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Association of N-acetyltransferase-2 Phenotype and CYP1A2 Activity with Cholangiocarcinoma in Thailand

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Abstract

Background/Aims: Arylamine N-acetyltransferase-2 and cytochrome P450 1A2 have been implicated in the carcinogen activation and conferred cancer susceptibility. Present study was set to determine whether acetylation phenotype and P450 1A2 were associated with cholangiocarcinoma.

Materials and Methods: Ninety-six unrelated Thais were recruited for the study: 52 healthy controls and 44 cholangiocarcinoma patients. The acetylation phenotype was assessed and P450 1A2 activity was determined in vivo using the caffeine metabolic ratio (CMR) after oral administration of a cup of coffee.

Results: The caffeine metabolic ratio in the control and patient groups varied widely and the frequency distribution was apparently non-normal. The CMR value was higher in the controls particularly in non-smokers. Taken individually rapid acetylator status was not significantly associated with cholangiocarcinoma, however the joint effect of acetylation and P450 1A2 activity resulted in a presented risk of cancer, odds ratio=3.5 (95% confident interval,1.0 to 12.1)

Conclusions: Arylamine N-acetyltransferase-2 and cytochrome P450 1A2 may play a role in presenting susceptibility to cholangiocarcinoma in Thai population.

The population of Northeast Thailand has one of the highest known rates of cholangiocarcinoma (CCA).[1] Potential risk factors for cholangiocarcinoma include opisthorchiasis[2,3] and endogenous nitrosation.[4,5] However, synergy between exposure to chemical carcinogens and infestation with the liver fluke Opisthorchis viverrini has been demonstrated in hamster models of hepatocarcinogenesis.[2,6] Xenobiotic metabolizing enzymes, cytochrome P450 2A5 (CYP2A5), a member of the cytochrome P450 superfamily, had been demonstrated to be up-regulated in the livers of fluke-infested hamsters.[7] Recently, CYP2A6 and CYP2E1 were also found up-regulated in CCA human liver tissues.[8] This may indicate xenobiotic metabolism and the genetic constituents of an individual partly confers the susceptibility to the cancer. Among the carcinogen metabolizing enzymes, arylamine N-acetyltransferase-2 (NAT2), one of the two NAT isoenzymes in humans, encodes the classical acetylation polymorphism yielding rapid, intermediate and slow acetylator phenotypes[9] and CYP1A2- the P450 enzyme, which primarily introduces or exposes a functional group on the drug molecule. These two enzymes are involved in the metabolic activation of several carcinogens, including aromatic and heterocyclic amines present in industry, cooked food and as environmental contaminants.[10,11] The metabolic activation pathways associated with carcinogenic aromatic and heterocyclic amines have been shown involved in N-oxidation, catalyzed primarily by CYP1A2, and subsequent O-esterification, often catalyzed by acetyltransferase-2 to form the N-acetoxy arylamine[12,13] that binds to the DNA to give carcinogen-DNA adducts.[14,15] Epidemiological studies support the notion that arylamine N-acetyltransferase polymorph-ism is associated with cancers of the colon, bladder, oral cavity and breast.[16-20]

On the basis of this metabolic activation pathway, individuals who possess both the rapid NAT-2 and rapid CYP1A2 phenotypes may be at a higher risk of developing certain types of cancer. The objective of this study was to assess the association of CYP1A2 activity and NAT2 phenotype polymorphism with CCA. In this preliminary report, NAT2 polymorph-ism was assessed by phenotyping using a well known NAT2 substrate, sulfamethazine.[21] CYP1A2 activity was determined in vivo by using caffeine as a metabolic probe[22,23] and the caffeine metabolic ratio (CMR) was employed as an index for individual CYP1A2 activity.

Materials and Methods

Subjects

Included in our study were 96 unrelated Thais living in the northeast Thailand: 52 control and 44 patients. To avoid confounding effects due to ethnic origin, our subjects were carefully recruited to be a relatively homogenous sample of Thais; subjects known from the interviews to be Chinese or other minority ethnic groups in this region were excluded. All of the patients were admitted to Srinagarind Hospital between October 1997 and December 1999 and were diagnosed as having intrahepatic cholangiocarcinoma (CCA) by histological criteria where liver specimens were obtained from partial hepatectomies. Control subjects were apparently healthy subjects with no current or previous diagnosis of any chronic diseases and were randomly selected from individuals who were of a similar aged as the CCA group, i.e. between 33-74 year old. All subjects were interviewed by investigators using a structured questionnaire that entailed personal history, previous illnesses, pertinent habits such as smoking, alcohol consumption and raw fish consumption and current drug usage. All participants were given an explanation of the study and written informed consent was obtained. The studied protocol was approved by the Ethical Research Committee, Khon Kaen University.

N-acetyltransferase-2 phenotype and CYP1A2 activity index were assessed in all subjects. They were asked to refrain from consuming any caffeinated food or drinks for at least 48 hours prior to the study. After fasting overnight, subjects were asked to drink a cup of coffee containing 3.3 mg of caffeine per kg body weight and a 250 mg capsule of sulfamethazine (USP) with a glass of water. Blood and urine samples were collected at 6 hours. Plasma samples were used to determine CYP1A2 activity by using an index of CMR which was calculated as the molar ratio of paraxanthine (PX) and caffeine: CMR = PX / Caffeine, where PX is the major metabolic product of caffeine metabolism.[24, 25]

Analysis

Caffeine and its metabolites were analyzed by

high performance liquid chromatography method previously described[23] with some modifications. Briefly, 400 mg of ammonium sulfate and 100 microliter of 0.1 M HCl containing β-hydroxyethyltheophylline as the internal standard were added into 300 microliter of sample and mixed well. The deproteinized sample was extracted with dichloromethane and isopropranol (85:15). The organic layer separated after centrifugation was evaporated by a heat block under a gentle stream of N_2 gas until dry. The residue was reconstituted with the mobile phase consisting of 10 mM ammonium acetate buffer pH 5, methanol and tetrahydrofuran (92:6.5:1.5) and injected into a Resolve column (C18, 5 µm, 3.9 × 300 mm), Waters Millipore Corporation, Milford, USA., equipped with an ultraviolet detector (Waters 490E Programmable multi-wavelength, U.S.A.) set at 276 nm. Within-day variation and between-day variation of the assays were assessed and found within 5 per cent.

Degree of acetylation capability was determined in the urine sample. Sulfamethazine and acetylated sulfamethazine were determined by a previously described method.[21] Acetylation capability was calculated as the percentage of acetylation of sulfamethazine. Frequency distribution plots of the percentage of acetylation and the number of subjects had a bimodal distribution with an antimode about 70 per cent, similar to our previous report.[26] Individuals with a degree of acetylation greater than 70 per cent were classified as the rapid (NAT2) acetylator phenotype, while those with a lesser degree were classified as the slow acetylator phenotype.[27]

Statistical Methods

Results are reported as the mean ± SD and compared using the Mann-Whitney U test and Student t-test. The data were examined for normality of distribution using probit plots, skewness and kurtosis and the Kolomogorov-Smirnov tests for normality. As smoking is known to modify CYP1A2 activity and fresh-water fish may be infested with the liver fluke, a known CCA risk,[6] cigarette smoke and fresh-water fish consumption habits in subjects were stratified in two levels, i.e. "non-smokers" (non-smokers + ex-smokers) versus "current smokers" and "well cooked" versus "raw" (mixed practices of well-cooked and raw), respectively.

To determine the effects of CYP1A2 (CMR index) and NAT2 phenotype on CCA, a multiple logistic regression was applied. Since both CMR and NAT2 are intrinsically continuous variables, they were examined separately for their linear relationship with CCA by plotting their values against log odds of CCA.[28] There was a clear departure from linearity for CMR and dichotomization for the data suggested by Mazumdar and Glassman[29] was the most appropriate method. The cut point for such dichotomization was estimated at 0.24. For NAT2, there was no clear pattern regarding linearity. Thus it was initially entered into the model as continuous. Later it was dichotomized as it did not affect the findings but simplified interpretation. The cut point was chosen based on the distribution of the data which appeared to be bimodal. Potential confounders included age, gender, body mass index, fish process, and cigarette smoking. Systematic investigation for interaction effects were done using both stratified analysis and model-fitting based on methods suggested by Kleinbaum.[30] All analyses were done using STATA statistical software. The level of significance was 0.05. All of the tests were two-sided.

Results

With regards to smoking status, fresh-water fish consumption practice and the prevalence of HBsAg positive (Table 1), subjects in both groups were not significantly differences. The caffeine metabolic ratio representing CYP1A2 activity varied 5.5 folds in control group, while this ratio in the patient group varied widely (i.e.up to 70-fold). Even when the outliers were

Table 1 Demographic data of subjects in the study

	CHCA	Control
Age (mean ± SD, in year)	53.9 + 10.0	51.4 + 9.7
Gender		
Female	16	10
Male	28	42
Smoking status		
Non-smoker/Ex-smoker	33	42
Current smoker	10	10
Fish consumption		
Cooked	5	12
Raw	39	40
HBsAg (+ve)	2/44	2/52

excluded, the variation was still as much as 17-fold. Frequency distributions of CMR in both groups were examined and did not appear to be normal. The Kolomogorov-Smirnov (K-S) statistical tests for normality indicated that CMR was significantly, non-normally distributed (for CCA group: K-S distance = 0.205, p = <0.001 and control group: K-S distance = 0.135, p = 0.019). The CMR of the controls was higher than in the CCA subjects (Figure 1, p = 0.004). When subjects were stratified according to their cigarette smoking habits, the CMR between the control and the CCA in smoker groups showed no significant difference (p = 0.21). However, for the non-smokers, the CMR was higher in the control group than in the CCA group (p = 0.004). The CMR was further examined to any association with the physiological parameters, however the CMR was evidently not affected by age or body mass (Table 2). The CMR is correlated weakly with liver function tests in the CCA group. However, if patients with evidence of jaundice were excluded, the

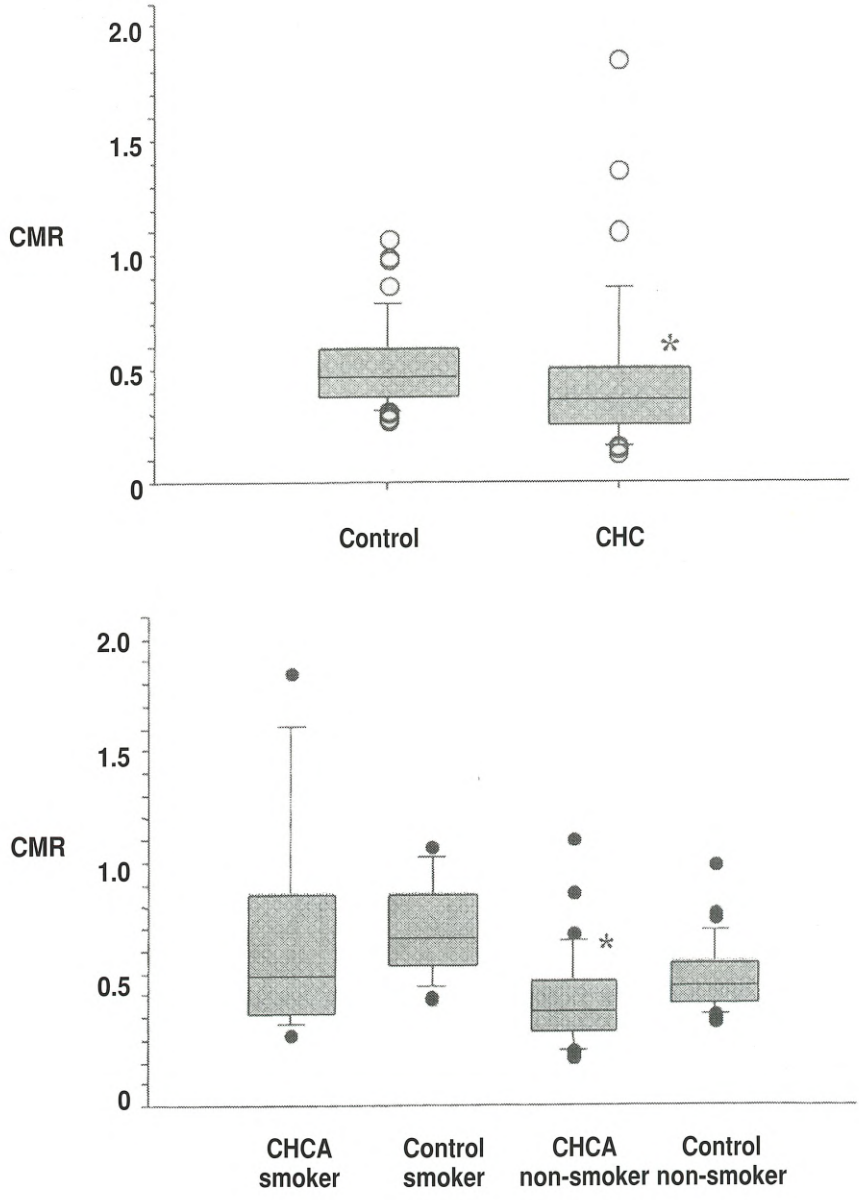

Fig. 1 Box plots of the CMR between control and CHCA subjects (top). Subjects were stratified according to smoking status (below).

Table 2 Correlation of the CMR and other physiological parameters

Parameters	CHCA		Control	
	r (n)		r (n)	
Age	0.000[a] (44)	P = 0.97	0.249 (52)	P = 0.08
BMI	0.002 (44)	P = 0.90	0.063 (52)	P = 0.65
Albumin	0.421 (44)	P = 0.004	0.02 (52)	P = 0.89
Total bilirubin	0.318 (40)	P = 0.046	0.232 (33)	P = 0.19

[a]*Pearson's correlation coefficient*

Table 3 Effect of NAT2 on CHCA according to the level of the CMR, adjusted for the effect of gender.

Effect of CMR	Cases (n = 44)	Controls (n = 52)	Crude OR	Adjusted OR	95%CI for adjusted OR
CMR 0.24					
NAT2 = rapid	82.6%	55.8%	3.8	3.5	1.0 to 12.1
NAT2 = slow	17.4%	44.2%	1	1	
CMR <0.24					
NAT2 = rapid	57.1%	77.8%	0.4	0.4	0.1 to 2.6
NAT2 = slow	42.9%	22.2%	1	1	

Likelihood ratio test for the effect of the interaction effect p-value = 0.04

correlation of the CMR and albumin or bilirubin was no longer discernible (r = 0.28 and 0.18 with p = 0.12 and 0.33, respectively for n = 33).

NAT2 phenotype among the controls was 60 per cent rapid acetylators, among the CCA group was 70 per cent, slightly higher than the control (by χ^2 test with p = 0.27). Subjects were subsequently classified into high CMR and low CMR values by using a cut-point of CMR = 0.24 as described in the Materials and Methods. Accordingly, 52 per cent of the CCA would belong to the high CMR group, while that for the controls was 83 per cent. The difference between two groups was statistically significant (χ^2 test, p = 0.001). The NAT2 alone was not significantly associated with CCA (p = 0.268) whereas the CMR was (p = 0.001). Their joint effect, however, suggested CMR was a significant effect modifier of the relationship between NAT2 and CCA (p = 0.03). That is, patients who have CMR 0.24 and rapid NAT2 phenotype demonstrated a higher risk of CCA (OR = 3.8; 95% CI: 1.0 to 12.3). In contrast, patients who have CMR <0.24, NAT2 = 1 demonstrated a protective effect against CCA (OR = 0.4; 95% CI: 0.1 to 2.1; Table 3).

In a multivariable analysis using logistic regression, age, gender, body mass index, fish process, and cigarette smoking caused no significant effect on the effects of CMR or the NAT2 on CCA. Gender was found unbalanced between the case and control groups and is known to be related to smoking, thus it was retained in the final model. By adjusting for the effects of gender, the interaction between the NAT2 and the CMR remained statistically significant (0.04). Among those who have CMR 0.24 and rapid NAT2 demonstrated a risk effect on CCA (OR = 3.5; 95%CI: 1.0 to 12.1). On the contrary, patients who have CMR <0.24, rapid NAT2 demonstrated a protective effect against CCA (OR = 0.4; 95% CI: 0.1 to 2.6).

Discussion

Arylamine N-acetyltransferase 2 is reportedly implicated in various types of malignancy, where slow acetylator phenotype is associated with bladder cancer[31,32] and rapid acetylator to colorectal cancer.[33,34] The mechanism for this association suggests that rapid acetylators are able to activate N-hydroxy-heterocyclic amine carcinogens more readily within the colon to their ultimate carcinogenic forms.[35] Moreover,

colorectal cancer risk is strongly elevated in individuals with the combined rapid phenotypes for CYP1A2 and NAT2, since CYP1A2 may be responsible for heterocyclic amine N-oxidation, a step toward the acetylation by NAT2.[15,36] Our present study showed a similar frequency of rapid acetylator as in a previous report[26] and a small increase in rapid NAT2 acetylator phenotype in CHCA. However when the analysis was restricted to the high CMR group, rapid acetylators significantly elevated the risk of CCA. This suggests that an interplay between the two phenotypes is necessary to confer such susceptibility to CCA.

Activity of CYP1A2 varied considerably among individuals. Genetic polymorphism of CYP1A2 has been reported but its relationship to phenotype is still unclear.[37] More recently, there are reports of single point mutations on the untranslated region of CYP1A2 which cause either a decrease of CYP1A2 activity[38] or an increase of the inducibility of CYP1A2 in cigarette smokers.[39] Nonetheless, the CMR is used as a reliable index for CYP1A2 activity[40] Environmental factors that could contribute to the variation are the exposure to inducers including tobacco smoke, grilled meat and cruciferous vegetables[37,41,42] and pathological status. Our results are consistent with previous reports that cigarette smoking is a strong inducer of CYP1A2.[43,44] CCA patients possessed a CYP1A2 activity comparable with the controls among the smokers, but the activity was lower in non-smokers. This suggests that the expression of CYP1A2 in the basal state of CCA is depressed. Factors that may be account for a depressed CYP1A2 expression include liver diseases.[45] Moreover, oxidative stress and various cytokines released during inflammatory conditions also are known to down-regulate the expression of CYP1A2.[46] It is conceivable that CHCA patients may be affected to a certain degree by oxidative stress.

Interpretation of present study was complicated by the fact that the CYP1A2 activity phenotype in CCA is probably affected by either intrinsic genetic background or concurrent disease. By excluding the outlier values from the logistic analysis of non-jaundice intrahepatic cholangiocarcinoma to assess the risk was largely unchanged. Cholangiocarcinoma may be diverse in aetiology and have marked differences in prognosis.[47,48] Since the number of cases in present study was limited, it is not possible to assess the association of other types of CCA with the present risk factors. In conclusion, present study suggests an association of the rapid NAT2 acetylator to the risk of cholangiocarcinoma in individuals with high CYP1A2 activity.

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