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NAT2 fast acetylator genotype is associated with an increased risk of lung cancer among never-smoking women in Taiwan

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Abstract

The correlation between cooking oil fumes, containing relatively higher amounts of heterocyclic amines, and female lung cancer has been revealed. The association of genetic polymorphisms of CYP1A2 and NAT2, two major enzymes responsible for the metabolism of heterocyclic amines, with lung cancer has been investigated with inconclusive results. In this study targeted on never-smoking population with 162 lung cancer patients and 208 non-cancer controls, while the distributions of CYP1A2 phenotypes in lung cancer patients were comparable to that in controls, NAT2 fast acetylators had an OR of 2.44 (95% CI 1.40–4.23, $P=0.002$) and 2.56 (95% CI 1.37–4.80, $P=0.003$) for lung cancer in overall and female cases, respectively, but not in males. These results suggested never-smoking females with NAT2 fast acetylator were more prone to lung cancer and reflected the possibility that exposure to heterocyclic amines may contribute to the female lung cancer development in Taiwan.
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1. Introduction

Lung cancer is a major cause of mortality worldwide, and it is the leading cause of cancer death with a steady increase rate every year in Taiwan [1,2]. Previous studies have pointed out that lung cancer was

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mainly caused by smoking [3], as observed in about 90% lung cancer incidence in western countries. However, the majority of Taiwanese female lung cancer patients with adenocarcinoma, were never-smokers while smoking has been referred to be the major causative factor for squamous carcinoma. Furthermore, an increasing incidence of adenocarcinoma without any increase in smoking percentage was observed for female patients. It was therefore reasonable to speculate that environmental factors other than cigarette smoking were involved in lung tumorigenesis in Taiwan.

Previous epidemiological studies have revealed the involvement of various environmental factors other than smoking in lung cancer, such as environmental exposure to passive smoking and cooking oil fumes (COF) [4]. Among those complicated ingredients in environmental exposures, aromatic heterocyclic amines (HCAs) are the major carcinogens and have been related to various human cancers, for examples, β-naphthylamine and 4-aminobiphenyl were considered to be associated with bladder cancer while HCAs was related to colon cancer [5–6]. Furthermore, heat-cooking meats and generated COF both contained a lot of HCAs [7–8] which had been confirmed to be mutagenic and carcinogenic in vivo and in vitro [9–10]. Importantly, most of HCAs were firstly subjected to an N-hydroxylation by hepatic cytochrome P450, mainly CYP1A subfamily, while CYP1A2 has a highest specificity and ability for N-hydroxylation. HCAs may also be directly oxidized in various tissues and then esterified by phase II enzymes, mainly NAT2, and eventually covalent bound with DNA after de-esterification to form nitrieum ion. Therefore, CYP1A2 and NAT2 both are crucial enzymes in the metabolic activation of heterocyclic amines [11–16].

Studies on molecular epidemiology have shown that inherited genetic traits may influence the susceptibility of an individual exposed to a specific toxic chemical. Numerous studies have also suggested various genetic polymorphisms for CYP1A2 and NAT2, which have great impacts on their expression levels and were involved in occurrences of various cancers. Studies have further revealed a higher risk for colorectal carcinoma of rapid metabolic phenotypes of CYP1A2 and/or NAT2 while slow NAT2 acetylator has been related to bladder cancer and prostate cancer,

and fast acetylators has been associated to breast cancer [17–24]. As for lung cancer, conflicting results have been reported [25–29] and these inconsistent results could be due to differences in ethnics, life styles or diets. Although a study of small scale has revealed that Chinese women with slow NAT2 and rapid CYP1A2 activity were at the highest risk for lung adenocarcinoma [25], the relationships of these polymorphisms with lung cancer were still uncertain. This study was aimed to investigate with a larger and never-smoking population to obtain a more comprehensive conclusion on this issue and to clarify the involvement of HCAs in female lung cancer in Taiwan.

2. Materials and methods

2.1. Study subjects

A total of 162 never-smoking patients with primary lung cancer were recruited from Veterans General Hospital-Taichung and Chung Shan Medical University Hospital into this study together. All cases also underwent a series of examination of pathological stages by board-certified pathologists. Meanwhile, 208 never-smoking controls with no history of cancer were collected from a community health survey. Demographic data for each individual covering age and gender were collected from patient interview and a review of the hospital charts with informed consent.

2.2. PCR-RFLP analysis for CYP1A2 and NAT2 genetic polymorphisms

Blood samples for every subject were collected intravenously and genomic DNA was extracted from peripheral blood cells by a conventional method and eventually dissolved in 20 μl of sterile distilled water. As for CYP1A2 genotyping, a PCR-RFLP method modified from a previously described procedure [30] was conducted. In each PCR reaction, 500 ng of DNA was added to a PCR mix containing 10 pmole each primer, 0.5 mM dNTPs, 5 μl PCR $10\times$ reaction buffer and 2.5U Taq polymerase and the used CYP1A2 primers were S (5′-GCTACACATGATC-GAGCTATAC) and As (5′-CAGGTCTCTTCACTG-TAAAGTTA). After an amplification of 30 cycles, PCR products were subjected to a DdeI digestion
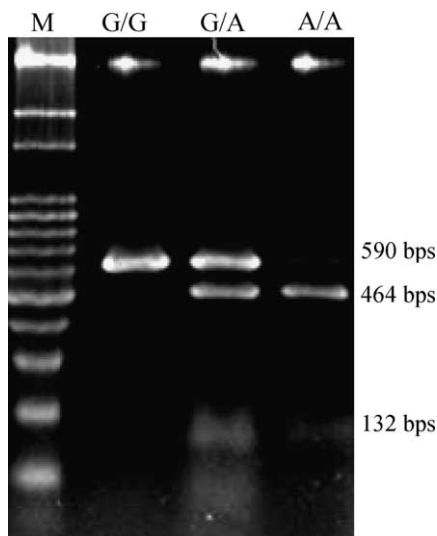
Fig. 1. PCR-RFLP analysis for CYP1A2 genotypes. Extracted genomic DNAs were subjected to PCR amplification specific for a CYP1A2 region followed by a DdeI digestion and then electrophoresis on a 2% agarose gel. The presence of a band (596 bps), 3 bands (596, 464, and 132 bps), and 2 bands (464 and 132 bps) indicated homozygous GG, heterozygous GA and homozygous AA, respectively.

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Genotyping analysis of *NAT2* polymorphism was conducted according to a previously described method [31]. Briefly, prepared genomic DNA was firstly amplified using the primers N5 (5′-GGAA-CAAATTGGACTTGG) and N4 (5′-TCT AGCA T GAATCACTCTGC) and then PCR product of 10 μl was subjected to a digestion with *KpnI* (M1 allele), *TaqI* (M2 allele), *BamHI* (M3 allele) or *MspI/AluI* (M4 allele). Restriction enzyme digested product was loaded onto a 2 or 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. The alleles detected were: *NAT2*4* (wild type); and the low-activity alleles, including *NAT2*5* (M1), NAT2*6 (M2), NAT2*7 (M3), NAT2*14 (M4). As previously defined [30–31], based on the number of low-activity alleles they carry, each individual was classified as a rapid acetylator (carrying 0 or 1 low-activity allele) or slow acetylator (carrying 2 low-activity alleles).

2.3. Statistical analysis

Statistical analysis of frequency distributions was done by χ^2-test, and the correlations between various genotypes of CYP1A2 and/or NAT2 and clinico-pathological parameters were analyzed by statistical software SPSS 10.0. Adjusted odd ratios (ORs) and a 95% confidence interval (95% CI) on lung cancer were evaluated for various factors using a multiple logistic regression model.

3. Results

3.1. The distribution of age, gender, CYP1A2 and NAT2 genetic polymorphisms in lung cancer cases and controls

In this case-control study targeted on never-smoking patients, 208 non-cancer controls, including 57 males and 151 females (with a mean age of 53.7 ± 10.9), and 162 primary lung cancer patients

Table 1
Distribution of CYP1A2 and NAT2 polymorphisms and related OR values for lung cancer in cases and controls

	Cases	Controls	Adjusted OR[a] (95% CI)
	$N = 162$	$N = 208$	
CYP1A2[b]			
High	88 (54.3%)	115 (55.3%)	0.90 (0.57–1.41)
Low	74 (45.7%)	93 (44.7%)	1.00
Female	109	151	
High	59 (54.1%)	79 (52.3%)	1.01 (0.59–1.71)
Low	50 (45.9%)	72 (47.7%)	1.00
Male	53	57	
High	29 (54.7%)	36 (63.2%)	0.67 (0.28–1.61)
Low	24 (45.9%)	21 (36.8%)	1.00
NAT2			
Fast	135 (83.3%)	144 (69.2%)	2.44 (1.40–4.23)
Slow	27 (16.7%)	64 (30.8%)	1.00
Female	109	151	
Fast	88 (80.7%)	99 (65.6%)	2.56 (1.37–4.80)
Slow	21 (19.3%)	52 (34.4%)	1.00
Male	53	57	
Fast	47 (88.7%)	45 (78.9%)	1.70 (0.51–5.71)
Slow	6 (11.3%)	12 (21.1%)	1.00

[a] OR had been adjusted for age.
[b] For CYP1A2, homogenous G/G genotype is referred to be a phenotype with high activity while G/A or A/A phenotype is regarded to be a low phenotype.

(126 adenocarcinoma, 34 squamous cell carcinoma, and 2 small cell carcinoma), including 53 males and 109 females (with a mean age of 63.2 ± 11.4), were recruited to study the involvements of genetic polymorphisms of CYP1A2 and NAT2 in lung tumorigenesis. The gender distribution in case group was comparable to that for the control groups.

Genomic DNAs, extracted from peripheral lymphocytes, were subjected to PCR amplification specific for a CYP1A2 region followed by a DdeI digestion as representative results shown in Fig. 1. As for activity, homozygous GG was referred to be high while G/A or AA was regarded to be low [28]. From the subsequent statistical analysis, the distributions of CYP1A2 high or low phenotypes in lung cancer patients were comparable to that in controls ($P = 0.85$, χ^2-test; Table 1). As for NAT2 genetic polymorphisms, representative data for M1–M4 allele in the PCR-RFLP analysis was shown in Fig. 2. Statistical analysis showed that the prevalence of these 4 variant alleles, M1–M4 was 2.4, 16.5, 9.9 and 0% in controls and 1.1, 13.8, 13.5 and 0% in cases, respectively. As for phenotypes, including fast (with 0 or only one variant allele) and slow (with 2 variant alleles), the prevalence of fast acetylators in cases were significantly higher than that in controls (83.3 vs. 69.2%;
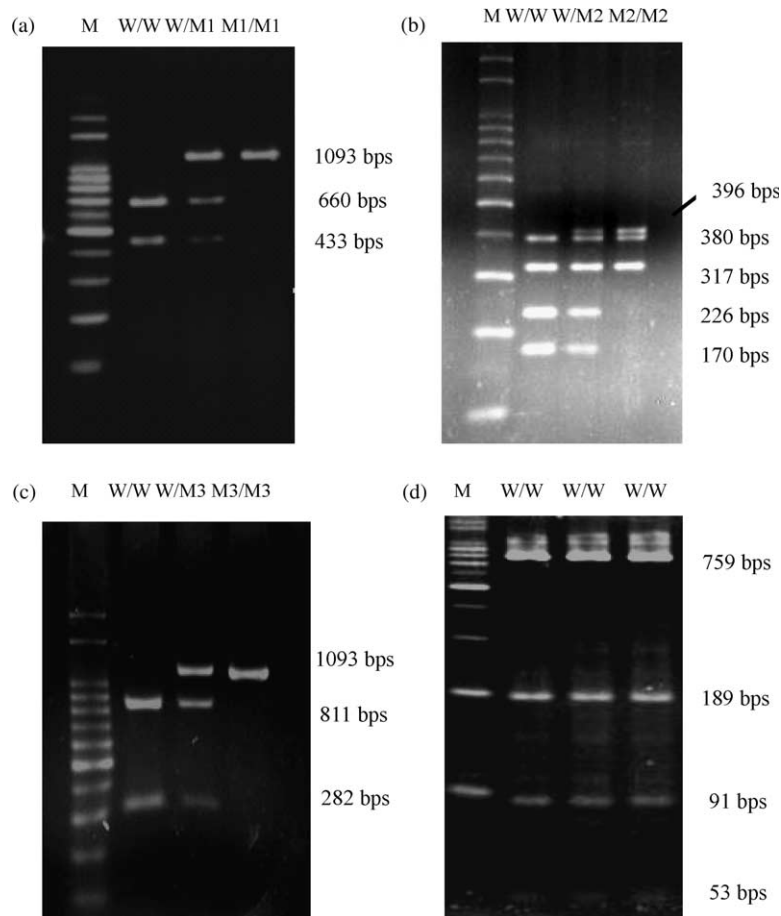

Fig. 2. PCR-RFLP analysis for NAT2 genetic polymorphisms. After PCR amplification, products were subjected to a digestion with KpnI (for M1 allele), TaqI (for M2 allele), BamHI (for M3 allele) or MspI/AluI (for M4 allele). Restriction enzyme digested product was loaded onto a 2 or 3% agarose gel. Representative results for various polymorphisms were shown in figures as indicated.

Table 2
The risk of NAT2 phenotypes for various types of lung cancer

NAT2 phenotype	Adenocarci-noma	Squamous carcinoma	Controls
	$N=126$	$N=34$	$N=208$
Fast	103 (81.7%)	30 (88.2%)	144 (69.2%)
Slow	23 (18.3%)	4 (11.8%)	64 (30.8%)
OR (95% CI)[a]	2.21 (1.24–3.95)[b]	3.01 (0.93–9.77)[c]	1[Ref]

[a] The OR refers to the risk of fast phenotype of NAT2 for lung cancer compared to that of slow phenotype with that in controls being 1.
[b] OR had been adjusted for age.
[c] OR had been adjusted for age and gender.

$P=0.002$). Furthermore, individuals with fast phenotypes experienced an elevated risk for lung cancer (OR 2.44, 95% CI 1.40–4.23), compared to those with slow phenotypes.

3.2. Gender difference in the distribution of NAT2 phenotypes and corresponding risk for lung cancers

Based on the significant difference in the distribution of NAT2 phenotypes between lung cancer cases and controls, a further analysis was performed by stratifying study subjects by gender. For females, the prevalence of fast acetylators in lung cancer cases was still higher than that of controls and experienced a significant OR of 2.56 (95% CI 1.37–4.80, Table 1). However, no statistical difference was observed for males (88.7 vs. 78.9% for cases vs. controls). In the other hand, such difference between genders was not observed for CYP1A2 since a comparable distribution

of CYP1A2 was still present for male or female (Table 1).

3.3. The risk of NAT2 phenotypes for various types of lung cancer

Following the discovery of gender difference in the distribution of NAT2 phenotypes, we further analyze if the distribution was different between various tumor types As the result shown in Table 2, the prevalence of fast acetylators in lung adenocarcinoma cases was significantly higher than that of controls (81.6 vs. 69.2%), and individuals carrying fast acetylator had an OR of 2.21 (95% CI 1.24–3.95, $P=0.007$) for lung adenocarcinoma, compared to those carrying slow acetylator. As for squamous carcinoma, individuals with fast acetylator had a higher OR of 3.01 (95% CI 0.93–9.77, $P=0.066$).

3.4. The risk of various NAT2 phenotypes for lung adenocarcinoma compared to gender matched controls

Since above statistical analysis has revealed a higher OR of individuals with fast NAT2 acetylators specific for adenocarcinoma, a further statistical analysis with gender matched controls was conducted to see if such tumor type specific OR was also exclusive for a certain gender As shown in Table 3, a significantly higher risk of fast NAT2 phenotypes for lung adenocarcinoma compared to that of slow phenotype was only seen in females with an OR of 2.73 (95% CI 1.40–5.33, $P=0.003$), but not in males.

Table 3
The risk of various NAT2 phenotypes for lung adenocarcinoma compared to matched controls

	Males		Females	
	Adenocarcinoma cases	Controls	Adenocarcinoma cases	Controls
NAT2 phenotype	$N=34$	$N=57$	$N=92$	$N=151$
Fast	28 (82.4%)	45 (78.9%)	75 (81.5%)	99 (65.5%)
Slow	6 (17.6%)	12 (21.1%)	17 (18.5%)	52 (34.5%)
OR (95% Cl) [a,b]	1.04 (0.31–3.52)	1[Ref]	2.73 (1.40–5.33)	1[Ref]

[a] The OR refers to the risk of fast phenotype of NAT2 for lung adenocarcinoma compared to that of slow phenotype with that in matched controls being 1.
[b] OR had been adjusted for age effect.

4. Discussion

Slow NAT2 acetylator, which can be determined by genotyping or by phenotyping, has been related to bladder cancer [22–24] and rapid metabolic phenotypes of CYP1A2 have been revealed to possess a higher risk for colorectal carcinoma [18]. However, the role of NAT2 in colon cancer was more controversial which may be due to an ethnic difference in the distribution of genetic polymorphisms or different mechanisms of susceptibility. Such involvement of these genetic polymorphisms in lung cancer may even be more complicated since lung is exposed to various environmental carcinogens, including polycyclic aromatic hydrocarbons and aromatic amines, from both inhalational and non-inhalational routes.

In the lung cancer incidence worldwide, a gender difference existed which was generally believed to be related to various factors, including smoking, hormonal effect and exposure profiles. In this aspect, Chinese women were quite unique for their high risk for lung cancer despite of a low smoking prevalence [32]. Furthermore, in our serial studies on lung cancer, we have identified several factors involved in the gender difference for lung cancer incidence, including HPV 16/18 infection, exposure to cooking oil fume, and p16 promoter hypermethylation. Various epidemiological studies have revealed a consistent finding that the risk of squamous carcinoma among current smokers was an order of magnitude higher than for adenocarcinoma. For examples, long duration of smoking (20 or more years) was associated with a two-fold increase in the risk of squamous cell carcinoma, but smoking was not associated with the risk of adenocarcinoma of the cervix. Furthermore, pathologic investigations indicated that Chinese women tend to contract lung adenocarcinoma, rather than squamous cell carcinoma, implying that the cause of lung cancer in Chinese women might not be smoking only. It was naturally reasonable to speculate that environmental factors other than cigarette smoking were related to lung cancer risk of Chinese women, and this hypothesis has been supported by a number of studies [32–35]. The proposed environmental factors associated with the incidence of lung cancer in Chinese women, including passive smoking, cooking practice, COF [4,36] were characterized for the complexity of their compositions. Previous studies indicated that various HCAs, a kind of carcinogen for rodents, had been found in these complexity environmental factors. In the other hand, CYP1A2 and NAT2 are vital enzymes in the metabolism of various HCAs, including 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), imidazoquinolines (IQ), and 2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline (MeIQx). Since females in our study group were mostly traditional Chinese women over 40, being housewives rather than workers, and they mainly lived in a traditional fashion to cook 3 meals in a day and 365 days during a year, it was reasonable to assume that they exposed to heterocyclic amines from limited sources while males tended to expose to various carcinogens, including occupational exposures. We thus hypothesized that an investigation on genetic polymorphisms of these relevant metabolic enzymes may shed light on the possible roles of HCA in lung carcinogenesis in this population.

In this PCR-RFLP study, the association of CYP1A2 and lung cancer susceptibility was firstly investigated. CYP1A2 was particularly interested because of its role in the activation of heterocyclic amines. One common CYP1A2 polymorphism (CYP1A2*F) has previously been associated with higher enzyme activity in smokers [37] suggesting that genetically determined variation in CYP1A2 expression may influence individual's susceptibility to smoking related cancers. In another study, by determining the metabolic activity for caffeine 3-demethylation, Nakajima and his colleagues [30] have evaluated the enzymatic activity of various CYP1A2 phenotypes and revealed that an allele with a G residue at the location of -2964 has a stronger activity than that with an A residue. However, in this study, we did not observe any significant difference in CYP1A2 distribution between cases and controls, nor between female and male subjects. A similar finding was also reported that the in vivo CTP1A2 activity level was not independently correlated with lung cancer [27].

As for NAT2, the highly polymorphic N-acetyltransferases are involved in both activation and inactivation reactions of numerous chemicals, such as tobacco derived aromatic amines. Furthermore, several previous studies have suggested that NAT2 fast acetylation was particularly associated with

an increased colorectal cancer risk in individuals who consume well-done meat, as a direct consequence of increased exposure to heterocyclic amine carcinogens [18,19,38]. In this study, we detected these study subjects using a PCR-RFLP assay that determined the four known NAT2 slow-acetylator alleles (M1–M4). The results showed that the risk for lung cancer of individuals with NAT2 fast acetylator was 2.44 folds of those with slow acetylator. Interestingly, the risk for lung cancer of female subjects with fast genotypes was 2.56 compared to that of female controls whereas no difference between male subjects and controls. Thus, it was possible that the involvement of NAT2 polymorphism is gender dependent. Furthermore, after being stratified by tumor types, it was revealed that individuals with NAT2 fast acetylator possessed a higher risk (OR = 2.21; 95%CI = 1.24–3.95) for lung adenocarcinoma, but not for lung squamous carcinoma. Because of the partially different etiology of these two lung cancer types, it is not surprising to find different susceptibility factor for each subgroup. However, we could not exclude the possibility that the insufficient sample size of squamous carcinoma samples in our study group may contribute to this tumor type-dependent difference. Further analysis revealed that such tumor type dependent difference was only found in female but not in male patients. It was therefore concluded that the involvement of NAT2 fast acetylator in lung tumorigenesis was mainly for never-smoking females rather than male lung cancer patients in Taiwan.

In the further statistical analysis for the combination of CYP1A2 and NAT2 polymorphisms, it was revealed that the risk of NAT2 fast acetylator was not synergistically added by CYP1A2 while the highest risk was for female subjects with CYP1A2 high activity and NAT2 fast acetylator (data not shown). Since NAT2 is mainly involved in the metabolism of HCAs, such as MeIQx, which is abundant in COF, the involvement of NAT2 genetic polymorphism in lung tumorigenesis further supported that the exposure to COF is associated to lung cancer incidences of Taiwanese women.

Furthermore, apart form acquiring from cooking fumes, HCAs may also be derived from diets. After being primarily metabolized in liver, these active metabolites may enter into lung via blood circulation to cause DNA damage and eventually lung tumor.

The abovementioned speculation has been preliminarily proven in animal models. Since CYP1A2 and NAT2 are also expressed in normal tissues of lung bronchial tubes and distal ends of pulmonary alveolus [39–41], although not as abundant as in liver, and may metabolize those inhaled cooking fumes and smokes and then result in tumor formation at these sites. Since most Taiwanese females were non-smokers, it was quite reasonable to speculate that the etiology of female lung cancer in Taiwan could be due to the exposure to HCAs, via inhalation or ingestion. It was noted that our data did not controvert the contribution of passive smoking in lung carcinogenesis of females since HCAs was also present in cigarette smokes [42]. Furthermore, in this study, a higher risk for lung cancer of subjects with NAT2 fast acetylator was only observed in females, a possible explanation was that females tend to expose to HCAs from limited and simplified sources, such as COF or passive smoking, while males expose to various and diversified carcinogens, including occupational exposures, which may complicate the association between NAT2 polymorphism and lung carcinogenesis of males. This female-only relationship reflected the possibility that contribution of exposure to heterocyclic amines from cooked meats and COF to female lung cancer development may be more significant than to male in Taiwan.

Although it was quite likely that the ultimate determinant of genetic susceptibility is complex, possibly arising from synergistic interactions between allelic variants of multiple genes including activating and detoxifying enzymes and environment carcinogen exposure, our results clearly indicated that NAT2 fast acetylator may be involved in lung tumorigenesis, exclusively for never-smoking female patients, in Taiwan.

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