Cancer Epidemiology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Risk factors for cholangiocarcinoma in high-risk area of Thailand: Role of lifestyle, diet and methylenetetrahydrofolate reductase polymorphisms

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ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form 24 November 2011
Accepted 26 November 2011
Available online xxx

Keywords:
Cholangiocarcinoma
Lifestyle
Diet
Methylenetetrahydrofolate reductase
Genetic polymorphisms

ABSTRACT

Background and aim: Cholangiocarcinoma (CCA) is the most common cancer in Northeast Thailand. Endemicity of *Opisthorchis viverrini* (OV) – a known carcinogen – is responsible, but although infection is very common, the lifetime risk of CCA is only 5%. Other co-factors must exist, including aspects of lifestyle or diet along with variations in genetic susceptibility to them. Change in methylenetetrahydrofolate reductase (MTHFR) activity may influence both DNA methylation and synthesis. This study investigates risk factors for CCA with a focus on lifestyle, diet and *MTHFR* polymorphisms. *Methods*: 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 OV eggs in stool. Lifestyle and dietary data were obtained at recruitment. *MTHFR* polymorphisms were analyzed using PCR with high resolution melting analysis. The associations were assessed using conditional logistic regression. *Results*: Consumption of alcohol, raw freshwater fish and beef sausage increased the risk of CCA, while fruit and/or vegetables consumption reduced risk. There were interactions between *MTHFR* and preserved freshwater fish and beef. These dietary items are either a source of OV or of pre-formed nitrosamine, folate and antioxidants that are of possible relevance in OV carcinogenesis. *Conclusions*: Primary prevention of CCA in high-risk population is based upon efforts to reduce OV infection. Reduced consumption of alcohol and preserved meats, and increased consumption of dietary folate, actions with a wider preventive potential, may also help in the reduction of CCA burden.

1. Introduction

Liver cancer is the fourth most common cause of death from cancer worldwide, with an estimated 696 000 deaths in 2008 [1]. It is the most common malignancy in Thailand, and data from population-based cancer registries show an increasing trend in both sexes in all centers [2]. In Northeast Thailand, the great majority of liver cancer cases are cholangiocarcinomas (CCA). In the Khon Kaen Cancer Registry, they comprise 83% of liver cancers in men, and 86% in women, with estimated age standardized incidence rates of 84.6 and 36.8 per 100 000 respectively [3].

In 1994, IARC concluded that there was sufficient evidence in humans for the carcinogenicity of infection with *Opisthorchis viverrini* (OV) with respect to CCA [4], and subsequent studies have confirmed this conclusion [5–8]. Nevertheless, since infection with the fluke is very common (24.5% prevalence among the adult population of Khon Kaen province, for example [6]) while the cumulative incidence of CCA (0–74) is only about 5% [9], it is clear that other co-factors must exist, including different patterns of lifestyle (e.g., tobacco and alcohol) or diet along with variations in genetic susceptibility to them. Previous studies have, for example, identified consumption of alcohol and fermented foods as risk factors [7] and of fruit as being protective [8] independent of OV infection. With respect to susceptibility to dietary cofactors, it is known that polymorphisms of the methylenetetrahydrofolate

Abbreviations: CCA, cholangiocarcinoma; OV, *Opisthorchis viverrini*; MTHFR, methylenetetrahydrofolate reductase; KKCS, Khon Kaen Cohort Study; KKCR, Khon Kaen Provincial Cancer Registry; FECT, formalin ethyl acetate concentration technique; PCR-HRM, polymerase chain reaction with high resolution melting analysis; OR, odds ratio; 95% CI, 95% confidence intervals.

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doi:10.1016/j.canep.2011.11.007

reductase (*MTHFR*) gene influence MTHFR activity [10,11], which is an important enzyme in folate metabolism affecting both DNA methylation and synthesis [12]. Low-activity variants of *MTHFR* C677T and A1298C are associated with decreased risks of colon cancer [13–15] and acute lymphocytic leukemia [16], while the same variants have also been linked with an increased risk of endometrial cancer [17], cervical intraepithelial neoplasia [18], esophageal squamous cell carcinoma [19], gastric cancer [20], and bladder cancer [21]. Until now, only one study has estimated the relationship between *MTHFR* polymorphisms and CCA risk in Korea [22]. No studies of this topic have been conducted in Thailand, where the incidence of CCA is highest in the world, and the relationship between *MTHFR* A1298C polymorphisms and the risk of CCA has not been studied at all. The present study, therefore, aimed to explore risk factors for CCA in high-risk area of Thailand, with a focus on lifestyle, diet, and polymorphisms in *MTHFR* C677T and A1298C.

2. Materials and methods

2.1. Study subjects

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 [23]. 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 and/or contrast radiology and/or tumor markers (such as CA19-9), as well as 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, 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/absence of OV eggs in the feces (detected by the formalin ethyl acetate concentration technique (FECT)) at recruitment. This research was approved by the Khon Kaen University Ethics Committee for Human Research (Reference No. HE512053).

2.2. Data collection

Data on cases and controls were taken from the questionnaire that was administered at the time of recruitment into the KKCS. The variables of interest were general (demographic) information on the study subjects, smoking, betel nut chewing, coffee/tea drinking, alcoholic beverage consumption, and food items and food consumption habits.

2.3. Assessment of cigarette smoking

A smoker was defined as someone who had ever smoked (filtered, unfiltered cigarettes and *Yamuan* – a home-made cheroot) on a daily basis. Smokers were asked at what age (years) they began smoking on a daily basis, frequency of smoking, and the average number of cigarettes smoked per unit of frequency. Former smokers were defined as individuals who had stopped smoking one or more years before interview, and could be classified according to the number of years since smoking cessation.

For the analysis of cigarette smoking, duration of smoking and average number of cigarettes per year were computed based on all smoking periods reported and dichotomized on the median for the control group. The average number of cigarettes was calculated as the annual cigarette smoking (filtered and unfiltered) plus 1.5 times annual *Yamuan* consumption. The 1.5 correction factor was used to allow for the bigger size of *Yamuan* compared with regular cigarettes [8,23]. The amount was divided based on the 50th percentile of the control group and categorized into non-smoker, low and high levels.

2.4. Alcohol consumption

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. Consumption of each subject was calculated as units of alcohol. A unit corresponds to 10 ml (approximately 8 g) of ethanol, and was determined by multiplying the volume of the drink (in milliliters) by its percentage and dividing by 1000 of the percentage of alcohol by volume was taken to be, for beer 5.0%, for *Sato* 7.0%, for white whisky 40% and for red whisky 35%.

2.5. Food consumption

The food frequency questionnaire was designed to include items that are common in the Thai diet [23]. For this study, the food frequency questionnaire consisted of 33 food items. The questions for each item consisted of consumption frequency in the four categories of non-consumer, <1/month and monthly, weekly, and daily, as well as amount (times) of consumption per unit of frequency. Analysis of types of dietary intake within the previous year was divided in three levels as never (non-consumer), low and high. Frequencies of each dietary intake and an amount of intake per year were computed based on each type of dietary intake reported and dichotomized on the median of the control group.

3. Laboratory methods

3.1. Specimen collection and DNA extraction

Blood samples (buffy coat) were available for 175 (80%) of 219 eligible CCA cases; and specimens were retrieved from the study bio-bank for them, and for 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).

3.2. PCR amplification and genetic polymorphisms detection

The polymerase chain reaction with high resolution melting analysis (PCR-HRM) technique of DNA amplification for *MTHFR* polymorphisms were performed in a 96-well plate in the Light-Cycler® 480 Real-Time PCR System 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 [24] to identify the three genotypes at each locus.

Amplification of *MTHFR* C677T and A1298C were modified as previously described [25]. 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 (Fig. 1(A) for *MTHFR* C677T and (B) for A1298C). Melting peaks of sequence variation were analyzed and compared with the wild-type sample. Different plot of melting

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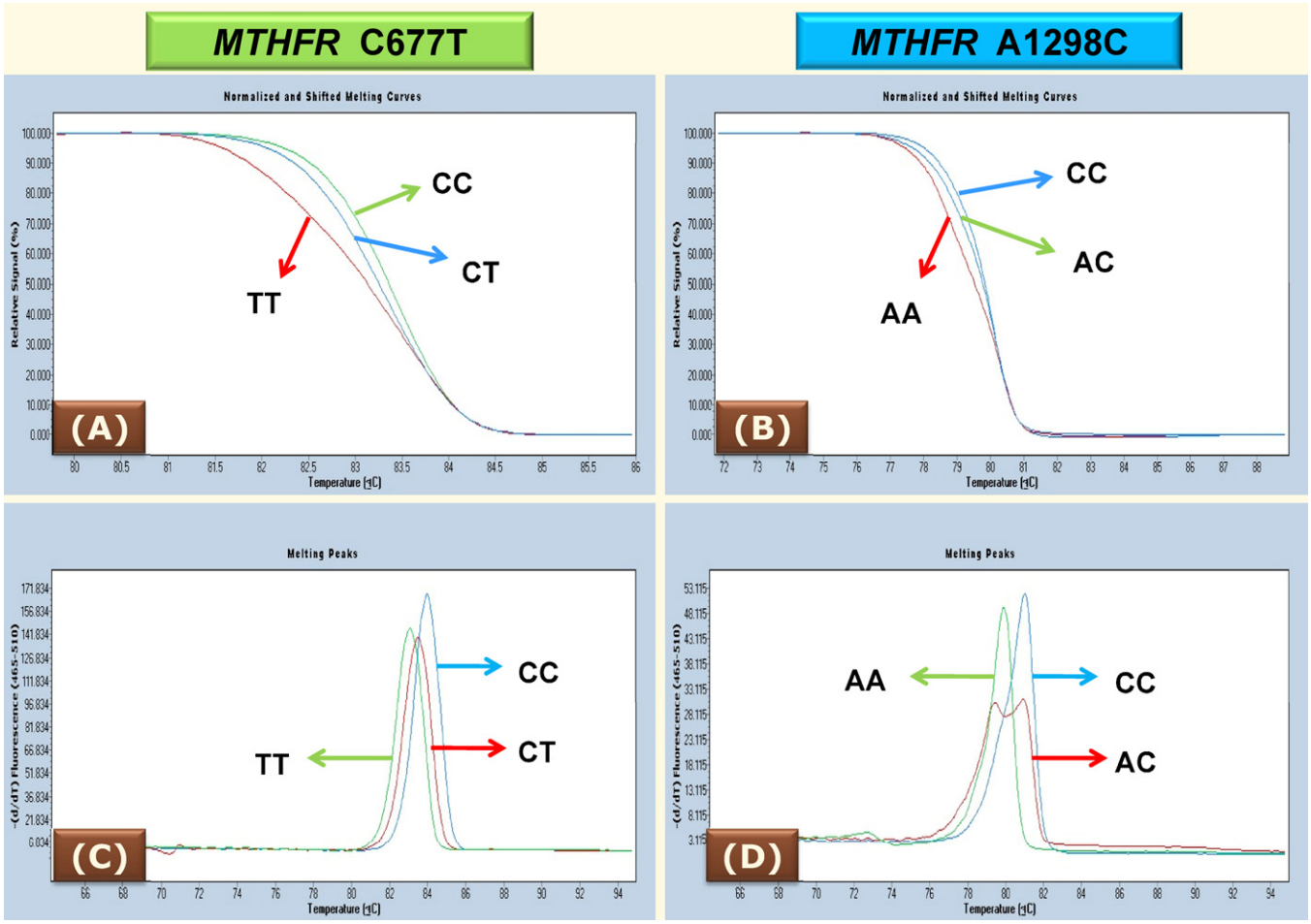

Fig. 1. Polymorphisms in *MTHFR* were analyzed using PCR with high resolution melting analysis.

peaks are illustrated in Fig. 1(C) for *MTHFR* C677T and (D) for A1298C.

3.3. Statistical analysis

To assess the strength of the associations between potential risk factors and risk of CCA, odds ratio (OR) with 95% confidence intervals (CIs) were estimated using conditional logistic regression. Univariate analysis was carried out to study the association between individual exposures and CCA risk using McNemar's Chi-square test and conditional logistic regression. Factors found to have a strong association with CCA in the univariate analysis ($P < 0.05$), and factors without association on univariate analysis but found to play important role as factors for CCA risk from the literature reviews were included in the multivariate analysis. Some of the dietary variables significant in univariate analysis could not be included because a multicolinearity with other apparently more relevant variables (dishes containing these dietary items). Possible modifications of the effects of potential risk factors by polymorphisms in *MTHFR* C677T and A1298C were also analyzed. A *P*-value <0.05 was considered statistically significant. All statistical analyzes were performed with a statistical package, STATA version 10 (Stata, College Station, TX).

4. Results

The case group comprised 92 females and 127 males, with a median age of 57 (minimum 31, maximum 69); there were two controls matched by age and sex for each case. Table 1 shows the distribution of *MTHFR* polymorphisms in cases and controls, and the associated odds ratios. There was an increased risk of CCA in individuals with the CC variant of A1298C. Table 1 also shows the joint distribution of the two polymorphisms. The combination of the CC variant of A1298 and the TT variant of C677 was significantly more frequent that expected among the controls (*P* for heterogeneity <0.001), but there were no interactions between the polymorphisms of *MTHFR* C677T and A1298C on CCA risk. In univariate analysis, there was a clear association with educational level: compared with the illiterate, subjects with primary school, and secondary or higher-level education had a reduced risk of CCA (Table 2). There were significant associations between cigarette smoking and alcohol consumption and risk of CCA (Table 2), and their joint effect appeared to be multiplicative with no interaction between them [data not shown]. With respect to the dietary variables, there were significant positive associations with consumption of freshwater fish (but not with sea fish, nor with shellfish), and with beef (but not with pork or poultry). Closer inspection showed that the association with beef was most marked – with clear dose–response effects – with beef prepared by fermentation (sour beef, *Naem*) and as beef sausage (*Sai-Krok*). Frequent consumption of the popular staple dish of Northeast Thailand – *Somtam* (raw papaya salad in fermented fish sauce) was also associated with increased risk, and a gradient of risk with increasing consumption was present. Conversely, frequent consumers of fruit and vegetables had a decreased risk of CCA.

Table 2 also shows the adjusted OR and 95% CIs from the multivariate analysis (including the 10 factors identified as increasing risk in univariate analysis). The factors that remained

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Table 1
Univariate analysis of polymorphisms of *MTHFR* on the risk of cholangiocarcinoma in Khon Kaen, Thailand.

Genetic polymorphisms		Cases		Controls		OR[a]	95% CI	*P*-value
		n = 175	%	*n* = 350	%			
MTHFR C677T polymorphism								
CC		74	42.3	160	45.7	1.0		
CT		57	32.6	110	31.4	1.1	0.74–1.74	0.55
TT		44	25.1	80	22.9	1.2	0.75–1.94	0.43
CT or TT (any T allele)		101	57.7	190	54.3	1.2	0.80–1.70	0.43
MTHFR A1298C polymorphism								
AA		84	48.0	189	54.0	1.0		
AC		60	34.3	125	35.7	1.1	0.73–1.64	0.67
CC		31	17.7	36	10.3	2.0	1.14–3.48	0.02
AC or CC (any C allele)		91	52.0	161	46.0	1.3	0.88–1.85	0.19
Joint effects								
MTHFR C677T	*MTHFR* A1298C							
CC	AA	43	24.6	96	27.4	1.0		
CC	AC	27	15.4	52	14.9	1.1	0.60–2.00	0.77
CC	CC	4	2.3	12	3.4	0.8	0.23–2.74	0.71
CT	AA	29	16.6	63	18.0	1.0	0.55–1.76	0.96
CT	AC	19	10.9	40	11.4	1.1	0.54–2.07	0.87
CT	CC	9	5.1	7	2.0	2.5	0.90–7.06	0.08
TT	AA	12	6.9	30	8.6	0.9	0.39–1.89	0.70
TT	AC	14	8.0	33	9.4	0.9	0.42–1.94	0.80
TT	CC	18	10.3	17	4.9	2.2	1.04–4.75	0.04
P-value for interaction								0.50

[a] Crude odds ratio from matched case–control analysis.

as significant risk factors were alcohol drinking, dish of raw freshwater fish (*Koi-Pla*) and beef sausage; while there were protective effects of total vegetable and fruit consumption.

Table 3 shows the results of interaction between *MTHFR* genotypes and some of the dietary items that appeared as risk factors for CCA in the analyzes above. There were interactions between polymorphism in *MTHFR* 677 and dish of semi-raw freshwater fish, sour beef and beef sausage (*P*-value for interaction 0.04, 0.004 and 0.01, respectively). For the *MTHFR* 1298, there were interactions with semi-raw freshwater fish and beef sausage with the same *P*-value for interaction (0.04). With respect to vegetables, fruit, or both, some of the combinations of polymorphisms and low consumption (same categories as in Table 2) were statistically significant (for example, the CC variants of both *MTHFR* 677 and 1298 and vegetable consumption), but there was no significant interaction overall. Neither was there any interaction between these polymorphisms and smoking or alcohol drinking.

Table 2
Distribution of cases and controls, and odds ratios for cholangiocarcinoma associated with demographic, lifestyle and dietary variables, and with *MTHFR* polymorphisms.

Variables	Cases		Controls		OR[a]	OR[b]	95% CI[c]	*P*-value
	n = 219	%	*n* = 438	%				
Education level								
Illiterate	26	11.9	15	3.4	1.0	1.0		
Primary school	185	84.5	398	90.9	0.3	2.6	0.56–12.02	0.22
Secondary school or higher	8	3.6	25	5.7	0.2	1.5	0.21–10.93	0.67
***MTHFR* C677T polymorphism**[h]								
CC	74	42.3	160	45.7	1.0	1.0		
CT	57	32.6	110	31.4	1.1	0.9	0.51–1.58	0.72
TT	44	25.1	80	22.9	1.2	0.9	0.42–1.80	0.71
***MTHFR* A1298C polymorphism**[h]								
AA	84	48.0	189	54.0	1.0	1.0		
AC	60	34.3	125	35.7	1.1	0.8	0.44–1.30	0.31
CC	31	17.7	36	10.3	2.0	1.3	0.58–3.02	0.51
Cigarette smoking								
Non-smoker	92	42.0	230	52.5	1.0	1.0		
Ex-smoker	36	16.4	53	12.1	2.8	1.3	0.56–3.14	0.52
Current smoker	91	41.6	155	35.4	2.8	1.2	0.49–2.82	0.71
Units of alcohol per month of all alcohol drinking								
Non-drinker	57	26.0	254	58.0	1.0	1.0		
<14	79	36.1	92	21.0	5.2	5.6	2.85–10.95	<0.001
≥14	83	37.9	92	21.0	6.7	9.5	4.55–19.79	<0.001
Dish of raw freshwater fish (*Koi-Pla*)[d]								
Non-consumer	41	18.7	153	34.9	1.0	1.0		
<1/month & monthly	79	36.1	196	44.8	1.2	1.6	0.74–3.49	0.23
Weekly	63	28.8	80	18.3	2.9	2.5	1.05–5.74	0.04
Daily	36	16.4	9	2.0	11.0	10.2	3.05–34.10	<0.001
Processed beef (beef sausage)[e]								
Non-consumer	20	9.1	89	20.3	1.0	1.0		
<1/month & monthly	39	17.8	92	21.0	1.8	1.0	0.37–2.56	0.97
Weekly	85	38.8	191	43.6	1.9	1.2	0.50–2.80	0.69
Daily	75	34.3	66	15.1	6.5	3.7	1.28–10.70	0.02
Frequency of *Somtam* consumption[d,e]								
Non-consumer	12	5.5	44	10.1	1.0	1.0		
<1/month & monthly	79	36.1	175	40.0	1.7	1.1	0.38–3.37	0.82
Weekly	98	44.8	176	40.2	2.2	0.9	0.33–2.53	0.87
Daily	30	13.6	43	9.7	2.8	1.9	0.55–6.62	0.31
Total vegetables[f] **(average times/month)**								
<52	136	62.1	214	48.9	1.0	1.0		
≥52	83	37.9	224	51.1	0.5	0.4	0.23–0.76	0.004
Total fruits[g] **(average times/month)**								
<35	131	59.8	217	49.5	1.0	1.0		
≥35	88	40.2	221	50.5	0.7	0.6	0.33–0.98	0.04

[a] Crude odds ratio from matched case–control analysis.
[b] Adjusted odds ratio from matched case–control analysis (adjusted for all other variables in the table).
[c] 95% confidence interval for OR.
[d] Source of liver fluke infection.
[e] Source of preformed nitrosamine, nitrate and nitrite contamination.
[f] Total vegetables: cabbage, lettuce, onion, parsley, morning glory, cucumber, eggplant, long bean and Thai seasonal vegetables.
[g] Total fruits: banana, water melon, melon, tamarind, mango and orange.
[h] DNA specimens for *MTHFR* genotyping were available for 175 out of 219 cases, and 350 out of 438 controls.

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Table 3
Gene–environmental interactions of *MTHFR* genotypes and dietary factors with the risk of cholangiocarcinoma in Khon Kaen, Thailand.

MTHFR 677	Dietary factors	Cases	Controls	OR[a]	95% CI	*P*-value	*P*-value[b]	*MTHFR* 1298	Dietary factors	Cases	Controls	OR[a]	95% CI	*P*-value	*P*-value[b]
		n = 175	*n* = 350							*n* = 175	*n* = 350				
677	Dish of semi-raw freshwater fish						0.04	1298	Dish of semi-raw freshwater fish						0.04
CC	≤Monthly	45	117	1.0				AA	≤Monthly	51	139	1.0			
CT	≤Monthly	31	77	1.0	0.55–1.67	0.88		AC	≤Monthly	37	89	1.0	0.61–1.68	0.97	
TT	≤Monthly	24	63	0.9	0.48–1.63	0.70		CC	≤Monthly	12	29	1.1	0.51–2.41	0.80	
CC	Weekly	20	37	1.3	0.69–2.61	0.38		AA	Weekly	22	46	1.3	0.72–2.45	0.36	
CT	Weekly	19	28	1.7	0.85–3.27	0.14		AC	Weekly	18	30	1.6	0.80–3.20	0.18	
TT	Weekly	14	16	2.3	0.97–5.24	0.06		CC	Weekly	13	5	8.9	2.42–32.51	0.001	
CC	Daily	9	6	3.5	1.13–10.58	0.03		AA	Daily	11	4	6.1	1.89–19.33	0.002	
CT	Daily	7	5	3.1	0.94–9.88	0.06		AC	Daily	5	6	1.9	0.56–6.53	0.30	
TT	Daily	6	1	13.6	1.60–115.56	0.02		CC	Daily	6	2	6.9	1.36–35.19	0.02	
677	Sour beef						0.004	1298	Sour beef						0.24
CC	≤Monthly	24	65	1.0				AA	≤Monthly	32	92	1.0			
CT	≤Monthly	19	57	0.8	0.41–1.73	0.64		AC	≤Monthly	19	52	1.0	0.49–1.91	0.93	
TT	≤Monthly	17	46	0.9	0.42–1.97	0.81		CC	≤Monthly	9	24	1.1	0.46–2.57	0.85	
CC	Weekly	23	64	0.9	0.47–1.80	0.87		AA	Weekly	31	64	1.5	0.80–2.84	0.20	
CT	Weekly	22	30	2.0	0.95–4.14	0.07		AC	Weekly	22	51	1.2	0.63–2.33	0.56	
TT	Weekly	19	29	1.6	0.73–3.40	0.25		CC	Weekly	11	8	3.9	1.37–11.23	0.01	
CC	Daily	27	31	2.8	1.30–5.80	0.01		AA	Daily	21	33	2.1	1.00–4.43	0.05	
CT	Daily	16	23	2.2	0.95–4.86	0.07		AC	Daily	19	22	3.0	1.35–6.61	0.01	
TT	Daily	8	5	5.0	1.45–17.15	0.01		CC	Daily	11	4	14.6	3.01–70.39	0.001	
677	Beef sausage						0.01	1298	Beef sausage						0.04
CC	≤Monthly	22	60	1.0				AA	≤Monthly	27	82	1.0			
CT	≤Monthly	20	46	1.1	0.51–2.37	0.82		AC	≤Monthly	19	43	1.3	0.63–2.55	0.51	
TT	≤Monthly	10	40	0.6	0.25–1.53	0.30		CC	≤Monthly	6	21	0.8	0.28–2.15	0.63	
CC	Weekly	22	68	0.9	0.45–1.83	0.80		AA	Weekly	32	79	1.3	0.71–2.45	0.39	
CT	Weekly	19	44	1.2	0.57–2.43	0.65		AC	Weekly	20	58	1.0	0.49–1.79	0.84	
TT	Weekly	25	36	1.6	0.80–3.31	0.18		CC	Weekly	14	11	3.8	1.48–9.89	0.01	
CC	Daily	30	32	3.3	1.51–7.07	0.003		AA	Daily	25	28	3.8	1.71–8.62	0.001	
CT	Daily	18	20	3.2	1.33–7.62	0.01		AC	Daily	21	24	3.5	1.56–7.85	0.002	
TT	Daily	9	4	8.3	2.23–30.82	0.002		CC	Daily	11	4	18.3	3.68–90.80	<0.001	

[a] Crude odds ratio from matched case–control analysis.
[b] *P*-value for interaction.

5. Discussion

The objectives of the present study were to investigate co-factors (genetic, lifestyle and diet) influencing the risk of CCA. OV is clearly the major risk factor explaining the frequency of CCA in Northeast Thailand, but this has been amply demonstrated in previous studies, and we were not interested in doing so again; therefore we matched cases and controls on OV status. Although the independent effect of OV cannot be evaluated with this design, it provides more efficiency in investigating other risk factors, alone or in combination with OV.

Smoking was not associated with CCA development in this present study (after adjustment for other variables in the multivariate analysis), which is consistent with the previous studies in Thailand [5,7,8,26] and elsewhere [27].

Alcohol drinking was significantly associated with an increased risk of CCA in previous studies in Thailand [5,7] and elsewhere [27,28]. Alcohol may affect metabolic pathways of endogenous and exogenous nitrosamines [7] and it may also exert its effect through associated deficiencies in nutrients, particularly folate [29]. In addition, it has been reported that alcohol (a folate antagonist) perturbs folate metabolism by reducing folate absorption, increasing folate excretion, or inhibiting methionine synthase [30]. The simultaneous removal of two uracil bases on opposite DNA strands may result in the formation of double-stranded breaks are important, because the accumulation of chromosome aberrations is a risk factors for cancer [31].

Consumption of fermented products (sour beef) or processed beef (beef sausage) was a significant risk factor for CCA in our study, but it is not clear result in the previous study [7]. It is possible that the previous study included the salty fish or meat, and fermented fish or pork in part of analysis but our study analyzed each variable separately.

Frequent consumers of fruit and vegetables had a tendency to have a decreased risk of CCA, as had been observed in previous reports [8,26]. Although the mechanism by which fruit and vegetables protect against different cancers is still not entirely clear, several lines of evidence suggest that this may be the result of many processes in restraining of endogenous carcinogenic formation, enzyme alteration, modification of carcinogen bioavailability, activation of activity in antiviral and antibacterial mechanisms, and strong stimulation of the immune system [32]. Fruit and vegetables are also important sources of vitamin C, which is effective in inhibiting endogenous nitrosamine production in subjects infected with OV [33].

In this present study, the genotype frequency of *MTHFR* 677 (T allele) was 54.3% in controls, which corresponds to a study from another Asian country – Korea (prevalence of T allele was 66.2%) [22]. Although the relationship between *MTHFR* A1298C polymorphisms and the risk of CCA have not been studied at all, our study showed the prevalence of C allele of *MTHFR* 1298 polymorphisms was 46% in controls.

Several studies have explored the associations between *MTHFR* polymorphisms and the risk of cancers, but they have showed inconsistent results. It may be the consequence of the various modifying effects that *MTHFR* polymorphisms have on the balance between DNA methylation and synthesis. The balance may be determined by lifestyle and dietary factors, especially alcohol (a folate antagonist), smoking (which impairs folate level), as well fruit and vegetables (dietary folate sources). Imbalance between DNA methylation and synthesis may cause tumor progression; for example, in subjects with the *MTHFR* 1298 CC variant and low

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folate intake both DNA methylation and DNA synthesis may be impaired and may increase CCA risk (in our study the OR was 2.4, 95% CI: 1.11–4.97). However when dietary folate intake is adequate, *MTHFR* 677 and 1298 wild-types may protect against CCA due to the sufficiency of enough methyl donors and promotion of DNA synthesis.

In conclusion, in a nested case–control within the KKCS covering the high-risk population, we have examined lifestyle, diet, and polymorphisms in *MTHFR* C677T and A1298C in relation to CCA risk, and we have found strong evidence that certain lifestyle and dietary factors play an important role in the etiology of CCA independently of the major risk factor, infection with OV. Importantly, our results also strongly suggest that polymorphisms in *MTHFR* genotypes act together with alcohol drinking and low folate intake to increase the risk of CCA. Primary prevention of CCA is based upon efforts to reduce infection with OV, particularly by discouraging consumption of foodstuffs containing raw or inadequately fermented freshwater fish. However, this has proved difficult to achieve, and the availability of simple and effective treatment for OV (Praziquantel) has resulted in chronic OV infection being replaced by a pattern of repeated cycles of infection and cure. Preventive measures might therefore also include encouraging the inhabitants in high-risk areas to increase consumption of vegetables and fruit and reduce consumption of alcohol and preserved meats. These actions would have the additional advantage of a preventive effect on other cancers and cardiovascular disease.

Conflict of interest

No potential conflicts of interest were disclosed.

Acknowledgements

This research was supported by the Office of the Higher Education Commission (N. Songserm was granted under the Strategic Scholarships for Frontier Research Networks for Ph.D. Program Thai Doctoral degree), the Thailand Research Fund (Research grant No. 530702), and Graduate School of Khon Kaen University. This work has also been supported by the UICC Yamagiwa-Yoshida Memorial International Cancer Study Grant (2010 YY1 Spring Selection) to enable N. Songserm to enhance levels of knowledge in cancer epidemiology at the Clinical Trial Service Unit & Epidemiological Studies Unit, University of Oxford, UK.

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