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

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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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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 ($\pm$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