4	3	0.9	4.7	1.30 to 25.33	0.01
GA	GG	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
AA	GG	2	1.4	2	0.6	7.0	1.61 to 63.46	0.004
P-value for interaction = 0.21								

*Crude odds ratio from matched case-control analysis.

†DNA samples for XRCC1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

‡DNA samples for OGG1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 2 Univariate analysis of cigarette smoking and alcohol drinking on the risk of cholangiocarcinoma in Khon Kaen, Thailand

Variables	Cases		Controls		OR*	95% CI	*P*-value
	n	%	n	%			
Cigarette smoking							
Non-smoker	92	42.0	230	52.5	1.0		
Smoker	127	58.0	208	47.5	2.7	1.57 to 4.50	<0.001
Age at which smoking started of all cigarettes (years)							
Non-smoker	92	42.0	230	52.5	1.0		
<17	28	12.8	48	11.0	2.9	1.38 to 5.91	0.01
17–19	35	16.0	44	10.1	3.6	1.85 to 7.17	<0.001
20–21	40	18.3	70	16.0	2.5	1.33 to 4.62	0.004
22+	24	11.0	46	10.5	2.4	1.19 to 4.73	0.01
P-value for trend = 0.002							
Alcohol consumption							
No	57	26.0	254	58.0	1.0		
Yes	162	74.0	184	42.0	5.7	3.61 to 9.04	<0.001
Units of alcohol per month of all alcohol consumption							
Non-drinker	57	26.0	254	58.0	1.0		
<14	79	36.1	92	21.0	5.2	3.15 to 8.43	<0.001
≥14	83	37.9	92	21.0	6.7	3.90 to 11.38	<0.001
Alcohol consumption and smoking							
No alcohol, non-smoker	42	19.2	178	40.6	1.0		
No alcohol, smoker	15	6.9	76	17.4	2.2	0.93 to 5.00	0.07
Alcohol drinker, non-smoker	50	22.8	52	11.9	5.8	3.12 to 10.78	<0.001
Alcohol drinker, smoker	112	51.1	132	30.1	10.4	5.07 to 21.16	<0.001
P-value for trend <0.001							
P-value for interaction = 0.68							

*Crude odds ratio from matched case-control analysis.

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Table 3 Cases and controls, and odds ratios for cholangiocarcinoma associated with smoking, alcohol drinking, and *XRCC1* and *OGG1* polymorphisms

Variables	Cases		Controls		OR*	OR†	95% CI‡	*P*-value
	n	%	n	%				
***XRCC1* G399A polymorphisms§**								
GG	62	38.8	149	44.0	1.0	1.0		
GA	94	58.8	169	49.9	1.3	1.2	0.76 to 1.94	0.43
AA	4	2.5	21	6.2	0.5	0.4	0.10 to 1.62	0.20
***OGG1* C326G polymorphisms¶**								
CC	34	23.4	95	28.9	1.0	1.0		
CG	109	75.2	229	69.6	1.4	1.2	0.70 to 2.07	0.49
GG	2	1.4	5	1.5	1.2	0.7	0.10 to 5.11	0.75
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Smoker	127	58.0	208	47.5	2.7	1.9	0.94 to 4.01	0.07
Units of alcohol per month of all alcohol consumption								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.2	2.74 to 9.95	<0.001
≥14	83	37.9	92	21.0	6.7	7.6	3.67 to 15.72	<0.001

*Crude odds ratio from matched case–control analysis.

†Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).

‡95% confidence interval for OR†.

§DNA samples for *XRCC*1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

¶DNA samples for *OGG*1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 4 Gene-environment interactions of *XRCC1* and *OGG1* polymorphisms together with smoking and alcohol drinking on cholangiocarcinoma risk in Khon Kaen, Thailand

XRCC1	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value	*OGG1*	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value
G399A	**All cigarettes smoking**					*0.87*†	**C326G**	**All cigarettes smoking**					*0.54*†
GG	Non-smoker	22	71	1.0			CC	Non-smoker	14	55	1.0		
GA	Non-smoker	48	99	1.5	0.82 to 2.79	0.18	CG	Non-smoker	49	122	1.5	0.77 to 2.99	0.22
AA	Non-smoker	2	11	0.6	0.11 to 2.74	0.47	GG	Non-smoker	1	1	—	—	—
GG	Smoker	40	78	2.8	1.30 to 5.91	0.01	CC	Smoker	20	40	3.1	1.17 to 8.38	0.02
GA	Smoker	46	70	3.4	1.60 to 7.28	0.001	CG	Smoker	60	107	4.3	1.79 to 10.21	0.001
AA	Smoker	2	10	1.2	0.21 to 6.29	0.86	GG	Smoker	1	4	1.5	0.14 to 15.44	0.74
G399A	**Units of alcohol per month of all alcohol drinking**					*0.80*†	**C326G**	**Units of alcohol per month of all alcohol drinking**					*0.74*†
GG	Non-drinker	16	83	1.0			CC	Non-drinker	10	65	1.0		
GA	Non-drinker	20	33	4.4	1.81 to 10.83	0.001	CG	Non-drinker	9	9	8.1	2.30 to 28.36	0.001
AA	Non-drinker	26	33	9.2	3.65 to 23.21	<0.001	GG	Non-drinker	15	21	8.6	2.62 to 28.02	<0.001
GG	<14	22	102	1.1	0.49 to 2.28	0.89	CC	<14	24	130	1.3	0.55 to 3.20	0.52
GA	<14	39	35	8.8	3.76 to 20.71	<0.001	CG	<14	46	58	7.0	2.85 to 17.39	<0.001
AA	<14	33	32	10.3	4.14 to 25.80	<0.001	GG	<14	39	41	11.2	4.18 to 30.17	<0.001
GG	≥14	1	12	0.4	0.05 to 3.71	0.44	CC	≥14	0	1	—	—	—
GA	≥14	2	3	2.8	0.31 to 25.41	0.36	CG	≥14	1	1	7.7	0.29 to 26.73	0.22
AA	≥14	1	6	2.5	0.25 to 23.99	0.44	GG	≥14	1	3	6.4	0.48 to 86.72	0.16

*Crude odds ratio from matched case-control analysis.

†*P*-value for interaction.

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Contributors NS, SP. CP, TE and SW conceived and designed the research. NS and PC performed the research. NS and SP carried out the analyses. All authors contributed to the writing and revisions of the manuscript and approved the final version.

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Data sharing statement No additional data are available.

Ethics approval Ethical approval was obtained from the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Figure Legends

Figure 1 Polymorphisms in *XRCC1* G399A and *OGG1* C326G were analyzed using PCR with high resolution melting analysis.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 (Internet). Lyon, France: IARC; 2010. http://globocan.iarc.fr (accessed 27 Mar 2013).
2. Khuhaprema T, Attasara P, Sriplung H, *et al*. *Cancer in Thailand Volume VI, 2004-2006.* Bangkok: NCIB, 2012.
3. Parkin DM, Ohshima H, Srivatanakul P, *et al*. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev* 1993;2:537–44.
4. Stern MC, Conti DV, Siegmund KD, *et al.* DNA repair single-nucleotide polymorphisms in colorectal cancer and their role as modifiers of the effect of cigarette smoking and alcohol in the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2007;16:2363–72.
5. Huang WY, Gao YT, Rashid A, *et al.* Selected base excision repair gene polymorphisms and susceptibility to biliary tract cancer and biliary stones: a population-based case-control study in China. *Carcinogenesis* 2008;29:100–5.
6. Zhang M, Huang WY, Andreotti G, *et al.* Variants of DNA repair genes and the risk of biliary tract cancers and stones: a population-based study in China. *Cancer Epidemiol Biomarkers Prev* 2008;17:2123–7.
7. Srivastava A, Srivastava K, Pandey SN, *et al*. Single-nucleotide polymorphisms of DNA repair genes *OGG1* and *XRCC1*: association with gallbladder cancer in North Indian population. *Ann Surg Oncol* 2009;16:1695–703.

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8. Thanan R, Murata M, Pinlaor S, *et al.* Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2008;17:518–24.

9. Dechakhamphu S, Pinlaor S, Sitthithaworn P, *et al*. Lipid peroxidation and etheno DNA adducts in white blood cells of liver fluke-infected patients: protection by plasma alpha-tocopherol and praziquantel. *Cancer Epidemiol Biomarkers Prev* 2010;19:310–8.

10. Zeng L, You G, Tanaka H, *et al.* Combined effects of polymorphisms of DNA-repair protein genes and metabolic enzyme genes on the risk of cholangiocarcinoma. *Jpn J Clin Oncol* 2013;43: 1190–4.

11. Songserm N, Promthet S, Sithithaworn P, *et al. MTHFR* Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand. *Asian Pac J Cancer Prev* 2011;12:1341–5.

12. Songserm N, Promthet S, Sithithaworn P, *et al.* Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms. *Cancer Epidemiol* 2012;36:e89–94.

13. Kosti O, Byrne C, Meeker KL, *et al.* Mutagen sensitivity, tobacco smoking and breast cancer risk: a case-control study. *Carcinogenesis* 2010;31:654–9.

14. Rouissi K, Ouerhani S, Hamrita B, *et al.* Smoking and polymorphisms in xenobiotic metabolism and DNA repair genes are additive risk factors affecting bladder cancer in Northern Tunisia. *Pathol Oncol Res* 2011;17:879–86.

15. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009;674:36–44.

16. Pelucchi C, Tramacere I, Boffetta P, *et al*. Alcohol consumption and cancer risk. *Nutr Cancer* 2011;63:983–90.

For peer review only

17. Parkin DM, Srivatanakul P, Khlat M, *et al.* Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991;48:323–8.

18. Chernrungroj G. Risk factors for cholangiocarcinoma: a case-control study (dissertation). Connecticut: Yale University, 2000.

19. Honjo S, Srivatanakul P, Sriplung H, *et al.* Genetic and environmental determinants of risk for cholangiocarcinoma via *Opisthorchis viverrini* in a densely infested area in Nakhon Phanom, northeast Thailand. *Int J Cancer* 2005;117:854–60.

20. Poomphakwaen K, Promthet S, Kamsa-Ard S, *et al.* Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. *Asian Pac J Cancer Prev* 2009;10:251–8.

21. Chalasani N, Baluyut A, Ismail A, *et al.* Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000;31:7–11.

22. Sriamporn S, Parkin DM, Pisani P, *et al.* A prospective study of diet, lifestyle, and genetic factors and the risk of cancer in Khon Kaen Province, northeast Thailand: description of the cohort. *Asian Pac J Cancer Prev* 2005;6:295–303.

23. Kietthubthew S, Sriplung H, Au WW, *et al*. Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand. *Int J Hyg Environ Health* 2006;209:21–9.

24. Sangrajrang S, Schmezer P, Burkholder I, *et al.* Polymorphisms in three base excision repair genes and breast cancer risk in Thai women. *Breast Cancer Res Treat* 2008;111:279–88.

25. Settheetham-Ishida W, Yuenyao P, Natphopsuk S, *et al*. Genetic Risk of DNA Repair Gene Polymorphisms (*XRCC1* and *XRCC3*) for High Risk Human Papillomavirus Negative Cervical Cancer in Northeast Thailand. *Asian Pac J Cancer Prev* 2011;12:963–6.

26. Klinchid J, Chewaskulyoung B, Saeteng S, *et al*. Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women. *Cancer Genet Cytogenet* 2009;195:143–9.

For peer review only

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27. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutat Res* 2003;526:93–125.

28. Lunn RM, Langlois RG, Hsieh LL, *et al*. *XRCC1* polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999;59:2557–61.

29. Duell EJ, Millikan RC, Pittman GS, *et al.* Polymorphisms in the DNA repair gene *XRCC1* and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:217–22.

30. Matullo G, Guarrera S, Carturan S, *et al.* DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer* 2001;92:562–7.

31. Vodicka P, Stetina R, Polakova V, *et al.* Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis* 2007;28:657–64.

For peer review only

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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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ABSTRACT

Objective: Cholangiocarcinoma (CCA) is the most common malignancy in a Northeast Thai population. Smoking and alcohol drinking are associated with production of free radical intermediates, which can cause several types of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. We therefore examined that whether polymorphisms in DNA base-excision repair (BER) genes, *XRCC1* G399A and *OGG1* C326G, were associated with CCA risk and whether they modified the effect of smoking and alcohol drinking in Thai population.

Design: A nested case-control study within cohort study was conducted: 219 subjects with primary CCA were each matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of *Opisthorchis viverrini* eggs in stools. Smoking and alcohol consumption were assessed on recruitment. Polymorphisms in BER genes were analyzed using a polymerase chain reaction with high resolution melting analysis. The associations were assessed using conditional logistic regression.

Results: Our results suggest that in Thai population polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, are associated with increased susceptibility to CCA, and that their role as modifiers of the effect of smoking and alcohol consumption influences the risk of CCA.

Conclusions: Better ways of reducing habitual smoking and alcohol consumption, targeted toward subgroups which are genetically susceptible, are recommended. CCA is multi-factorial disease, and a comprehensive approach is needed for its effective prevention. This approach would also have the additional advantage of reducing the onset of other cancers.

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Strengths and limitations of this study

- This was a nested case-control study within the cohort study. This design has the advantage of minimising a systematic recall bias which can occur when the receipt of diagnosis of cancer leads to subjects modifying their self-reported personal histories, in this case, their recall of cigarette smoking and alcohol consumption. This was prevented in the present study because subjects were interviewed at the time of recruitment, before case or control status was defined.
- The diagnosis of cholangiocarcinoma was not histologically confirmed in all the cases.
- This study did not include the population aged 70 years and above.
- In future research, larger samples are needed to increase the power of the studies.

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INTRODUCTION

The data from GLOBOCAN 2008 shows that liver cancer is the fourth most common cause of death from cancer worldwide.[1] It is the most common cancer in Northeast Thailand, where the great majority of cases are cholangiocarcinomas (CCA).[2] Data from the Khon Kaen Cancer Registry (KKCR) have shown that the estimated age-standardised incidence rates for men and women are 84.6 and 36.8 per 100 000, respectively.[3]

Among several types of DNA damage induced by carcinogens from tobacco and alcohol are oxidative base damage and single-stand breaks, which are repaired by the base-excision repair pathway (BER).[4] Genetic variants and polymorphisms in DNA repair pathways have been reported to affect the levels of DNA lesions, which accumulate in the epithelial tissue of the hepatobiliary tract system, thus influencing the susceptibility to biliary tract cancers and stones in a Chinese population[5, 6] and gallbladder cancer in an Indian population.[7] There were very few studies on this topic in relation to CCA in Thai population, but there have been studies, which clarify the role of the accumulation of levels of oxidative DNA lesions in liver fluke-infected- and CCA patients[8-10]; these studies concluded that some biomarkers from oxidative DNA lesions induced by liver fluke infection – a major risk factor – may play an important role in CCA development. However, the results from our previous study showed that not only liver fluke infection but also certain lifestyle and dietary co-factors were associated with increased risk of CCA.[11, 12] Long-term smoking[13, 14] and alcohol drinking[4, 15, 16] are associated with production of free radical intermediates, which can cause several kinds of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. With respect to genetic factors, a number of polymorphisms in various DNA repair genes have been discovered, and it is possible that these polymorphisms may affect DNA repair capacity and thus modulate susceptibility to cancer. Investigations are therefore needed into the different patterns of

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3 smoking and alcohol drinking in relation to susceptibility to CCA, along with DNA repair
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5 capacity induced by genetic variations or polymorphisms.

Although cigarette smoking was not associated with CCA development in the previous studies conducted in Thailand[12, 17-20] and elsewhere,[21] a modifying effect by DNA repair genes remains a possibility. With respect to the use of alcohol, this was related to a substantially increased risk of CCA in our previous study.[12] At the molecular level, it is known that single-nucleotide polymorphisms (SNPs) in genes participate biochemically in the base-excision repair pathway (X-ray repair cross-complementing group 1: *XRCC1* and 8-oxoguanine DNA glycosylase 1: *OGG1*), which repairs smoking- and alcohol-induced oxidative DNA base modifications.[4]

In this present study, we therefore investigated whether polymorphisms in DNA base-excision repair genes (*XRCC1* G399A and *OGG1* C326G) are associated with CCA risk and whether their effects are modified by cigarette smoking and alcohol drinking in a Northeastern Thai population residing in a high-incidence area of opisthorchiasis-related CCA. The selection of these SNPs in DNA base-excision repair genes was based on their putative effect on protein function and/or previous evidence of associations with the risk of hepatobiliary tract cancers in Asian, Chinese and Indian populations[5-7]and the combined effects of a DNA-repair gene and metabolic gene polymorphisms on CCA risk in Thailand.[10] However, these studies involved only gene-gene interactions. There is need for studies of gene-environment interactions, especially between DNA-repair genes and exposure to the carcinogenic effects of tobacco/alcohol carcinogens in relation to CCA risk.

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MATERIALS AND METHODS

Study subjects

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS). Details of the KKCS and the selection of CCA cases and controls used in the present study have been previously reported.[11, 12, 22] Briefly, 219 cohort members who had developed a primary CCA, diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology, six or more months after enrollment were identified. The vital status and date of death of potential cases were ascertained by linkage to the files of deaths in Thailand, which are held in the database of the National Health Security Office and the demographic database of Ministry of Interior. All cases had died within two years of diagnosis. Two non-cancer controls from the same cohort population were randomly selected for matching with each case on sex, age at recruitment (±3 years) and presence/absence of liver fluke eggs in the faeces at recruitment (detected by the formalin ethyl acetate concentration technique). This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Assessment of cigarette smoking and alcohol drinking

Definitions of alcohol use, cigarette smoking and levels of consumption of alcohol were the same as those previously described.[12] A smoker was defined as someone who had ever smoked any type of cigarette on a daily basis. However, in the present study smokers were categorized into four groups according to age when they began smoking. The 1st, 2nd and 3rd quartiles obtained from the control group frequency distribution for age of starting to smoke were used as the cut-offs for the four groups.

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Ever drinkers were defined as those who consumed at least one type of alcoholic beverage (beer, *Sato*, white whisky, red whisky and other whiskies) at least once a month; those drinking less than this were defined as non-drinkers. The consumption of each subject was calculated in terms of units of alcohol, where a unit corresponds to 10 ml (approximately 8 gm) of ethanol. The number of units of alcohol in a drink was determined by multiplying volume of the drink (in milliliters) by its alcohol percentage (beer 5.0%, *Sato* 7.0%, white whisky 40%, red whisky 35%) and dividing by 1000. Drinkers were dichotomized according to units of alcohol consumed per month on the basis of the median monthly consumption for the controls.

Laboratory methods

Specimen collection and DNA extraction

Blood samples (buffy coat) were available for 175 (80%) of the 219 eligible CCA cases; specimens were retrieved from the KKCS bio-bank for these cases and for the 350 matched controls. Genomic DNA was extracted from buffy coat fractions using the standard protocols of Genomic DNA Mini Kit with Proteinase K (Geneaid Biotech). Of those with DNA samples, genotyping was successfully carried out for 95% (499 out of 525) of all samples for *XRCC1* and for 90% (474 out of 525) of all samples for *OGG1*. Laboratory personnel were blinded to the case-control status of the available samples.

PCR amplification and genetic polymorphisms detection

The real-time polymerase chain reactions with high resolution melting analysis (Real-time PCR-HRM) technique of DNA amplification for the DNA base-excision repair genes (*XRCC1* and *OGG1*) polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System.

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The amplification of *XRCC1* G399A used two primers, [F]:5′-AGT GGG TGC TGG ACT GTC-3′ and [R]:5′-TTG CCC AGC ACA GGA TAA-3′, and was performed in a LightCycler® 480 Real-Time PCR System with a final volume of 20 µl containing 10 µl of master mix, 4.4 µl of H_2O, 3 mM of $MgCl_2$, 0.3 µM of each primer and 200 ng of the DNA template. The amplification of the *OGG1* C326G was performed in the same way, but the primers were [F]:5′-CCT ACA GGT GCT GTT CAG T-3′ and [R]:5′-CCT TTG GAA CCC TTT CTG-3′.

HRM data were analyzed using the LightCycler 480® Gene Scanning Software version 1.5 (Roche). Normalized and temperature-shifted melting curves carrying a sequence variation were evaluated and compared with the wild-type sample. Sequence variations were distinguished by different shape of melting curves (Figure 1(A) for *XRCC1* G399A and (B) for *OGG1* C326G). Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plots were distinguished by different melting peaks for each genotype (Figure 1(C) for *XRCC1* G399A and (D) for *OGG1* C326G). To improve the genotyping quality and validation, genotyping of 10% of random samples was confirmed by the PCR with restriction fragment length polymorphism techniques (PCR-RFLP).

Statistical analysis

To assess the strength of the associations between polymorphisms in DNA base-excision repair genes (*XRCC1* and *OGG1*) and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. A univariate analysis using McNemar’s chi-square test and conditional logistic regression was carried out to explore the associations between DNA-repair genes polymorphisms, smoking and alcohol drinking, and the risk of CCA. Factors found to have a strong association with CCA in the univariate analysis (p

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<0.05), and factors without association in the univariate analysis, but found to play important roles as factors for CCA risk from literature reviews, were included in the multivariate analysis. Possible modifications of the effects of smoking and alcohol drinking by polymorphisms in *XRCC1* G399A and *OGG1* C326G were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

RESULTS

There were 219 cohort members who had developed a primary CCA six or more months after enrollment into the KKCS. They comprised 92 females and 127 males, with a median age of 57 years (minimum 31, maximum 69). There were two controls matched by age and sex for each case. Table 1 shows the distribution of *XRCC1* G399A and *OGG1* C326G polymorphisms in cases and controls, the joint distribution of those polymorphisms, and the associated odds ratios. While there were no associations between any of these individual polymorphisms and the risk of CCA, the combinations of *XRCC1* GA heterozygous and *OGG1* CC wild-type or CG heterozygous, and of *XRCC1* GG wild-type and *OGG1* CG heterozygous, were significantly related to a reduced risk of CCA (*P*-value <0.001). Conversely, the combinations of the *OGG1* GG mutant, homozygous with all three genotypes of *XRCC1* (wild-type, heterozygous and mutant homozygous), were significantly associated with an increased risk of CCA (*P*-value = 0.01, 0.03 and 0.004, respectively), but there were no interactions between the polymorphisms of *XRCC1* G399A and *OGG1* C326G on CCA risk.

In the univariate analysis (Table 2), there were significant associations between cigarette smoking and alcohol consumption and the risk of CCA, and their joint effect appeared to be

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multiplicative with no interactions between them. There was trend in risk with smoking frequency or number of years of habitual smoking (*P*-value for trend = 0.002).

In the multivariate analysis (Table 3), shows the adjusted ORs and 95% CIs from the multivariate analysis. There was a clear association with alcohol drinking: compared with non-drinkers, individuals who consumed <14 units of alcohol per month had an increased risk of CCA (OR=5.2, 95% CI 2.74 to 9.95), and the risk was higher still in those who consumed ≥14 units of alcohol per month (OR=7.6, 95% CI 3.67 to 15.72).

Table 4 shows the results of gene-environment interaction of the *XRCC1* and *OGG1* polymorphisms along with smoking and alcohol drinking on the risk of CCA. When compared with the reference subjects (nonsmokers with the *XRCC1* GG wild-type), smokers carrying the *XRCC1* GG wild-type had an increased susceptibility to CCA (OR=2.8, 95% CI 1.30 to 5.91), and this was higher still in smokers with GA heterozygous (OR=3.4, 95% CI 1.60 to 7.28). Moreover, smokers with the *OGG1* CC wild-type and CG heterozygous had an increased risk of CCA compared with nonsmokers with the wild-type. With respect to alcohol consumption, some combinations of alcohol drinking and mutant polymorphisms of both *XRCC1* and *OGG1* were statistically significant. For example, compared with the reference group (nondrinkers with the *XRCC1* GG wild-type), subjects with the *XRCC1* GA heterozygous who were nondrinkers or drinkers (<14 units of alcohol per month) had an increased susceptibility to CCA (4.4-fold increased to 8.8-fold). Similarly, subjects with the *XRCC1* AA variant genotype had an increased risk of CCA (9.2-fold in nondrinkers increased to 10.3-fold in drinkers of <14 units of alcohol per month). Again, there were no interactions between *XRCC1* or *OGG1* polymorphisms and smoking or alcohol drinking.

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DISCUSSION

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS).[11, 12, 22] The design of this study minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. This design is very useful for examining the role of self-reported factors such as cigarette smoking and alcohol consumption, which are modified when cancer is present.

Smoking was not associated with CCA development in this present study (after adjustment for all other variables in the multivariate analysis) – polymorphisms in *XRCC1* and *OGG1*, cigarette smoking and units of alcohol per month of all alcohol consumption.

Alcohol consumption was significantly associated with an increased risk of CCA in previous studies in Thailand[18, 19] and elsewhere.[21] Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines[16, 19] and it is associated with production of free radical intermediate, which can cause several kinds of DNA lesions.[4, 15, 16] Reduced repair of these DNA lesions would therefore constitute a major risk factor for cancer development.[15, 16]

The genotype frequencies of *XRCC1* and *OGG1* polymorphisms found in the controls for the present study were consistent with those found in other studies in Thailand: the prevalence of G allele of *XRCC1* codon 399 was 68.88% in the present study compared with 36.59%–73.20% in other studies[10, 23-25] and A allele of *XRCC1* codon 399 was 31.12% in controls (prevalence of A allele was 26.80%–52.56%). In our analysis of the *OGG1* codon 326, the genotype distribution in the controls deviated from the Hardy–Weinberg equilibrium. Genotype frequency of *OGG1* C326G in controls was slightly higher than rates noted in reports from other studies in Thailand.[10, 24, 26] The C allele of *OGG1* codon 326 was 63.68% in controls (C allele prevalence was between 50.12%–55.00%) and G allele of *OGG1* codon 326 was 36.32% in controls (prevalence of G allele in *OGG1* codon 326 was between 45.00%–49.88%).

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XRCC1 and *OGG1* are major DNA repair genes involved in BER. Mutations and polymorphisms in DNA repair genes, which affect the efficiency of DNA damage repair, may increase an individual's susceptibility to several cancers[23-26] including cancers of the hepatobiliary tract.[5-7] However, no studies on this topic have been conducted in relation to CCA susceptibility and especially not in Thailand where the incidence of CCA is highest in the world.[17] However, there are four studies in a Thai population, which investigated relationships between the BER gene polymorphisms and other common cancers. Whereas *XRCC1* G399A polymorphisms tend to be associated with a reduced risk of oral squamous cell carcinoma,[23] they are associated with increased risks of breast[24] and cervical cancers,[25] and *OGG1* C326G polymorphisms tend be related to increased risks of breast[24] and lung cancers.[26]

Several studies have explored the associations between polymorphisms of the DNA repair genes involved in BER (*XRCC1* G399A and *OGG1* C326G) and the risk of various cancers, but they have shown inconsistent results. This may be the consequence of the different modifying effects, which these polymorphisms have on the balance during the DNA repair process. Imbalance in the tightly regulated process can be caused by SNPs and will result in deficient DNA repair and an increase in DNA breaks.[27] The *XRCC1* codon 399 A allele and the *OGG1* 326 G allele have been associated with a reduced capacity of the DNA repair process as detected by the persistence of DNA bulky adducts[28-30] and γ-irradiation induced oxidative DNA damage.[31] In addition, the balance may be modified by smoking- and alcohol-induced oxidative DNA base modifications interacting with the repair inefficiency of BER polymorphisms[4]; for example, the *XRCC1* GA variant heterozygous carriers, who were nondrinkers or drinkers of <14 units of alcohol per month, had an increased susceptibility to CCA (4.4-fold in nondrinkers increase to

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8.8-fold in drinkers) when compared with nondrinkers carrying the *XRCC1* GG wild-type (Table 4).

In conclusion, this study is the first report of possible associations between the main polymorphisms in BER genes (*XRCC1* G399A, *OGG1* C326G), the joint effects of both genes, and their modification of the effects of cigarette smoking and alcohol drinking on CCA risk in a Thai population. Our results suggest that polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, were associated with increased susceptibility to CCA. CCA is a multifaceted disease requiring a broad range of preventative actions. In the context of the present study, there is a need for more effective programmes for smoking cessation and reducing alcohol consumption, targeted especially at subgroups which are genetically at particular risk for CCA. Such an approach could also help in the prevention of other forms of cancer.

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Table 1 Univariate analysis of *XRCC1* and *OGG1* polymorphisms with the risk of cholangiocarcinoma in Khon Kaen, Thailand

Genetic polymorphisms		Cases		Controls		OR*	95% CI	*P*-value
		n	%	n	%			
***XRCC1* G399A†**								
GG		62	38.8	149	44.0	1.0		
GA		94	58.8	169	49.9	1.3	0.89 to 1.97	0.17
AA		4	2.5	21	6.2	0.5	0.16 to 1.42	0.18
GA or AA (any A allele)		98	61.3	190	56.1	1.2	0.82 to 1.78	0.33
***OGG1* C326G‡**								
CC		34	23.5	95	28.9	1.0		
CG		109	75.2	229	69.6	1.4	0.86 to 2.23	0.18
GG		2	1.4	5	1.5	1.2	0.22 to 7.03	0.80
CG or GG (any G allele)		111	76.6	234	71.1	1.4	0.86 to 2.22	0.18
Joint effects								
***XRCC1* G399A**	***OGG1* C326G**							
GG	CC	14	9.7	41	12.7	1.0		
GA	CC	19	13.1	47	14.5	0.3	0.15 to 0.55	<0.001
AA	CC	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
GG	CG	42	29.0	100	30.9	0.1	0.07 to 0.25	<0.001
GA	CG	56	38.6	108	33.3	0.1	0.07 to 0.22	<0.001
AA	CG	3	2.1	15	4.6	0.9	0.42 to 2.07	1.00
GG	GG	5	3.4	3	0.9	4.7	1.30 to 25.33	0.01
GA	GG	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
AA	GG	2	1.4	2	0.6	7.0	1.61 to 63.46	0.004
P-value for interaction = 0.21								

*Crude odds ratio from matched case-control analysis.

†DNA samples for XRCC1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

‡DNA samples for OGG1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 2 Univariate analysis of cigarette smoking and alcohol drinking on the risk of cholangiocarcinoma in Khon Kaen, Thailand

Variables	Cases		Controls		OR*	95% CI	*P*-value
	n	%	n	%			
Cigarette smoking							
Non-smoker	92	42.0	230	52.5	1.0		
Smoker	127	58.0	208	47.5	2.7	1.57 to 4.50	<0.001
Age at which smoking started of all cigarettes (years)							
Non-smoker	92	42.0	230	52.5	1.0		
<17	28	12.8	48	11.0	2.9	1.38 to 5.91	0.01
17–19	35	16.0	44	10.1	3.6	1.85 to 7.17	<0.001
20–21	40	18.3	70	16.0	2.5	1.33 to 4.62	0.004
22+	24	11.0	46	10.5	2.4	1.19 to 4.73	0.01
P-value for trend = 0.002							
Alcohol consumption							
No	57	26.0	254	58.0	1.0		
Yes	162	74.0	184	42.0	5.7	3.61 to 9.04	<0.001
Units of alcohol per month of all alcohol consumption							
Non-drinker	57	26.0	254	58.0	1.0		
<14	79	36.1	92	21.0	5.2	3.15 to 8.43	<0.001
≥14	83	37.9	92	21.0	6.7	3.90 to 11.38	<0.001
Alcohol consumption and smoking							
No alcohol, non-smoker	42	19.2	178	40.6	1.0		
No alcohol, smoker	15	6.9	76	17.4	2.2	0.93 to 5.00	0.07
Alcohol drinker, non-smoker	50	22.8	52	11.9	5.8	3.12 to 10.78	<0.001
Alcohol drinker, smoker	112	51.1	132	30.1	10.4	5.07 to 21.16	<0.001
P-value for trend <0.001							
P-value for interaction = 0.68							

*Crude odds ratio from matched case-control analysis.

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Table 3 Cases and controls, and odds ratios for cholangiocarcinoma associated with smoking, alcohol drinking, and *XRCC1* and *OGG1* polymorphisms

Variables	Cases		Controls		OR*	OR†	95% CI‡	*P*-value
	n	%	n	%				
***XRCC1* G399A polymorphisms§**								
GG	62	38.8	149	44.0	1.0	1.0		
GA	94	58.8	169	49.9	1.3	1.2	0.76 to 1.94	0.43
AA	4	2.5	21	6.2	0.5	0.4	0.10 to 1.62	0.20
***OGG1* C326G polymorphisms¶**								
CC	34	23.4	95	28.9	1.0	1.0		
CG	109	75.2	229	69.6	1.4	1.2	0.70 to 2.07	0.49
GG	2	1.4	5	1.5	1.2	0.7	0.10 to 5.11	0.75
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Smoker	127	58.0	208	47.5	2.7	1.9	0.94 to 4.01	0.07
Units of alcohol per month of all alcohol consumption								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.2	2.74 to 9.95	<0.001
≥14	83	37.9	92	21.0	6.7	7.6	3.67 to 15.72	<0.001

*Crude odds ratio from matched case–control analysis.

†Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).

‡95% confidence interval for OR†.

§DNA samples for *XRCC*1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

¶DNA samples for *OGG*1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 4 Gene-environment interactions of *XRCC1* and *OGG1* polymorphisms together with smoking and alcohol drinking on cholangiocarcinoma risk in Khon Kaen, Thailand

XRCC1	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value	*OGG1*	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value
G399A	**All cigarettes smoking**					*0.87*†	**C326G**	**All cigarettes smoking**					*0.54*†
GG	Non-smoker	22	71	1.0			CC	Non-smoker	14	55	1.0		
GA	Non-smoker	48	99	1.5	0.82 to 2.79	0.18	CG	Non-smoker	49	122	1.5	0.77 to 2.99	0.22
AA	Non-smoker	2	11	0.6	0.11 to 2.74	0.47	GG	Non-smoker	1	1	–	–	–
GG	Smoker	40	78	2.8	1.30 to 5.91	0.01	CC	Smoker	20	40	3.1	1.17 to 8.38	0.02
GA	Smoker	46	70	3.4	1.60 to 7.28	0.001	CG	Smoker	60	107	4.3	1.79 to 10.21	0.001
AA	Smoker	2	10	1.2	0.21 to 6.29	0.86	GG	Smoker	1	4	1.5	0.14 to 15.44	0.74
G399A	**Units of alcohol per month of all alcohol drinking**					*0.80*†	**C326G**	**Units of alcohol per month of all alcohol drinking**					*0.74*†
GG	Non-drinker	16	83	1.0			CC	Non-drinker	10	65	1.0		
GA	Non-drinker	20	33	4.4	1.81 to 10.83	0.001	CG	Non-drinker	9	9	8.1	2.30 to 28.36	0.001
AA	Non-drinker	26	33	9.2	3.65 to 23.21	<0.001	GG	Non-drinker	15	21	8.6	2.62 to 28.02	<0.001
GG	<14	22	102	1.1	0.49 to 2.28	0.89	CC	<14	24	130	1.3	0.55 to 3.20	0.52
GA	<14	39	35	8.8	3.76 to 20.71	<0.001	CG	<14	46	58	7.0	2.85 to 17.39	<0.001
AA	<14	33	32	10.3	4.14 to 25.80	<0.001	GG	<14	39	41	11.2	4.18 to 30.17	<0.001
GG	≥14	1	12	0.4	0.05 to 3.71	0.44	CC	≥14	0	1	–	–	–
GA	≥14	2	3	2.8	0.31 to 25.41	0.36	CG	≥14	1	1	7.7	0.29 to 26.73	0.22
AA	≥14	1	6	2.5	0.25 to 23.99	0.44	GG	≥14	1	3	6.4	0.48 to 86.72	0.16

*Crude odds ratio from matched case-control analysis.

†*P*-value for interaction.

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Contributors NS, SP. CP, TE and SW conceived and designed the research. NS and PC performed the research. NS and SP carried out the analyses. All authors contributed to the writing and revisions of the manuscript and approved the final version.

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Data sharing statement No additional data are available.

Ethics approval Ethical approval was obtained from the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

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Figure Legends

Figure 1 Polymorphisms in *XRCC1* G399A and *OGG1* C326G were analyzed using PCR with high resolution melting analysis.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 (Internet). Lyon, France: IARC; 2010. http://globocan.iarc.fr (accessed 27 Mar 2013).
2. Khuhaprema T, Attasara P, Sriplung H, *et al*. *Cancer in Thailand Volume VI, 2004-2006.* Bangkok: NCIB, 2012.
3. Parkin DM, Ohshima H, Srivatanakul P, *et al*. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev* 1993;2:537–44.
4. Stern MC, Conti DV, Siegmund KD, *et al.* DNA repair single-nucleotide polymorphisms in colorectal cancer and their role as modifiers of the effect of cigarette smoking and alcohol in the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2007;16:2363–72.
5. Huang WY, Gao YT, Rashid A, *et al.* Selected base excision repair gene polymorphisms and susceptibility to biliary tract cancer and biliary stones: a population-based case-control study in China. *Carcinogenesis* 2008;29:100–5.
6. Zhang M, Huang WY, Andreotti G, *et al.* Variants of DNA repair genes and the risk of biliary tract cancers and stones: a population-based study in China. *Cancer Epidemiol Biomarkers Prev* 2008;17:2123–7.
7. Srivastava A, Srivastava K, Pandey SN, *et al*. Single-nucleotide polymorphisms of DNA repair genes *OGG1* and *XRCC1*: association with gallbladder cancer in North Indian population. *Ann Surg Oncol* 2009;16:1695–703.

For peer review only

8. Thanan R, Murata M, Pinlaor S, *et al.* Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2008;17:518–24.
9. Dechakhamphu S, Pinlaor S, Sitthithaworn P, *et al*. Lipid peroxidation and etheno DNA adducts in white blood cells of liver fluke-infected patients: protection by plasma alpha-tocopherol and praziquantel. *Cancer Epidemiol Biomarkers Prev* 2010;19:310–8.
10. Zeng L, You G, Tanaka H, *et al.* Combined effects of polymorphisms of DNA-repair protein genes and metabolic enzyme genes on the risk of cholangiocarcinoma. *Jpn J Clin Oncol* 2013;43: 1190–4.
11. Songserm N, Promthet S, Sithithaworn P, *et al. MTHFR* Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand. *Asian Pac J Cancer Prev* 2011;12:1341–5.
12. Songserm N, Promthet S, Sithithaworn P, *et al.* Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms. *Cancer Epidemiol* 2012;36:e89–94.
13. Kosti O, Byrne C, Meeker KL, *et al.* Mutagen sensitivity, tobacco smoking and breast cancer risk: a case-control study. *Carcinogenesis* 2010;31:654–9.
14. Rouissi K, Ouerhani S, Hamrita B, *et al.* Smoking and polymorphisms in xenobiotic metabolism and DNA repair genes are additive risk factors affecting bladder cancer in Northern Tunisia. *Pathol Oncol Res* 2011;17:879–86.
15. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009;674:36–44.
16. Pelucchi C, Tramacere I, Boffetta P, *et al*. Alcohol consumption and cancer risk. *Nutr Cancer* 2011;63:983–90.

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17. Parkin DM, Srivatanakul P, Khlat M, *et al.* Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991;48:323–8.
18. Chernrungroj G. Risk factors for cholangiocarcinoma: a case-control study (dissertation). Connecticut: Yale University, 2000.
19. Honjo S, Srivatanakul P, Sriplung H, *et al.* Genetic and environmental determinants of risk for cholangiocarcinoma via *Opisthorchis viverrini* in a densely infested area in Nakhon Phanom, northeast Thailand. *Int J Cancer* 2005;117:854–60.
20. Poomphakwaen K, Promthet S, Kamsa-Ard S, *et al.* Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. *Asian Pac J Cancer Prev* 2009;10:251–8.
21. Chalasani N, Baluyut A, Ismail A, *et al.* Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000;31:7–11.
22. Sriamporn S, Parkin DM, Pisani P, *et al.* A prospective study of diet, lifestyle, and genetic factors and the risk of cancer in Khon Kaen Province, northeast Thailand: description of the cohort. *Asian Pac J Cancer Prev* 2005;6:295–303.
23. Kietthubthew S, Sriplung H, Au WW, *et al*. Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand. *Int J Hyg Environ Health* 2006;209:21–9.
24. Sangrajrang S, Schmezer P, Burkholder I, *et al.* Polymorphisms in three base excision repair genes and breast cancer risk in Thai women. *Breast Cancer Res Treat* 2008;111:279–88.
25. Settheetham-Ishida W, Yuenyao P, Natphopsuk S, *et al*. Genetic Risk of DNA Repair Gene Polymorphisms (*XRCC1* and *XRCC3*) for High Risk Human Papillomavirus Negative Cervical Cancer in Northeast Thailand. *Asian Pac J Cancer Prev* 2011;12:963–6.
26. Klinchid J, Chewaskulyoung B, Saeteng S, *et al*. Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women. *Cancer Genet Cytogenet* 2009;195:143–9.

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27. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutat Res* 2003;526:93–125.

28. Lunn RM, Langlois RG, Hsieh LL, *et al*. *XRCC1* polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999;59:2557–61.

29. Duell EJ, Millikan RC, Pittman GS, *et al.* Polymorphisms in the DNA repair gene *XRCC1* and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:217–22.

30. Matullo G, Guarrera S, Carturan S, *et al.* DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer* 2001;92:562–7.

31. Vodicka P, Stetina R, Polakova V, *et al.* Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis* 2007;28:657–64.

For peer review only

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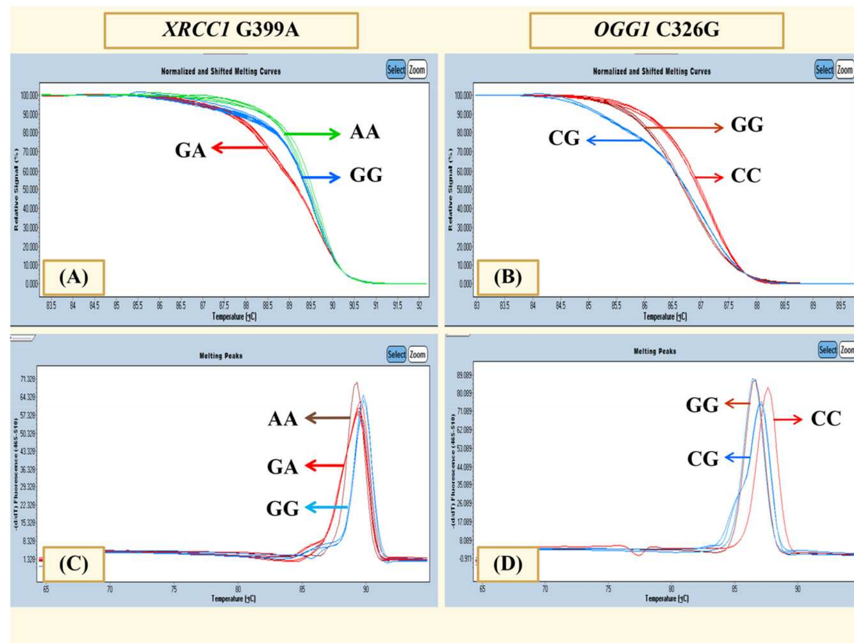

Figure 1 Polymorphisms in XRCC1 G399A and OGG1 C326G were analyzed using PCR with high resolution melting analysis.
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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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ABSTRACT

Objective: Cholangiocarcinoma (CCA) is the most common malignancy in a Northeast Thai population. Smoking and alcohol drinking are associated with production of free radical intermediates, which can cause several types of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. We therefore examined that whether polymorphisms in DNA base-excision repair (BER) genes, *XRCC1* G399A and *OGG1* C326G, were associated with CCA risk and whether they modified the effect of smoking and alcohol drinking in Thai population.

Design: A nested case-control study within cohort study was conducted: 219 subjects with primary CCA were each matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of *Opisthorchis viverrini* eggs in stools. Smoking and alcohol consumption were assessed on recruitment. Polymorphisms in BER genes were analyzed using a polymerase chain reaction with high resolution melting analysis. The associations were assessed using conditional logistic regression.

Results: Our results suggest that in Thai population polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, are associated with increased susceptibility to CCA, and that their role as modifiers of the effect of smoking and alcohol consumption influences the risk of CCA.

Conclusions: Better ways of reducing habitual smoking and alcohol consumption, targeted toward subgroups which are genetically susceptible, are recommended. CCA is multi-factorial disease, and a comprehensive approach is needed for its effective prevention. This approach would also have the additional advantage of reducing the onset of other cancers.

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Strengths and limitations of this study

- This was a nested case-control study within the cohort study. This design has the advantage of minimising a systematic recall bias which can occur when the receipt of diagnosis of cancer leads to subjects modifying their self-reported personal histories, in this case, their recall of cigarette smoking and alcohol consumption. This was prevented in the present study because subjects were interviewed at the time of recruitment, before case or control status was defined.
- The diagnosis of cholangiocarcinoma was not histologically confirmed in all the cases.
- This study did not include the population aged 70 years and above.
- In future research, larger samples are needed to increase the power of the studies.

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INTRODUCTION

The data from GLOBOCAN 2008 shows that liver cancer is the fourth most common cause of death from cancer worldwide.[1] It is the most common cancer in Northeast Thailand, where the great majority of cases are cholangiocarcinomas (CCA).[2] Data from the Khon Kaen Cancer Registry (KKCR) have shown that the estimated age-standardised incidence rates for men and women are 84.6 and 36.8 per 100 000, respectively.[3]

Among several types of DNA damage induced by carcinogens from tobacco and alcohol are oxidative base damage and single-stand breaks, which are repaired by the base-excision repair pathway (BER).[4] Genetic variants and polymorphisms in DNA repair pathways have been reported to affect the levels of DNA lesions, which accumulate in the epithelial tissue of the hepatobiliary tract system, thus influencing the susceptibility to biliary tract cancers and stones in a Chinese population[5, 6] and gallbladder cancer in an Indian population.[7] There were very few studies on this topic in relation to CCA in Thai population, but there have been studies, which clarify the role of the accumulation of levels of oxidative DNA lesions in liver fluke-infected- and CCA patients[8-10]; these studies concluded that some biomarkers from oxidative DNA lesions induced by liver fluke infection – a major risk factor – may play an important role in CCA development. However, the results from our previous study showed that not only liver fluke infection but also certain lifestyle and dietary co-factors were associated with increased risk of CCA.[11, 12] Long-term smoking[13, 14] and alcohol drinking[4, 15, 16] are associated with production of free radical intermediates, which can cause several kinds of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. With respect to genetic factors, a number of polymorphisms in various DNA repair genes have been discovered, and it is possible that these polymorphisms may affect DNA repair capacity and thus modulate susceptibility to cancer. Investigations are therefore needed into the different patterns of

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smoking and alcohol drinking in relation to susceptibility to CCA, along with DNA repair capacity induced by genetic variations or polymorphisms.

Although cigarette smoking was not associated with CCA development in the previous studies conducted in Thailand[12, 17-20] and elsewhere,[21] a modifying effect by DNA repair genes remains a possibility. With respect to the use of alcohol, this was related to a substantially increased risk of CCA in our previous study.[12] At the molecular level, it is known that single-nucleotide polymorphisms (SNPs) in genes participate biochemically in the base-excision repair pathway (X-ray repair cross-complementing group 1: *XRCC1* and 8-oxoguanine DNA glycosylase 1: *OGG1*), which repairs smoking- and alcohol-induced oxidative DNA base modifications.[4]

In this present study, we therefore investigated whether polymorphisms in DNA base-excision repair genes (*XRCC1* G399A and *OGG1* C326G) are associated with CCA risk and whether their effects are modified by cigarette smoking and alcohol drinking in a Northeastern Thai population residing in a high-incidence area of opisthorchiasis-related CCA. The selection of these SNPs in DNA base-excision repair genes was based on their putative effect on protein function and/or previous evidence of associations with the risk of hepatobiliary tract cancers in Asian, Chinese and Indian populations[5-7]and the combined effects of a DNA-repair gene and metabolic gene polymorphisms on CCA risk in Thailand.[10] However, these studies involved only gene-gene interactions. There is need for studies of gene-environment interactions, especially between DNA-repair genes and exposure to the carcinogenic effects of tobacco/alcohol carcinogens in relation to CCA risk.

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MATERIALS AND METHODS

Study subjects

This was a case-control study nested within the 23 584 subjects of the Khon Kaen Cohort Study (KKCS). Details of the KKCS and the selection of CCA cases and controls used in the present study have been previously reported.[11, 12, 22] Briefly, 219 (0.9%) cohort members who had developed a primary CCA, diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology, six or more months after enrollment were identified. The vital status and date of death of potential cases were ascertained by linkage to the files of deaths in Thailand, which are held in the database of the National Health Security Office and the demographic database of Ministry of Interior. All cases had died within two years of diagnosis. Two non-cancer controls from the same cohort population were randomly selected for matching with each case on sex, age at recruitment (±3 years) and presence/absence of liver fluke eggs in the faeces at recruitment (detected by the formalin ethyl acetate concentration technique). This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Assessment of cigarette smoking and alcohol drinking

Definitions of alcohol use, cigarette smoking and levels of consumption of alcohol were the same as those previously described.[12] A smoker was defined as someone who had ever smoked any type of cigarette on a daily basis. However, in the present study smokers were categorized into four groups according to age when they began smoking. The 1st, 2nd and 3rd quartiles obtained from the control group frequency distribution for age of starting to smoke were used as the cut-offs for the four groups.

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Ever drinkers were defined as those who consumed at least one type of alcoholic beverage (beer, *Sato*, white whisky, red whisky and other whiskies) at least once a month; those drinking less than this were defined as non-drinkers. The consumption of each subject was calculated in terms of units of alcohol, where a unit corresponds to 10 ml (approximately 8 gm) of ethanol. The number of units of alcohol in a drink was determined by multiplying volume of the drink (in milliliters) by its alcohol percentage (beer 5.0%, *Sato* 7.0%, white whisky 40%, red whisky 35%) and dividing by 1000. Drinkers were dichotomized according to units of alcohol consumed per month on the basis of the median monthly consumption for the controls.

Laboratory methods

Specimen collection and DNA extraction

Blood samples (buffy coat) were available for 175 (80%) of the 219 eligible CCA cases; specimens were retrieved from the KKCS bio-bank for these cases and for the 350 matched controls. Genomic DNA was extracted from buffy coat fractions using the standard protocols of Genomic DNA Mini Kit with Proteinase K (Geneaid Biotech). Of those with DNA samples, genotyping was successfully carried out for 95% (499 out of 525) of all samples for *XRCC1* and for 90% (474 out of 525) of all samples for *OGG1*. Laboratory personnel were blinded to the case-control status of the available samples.

PCR amplification and genetic polymorphisms detection

The real-time polymerase chain reactions with high resolution melting analysis (Real-time PCR-HRM) technique of DNA amplification for the DNA base-excision repair genes (*XRCC1* and *OGG1*) polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System.

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The amplification of *XRCC1* G399A used two primers, [F]:5′-AGT GGG TGC TGG ACT GTC-3′ and [R]:5′-TTG CCC AGC ACA GGA TAA-3′, and was performed in a LightCycler® 480 Real-Time PCR System with a final volume of 20 µl containing 10 µl of master mix, 4.4 µl of H_2O, 3 mM of $MgCl_2$, 0.3 µM of each primer and 200 ng of the DNA template. The amplification of the *OGG1* C326G was performed in the same way, but the primers were [F]:5′-CCT ACA GGT GCT GTT CAG T-3′ and [R]:5′-CCT TTG GAA CCC TTT CTG-3′.

HRM data were analyzed using the LightCycler 480® Gene Scanning Software version 1.5 (Roche). Normalized and temperature-shifted melting curves carrying a sequence variation were evaluated and compared with the wild-type sample. Sequence variations were distinguished by different shape of melting curves (Figure 1(A) for *XRCC1* G399A and (B) for *OGG1* C326G). Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plots were distinguished by different melting peaks for each genotype (Figure 1(C) for *XRCC1* G399A and (D) for *OGG1* C326G). To improve the genotyping quality and validation, genotyping of 10% of random samples was confirmed by the PCR with restriction fragment length polymorphism techniques (PCR-RFLP).

Statistical analysis

To assess the strength of the associations between polymorphisms in DNA base-excision repair genes (*XRCC1* and *OGG1*) and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. A univariate analysis using McNemar's chi-square test and conditional logistic regression was carried out to explore the associations between DNA-repair genes polymorphisms, smoking and alcohol drinking, and the risk of CCA. Factors found to have a strong association with CCA in the univariate analysis (p

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<0.05), and factors without association in the univariate analysis, but found to play important roles as factors for CCA risk from literature reviews, were included in the multivariate analysis. Possible modifications of the effects of smoking and alcohol drinking by polymorphisms in *XRCC1* G399A and *OGG1* C326G were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

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RESULTS

There were 219 cohort members who had developed a primary CCA six or more months after enrollment into the KKCS. They comprised 92 females and 127 males, with a median age of 57 years (minimum 31, maximum 69). There were two controls matched by age and sex for each case. Table 1 shows the distribution of *XRCC1* G399A and *OGG1* C326G polymorphisms in cases and controls, the joint distribution of those polymorphisms, and the associated odds ratios. While there were no associations between any of these individual polymorphisms and the risk of CCA, the combinations of *XRCC1* GA heterozygous and *OGG1* CC wild-type or CG heterozygous, and of *XRCC1* GG wild-type and *OGG1* CG heterozygous, were significantly related to a reduced risk of CCA (*P*-value <0.001). Conversely, the combinations of the *OGG1* GG mutant, homozygous with all three genotypes of *XRCC1* (wild-type, heterozygous and mutant homozygous), were significantly associated with an increased risk of CCA (*P*-value = 0.01, 0.03 and 0.004, respectively), but there were no interactions between the polymorphisms of *XRCC1* G399A and *OGG1* C326G on CCA risk.

In the univariate analysis (Table 2), there were significant associations between cigarette smoking and alcohol consumption and the risk of CCA, and their joint effect appeared to be

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multiplicative with no interactions between them. There was trend in risk with smoking frequency or number of years of habitual smoking (*P*-value for trend = 0.002).

In the multivariate analysis (Table 3), shows the adjusted ORs and 95% CIs from the multivariate analysis. There was a clear association with alcohol drinking: compared with non-drinkers, individuals who consumed <14 units of alcohol per month had an increased risk of CCA (OR=5.2, 95% CI 2.74 to 9.95), and the risk was higher still in those who consumed ≥14 units of alcohol per month (OR=7.6, 95% CI 3.67 to 15.72).

Table 4 shows the results of gene-environment interaction of the *XRCC1* and *OGG1* polymorphisms along with smoking and alcohol drinking on the risk of CCA. When compared with the reference subjects (nonsmokers with the *XRCC1* GG wild-type), smokers carrying the *XRCC1* GG wild-type had an increased susceptibility to CCA (OR=2.8, 95% CI 1.30 to 5.91), and this was higher still in smokers with GA heterozygous (OR=3.4, 95% CI 1.60 to 7.28). Moreover, smokers with the *OGG1* CC wild-type and CG heterozygous had an increased risk of CCA compared with nonsmokers with the wild-type. With respect to alcohol consumption, some combinations of alcohol drinking and mutant polymorphisms of both *XRCC1* and *OGG1* were statistically significant. For example, compared with the reference group (nondrinkers with the *XRCC1* GG wild-type), subjects with the *XRCC1* GA heterozygous who were nondrinkers or drinkers (<14 units of alcohol per month) had an increased susceptibility to CCA (4.4-fold increased to 8.8-fold). Similarly, subjects with the *XRCC1* AA variant genotype had an increased risk of CCA (9.2-fold in nondrinkers increased to 10.3-fold in drinkers of <14 units of alcohol per month). Again, there were no interactions between *XRCC1* or *OGG1* polymorphisms and smoking or alcohol drinking.

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DISCUSSION

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS).[11, 12, 22] The design of this study minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. This design is very useful for examining the role of self-reported factors such as cigarette smoking and alcohol consumption, which are modified when cancer is present.

Smoking was not associated with CCA development in this present study (after adjustment for all other variables in the multivariate analysis) – polymorphisms in *XRCC1* and *OGG1*, cigarette smoking and units of alcohol per month of all alcohol consumption.

Alcohol consumption was significantly associated with an increased risk of CCA in previous studies in Thailand[18, 19] and elsewhere.[21] Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines[16, 19] and it is associated with production of free radical intermediate, which can cause several kinds of DNA lesions.[4, 15, 16] Reduced repair of these DNA lesions would therefore constitute a major risk factor for cancer development.[15, 16]

The genotype frequencies of *XRCC1* and *OGG1* polymorphisms found in the controls for the present study were consistent with those found in other studies in Thailand: the prevalence of G allele of *XRCC1* codon 399 was 68.88% in the present study compared with 36.59%–73.20% in other studies[10, 23-25] and A allele of *XRCC1* codon 399 was 31.12% in controls (prevalence of A allele was 26.80%–52.56%). In our analysis of the *OGG1* codon 326, the genotype distribution in the controls deviated from the Hardy–Weinberg equilibrium. Genotype frequency of *OGG1* C326G in controls was slightly higher than rates noted in reports from other studies in Thailand.[10, 24, 26] The C allele of *OGG1* codon 326 was 63.68% in controls (C allele prevalence was between 50.12%–55.00%) and G allele of *OGG1* codon 326 was 36.32% in controls (prevalence of G allele in *OGG1* codon 326 was between 45.00%–49.88%).

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XRCC1 and *OGG1* are major DNA repair genes involved in BER. Mutations and polymorphisms in DNA repair genes, which affect the efficiency of DNA damage repair, may increase an individual's susceptibility to several cancers[23-26] including cancers of the hepatobiliary tract.[5-7] However, no studies on this topic have been conducted in relation to CCA susceptibility and especially not in Thailand where the incidence of CCA is highest in the world.[17] However, there are four studies in a Thai population, which investigated relationships between the BER gene polymorphisms and other common cancers. Whereas *XRCC1* G399A polymorphisms tend to be associated with a reduced risk of oral squamous cell carcinoma,[23] they are associated with increased risks of breast[24] and cervical cancers,[25] and *OGG1* C326G polymorphisms tend be related to increased risks of breast[24] and lung cancers.[26]

Several studies have explored the associations between polymorphisms of the DNA repair genes involved in BER (*XRCC1* G399A and *OGG1* C326G) and the risk of various cancers, but they have shown inconsistent results. This may be the consequence of the different modifying effects, which these polymorphisms have on the balance during the DNA repair process. Imbalance in the tightly regulated process can be caused by SNPs and will result in deficient DNA repair and an increase in DNA breaks.[27] The *XRCC1* codon 399 A allele and the *OGG1* 326 G allele have been associated with a reduced capacity of the DNA repair process as detected by the persistence of DNA bulky adducts[28-30] and γ-irradiation induced oxidative DNA damage.[31] In addition, the balance may be modified by smoking- and alcohol-induced oxidative DNA base modifications interacting with the repair inefficiency of BER polymorphisms[4]; for example, the *XRCC1* GA variant heterozygous carriers, who were nondrinkers or drinkers of <14 units of alcohol per month, had an increased susceptibility to CCA (4.4-fold in nondrinkers increase to

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8.8-fold in drinkers) when compared with nondrinkers carrying the *XRCC1* GG wild-type (Table 4).

In conclusion, this study is the first report of possible associations between the main polymorphisms in BER genes (*XRCC1* G399A, *OGG1* C326G), the joint effects of both genes, and their modification of the effects of cigarette smoking and alcohol drinking on CCA risk in a Thai population. Our results suggest that polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, were associated with increased susceptibility to CCA. CCA is a multifaceted disease requiring a broad range of preventative actions. In the context of the present study, there is a need for more effective programmes for smoking cessation and reducing alcohol consumption, targeted especially at subgroups which are genetically at particular risk for CCA. Such an approach could also help in the prevention of other forms of cancer.

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Table 1 Univariate analysis of *XRCC1* and *OGG1* polymorphisms with the risk of cholangiocarcinoma in Khon Kaen, Thailand

Genetic polymorphisms		Cases		Controls		OR*	95% CI	*P*-value
		n	%	n	%			
***XRCC1* G399A†**								
GG		62	38.8	149	44.0	1.0		
GA		94	58.8	169	49.9	1.3	0.89 to 1.97	0.17
AA		4	2.5	21	6.2	0.5	0.16 to 1.42	0.18
GA or AA (any A allele)		98	61.3	190	56.1	1.2	0.82 to 1.78	0.33
***OGG1* C326G‡**								
CC		34	23.5	95	28.9	1.0		
CG		109	75.2	229	69.6	1.4	0.86 to 2.23	0.18
GG		2	1.4	5	1.5	1.2	0.22 to 7.03	0.80
CG or GG (any G allele)		111	76.6	234	71.1	1.4	0.86 to 2.22	0.18
Joint effects								
***XRCC1* G399A**	***OGG1* C326G**							
GG	CC	14	9.7	41	12.7	1.0		
GA	CC	19	13.1	47	14.5	0.3	0.15 to 0.55	<0.001
AA	CC	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
GG	CG	42	29.0	100	30.9	0.1	0.07 to 0.25	<0.001
GA	CG	56	38.6	108	33.3	0.1	0.07 to 0.22	<0.001
AA	CG	3	2.1	15	4.6	0.9	0.42 to 2.07	1.00
GG	GG	5	3.4	3	0.9	4.7	1.30 to 25.33	0.01
GA	GG	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
AA	GG	2	1.4	2	0.6	7.0	1.61 to 63.46	0.004
P-value for interaction = 0.21								

*Crude odds ratio from matched case-control analysis.

†DNA samples for XRCC1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

‡DNA samples for OGG1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 2 Univariate analysis of cigarette smoking and alcohol drinking on the risk of cholangiocarcinoma in Khon Kaen, Thailand

Variables	Cases		Controls		OR*	95% CI	*P*-value
	n	%	n	%			
Cigarette smoking							
Non-smoker	92	42.0	230	52.5	1.0		
Smoker	127	58.0	208	47.5	2.7	1.57 to 4.50	<0.001
Age at which smoking started of all cigarettes (years)							
Non-smoker	92	42.0	230	52.5	1.0		
<17	28	12.8	48	11.0	2.9	1.38 to 5.91	0.01
17–19	35	16.0	44	10.1	3.6	1.85 to 7.17	<0.001
20–21	40	18.3	70	16.0	2.5	1.33 to 4.62	0.004
22+	24	11.0	46	10.5	2.4	1.19 to 4.73	0.01
P-value for trend = 0.002							
Alcohol consumption							
No	57	26.0	254	58.0	1.0		
Yes	162	74.0	184	42.0	5.7	3.61 to 9.04	<0.001
Units of alcohol per month of all alcohol consumption							
Non-drinker	57	26.0	254	58.0	1.0		
<14	79	36.1	92	21.0	5.2	3.15 to 8.43	<0.001
≥14	83	37.9	92	21.0	6.7	3.90 to 11.38	<0.001
Alcohol consumption and smoking							
No alcohol, non-smoker	42	19.2	178	40.6	1.0		
No alcohol, smoker	15	6.9	76	17.4	2.2	0.93 to 5.00	0.07
Alcohol drinker, non-smoker	50	22.8	52	11.9	5.8	3.12 to 10.78	<0.001
Alcohol drinker, smoker	112	51.1	132	30.1	10.4	5.07 to 21.16	<0.001
P-value for trend <0.001							
P-value for interaction = 0.68							

*Crude odds ratio from matched case-control analysis.

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Table 3 Cases and controls, and odds ratios for cholangiocarcinoma associated with smoking, alcohol drinking, and *XRCC1* and *OGG1* polymorphisms

Variables	Cases		Controls		OR*	OR†	95% CI‡	*P*-value
	n	%	n	%				
***XRCC1* G399A polymorphisms§**								
GG	62	38.8	149	44.0	1.0	1.0		
GA	94	58.8	169	49.9	1.3	1.2	0.76 to 1.94	0.43
AA	4	2.5	21	6.2	0.5	0.4	0.10 to 1.62	0.20
***OGG1* C326G polymorphisms¶**								
CC	34	23.4	95	28.9	1.0	1.0		
CG	109	75.2	229	69.6	1.4	1.2	0.70 to 2.07	0.49
GG	2	1.4	5	1.5	1.2	0.7	0.10 to 5.11	0.75
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Smoker	127	58.0	208	47.5	2.7	1.9	0.94 to 4.01	0.07
Units of alcohol per month of all alcohol consumption								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.2	2.74 to 9.95	<0.001
≥14	83	37.9	92	21.0	6.7	7.6	3.67 to 15.72	<0.001

*Crude odds ratio from matched case–control analysis.

†Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).

‡95% confidence interval for OR†.

§DNA samples for *XRCC*1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

¶DNA samples for *OGG*1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 4 Gene-environment interactions of *XRCC1* and *OGG1* polymorphisms together with smoking and alcohol drinking on cholangiocarcinoma risk in Khon Kaen, Thailand

XRCC1	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value	*OGG1*	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value
G399A	**All cigarettes smoking**					*0.87*†	**C326G**	**All cigarettes smoking**					*0.54*†
GG	Non-smoker	22	71	1.0			CC	Non-smoker	14	55	1.0		
GA	Non-smoker	48	99	1.5	0.82 to 2.79	0.18	CG	Non-smoker	49	122	1.5	0.77 to 2.99	0.22
AA	Non-smoker	2	11	0.6	0.11 to 2.74	0.47	GG	Non-smoker	1	1	—	—	—
GG	Smoker	40	78	2.8	1.30 to 5.91	0.01	CC	Smoker	20	40	3.1	1.17 to 8.38	0.02
GA	Smoker	46	70	3.4	1.60 to 7.28	0.001	CG	Smoker	60	107	4.3	1.79 to 10.21	0.001
AA	Smoker	2	10	1.2	0.21 to 6.29	0.86	GG	Smoker	1	4	1.5	0.14 to 15.44	0.74
G399A	**Units of alcohol per month of all alcohol drinking**					*0.80*†	**C326G**	**Units of alcohol per month of all alcohol drinking**					*0.74*†
GG	Non-drinker	16	83	1.0			CC	Non-drinker	10	65	1.0		
GA	Non-drinker	20	33	4.4	1.81 to 10.83	0.001	CG	Non-drinker	9	9	8.1	2.30 to 28.36	0.001
AA	Non-drinker	26	33	9.2	3.65 to 23.21	<0.001	GG	Non-drinker	15	21	8.6	2.62 to 28.02	<0.001
GG	<14	22	102	1.1	0.49 to 2.28	0.89	CC	<14	24	130	1.3	0.55 to 3.20	0.52
GA	<14	39	35	8.8	3.76 to 20.71	<0.001	CG	<14	46	58	7.0	2.85 to 17.39	<0.001
AA	<14	33	32	10.3	4.14 to 25.80	<0.001	GG	<14	39	41	11.2	4.18 to 30.17	<0.001
GG	≥14	1	12	0.4	0.05 to 3.71	0.44	CC	≥14	0	1	—	—	—
GA	≥14	2	3	2.8	0.31 to 25.41	0.36	CG	≥14	1	1	7.7	0.29 to 26.73	0.22
AA	≥14	1	6	2.5	0.25 to 23.99	0.44	GG	≥14	1	3	6.4	0.48 to 86.72	0.16

*Crude odds ratio from matched case-control analysis.

†*P*-value for interaction.

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Figure Legends

Figure 1 Polymorphisms in *XRCC1* G399A and *OGG1* C326G were analyzed using PCR with high resolution melting analysis.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 (Internet). Lyon, France: IARC; 2010. http://globocan.iarc.fr (accessed 27 Mar 2013).
2. Khuhaprema T, Attasara P, Sriplung H, *et al*. *Cancer in Thailand Volume VI, 2004-2006.* Bangkok: NCIB, 2012.
3. Parkin DM, Ohshima H, Srivatanakul P, *et al*. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev* 1993;2:537–44.
4. Stern MC, Conti DV, Siegmund KD, *et al.* DNA repair single-nucleotide polymorphisms in colorectal cancer and their role as modifiers of the effect of cigarette smoking and alcohol in the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2007;16:2363–72.
5. Huang WY, Gao YT, Rashid A, *et al.* Selected base excision repair gene polymorphisms and susceptibility to biliary tract cancer and biliary stones: a population-based case-control study in China. *Carcinogenesis* 2008;29:100–5.
6. Zhang M, Huang WY, Andreotti G, *et al.* Variants of DNA repair genes and the risk of biliary tract cancers and stones: a population-based study in China. *Cancer Epidemiol Biomarkers Prev* 2008;17:2123–7.
7. Srivastava A, Srivastava K, Pandey SN, *et al*. Single-nucleotide polymorphisms of DNA repair genes *OGG1* and *XRCC1*: association with gallbladder cancer in North Indian population. *Ann Surg Oncol* 2009;16:1695–703.

For peer review only

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8. Thanan R, Murata M, Pinlaor S, *et al.* Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2008;17:518–24.

9. Dechakhamphu S, Pinlaor S, Sitthithaworn P, *et al*. Lipid peroxidation and etheno DNA adducts in white blood cells of liver fluke-infected patients: protection by plasma alpha-tocopherol and praziquantel. *Cancer Epidemiol Biomarkers Prev* 2010;19:310–8.

10. Zeng L, You G, Tanaka H, *et al.* Combined effects of polymorphisms of DNA-repair protein genes and metabolic enzyme genes on the risk of cholangiocarcinoma. *Jpn J Clin Oncol* 2013;43: 1190–4.

11. Songserm N, Promthet S, Sithithaworn P, *et al. MTHFR* Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand. *Asian Pac J Cancer Prev* 2011;12:1341–5.

12. Songserm N, Promthet S, Sithithaworn P, *et al.* Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms. *Cancer Epidemiol* 2012;36:e89–94.

13. Kosti O, Byrne C, Meeker KL, *et al.* Mutagen sensitivity, tobacco smoking and breast cancer risk: a case-control study. *Carcinogenesis* 2010;31:654–9.

14. Rouissi K, Ouerhani S, Hamrita B, *et al.* Smoking and polymorphisms in xenobiotic metabolism and DNA repair genes are additive risk factors affecting bladder cancer in Northern Tunisia. *Pathol Oncol Res* 2011;17:879–86.

15. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009;674:36–44.

16. Pelucchi C, Tramacere I, Boffetta P, *et al*. Alcohol consumption and cancer risk. *Nutr Cancer* 2011;63:983–90.

For peer review only

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17. Parkin DM, Srivatanakul P, Khlat M, *et al.* Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991;48:323–8.
18. Chernrungroj G. Risk factors for cholangiocarcinoma: a case-control study (dissertation). Connecticut: Yale University, 2000.
19. Honjo S, Srivatanakul P, Sriplung H, *et al.* Genetic and environmental determinants of risk for cholangiocarcinoma via *Opisthorchis viverrini* in a densely infested area in Nakhon Phanom, northeast Thailand. *Int J Cancer* 2005;117:854–60.
20. Poomphakwaen K, Promthet S, Kamsa-Ard S, *et al.* Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. *Asian Pac J Cancer Prev* 2009;10:251–8.
21. Chalasani N, Baluyut A, Ismail A, *et al.* Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000;31:7–11.
22. Sriamporn S, Parkin DM, Pisani P, *et al.* A prospective study of diet, lifestyle, and genetic factors and the risk of cancer in Khon Kaen Province, northeast Thailand: description of the cohort. *Asian Pac J Cancer Prev* 2005;6:295–303.
23. Kietthubthew S, Sriplung H, Au WW, *et al*. Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand. *Int J Hyg Environ Health* 2006;209:21–9.
24. Sangrajrang S, Schmezer P, Burkholder I, *et al.* Polymorphisms in three base excision repair genes and breast cancer risk in Thai women. *Breast Cancer Res Treat* 2008;111:279–88.
25. Settheetham-Ishida W, Yuenyao P, Natphopsuk S, *et al*. Genetic Risk of DNA Repair Gene Polymorphisms (*XRCC1* and *XRCC3*) for High Risk Human Papillomavirus Negative Cervical Cancer in Northeast Thailand. *Asian Pac J Cancer Prev* 2011;12:963–6.
26. Klinchid J, Chewaskulyoung B, Saeteng S, *et al*. Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women. *Cancer Genet Cytogenet* 2009;195:143–9.

For peer review only

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27. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutat Res* 2003;526:93–125.

28. Lunn RM, Langlois RG, Hsieh LL, *et al*. *XRCC1* polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999;59:2557–61.

29. Duell EJ, Millikan RC, Pittman GS, *et al.* Polymorphisms in the DNA repair gene *XRCC1* and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:217–22.

30. Matullo G, Guarrera S, Carturan S, *et al.* DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer* 2001;92:562–7.

31. Vodicka P, Stetina R, Polakova V, *et al.* Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis* 2007;28:657–64.

For peer review only

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Gene-environment interaction involved in cholangiocarcinoma in Thai population: polymorphisms of DNA repair genes, smoking and use of alcohol

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ABSTRACT

Objective: Cholangiocarcinoma (CCA) is the most common malignancy in a Northeast Thai population. Smoking and alcohol drinking are associated with production of free radical intermediates, which can cause several types of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. We therefore examined that whether polymorphisms in DNA base-excision repair (BER) genes, *XRCC1* G399A and *OGG1* C326G, were associated with CCA risk and whether they modified the effect of smoking and alcohol drinking in Thai population.

Design: A nested case-control study within cohort study was conducted: 219 subjects with primary CCA were each matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of *Opisthorchis viverrini* eggs in stools. Smoking and alcohol consumption were assessed on recruitment. Polymorphisms in BER genes were analyzed using a polymerase chain reaction with high resolution melting analysis. The associations were assessed using conditional logistic regression.

Results: Our results suggest that in Thai population polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, are associated with increased susceptibility to CCA, and that their role as modifiers of the effect of smoking and alcohol consumption influences the risk of CCA.

Conclusions: Better ways of reducing habitual smoking and alcohol consumption, targeted toward subgroups which are genetically susceptible, are recommended. CCA is multi-factorial disease, and a comprehensive approach is needed for its effective prevention. This approach would also have the additional advantage of reducing the onset of other cancers.

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Strengths and limitations of this study

- This was a nested case-control study within the cohort study. This design has the advantage of minimising a systematic recall bias which can occur when the receipt of diagnosis of cancer leads to subjects modifying their self-reported personal histories, in this case, their recall of cigarette smoking and alcohol consumption. This was prevented in the present study because subjects were interviewed at the time of recruitment, before case or control status was defined.
- The diagnosis of cholangiocarcinoma was not histologically confirmed in all the cases.
- This study did not include the population aged 70 years and above.
- In future research, larger samples are needed to increase the power of the studies.

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INTRODUCTION

The data from GLOBOCAN 2008 shows that liver cancer is the fourth most common cause of death from cancer worldwide.[1] It is the most common cancer in Northeast Thailand, where the great majority of cases are cholangiocarcinomas (CCA).[2] Data from the Khon Kaen Cancer Registry (KKCR) have shown that the estimated age-standardised incidence rates for men and women are 84.6 and 36.8 per 100 000, respectively.[3]

Among several types of DNA damage induced by carcinogens from tobacco and alcohol are oxidative base damage and single-stand breaks, which are repaired by the base-excision repair pathway (BER).[4] Genetic variants and polymorphisms in DNA repair pathways have been reported to affect the levels of DNA lesions, which accumulate in the epithelial tissue of the hepatobiliary tract system, thus influencing the susceptibility to biliary tract cancers and stones in a Chinese population[5, 6] and gallbladder cancer in an Indian population.[7] There were very few studies on this topic in relation to CCA in Thai population, but there have been studies, which clarify the role of the accumulation of levels of oxidative DNA lesions in liver fluke-infected- and CCA patients[8-10]; these studies concluded that some biomarkers from oxidative DNA lesions induced by liver fluke infection – a major risk factor – may play an important role in CCA development. However, the results from our previous study showed that not only liver fluke infection but also certain lifestyle and dietary co-factors were associated with increased risk of CCA.[11, 12] Long-term smoking[13, 14] and alcohol drinking[4, 15, 16] are associated with production of free radical intermediates, which can cause several kinds of DNA lesions. Reduced repair of these DNA lesions would constitute an important risk factor for cancer development. With respect to genetic factors, a number of polymorphisms in various DNA repair genes have been discovered, and it is possible that these polymorphisms may affect DNA repair capacity and thus modulate susceptibility to cancer. Investigations are therefore needed into the different patterns of

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smoking and alcohol drinking in relation to susceptibility to CCA, along with DNA repair capacity induced by genetic variations or polymorphisms.

Although cigarette smoking was not associated with CCA development in the previous studies conducted in Thailand[12, 17-20] and elsewhere,[21] a modifying effect by DNA repair genes remains a possibility. With respect to the use of alcohol, this was related to a substantially increased risk of CCA in our previous study.[12] At the molecular level, it is known that single-nucleotide polymorphisms (SNPs) in genes participate biochemically in the base-excision repair pathway (X-ray repair cross-complementing group 1: *XRCC1* and 8-oxoguanine DNA glycosylase 1: *OGG1*), which repairs smoking- and alcohol-induced oxidative DNA base modifications.[4]

In this present study, we therefore investigated whether polymorphisms in DNA base-excision repair genes (*XRCC1* G399A and *OGG1* C326G) are associated with CCA risk and whether their effects are modified by cigarette smoking and alcohol drinking in a Northeastern Thai population residing in a high-incidence area of opisthorchiasis-related CCA. The selection of these SNPs in DNA base-excision repair genes was based on their putative effect on protein function and/or previous evidence of associations with the risk of hepatobiliary tract cancers in Asian, Chinese and Indian populations[5-7]and the combined effects of a DNA-repair gene and metabolic gene polymorphisms on CCA risk in Thailand.[10] However, these studies involved only gene-gene interactions. There is need for studies of gene-environment interactions, especially between DNA-repair genes and exposure to the carcinogenic effects of tobacco/alcohol carcinogens in relation to CCA risk.

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MATERIALS AND METHODS

Study subjects

This was a case-control study nested within the 23 584 subjects of the Khon Kaen Cohort Study (KKCS). Details of the KKCS and the selection of CCA cases and controls used in the present study have been previously reported.[11, 12, 22] Briefly, 219 (0.9%) cohort members who had developed a primary CCA, diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology, six or more months after enrollment were identified. The vital status and date of death of potential cases were ascertained by linkage to the files of deaths in Thailand, which are held in the database of the National Health Security Office and the demographic database of Ministry of Interior. All cases had died within two years of diagnosis. Two non-cancer controls from the same cohort population were randomly selected for matching with each case on sex, age at recruitment (±3 years) and presence/absence of liver fluke eggs in the faeces at recruitment (detected by the formalin ethyl acetate concentration technique). This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

Assessment of cigarette smoking and alcohol drinking

Definitions of alcohol use, cigarette smoking and levels of consumption of alcohol were the same as those previously described.[12] A smoker was defined as someone who had ever smoked any type of cigarette on a daily basis. However, in the present study smokers were categorized into four groups according to age when they began smoking. The 1st, 2nd and 3rd quartiles obtained from the control group frequency distribution for age of starting to smoke were used as the cut-offs for the four groups.

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Ever drinkers were defined as those who consumed at least one type of alcoholic beverage (beer, *Sato*, white whisky, red whisky and other whiskies) at least once a month; those drinking less than this were defined as non-drinkers. The consumption of each subject was calculated in terms of units of alcohol, where a unit corresponds to 10 ml (approximately 8 gm) of ethanol. The number of units of alcohol in a drink was determined by multiplying volume of the drink (in milliliters) by its alcohol percentage (beer 5.0%, *Sato* 7.0%, white whisky 40%, red whisky 35%) and dividing by 1000. Drinkers were dichotomized according to units of alcohol consumed per month on the basis of the median monthly consumption for the controls.

Laboratory methods

Specimen collection and DNA extraction

Blood samples (buffy coat) were available for 175 (80%) of the 219 eligible CCA cases; specimens were retrieved from the KKCS bio-bank for these cases and for the 350 matched controls. Genomic DNA was extracted from buffy coat fractions using the standard protocols of Genomic DNA Mini Kit with Proteinase K (Geneaid Biotech). Of those with DNA samples, genotyping was successfully carried out for 95% (499 out of 525) of all samples for *XRCC1* and for 90% (474 out of 525) of all samples for *OGG1*. Laboratory personnel were blinded to the case-control status of the available samples.

PCR amplification and genetic polymorphisms detection

The real-time polymerase chain reactions with high resolution melting analysis (Real-time PCR-HRM) technique of DNA amplification for the DNA base-excision repair genes (*XRCC1* and *OGG1*) polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System.

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The amplification of *XRCC1* G399A used two primers, [F]:5′-AGT GGG TGC TGG ACT GTC-3′ and [R]:5′-TTG CCC AGC ACA GGA TAA-3′, and was performed in a LightCycler® 480 Real-Time PCR System with a final volume of 20 µl containing 10 µl of master mix, 4.4 µl of H_2O, 3 mM of $MgCl_2$, 0.3 µM of each primer and 200 ng of the DNA template. The amplification of the *OGG1* C326G was performed in the same way, but the primers were [F]:5′-CCT ACA GGT GCT GTT CAG T-3′ and [R]:5′-CCT TTG GAA CCC TTT CTG-3′.

HRM data were analyzed using the LightCycler 480® Gene Scanning Software version 1.5 (Roche). Normalized and temperature-shifted melting curves carrying a sequence variation were evaluated and compared with the wild-type sample. Sequence variations were distinguished by different shape of melting curves (Figure 1(A) for *XRCC1* G399A and (B) for *OGG1* C326G). Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plots were distinguished by different melting peaks for each genotype (Figure 1(C) for *XRCC1* G399A and (D) for *OGG1* C326G). To improve the genotyping quality and validation, genotyping of 10% of random samples was confirmed by the PCR with restriction fragment length polymorphism techniques (PCR-RFLP).

Statistical analysis

To assess the strength of the associations between polymorphisms in DNA base-excision repair genes (*XRCC1* and *OGG1*) and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. A univariate analysis using McNemar's chi-square test and conditional logistic regression was carried out to explore the associations between DNA-repair genes polymorphisms, smoking and alcohol drinking, and the risk of CCA. Factors found to have a strong association with CCA in the univariate analysis (p

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3 <0.05), and factors without association in the univariate analysis, but found to play important roles as factors for CCA risk from literature reviews, were included in the multivariate analysis. Possible modifications of the effects of smoking and alcohol drinking by polymorphisms in *XRCC1* G399A and *OGG1* C326G were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

RESULTS

There were 219 cohort members who had developed a primary CCA six or more months after enrollment into the KKCS. They comprised 92 females and 127 males, with a median age of 57 years (minimum 31, maximum 69). There were two controls matched by age and sex for each case. Table 1 shows the distribution of *XRCC1* G399A and *OGG1* C326G polymorphisms in cases and controls, the joint distribution of those polymorphisms, and the associated odds ratios. While there were no associations between any of these individual polymorphisms and the risk of CCA, the combinations of *XRCC1* GA heterozygous and *OGG1* CC wild-type or CG heterozygous, and of *XRCC1* GG wild-type and *OGG1* CG heterozygous, were significantly related to a reduced risk of CCA (*P*-value <0.001). Conversely, the combinations of the *OGG1* GG mutant, homozygous with all three genotypes of *XRCC1* (wild-type, heterozygous and mutant homozygous), were significantly associated with an increased risk of CCA (*P*-value = 0.01, 0.03 and 0.004, respectively), but there were no interactions between the polymorphisms of *XRCC1* G399A and *OGG1* C326G on CCA risk.

In the univariate analysis (Table 2), there were significant associations between cigarette smoking and alcohol consumption and the risk of CCA, and their joint effect appeared to be

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multiplicative with no interactions between them. There was trend in risk with smoking frequency or number of years of habitual smoking (*P*-value for trend = 0.002).

In the multivariate analysis (Table 3), shows the adjusted ORs and 95% CIs from the multivariate analysis. There was a clear association with alcohol drinking: compared with non-drinkers, individuals who consumed <14 units of alcohol per month had an increased risk of CCA (OR=5.2, 95% CI 2.74 to 9.95), and the risk was higher still in those who consumed ≥14 units of alcohol per month (OR=7.6, 95% CI 3.67 to 15.72).

Table 4 shows the results of gene-environment interaction of the *XRCC1* and *OGG1* polymorphisms along with smoking and alcohol drinking on the risk of CCA. When compared with the reference subjects (nonsmokers with the *XRCC1* GG wild-type), smokers carrying the *XRCC1* GG wild-type had an increased susceptibility to CCA (OR=2.8, 95% CI 1.30 to 5.91), and this was higher still in smokers with GA heterozygous (OR=3.4, 95% CI 1.60 to 7.28). Moreover, smokers with the *OGG1* CC wild-type and CG heterozygous had an increased risk of CCA compared with nonsmokers with the wild-type. With respect to alcohol consumption, some combinations of alcohol drinking and mutant polymorphisms of both *XRCC1* and *OGG1* were statistically significant. For example, compared with the reference group (nondrinkers with the *XRCC1* GG wild-type), subjects with the *XRCC1* GA heterozygous who were nondrinkers or drinkers (<14 units of alcohol per month) had an increased susceptibility to CCA (4.4-fold increased to 8.8-fold). Similarly, subjects with the *XRCC1* AA variant genotype had an increased risk of CCA (9.2-fold in nondrinkers increased to 10.3-fold in drinkers of <14 units of alcohol per month). Again, there were no interactions between *XRCC1* or *OGG1* polymorphisms and smoking or alcohol drinking.

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DISCUSSION

This was a case-control study nested within the 23 584 Khon Kaen Cohort Study (KKCS).[11, 12, 22] The design of this study minimises recall bias of cigarette smoking and alcohol drinking, as subjects were interviewed at the time of recruitment, before case or control status was defined. This design is very useful for examining the role of self-reported factors such as cigarette smoking and alcohol consumption, which are modified when cancer is present.

Smoking was not associated with CCA development in this present study (after adjustment for all other variables in the multivariate analysis) – polymorphisms in *XRCC1* and *OGG1*, cigarette smoking and units of alcohol per month of all alcohol consumption.

Alcohol consumption was significantly associated with an increased risk of CCA in previous studies in Thailand[18, 19] and elsewhere.[21] Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines[16, 19] and it is associated with production of free radical intermediate, which can cause several kinds of DNA lesions.[4, 15, 16] Reduced repair of these DNA lesions would therefore constitute a major risk factor for cancer development.[15, 16]

The genotype frequencies of *XRCC1* and *OGG1* polymorphisms found in the controls for the present study were consistent with those found in other studies in Thailand: the prevalence of G allele of *XRCC1* codon 399 was 68.88% in the present study compared with 36.59%–73.20% in other studies[10, 23-25] and A allele of *XRCC1* codon 399 was 31.12% in controls (prevalence of A allele was 26.80%–52.56%). In our analysis of the *OGG1* codon 326, the genotype distribution in the controls deviated from the Hardy–Weinberg equilibrium. Genotype frequency of *OGG1* C326G in controls was slightly higher than rates noted in reports from other studies in Thailand.[10, 24, 26] The C allele of *OGG1* codon 326 was 63.68% in controls (C allele prevalence was between 50.12%–55.00%) and G allele of *OGG1* codon 326 was 36.32% in controls (prevalence of G allele in *OGG1* codon 326 was between 45.00%–49.88%).

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3 *XRCC1* and *OGG1* are major DNA repair genes involved in BER. Mutations and polymorphisms in DNA repair genes, which affect the efficiency of DNA damage repair, may increase an individual's susceptibility to several cancers[23-26] including cancers of the hepatobiliary tract.[5-7] However, no studies on this topic have been conducted in relation to CCA susceptibility and especially not in Thailand where the incidence of CCA is highest in the world.[17] However, there are four studies in a Thai population, which investigated relationships between the BER gene polymorphisms and other common cancers. Whereas *XRCC1* G399A polymorphisms tend to be associated with a reduced risk of oral squamous cell carcinoma,[23] they are associated with increased risks of breast[24] and cervical cancers,[25] and *OGG1* C326G polymorphisms tend be related to increased risks of breast[24] and lung cancers.[26]

Several studies have explored the associations between polymorphisms of the DNA repair genes involved in BER (*XRCC1* G399A and *OGG1* C326G) and the risk of various cancers, but they have shown inconsistent results. This may be the consequence of the different modifying effects, which these polymorphisms have on the balance during the DNA repair process. Imbalance in the tightly regulated process can be caused by SNPs and will result in deficient DNA repair and an increase in DNA breaks.[27] The *XRCC1* codon 399 A allele and the *OGG1* 326 G allele have been associated with a reduced capacity of the DNA repair process as detected by the persistence of DNA bulky adducts[28-30] and γ-irradiation induced oxidative DNA damage.[31] In addition, the balance may be modified by smoking- and alcohol-induced oxidative DNA base modifications interacting with the repair inefficiency of BER polymorphisms[4]; for example, the *XRCC1* GA variant heterozygous carriers, who were nondrinkers or drinkers of <14 units of alcohol per month, had an increased susceptibility to CCA (4.4-fold in nondrinkers increase to

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8.8-fold in drinkers) when compared with nondrinkers carrying the *XRCC1* GG wild-type (Table 4).

In conclusion, this study is the first report of possible associations between the main polymorphisms in BER genes (*XRCC1* G399A, *OGG1* C326G), the joint effects of both genes, and their modification of the effects of cigarette smoking and alcohol drinking on CCA risk in a Thai population. Our results suggest that polymorphisms in *XRCC1* and *OGG1* genes, particularly in combination, were associated with increased susceptibility to CCA. CCA is a multifaceted disease requiring a broad range of preventative actions. In the context of the present study, there is a need for more effective programmes for smoking cessation and reducing alcohol consumption, targeted especially at subgroups which are genetically at particular risk for CCA. Such an approach could also help in the prevention of other forms of cancer.

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Table 1 Univariate analysis of *XRCC1* and *OGG1* polymorphisms with the risk of cholangiocarcinoma in Khon Kaen, Thailand

Genetic polymorphisms		Cases		Controls		OR*	95% CI	*P*-value
		n	%	n	%			
***XRCC1* G399A†**								
GG		62	38.8	149	44.0	1.0		
GA		94	58.8	169	49.9	1.3	0.89 to 1.97	0.17
AA		4	2.5	21	6.2	0.5	0.16 to 1.42	0.18
GA or AA (any A allele)		98	61.3	190	56.1	1.2	0.82 to 1.78	0.33
***OGG1* C326G‡**								
CC		34	23.5	95	28.9	1.0		
CG		109	75.2	229	69.6	1.4	0.86 to 2.23	0.18
GG		2	1.4	5	1.5	1.2	0.22 to 7.03	0.80
CG or GG (any G allele)		111	76.6	234	71.1	1.4	0.86 to 2.22	0.18
Joint effects								
***XRCC1* G399A**	***OGG1* C326G**							
GG	CC	14	9.7	41	12.7	1.0		
GA	CC	19	13.1	47	14.5	0.3	0.15 to 0.55	<0.001
AA	CC	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
GG	CG	42	29.0	100	30.9	0.1	0.07 to 0.25	<0.001
GA	CG	56	38.6	108	33.3	0.1	0.07 to 0.22	<0.001
AA	CG	3	2.1	15	4.6	0.9	0.42 to 2.07	1.00
GG	GG	5	3.4	3	0.9	4.7	1.30 to 25.33	0.01
GA	GG	2	1.4	4	1.2	3.5	1.10 to 14.60	0.03
AA	GG	2	1.4	2	0.6	7.0	1.61 to 63.46	0.004
P-value for interaction = 0.21								

*Crude odds ratio from matched case-control analysis.

†DNA samples for XRCC1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

‡DNA samples for OGG1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 2 Univariate analysis of cigarette smoking and alcohol drinking on the risk of cholangiocarcinoma in Khon Kaen, Thailand

Variables	Cases		Controls		OR*	95% CI	*P*-value
	n	%	n	%			
Cigarette smoking							
Non-smoker	92	42.0	230	52.5	1.0		
Smoker	127	58.0	208	47.5	2.7	1.57 to 4.50	<0.001
Age at which smoking started of all cigarettes (years)							
Non-smoker	92	42.0	230	52.5	1.0		
<17	28	12.8	48	11.0	2.9	1.38 to 5.91	0.01
17–19	35	16.0	44	10.1	3.6	1.85 to 7.17	<0.001
20–21	40	18.3	70	16.0	2.5	1.33 to 4.62	0.004
22+	24	11.0	46	10.5	2.4	1.19 to 4.73	0.01
P-value for trend = 0.002							
Alcohol consumption							
No	57	26.0	254	58.0	1.0		
Yes	162	74.0	184	42.0	5.7	3.61 to 9.04	<0.001
Units of alcohol per month of all alcohol consumption							
Non-drinker	57	26.0	254	58.0	1.0		
<14	79	36.1	92	21.0	5.2	3.15 to 8.43	<0.001
≥14	83	37.9	92	21.0	6.7	3.90 to 11.38	<0.001
Alcohol consumption and smoking							
No alcohol, non-smoker	42	19.2	178	40.6	1.0		
No alcohol, smoker	15	6.9	76	17.4	2.2	0.93 to 5.00	0.07
Alcohol drinker, non-smoker	50	22.8	52	11.9	5.8	3.12 to 10.78	<0.001
Alcohol drinker, smoker	112	51.1	132	30.1	10.4	5.07 to 21.16	<0.001
P-value for trend <0.001							
P-value for interaction = 0.68							

*Crude odds ratio from matched case-control analysis.

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Table 3 Cases and controls, and odds ratios for cholangiocarcinoma associated with smoking, alcohol drinking, and *XRCC1* and *OGG1* polymorphisms

Variables	Cases		Controls		OR*	OR†	95% CI‡	*P*-value
	n	%	n	%				
***XRCC1* G399A polymorphisms§**								
GG	62	38.8	149	44.0	1.0	1.0		
GA	94	58.8	169	49.9	1.3	1.2	0.76 to 1.94	0.43
AA	4	2.5	21	6.2	0.5	0.4	0.10 to 1.62	0.20
***OGG1* C326G polymorphisms¶**								
CC	34	23.4	95	28.9	1.0	1.0		
CG	109	75.2	229	69.6	1.4	1.2	0.70 to 2.07	0.49
GG	2	1.4	5	1.5	1.2	0.7	0.10 to 5.11	0.75
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Smoker	127	58.0	208	47.5	2.7	1.9	0.94 to 4.01	0.07
Units of alcohol per month of all alcohol consumption								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.2	2.74 to 9.95	<0.001
≥14	83	37.9	92	21.0	6.7	7.6	3.67 to 15.72	<0.001

*Crude odds ratio from matched case–control analysis.

†Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).

‡95% confidence interval for OR†.

§DNA samples for *XRCC*1 genotyping were successfully carried out for 160 out of 175 cases, and 339 out of 350 controls.

¶DNA samples for *OGG*1 genotyping were successfully carried out for 145 out of 175 cases, and 329 out of 350 controls.

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Table 4 Gene-environment interactions of *XRCC1* and *OGG1* polymorphisms together with smoking and alcohol drinking on cholangiocarcinoma risk in Khon Kaen, Thailand

XRCC1	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value	*OGG1*	Smoking / Use of alcohol	Cases n	Controls n	OR*	95% CI	*P*-value
G399A	**All cigarettes smoking**					*0.87*†	**C326G**	**All cigarettes smoking**					*0.54*†
GG	Non-smoker	22	71	1.0			CC	Non-smoker	14	55	1.0		
GA	Non-smoker	48	99	1.5	0.82 to 2.79	0.18	CG	Non-smoker	49	122	1.5	0.77 to 2.99	0.22
AA	Non-smoker	2	11	0.6	0.11 to 2.74	0.47	GG	Non-smoker	1	1	—	—	—
GG	Smoker	40	78	2.8	1.30 to 5.91	0.01	CC	Smoker	20	40	3.1	1.17 to 8.38	0.02
GA	Smoker	46	70	3.4	1.60 to 7.28	0.001	CG	Smoker	60	107	4.3	1.79 to 10.21	0.001
AA	Smoker	2	10	1.2	0.21 to 6.29	0.86	GG	Smoker	1	4	1.5	0.14 to 15.44	0.74
G399A	**Units of alcohol per month of all alcohol drinking**					*0.80*†	**C326G**	**Units of alcohol per month of all alcohol drinking**					*0.74*†
GG	Non-drinker	16	83	1.0			CC	Non-drinker	10	65	1.0		
GA	Non-drinker	20	33	4.4	1.81 to 10.83	0.001	CG	Non-drinker	9	9	8.1	2.30 to 28.36	0.001
AA	Non-drinker	26	33	9.2	3.65 to 23.21	<0.001	GG	Non-drinker	15	21	8.6	2.62 to 28.02	<0.001
GG	<14	22	102	1.1	0.49 to 2.28	0.89	CC	<14	24	130	1.3	0.55 to 3.20	0.52
GA	<14	39	35	8.8	3.76 to 20.71	<0.001	CG	<14	46	58	7.0	2.85 to 17.39	<0.001
AA	<14	33	32	10.3	4.14 to 25.80	<0.001	GG	<14	39	41	11.2	4.18 to 30.17	<0.001
GG	≥14	1	12	0.4	0.05 to 3.71	0.44	CC	≥14	0	1	—	—	—
GA	≥14	2	3	2.8	0.31 to 25.41	0.36	CG	≥14	1	1	7.7	0.29 to 26.73	0.22
AA	≥14	1	6	2.5	0.25 to 23.99	0.44	GG	≥14	1	3	6.4	0.48 to 86.72	0.16

*Crude odds ratio from matched case-control analysis.

†*P*-value for interaction.

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Contributors NS, SP. CP, TE and SW conceived and designed the research. NS and PC performed the research. NS and SP carried out the analyses. All authors contributed to the writing and revisions of the manuscript and approved the final version.

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Data sharing statement No additional data are available.

Ethics approval Ethical approval was obtained from the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

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Figure Legends

Figure 1 Polymorphisms in *XRCC1* G399A and *OGG1* C326G were analyzed using PCR with high resolution melting analysis.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 (Internet). Lyon, France: IARC; 2010. http://globocan.iarc.fr (accessed 27 Mar 2013).

2. Khuhaprema T, Attasara P, Sriplung H, *et al*. *Cancer in Thailand Volume VI, 2004-2006.* Bangkok: NCIB, 2012.

3. Parkin DM, Ohshima H, Srivatanakul P, *et al*. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev* 1993;2:537–44.

4. Stern MC, Conti DV, Siegmund KD, *et al.* DNA repair single-nucleotide polymorphisms in colorectal cancer and their role as modifiers of the effect of cigarette smoking and alcohol in the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2007;16:2363–72.

5. Huang WY, Gao YT, Rashid A, *et al.* Selected base excision repair gene polymorphisms and susceptibility to biliary tract cancer and biliary stones: a population-based case-control study in China. *Carcinogenesis* 2008;29:100–5.

6. Zhang M, Huang WY, Andreotti G, *et al.* Variants of DNA repair genes and the risk of biliary tract cancers and stones: a population-based study in China. *Cancer Epidemiol Biomarkers Prev* 2008;17:2123–7.

7. Srivastava A, Srivastava K, Pandey SN, *et al*. Single-nucleotide polymorphisms of DNA repair genes *OGG1* and *XRCC1*: association with gallbladder cancer in North Indian population. *Ann Surg Oncol* 2009;16:1695–703.

For peer review only

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8. Thanan R, Murata M, Pinlaor S, *et al.* Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2008;17:518–24.

9. Dechakhamphu S, Pinlaor S, Sitthithaworn P, *et al*. Lipid peroxidation and etheno DNA adducts in white blood cells of liver fluke-infected patients: protection by plasma alpha-tocopherol and praziquantel. *Cancer Epidemiol Biomarkers Prev* 2010;19:310–8.

10. Zeng L, You G, Tanaka H, *et al.* Combined effects of polymorphisms of DNA-repair protein genes and metabolic enzyme genes on the risk of cholangiocarcinoma. *Jpn J Clin Oncol* 2013;43: 1190–4.

11. Songserm N, Promthet S, Sithithaworn P, *et al. MTHFR* Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand. *Asian Pac J Cancer Prev* 2011;12:1341–5.

12. Songserm N, Promthet S, Sithithaworn P, *et al.* Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms. *Cancer Epidemiol* 2012;36:e89–94.

13. Kosti O, Byrne C, Meeker KL, *et al.* Mutagen sensitivity, tobacco smoking and breast cancer risk: a case-control study. *Carcinogenesis* 2010;31:654–9.

14. Rouissi K, Ouerhani S, Hamrita B, *et al.* Smoking and polymorphisms in xenobiotic metabolism and DNA repair genes are additive risk factors affecting bladder cancer in Northern Tunisia. *Pathol Oncol Res* 2011;17:879–86.

15. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009;674:36–44.

16. Pelucchi C, Tramacere I, Boffetta P, *et al*. Alcohol consumption and cancer risk. *Nutr Cancer* 2011;63:983–90.

For peer review only

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17. Parkin DM, Srivatanakul P, Khlat M, *et al.* Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991;48:323–8.

18. Chernrungroj G. Risk factors for cholangiocarcinoma: a case-control study (dissertation). Connecticut: Yale University, 2000.

19. Honjo S, Srivatanakul P, Sriplung H, *et al.* Genetic and environmental determinants of risk for cholangiocarcinoma via *Opisthorchis viverrini* in a densely infested area in Nakhon Phanom, northeast Thailand. *Int J Cancer* 2005;117:854–60.

20. Poomphakwaen K, Promthet S, Kamsa-Ard S, *et al.* Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. *Asian Pac J Cancer Prev* 2009;10:251–8.

21. Chalasani N, Baluyut A, Ismail A, *et al.* Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000;31:7–11.

22. Sriamporn S, Parkin DM, Pisani P, *et al.* A prospective study of diet, lifestyle, and genetic factors and the risk of cancer in Khon Kaen Province, northeast Thailand: description of the cohort. *Asian Pac J Cancer Prev* 2005;6:295–303.

23. Kietthubthew S, Sriplung H, Au WW, *et al*. Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand. *Int J Hyg Environ Health* 2006;209:21–9.

24. Sangrajrang S, Schmezer P, Burkholder I, *et al.* Polymorphisms in three base excision repair genes and breast cancer risk in Thai women. *Breast Cancer Res Treat* 2008;111:279–88.

25. Settheetham-Ishida W, Yuenyao P, Natphopsuk S, *et al*. Genetic Risk of DNA Repair Gene Polymorphisms (*XRCC1* and *XRCC3*) for High Risk Human Papillomavirus Negative Cervical Cancer in Northeast Thailand. *Asian Pac J Cancer Prev* 2011;12:963–6.

26. Klinchid J, Chewaskulyoung B, Saeteng S, *et al*. Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women. *Cancer Genet Cytogenet* 2009;195:143–9.

For peer review only

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27. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutat Res* 2003;526:93–125.

28. Lunn RM, Langlois RG, Hsieh LL, *et al*. *XRCC1* polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999;59:2557–61.

29. Duell EJ, Millikan RC, Pittman GS, *et al.* Polymorphisms in the DNA repair gene *XRCC1* and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:217–22.

30. Matullo G, Guarrera S, Carturan S, *et al.* DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer* 2001;92:562–7.

31. Vodicka P, Stetina R, Polakova V, *et al.* Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis* 2007;28:657–64.

For peer review only

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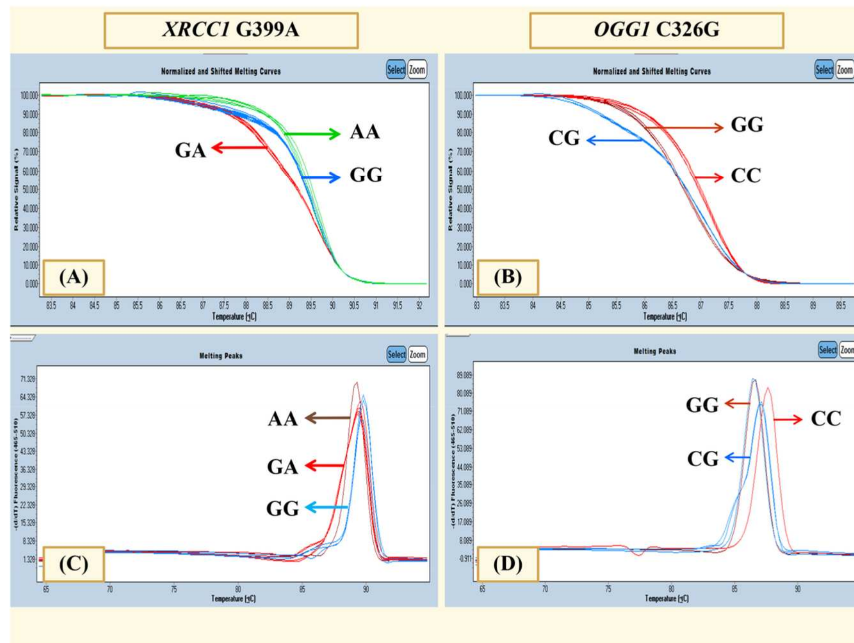

Figure 1 Polymorphisms in XRCC1 G399A and OGG1 C326G were analyzed using PCR with high resolution melting analysis.
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