RESEARCH COMMUNICATION

MTHFR Polymorphisms and *Opisthorchis viverrini* Infection: a Relationship with Increased Susceptibility to Cholangiocarcinoma in Thailand

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Abstract

***Opisthorchis viverrini* (OV) infection is the major risk factor for cholangiocarcinoma (CCA). Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism. Change in MTHFR activity may influence both DNA methylation and synthesis, crucial steps in carcinogenesis. This study aimed to investigate the association between *MTHFR* polymorphisms and OV infection with CCA risk in a high-incidence area of Thailand. A nested case-control study within cohort study was carried out: 219 subjects with primary CCA were matched with two non-cancer controls from the same cohort on sex, age at recruitment and presence/absence of OV eggs in stool. At the time of recruitment information on consumption of foodstuffs potentially contaminated by OV was obtained by questionnaire. *MTHFR* polymorphisms were analyzed using PCR with high resolution melting analysis. Associations between variables and the risk of CCA were assessed using conditional logistic regression. Risk of CCA was related to consumption of a dish of raw freshwater fish (Koi-Pla) with clear dose-response effects, and there were joint effects on CCA risk between *MTHFR* polymorphisms and consumption of dishes containing raw- and/or semi-raw freshwater fish. This study provides evidence to support a relationship of increased susceptibility to CCA in individuals with *MTHFR* variants, especially for those individuals who have OV infection or consume semi-raw freshwater fish (acting either as a source of OV or of pre-formed nitrosamine). Folate may play an important role in OV-related cholangiocarcinogenesis by upsetting the balance between DNA methylation and synthesis in the folate pathway.**

Keywords: Methylenetetrahydrofolate reductase -polymorphisms - *Opisthorchis viverrini* - cholangiocarcinoma

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Introduction

Liver cancer is the most common type of malignancy in Thailand, the great majority of cases being cholangiocarcinomas (CCA) (Khuhaprema et al., 2007). In the Khon Kaen Cancer Registry (KKCR), they comprise 83% in men, and 86% in women, with estimated age standardised incidence rates of 84.6 and 36.8 per 100,000, respectively (Parkin et al., 1993). Infection with *Opisthorchis viverrini* (OV) is known to underlie the high risk of CCA in north-east Thailand (IARC, 1994), but the correlation between risk and infection is far from perfect, at both individual and community levels (Sriamporn et al., 2004), so that other co-factors, environmental and genetic, almost certainly play a role in aetiology.

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism (Choi et al., 2002), and a change in MTHFR activity may thus influence both DNA methylation and DNA synthesis. Two common polymorphisms in the *MTHFR* gene (C677T and A1298C) have been characterized (Frosst et al., 1995; Weisberg et al., 1998), and have attracted a great deal of attention with regard to cancer risk, although the results have been conflicting. There is only one epidemiological study (from Korea) on the association between *MTHFR* polymorphisms and CCA risk, which suggested that the combination of *MTHFR* 677CC with the thymidylate synthase enhancer region (*TSER*) 2R(+) genotype increased the risk (Ko et al., 2006). No studies of this topic have been conducted in Thailand, where the incidence of CCA is the highest in the world. Therefore, the present study aimed to examine the association between *MTHFR* polymorphisms and OV infection in influencing the risk of CCA in a high-incidence area of Thailand.

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Materials and Methods

This study is a nested case-control study within a cohort study. Cases of CCA (ICD-10: 22.1) and a sample of non-affected controls were drawn from subjects enrolled in the Khon Kaen Cohort Study (KKCS), details of which have been published previously (Sriamporn et al., 2005).

Study subjects

219 cohort members who had developed a primary CCA six or more months after enrollment were identified. Since CCA is rarely diagnosed by liver biopsy and histopathology, the criteria for inclusion as a case included diagnosis at least by ultrasound, with or without contrast radiology, tumour markers (such as CA19-9) or histopathology. The vital status and date of death of potential cases was ascertained by linkage to the file of deaths in Thailand, in the database of the National Health Security Office (NHSO), together with the demographic database of Ministry of Interior. All cases had died within 2 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 or absence of OV eggs in the faeces at the time of recruitment.

Data collection

Data on cases and controls were taken from the questionnaire administered at the time of recruitment into the KKCS. The food frequency questionnaire (FFQ) was designed to include items that are common in the Thai diet but this present study focused on those dietary items that are known to be vehicles for infection with OV: dishes containing raw fish (*Koi-Pla*) or semi-cooked fish (*Pla-Chom*/*Pla-Choa*). The FFQ allowed consumption to be classified into four categories: non-consumer, <1/ month and monthly, weekly, and daily, as well as amount (times) of consumption per unit of frequency. Frequencies of each dietary intake item and the annual intake were computed and dichotomized with regard to the median of the control group.

Detection of OV eggs in stools

Stool specimens were obtained from all subjects at the time of recruitment and examined for OV within 24 hour by the formalin ethyl acetate concentration technique (FECT) (Elkins et al., 1990; Sriamporn et al., 2005).

Analysis of MTHFR genetic polymorphisms

Genomic DNA was extracted from buffy coat fractions of CCA cases (175 (80%) of 219 eligible CCA cases) and 350 matched controls using the standard protocols of Genomic DNA mini Kit with Proteinase K (Geneaid Biotech).

The polymerase chain reaction with high resolution melting analysis (PCR-HRM) technique of DNA amplification for *MTHFR* C677T and A1298C polymorphisms were performed in a 96-well plate in the LightCycler® 480 Real-Time PCR System. The LightCycler® 480 High Resolution Melting Master (Roche) was used in a final volume of 20 μl containing 10 μl of master mix, 5.2 μl of H_2O, 2 mM of $MgCl_2$, 0.4 μM of each primer and 200 ng of the DNA template. Experimental samples were compared with the positive standard controls according to previous our study (Promthet et al., 2010) to identify the three genotypes at each locus. Amplification of *MTHFR* polymorphisms was modified as previously described (Seipp et al., 2008).

Statistical analysis

The distributions of general characteristics, OV infection and prevalence of genotypes for the *MTHFR* C677T and A1298C polymorphisms were analyzed in cases and their matched controls by using the McNemar test. To assess the strength of the associations between potential risk factors and the risk of CCA, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. Possible modifications of the effects of OV infection and consumption of OV-contaminated foodstuffs by polymorphisms in *MTHFR* C677T and A1298C were also analyzed. A P-value <0.05 was considered statistically significant.

Results

The case group comprised 92 females and 127 males, with a median age of 57 years. OV eggs were detected in the stool of 70 cases (37.8%). There were two controls matched by sex, age at recruitment and status of OV infection (detection of OV eggs in stool) for each case. With respect to demographic factors, there was no association between risk of CCA and marital status (79% of cases and 83% controls were married) or occupation (89% of cases and 91% of controls were farmers). However, there was a clear association with educational level: compared with the illiterate, subjects with primary school education had a reduced risk of CCA (OR = 0.3, 95% CI: 0.15-0.54), and this was lower still in those with secondary or higher-level education (OR = 0.2, 95% CI: 0.17-0.53). With respect to *MTHFR* polymorphisms, there was an increased risk of CCA in individuals with the CC variant of A1298C (OR = 2.0, 95% CI: 1.14-3.48).

There were no differences in the odds ratios associated with the different *MTHFR* polymorphisms between subjects who were positive and those who were negative for OV eggs in stool. However, positive OV subjects who carrying the *MTHFR* 1298 CC variants had an increased susceptibility to CCA compared with negative OV subjects who carrying the wild-type.

Because of the matched design, the effect of OV infection on risk of CCA cannot be evaluated. However, even when cases and controls were stratified by OV infection status, consumption of the two dietary items likely to contain the encysted phase of OV (*Koi-Pla*, and *Pla-Chom*/*Pla-Choa*) was strongly related to the risk of CCA. What is more, there was a significant interaction between them, with rather higher risks associated with the dietary items in OV positive, compared with OV negative subjects.

Table 1 shows the results of interaction between *MTHFR* polymorphisms and consumption of the two

Table 1. Interactions of *MTHFR* Polymorphisms and OV-contaminated Foodstuffs with the Risk of Cholangiocarcinoma in Khon Kaen, Thailand

MTHFR	Dietary factor	No.*		OR[a]	95% CI	P-value
677	Consumption of *koi pla*					0.10[b]
CC	Nonconsumer	13	78	1.0		
CT	Nonconsumer	9	31	1.7	0.65-4.55	0.28
TT	Nonconsumer	4	7	2.6	0.64-10.5	0.18
CC	<1/month	21	51	2.1	0.87-5.04	0.10
CT	<1/month	27	49	2.6	1.07-6.34	0.04
TT	<1/month	25	64	1.9	0.78-4.47	0.16
CC	Weekly	29	29	6.1	2.55-14.7	<0.001
CT	Weekly	13	25	3.0	1.15-7.88	0.03
TT	Weekly	9	9	6.1	1.70-21.7	0.01
CC	Daily	11	2	22.1	4.34- 113	<0.001
CT	Daily	8	5	6.4	1.82-22.9	0.004
TT	Daily	6	0	-	-	-
677	Consumption of *pla-chom/pla-choa*					0.04[b]
CC	Nonconsumer	13	78	1.0		
CT	Nonconsumer	9	31	1.9	0.73-5.17	0.18
TT	Nonconsumer	4	7	3.6	0.87-14.9	0.08
CC	<1/month	32	39	6.2	2.62-14.8	<0.001
CT	<1/month	22	46	3.6	1.47-8.90	0.01
TT	<1/month	20	56	2.6	1.06-6.23	0.04
CC	Weekly	20	37	3.3	1.43-7.71	0.01
CT	Weekly	19	28	4.8	1.98-11.8	0.001
TT	Weekly	14	16	6.5	2.32-18.2	<0.001
CC	Daily	9	6	7.7	2.22-27.0	0.001
CT	Daily	7	5	6.9	1.86-25.9	0.004
TT	Daily	6	1	31.1	3.33- 291	0.003
1298	Consumption of *koi pla*					0.13[b]
AA	Nonconsumer	12	69	1.0		
AC	Nonconsumer	9	40	1.0	0.35-2.70	0.96
CC	Nonconsumer	5	7	5.1	0.95-27.3	0.06
AA	<1/month	36	87	1.6	0.69-3.76	0.28
AC	<1/month	30	58	1.9	0.75-4.68	0.18
CC	<1/month	7	19	1.4	0.45-4.61	0.54
AA	Weekly	24	31	4.1	1.69-10.0	0.002
AC	Weekly	16	22	3.0	1.18-7.52	0.02
CC	Weekly	11	10	5.7	1.72-18.6	0.004
AA	Daily	12	2	19.1	3.82-95.9	<0.001
AC	Daily	5	5	4.5	1.07-18.5	0.04
CC	Daily	8	0	-	-	-
1298	Consumption of *pla-chom/pla-choa*					0.04[b]
AA	Nonconsumer	12	69	1.0		
AC	Nonconsumer	9	40	1.2	0.45-3.42	0.67
CC	Nonconsumer	5	7	4.2	0.90-19.5	0.07
AA	<1/month	39	70	3.4	1.45-7.79	0.01
AC	<1/month	28	49	3.3	1.39-7.99	0.01
CC	<1/month	7	22	2.1	0.66-6.32	0.21
AA	Weekly	22	46	2.8	1.21-6.46	0.02
AC	Weekly	18	30	3.6	1.42-9.05	0.01
CC	Weekly	13	5	18.2	4.36-76.2	<0.001
AA	Daily	11	4	9.7	2.74-34.4	<0.001
AC	Daily	5	6	4.1	1.04-16.3	0.04
CC	Daily	6	2	12.3	2.16-70.3	0.01

*Numbers, cases 175 and controls 350; OV, *Opisthorchis viverrini*; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; 95% CI, 95% confidence intervals; [a]Crude odds ratio from matched case-control analysis; [b]P-value for interaction

potential vehicles of OV infection, with the risk of CCA. There were interactions between polymorphisms in both MTHFR 677 and 1298 and consumption of Pla-Chom/ Pla-Choa with the same P-value (0.04).

Discussion

The design of the present study - a nested case-control study within a cohort study - minimises recall bias of dietary factors (OV-contaminated foodstuffs), as subjects were interviewed at the time of recruitment, before case or control status was defined. Although this design is not important for studying the role of genetic factors, it is very useful for examining the role of other factors, especially diet, which are modified when cancer is present.

OV infection is strongly associated with the consumption of dishes containing raw- (*Koi-Pla*) and/or semi-raw freshwater fish (*Pla-Chom*/*Pla-Choa*) in this present study as in previous studies (Upatham et al., 1984; Honjo et al., 2005; Poomphakwaen et al., 2009). These dietary items are the usual vehicles of OV infection in the population under study (Sithithaworn et al., 2003; Sripa et al., 2007). In the present study, we found evidence that consumption of *Koi-Pla* and *Pla-Chom*/*Pla-Choa* was strongly related to the risk of developing CCA, with clear dose-response effects, independently of OV infection status although there was some interaction with the latter: *MTHFR* polymorphisms act together with consumption of *Pla-Chom*/*Pla-Choa* in increasing susceptibility to CCA.

There are two possible explanations for these observations. One is that a single egg count at the time of study enrollment does not capture adequately past exposure to OV in cohort subjects. In fact, this is entirely plausible, and especially so since successful treatment and subsequent re-infection have become common with the advent of relatively simple and effective treatment, in the form of the drug Praziquantel. In a previous study, indeed, Praziquantel was found to be a risk factor for CCA (Elkins et al., 1996; Honjo et al., 2005) suggesting that subjects who take the drug frequently do so because of repeated exposure to infection. Alternatively, other constituents of these dietary items may provide a risk additional to the OV metacercaria within the fish that they contain. One candidate is carcinogenic N-nitroso compounds which have been shown to be present in the foodstuffs prepared from fermentation of freshwater fish (Mitacek et al., 1999). Many authors have suggested that N-nitroso compounds in fermented foods are primary carcinogens leading to CCA development (Migasena et al., 1980), and there is abundant evidence from animal experiments of the joint effects of N-nitroso compounds and OV infection in carcinogenesis (Flavell et al., 1983; Thamavit et al., 1987).

Several studies have explored the possible associations between polymorphisms in *MTHFR* and the risk of certain cancers, but they have showed inconsistent results. This may be the consequence of the various modifying effects that *MTHFR* polymorphisms have on the balance between DNA methylation and DNA synthesis. The balance may be determined by dietary factors, especially dietary folate. Imbalance between DNA methylation and DNA synthesis may cause of cancer development (Kim, 2000). Low enzyme activity of MTHFR leads to higher plasma homocysteine levels, leading to a great pool of methylenetetrahydrofolate (methylene-THF). Increased methylene-THF in the DNA synthesis pathway reduces misincorporation of uracil in DNA. Hence from the DNA

methylation standpoint, we have found that subjects carrying *MTHFR* 1298 CC homozygous variants increase the risk of CCA when compared with wild-type (OR = 2.0, 95% CI: 1.14-3.48), whereas from the DNA synthesis standpoint, we have explained from our another study, the result showed that *MTHFR* 677 and 1298 variants carriers may have a protective effect on the risk of colon cancer in Thai population (Promthet et al., 2010).

This study has some limitations. In the KKCS, subjects were interviewed and tested for OV infection status by faecal egg counts only on entry, there were no repeat examinations. With respect to intensity and duration of infection with OV – probably the most relevant factors influencing risk of CCA – a single exposure measure is likely to be inadequate, as already noted. Similarly, although evaluation of consumption of the dietary items aimed to collect "usual" habits, prior to interview, misclassification is probable, and there is in addition the possibility of change in dietary habits post interview. The basis of diagnosis of CCA was rarely by histology, so that it is possible that other cases of fatal liver disease showing features of biliary obstruction, with a low serum alpha-fetoprotein, were included in the case group.

In conclusion, this study provides evidence to support an increased susceptibility to CCA in individuals with *MTHFR* variants, especially for those individuals infected by OV, or consuming semi-raw freshwater fish (either a source of OV or of pre-formed nitrosamine). Folate may play an important role in OV-related cholangiocarcinogenesis via unbalancing between DNA methylation and DNA synthesis in the folate pathway. The associations of CCA with polymorphisms of *MTHFR* will be helpful in suggesting the likely mechanisms of OV-related carcinogenesis, and provide a basis for further study, and possible preventive strategies. A strong association with one or more polymorphisms could conceivably be used to identify individuals at high risk of developing CCA. Such individuals could be prioritised for regular testing for OV infection and treatment as appropriate.

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References

Choi SW, Mason JB (2002). Folate status: effects on pathways of colorectal carcinogenesis. *J Nutr*, **132**, 2413S-2418S.

Elkins DB, Haswell-Elkins MR, Mairiang E, et al (1990). A high frequency of hepatobiliary disease and suspected cholangiocarcinoma associated with heavy *Opisthorchis viverrini* infection in a small community in north-east Thailand. *Trans R Soc Trop Med Hyg*, **84**, 715-9.

Elkins DB, Mairiang E, Sithithaworn P, et al (1996). Cross-sectional patterns of hepatobiliary abnormalities and possible precursor conditions of cholangiocarcinoma associated with *Opisthorchis viverrini* infection in humans. *Am J Trop Med Hyg*, **55**, 295-301.

Flavell DJ, Lucas SB (1983). Promotion of N-nitrosodimethylamine-initiated bile duct carcinogenesis in the hamster by the human liver fluke, *Opisthorchis viverrini*. *Carcinogenesis*, **4**, 927-30.

Frosst P, Blom HJ, Milos R, et al (1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*, **10**, 111-3.

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

IARC (1994). Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum*, **61**, 1-241.

Khuhaprema T, Srivatanakul P, Sriplung H, et al (2007). Cancer in Thailand, Vol. IV, 1998-2000. Bangkok, Bangkok Medical Publisher.

Kim YI (2000). Methylenetetrahydrofolate reductase polymorphisms, folate, and cancer risk: a paradigm of gene-nutrient interactions in carcinogenesis. *Nutr Rev*, **58**, 205-9.

Ko KH, Kim NK, Yim DJ, et al (2006). Polymorphisms of 5,10-methylenetetrahydrofolate reductase (*MTHFR* C677T) and thymidylate synthase enhancer region (*TSER*) as a risk factor of cholangiocarcinoma in a Korean population. *Anticancer Res*, **26**, 4229-33.

Migasena P, Reaunsuwan W, Changbumrung S (1980). Nitrates and nitrites in local Thai preserved protein foods. *J Med Assoc Thai*, **63**, 500-5.

Mitacek EJ, Brunnemann KD, Suttajit M, et al (1999). Exposure to N-nitroso compounds in a population of high liver cancer regions in Thailand: volatile nitrosamine (VNA) levels in Thai food. *Food Chem Toxicol*, **37**, 297-305.

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

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

Promthet SS, Pientong C, Ekalaksananan T, et al (2010). Risk factors for colon cancer in Northeastern Thailand: interaction of *MTHFR* codon 677 and 1298 genotypes with environmental factors. *J Epidemiol*, **20**, 329-38.

Seipp MT, Pattison D, Durtschi JD, et al (2008). Quadruplex genotyping of *F5*, *F2*, and variants in a single closed tube by high-resolution amplicon melting. *Clin Chem*, **54**, 108-15.

Sithithaworn P, Haswell-Elkins M (2003). Epidemiology of *Opisthorchis viverrini*. *Acta Trop*, **88**, 187-94.

Sriamporn S, Parkin DM, Pisani P, et al (2005). 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*, **6**, 295-303.

Sriamporn S, Pisani P, Pipitgool V, et al (2004). Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop Med Int Health*, **9**, 588-94.

Sripa B, Kaewkes S, Sithithaworn P, et al (2007). Liver fluke induces cholangiocarcinoma. *PLoS Med*, **4**, e201.

Thamavit W, Kongkanuntn R, Tiwawech D, et al (1987). Level of *Opisthorchis* infestation and carcinogen dose-dependence of cholangiocarcinoma induction in Syrian golden hamsters. *Virchows Arch B Cell Pathol Incl Mol Pathol*, **54**, 52-8.

Upatham ES, Viyanant V, Kurathong S, et al (1984). Relationship between prevalence and intensity of *Opisthorchis viverrini* infection, and clinical symptoms and signs in a rural community in north-east Thailand. *Bull World Health Organ*, **62**, 451-61.

Weisberg I, Tran P, Christensen B, et al (1998). A second genetic polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) associated with decreased enzyme activity. *Mol Genet Metab*, **64**, 169-72.