RESEARCH ARTICLE

Association of CYP2E1 and NAT2 Polymorphisms with Lung Cancer Susceptibility among Mongolian and Han Populations in the Inner Mongolian Region

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Abstract

Purpose: To explore associations of CYP2E1 and NAT2 polymorphisms with lung cancer susceptibility among Mongolian and Han populations in the Inner Mongolian region. Materials and Methods: CYP2E1 and NAT2 polymorphisms were detected by PCR-RFLP in 930 lung cancer patients and 1000 controls. Results: (1) Disequilibrium of the distribution of NAT2 polymorphism was found in lung cancer patients among Han and Mongolian populations (p=0.031). (2) Lung cancer risk was higher in individuals with c1, D allele of CYP2E1 RsaI/PstI, DraI polymorphisms and slow acetylation of NAT2 (c1 compared with c2, OR=1.382, 95%CI: 1.178-1.587, p=0.003; D compared with C, OR=1.241, 95%CI: 1.053-1.419, P<0.001; slow acetylation compared with rapid acetylation, OR=1.359, 95%CI:1.042-1.768, p=0.056) (3) Compared with c2/c2 and rapid acetylation, c1/c1 together with slow acetylation synergetically increased risk of lung cancer 2.83 fold. (4) Smokers with CYP2E1 c1/c1, DD, and NAT2 slow acetylation have 2.365, 1.916, 1.841 fold lung cancer risk than others with c2/c2, CC and NAT2 rapid acetylation, respectively. (5) Han smokers with NAT2 slow acetylation have 1.974 fold lung cancer risk than others with rapid acetylation. Conclusions: Disequilibrium distribution of NAT2 polymorphism was found in lung cancer patients among Han and Mongolian populations. Besides, Han smokers with NAT2 slow acetylation may have higher lung cancer risk compared with rapid acetylation couterparts. CYP2E1 c1/c1, DD and NAT2 slow acetylation, especially combined with smoking, contributes to the development of lung cancer. CYP2E1 c1/c1 or DD genotype and NAT2 slow acetylation have strong synergistic action in increasing lung cancer risk.

Keywords: CYP2E1 - NAT2 - genetic polymorphism - lung cancer - susceptibility

Asian Pac J Cancer Prev, **15 (21)**, 9203-9210

Introduction

According to the International Agency for Research on Cancer (IARC) GLOBOCAN World Cancer Report, lung cancer affects more than 1 million people a year and certainly stands to be a leading cause of cancer mortality worldwide (Jemal et al., 2011). As the highly developed technology of prevention and treatment, lung cancer incidence has declined in several regions but has yet to peak in many other parts of the world, particularly China (Chen et al., 2014). The carcinogenesis of lung cancer may result from a variety of triggers, among which tobacco consumption was indicated to be one of the primary agents. While there exists a fact that never-smoking patients account for 15% of lung cancer patients (Hadoux et al., 2011). Therefore, we indicated that, to a large extent, individual susceptibility leading to this difference in the occurrence of lung cancer. Epidemiologic studies showed that the difference of individual susceptibility to diseases can explains as different genetic origin. A number of studies have concluded that susceptibility to lung cancer is affected by polymorphisms of metabolic enzymes genes, modulating the levels of metabolic activation and detoxification of carcinogens (Ragin et al., 2010; Wright et al., 2010).

Polymorphisms in the carcinogen-metabolizing genes have been analyzed on individual basis (Bruske-Hohlfeld et al., 2009). Several studies have addressed the relationship between the genetic polymorphisms of enzymes involved in the metabolic activation of carcinogens and the occurrence of lung cancer (Zhan et al., 2010). CYP2E1 gene is an ethanol-induced gene which is capable of catalyzing the biotransformation of not only ethanol, but also tobacco related carcinogens (N-nitrosamins, benzene, urethane, butadiene and styrene) and more than 80 low- molecular substances which are considered as potential carcinogens. Cytochrome P450 2E1 (CYP2E1) is mapped in the 10q24.3 region of chromosome. CYP2E1 Rsa I/Pst I polymorphism has a G→C substitution at 1293bp in 5'-untranslated region of

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CYP2E1 gene and introduces Pst I restriction enzyme site. CYP2E1 Dra I polymorphism has a T→A substitution at 7632bp in sixth intron and destroys the Dra I restriction enzyme site (Crabb et al., 2007).

Furthermore, Nacetyltransferase-2 (NAT2) is a polymorphic gene involves in the catalyzing of N-acetylation (deactivation) and O-acetylation (activation) process of a variety of heterocyclicamine (HCAs), polycyclic aromatic hydrocarbon (PAHs) and other carcinogens (Wang et al., 2005). Different metabolic phenotypes are decided by the point mutation in coding region or non-coding region on NAT2 M1, M2, M3. The three overriding kinds of mutant alleles, called slow acetylation genotype can lead to the decline of enzyme's activity, determined by the detected C→T substitution at 481bp, the G→A substitution at 590bp and the G→A substitution at 857bp. Individuals with at least one wild-type allele (Wt/Mx and Wt/Wt) are classified as rapid, and homozygotic type (Mx/Mx) is classified as slow (Walraven JM et al., 2008).

Although, previous studies somehow identified the functional role of CYP2E1 or NAT2 polymorphism and lung cancer risk, several studies also showed the synergistic interaction between gene polymorphisms and chemical or environmental cancerogens, such as cigarettes smoking, which has a trend to increase the risk of lung cancer (Tian et al., 2014; Cao et al., 2014). Besides, many studies aim at exploring the gene-gene interaction among metabolic enzyme genes (Singh et al., 2011; Ying et al., 2013; Gao et al., 2014), but interaction between CYP2E1 and NAT2 polymorphism has not been reported.

In Inner Mongolia region of China, Mongolia population reaches to 17.11% of total and Han population accounting for 79.54% according to the demographic census in 2011. Su XL et al. have concluded the equalized distribution of CYP2E1 RsaI/PstI and Dra I polymorphism (Su XL et al., 2011). While, far from now on, there is no researchers studied NAT2 Wt/Mx polymorphisms distribution among Han and Mongolia population. In our research, we enlarged the sample size of subjects in order to further explore the variance of heredity among Han and Mongolia population and to assess the linkage between CYP2E1, NAT2 polymorphisms and lung cancer risk. Besides, synergetic interaction between genotypes and potential risk factors (such as smoking and alcohol intake) and gene-gene interaction were also estimated based on current data.

Materials and Methods

Subject characteristics

The human subject's protocol for this study was reviewed and approved by the Ethics Committee of Inner Mongolian Medical University and Huimin District Hospital of Hohhot.

The present study included 930 lung cancer patients and 1000 healthy controls. Lung cancer cases were recruited from patients undergoing bronchoscopy in the first Affiliated Hospital of Inner Mongolian Medical University and Huimin District Hospital of Hohhot from January 2000 to April 2014. There were no restrictions on age, sex, histology, or stage, but, all patients included in this research had histopathologically confirmed and previously untreated (by chemotherapy or radiotherapy) lung cancer. What's more, individuals who had any prior history of other cancer type or cancer-prone diseases were excluded. Control subjects were selected from a pool of healthy volunteers who visited the healthy check-up center at the aforementioned hospitals during the same period of time. Controls were frequency matched to the cases based on age (±5 years old) and gender. After subjects provided signed informed consent, trained researchers interviewed all subjects using a structured questionnaire including questions on demographics and risk variables, such as gender, age, smoking habits, alcohol consumption, dwelling, education and histological type. Participants who had smoked at least once a day for more than one year in his or her life time were regarded as smokers and the rest were considered as non-smokers. Drinkers are defined as who have average consumption of alcohol (≥36%) daily≥100ml and the rest are non-drinkers. As one of the main purposes of our research is to explore the difference of genotype distribution and lung cancer susceptibility between Han and Mongolia population, therefore, quantitatively equal lung cancer cases and controls with regard to nationality were taken into consideration.

DNA extraction and genotyping

3~5 ml peripheral venous blood was drawn from per person into sodium citrate solution, and then stored at -80°C. DNA extraction was performed according to the manufacturer`s protocol for Qiagen DNA extraction kits (qiagen, Hilden, NRW, Germany). The content of DNA was quantified by spectrophotometric absorption (Nanodrop Spectrophotometer™ 2000c, Thermo, USA). Polymerase chain reaction (PCR) was performed using an Thermal Cycler (Applied Biosystems, Foster City, CA, USA). CYP2E1 Pst I/Rsa I, Dra I and NAT2 genotypes were determined using a PCR-RFLP assay. Amplifications were carried out and PCR products were generated using the specific forward and reverse primers.

PCR was used to amplify two segments of CYP2E1, which one has both Pst I and Rsa I site, the other one has Dra I site. The following primers were used: 5'-CCAGTCGAGTCTACATTGTCA-3' and 5'-TTCATTCTGTCTTCTAACTGG-3'; 5'-AGTCGACATGTGATGGATCCA-3' and 5'-GACAGGGTTTCATCATGTTGG-3'respectively. We amplify the segments of NAT2 used primer 5'-CTTCTCCTGCAGGTGACCAT-3' and 5'-GAAGCAGAGTGATTCATGCT -3'. PCR was performed in 50μl reaction mixtures containing 18μl of ddH2O, 25μl of 2×Taq Master Mix, 2μl of forward primer and 2μl of reverse primer, 3μl of template DNA. After an initial denaturation at 94°C for 5min, the DNA was amplified by 35 cycles of 60s at 94°C, 60s at 59°C, 60s at 72°C, followed by a final extension step of 7min at 72°C. CYP2E1 PCR products were digested with Rsa I and Dra I (Thermo), NAT2 PCR products were digested with Kpn I,Taq I,Bam HI (Thermo), respectively, in 37°C water bath for 18h, the PCR products with Taq I in 65°C water bath for 18h. Digestion products were resolved

using 2% agarose gel under 80V for 45min and analyze by MultiSpectral imaging instrument (UVP, Upland, CA, USA).

Statistical analysis

Demographic and clinical data between lung cancer and control groups were compared by Two-sided χ^2 test for categorical variables and student's t text for continuous variables. χ^2 test was used to detect whether there were significant difference between the genotype and allele frequencies among Han and Mongolian population both in case and control groups. χ^2 test was also used to identify whether genotype distribution in controls accord with Hardy-Weinberg equilibrium. Odds ratios (OR), 95% confidence intervals (CIs) were calculated to assess the association between polymorphism and lung cancer risk by logistic regression analysis, adjusting for potential confounding factors. The associations between lung cancer and selected risk factors

(smoking statue, alcohol intake and histological type) were also investigated using stratified analysis. Besides, in the part of gene-gene interaction, two of protective genotypes or phenotype selected in the test of 'The Association of CYP2E1 Rsa I/Pst I, Dra I, NAT2 polymorphism with lung cancer susceptibility (Table 3)' were combined as a reference (assumed as the most safe situation), other combinations of genotypes or phenotype compared with the reference to computing OR which was used to assess the strength of gene-gene synergetic interaction. All P values were based on two-sided tests and $p<0.05$ were considered statistically significant. Analyses were performed by SPSS (Version 20.0)

Results

Subjects characteristics

The demographic and clinic data of all enrolled 930 lung cancer patients and 1000 controls were shown in Table 1. The percentage of Han and Mongolia population among cases and controls were equal. Although there existed different degree of the loss of the follow-up data on several variables, the missing quantity accounting for the overall cases or controls was less than 5%. There was no significant differences between the cases and controls in mean age or gender, suggesting the adequate matching based on these two variables. Lung cancer patients had a significant higher prevalence of smoking habit than controls (smoking status, $p=0.004$). Besides smoking, other factors, such as alcohol intake ($p=0.093$), dwelling ($p=0.470$), income ($p=0.613$) and education ($p=0.720$) did not show significant variance between cases and controls. Among all the 930 lung cancer cases, adenocarcinoma, squamous cell carcinoma, small cell carcinoma and LCLC histological type of lung cancer represented 22.8%, 51.2%, 17.4%, and 7.0% respectively. Genotype and allele distribution in all control groups were in accordance with Hardy-Weinberg equilibrium ($p>0.05$).

PCR-product

The DNA fragment of the CYP2E1 gene was 413bp after PCR amplification with primer Rsa I/Pst I.

Table 1. Demographic Characteristics of Patients and Controls

Varibles		Controls N=1000 (n%)	Cases N=930 (n%)	p[a]
Nationality	Han	500 (50.0)	465 (50.0)	
	Mongolian	500 (50.0)	465 (50.0)	-
Gender	Male	618 (61.8)	602 (64.7)	0.186
	Female	382 (31.2)	328 (35.3)	
Age(years)	<50	406 (40.6)	346 (37.20)	0.135
	≥50	594 (59.4)	584 (62.8)	
Mean age in years(SD)[e]		58.07±10.03	60.24±10.12	0.412
Smoking status[b]				0.004*
	Smoker	651 (66.6)	661 (72.8)	
	Non-smoker	327 (33.4)	247 (27.2)	
Alcohol intake[c]				0.093
	Ex- or current drinker	564 (57.4)	562 (61.2)	
	Non-drinker	419 (42.6)	356 (38.8)	
Dwelling				0.470
	Rural	442 (45.6)	406 (52.7)	
	Urban	528 (54.4)	512 (55.8)	
Income(yuan/month)				0.613
	≤2000RMB	502 (50.6)	490 (52.7)	
	2000-5000RMB	378 (38.1)	344 (37.0)	
	≥5000RMB	112 (11.3)	96 (10.3)	
Education				0.720
	Never	18 (1.9)	20 (2.2)	
	Elementary school	439 (45.3)	432 (46.6)	
	Junior school	386 (39.8)	370 (39.9)	
	Senior school and upwards	126 (13.0)	106 (11.4)	
Histological type[d]				
	Adenocarcinoma		212 (22.8)	
	Squamous cell carcinoma		476 (51.2)	
	Small cell carcinoma		162 (17.4)	
	LCLC		65 (7.0)	

Compraed with control group,$p<0.05$; [a]Two-sided χ^2 test for categorical variables and student's t text for continuous variables; [b,c,d]Variables selected for stratification analysis, $p<0.05$; [e,f]Mean±SD

Table 2. Genotype Distribution of CYP2E1 Pst I/ Rsa I, Dra I, NAT2 Polymorphisms in Controls and Lung Cancer Patients among Mongolian and Han Population

Genotype	Controls[b] N=1000(%)		p[a]	Cases N=930(%)		p[a]
	Han	Mongolian		Han	Mongolian	
CYP2E1Pst I/Rsa I Genotype						
c2/c2	38(7.6)	37(7.4)		24(5.2)	19(4.1)	
c1/c2	198(39.6)	186(37.2)		164(35.3)	154(33.1)	
c1/c1	264(52.8)	277(55.4)	0.807	277(59.6)	292(62.8)	0.524
Alleles						
c2	274(27.4)	260(26.0)		212(22.8)	192(20.6)	
c1	726(72.6)	740(74.0)	0.479	718(77.2)	738(79.4)	0.261
CYP2E1 Dra I Genotype						
CC	45(9.0)	40(8.0)		25 (5.4)	24(5.2)	
CD	208(41.6)	204(40.8)		180(38.7)	170(36.6)	
DD	247(49.4)	256(51.2)	0.781	245(52.7)	256(55.1)	0.76
Alleles						
C	298(29.8)	284(28.4)		230(24.7)	218(23.4)	
D	702(70.2)	716(71.6)	0.491	670(72.0)	682(73.3)	0.513
NAT2 Genotype						
Wt/Wt	211(42.2)	224(44.8)		172(37.0)	195(41.9)	
Wt/Mx	223(44.6)	229(45.8)		212(45.6)	216(46.5)	
Mx/Mx	66(13.2)	47(9.4)	0.159	81(17.4)	54(11.6)	0.031*
Alleles						
Wt	355(35.5)	323(32.3)	0.143	374(40.2)	324(34.8)	0.019*
Mx	645(64.5)	677(67.7)		556(59.8)	606(65.2)	

*Compared with Han population, *$p<0.05$; [a]P value for Two-sided χ^2 test; [b]Test for Hardy-Weinberg equilibrium in control group and all p-value>0.05.

Table 3. The Association of CYP2E1 Rsa I/Pst I, Dra I, NAT2 polymorphism with lung cancer susceptibility

Genotype	Controls N=1000(%)	Cases N=930(%)	OR[a] (95%CI)	*p*
CYP2E1 Rsa I/Pst I				
genotype comparison				
c2/c2	75(7.5)	43(4.6)	1(ref)	
c1/c2	384(38.4)	318(34.2)	1.417(0.925-2.018)	0.076*
c1/c1	541(54.1)	569(61.2)	1.893(1.237-2.941)	0.003*
allele comparison				
c2	534(26.7)	404(43.4)	1(ref)	
c1	1466(73.3)	1456(56.6)	1.382(1.178-1.587)	<0.001*
CYP2E1 Dra I				
genotype comparison				
CC	85(8.5)	49(5.3)	1(ref)	
DC	412(41.2)	350(37.6)	1.472(1.068-2.185)	0.028*
DD	503(50.3)	531(57.1)	1.678(1.192-2.453)	0.002*
allele comparison				
C	582(29.1)	448(22.4)	1(ref)	
D	1418(70.9)	1412(70.6)	1.241(1.053-1.419)	<0.001*
NAT2				
rapid acetylation[b]	887(40.6)	795(37.2)	1(ref)	
slow acetylation[c]	113(59.4)	135(62.8)	1.359(1.042-1.768)	0.056*
Wt	1322(66.1)	1162(62.5)	1(ref)	
M_x	678(33.9)	698(37.5)	1.145(1.004-1.304)	0.018*

*Compraed with control group,*P<0.05; [a]Odds Ratio adjusted by age, gender, smoking status, alcohol intake

Table 4. Gene-Gene Interaction between CYP2E1 and NAT2 Polymorphisms

Genotype[a]		Control (N)	Cases (N)	OR[b] (95%Cl)	*p*
CYP2E1 Rsa I/Pst I	NAT2				
c2/c2	rapid acetylation	17	24	Ref(1)	
c1/c2	rapid acetylation	34	45	0.896(0.413-1.903)	0.783
c1/c1	rapid acetylation	65	88	0.954(0.486-1.926)	0.905
c2/c2	slow acetylation	13	19	1.043(0.413-2.661)	0.946
c1/c2	slow acetylation	11	33	2.131(0.865-4.123)	0.107
c1/c1	slow acetylation	9	41	2.831(1.085-7.387)	0.030*
CYP2E1 Dra I	NAT2				
CC	rapid acetylation	11	9	Ref(1)	
DC	rapid acetylation	40	34	1.031(0.382-2.781)	0.936
DD	rapid acetylation	63	58	1.116(0.426-2.806)	0.805
CC	slow acetylation	10	10	1.225(0.356-4.241)	0.753
DC	slow acetylation	32	29	1.936(0.712-5.296)	0.198
DD	slow acetylation	21	39	2.284(0.843-6.352)	0.112

*Compraed with control group,*P<0.05; [a]Risk grnotype or phenotype: selected in Table 3; [b]OR: Odds Ratio adjusted by age, gender, smoking status, alcohol intake

Homozygous wild-type (c1/c1) containing restriction site in each DNA chain become into two fragments of 352bp and 61bp after being digested with Rsa I. Homozygotic mutant (c2/c2) had a fragment of 413bp. Heterozygote (c1/c2) containing restriction enzyme digest site in one of DNA chain become into 3 fragment of 413bp, 352bp and 61bp.

The DNA fragment of the CYP2E1 gene was 376bp after PCR amplification with primer Dra I. Homozygous wild type (DD) containing restriction enzyme digest site in each DNA chain become into two fragments of 251bp and 125bp after being digested with Dra I. Homozygotic mutant (CC) without a restriction site only had a fragment of 376bp. Heterozygote (DC) containing restriction enzyme digest site in one of DNA chain become into 3 fragment of 376bp, 251bp and 125bp.

The DNA fragment of the NAT2 gene was 815bp after PCR amplification with primer NAT2. Homozygous wild type (Wt/Wt) become into two fragments of 656 and 159bp after being digested with Kpn I. Homozygotic mutant (M1/M1) only had a fragment of 815bp. Heterozygote (Wt/M1) become into 3 fragment of 815bp, 656bp and 159bp.

Homozygous wild type (Wt/Wt) become into four fragments of 378bp, 227bp, 169bp, 41bp after being digested with Taq I; Homozygotic mutant (M2/M2) without a 590 site only had three fragments of 396bp, 378bp, 41bp; Heterozygotic mutant (Wt/M2) containing restriction enzyme digest site in one of DNA chain become into 5 fragment of 396bp, 378bp, 227bp, 169, 41bp.

Homozygous wild type (Wt/Wt) become into two fragments of 535bp, 280bp after being digested with Bam HI; Homozygotic mutant (M3/M3) only had the fragment of 815 bp; Heterozygotic mutant (Wt/Mx) containing restriction enzyme digest site in one of DNA chain become into 3 fragments of 815 bp, 535bp and 280bp.

Genotype distribution among Han and Mongolia population

The genotype and allele distribution of CYP2E1 Pst I/Rsa I, Dra I and NAT2 polymorphisms among Han and Mongolia population were shown in Table 2. It showed that the distribution of NAT2 genotypes was in disequilibrium, the Mx allele in Han lung cancer cases was significantly higher than Mongolian patients (p=0.019). While, this

Table 6. The difference among Mongolian and Han population for Association between NAT2 Polymorphism, Smoking, and Lung Cancer Susceptibility

Genotype	Han population (n) Control	Lung cancer	OR	p[a]	Mongolian population (n) Control	Lung cancer	OR	p[a]
NAT2								
rapid acetylation	434	384	1(ref)		453	411	1(ref)	
slow acetylation	66	81	1.364 (0.943-1.945)	0.068	47	54	1.238 (0.818-1.893)	0.152
Smoking statusa								
Smoker								
rapid acetylation	284	283	1(ref)		315	232	1(ref)	
slow acetylation	31	60	1.974 (1.251-3.121)	0.004*	21	26	1.712 (0.959-3.094)	0.101
Non-smoker								
rapid acetylation	150	104	1(ref)		120	97	1(ref)	
slow acetylation	31	18	0.871 (0.481-1.602)	0.612	26	28	1.359 (0.764-2.457)	0.344

*Compraed with control group,*P<0.05; [a]Odds Ratio adjusted by age, gender, smoking status, alcohol intake

Table 5. Stratification Analysis of CYP2E1 Rsa I/Pst I, CYP2E1 Dra I and NAT2 Mx/Wt Polymorphisms and Lung Cancer

Variables	CYP2E1 Rsa I/Pst I Controls/Cases			ORa (95%CI)	p^a	OR^b (95%CI)	p^b	CYP2E1 Dra I Controls/Cases			OR^c (95%CI)	p^c	OR^d (95%CI)	p^d	NAT2 Controls/Cases		OR^e (95%CI)	p^e
	c2/c2	c1/c2	c1/c1					CC	CD	DD					rapid acetylation	slow acetylation		
Smoking status																		
Smoker				1.897		2.365					1.523		1.916		587	74	1.841	
	41/20	274/258	336/383	(1.095-3.376)	0.027*	(1.325-4.148)	0.002*	39/23	244/212	368/426	(0.876-2.647)	0.178	(1.106-3.268)	0.013*			(1.233-2.759)	0.002*
Non-smoker				0.957		1.434					1.154		1.574		182	61	1.238	
	34/22	109/60	184/165	(0.567-1.646)	0.604	(0.823-2.54)	0.306	42/24	121/78	164/145	(0.674-2.048)	0.796	(0.812-2.588)	0.082			(0.831-1.846)	0.237
Alcohol intake																		
Drinker				1.488		1.687					1.548		1.612				1.364	
	35/23	198/187	331/352	(0.876-2.587)	0.214	(0.104-2.813)	0.104	38/25	237/246	289/291	(0.906-2.658)	0.091	(0.954-2.634)	0.113	501/494	63/68	(0.844-2.106)	0.165
Non-drinker				1.106						214/231	0.846		1.424		379/300	50/56	1.109	
	28/20	182/124	209/212	(0.578-1.821)	0.887	(0.875-2.787)	0.262	30/23	175/96		(0.412-1.403)	0.264	(0.806-2.513)	0.272			(0.806-1.596)	0.623
Histological type																		
(Overall controls)	75	384	541					85	412	503					887	113		
Adenocarcinoma				1.345		1.824				118	1.753		1.823		182	30	1.265	
	10	67	135	(0.607-3.478)	0.423	(0.914-3.487)	0.075	9	86		(0.907-3.818)	0.423	(0.893-3.756)	0.101			(0.808-1.967)	0.152
Squamous cell carcinoma				1.641		1.704					1.773		1.967		410	66	1.096	
	23	188	265	(1.016-2.887)	0.087	(1.042-2.814)	0.059	20	180	275	(0.986-2.917)	0.031	(1.161-3.212)	0.013*			(0.762-1.521)	0.521
Small cell carcinoma				0.864		1.833					1.006		1.348		138	24	1.343	
	9	36	117	(0.415-1.754)	0.547	(0.871-3.581)	0.134	10	52	100	(0.468-2.067)	0.954	(0.587-2.747)	0.352			(0.967-2.152)	0.193
LCLC				1.448		1.857					0.823		0.674		53	12	1.758	
	3	21	41	(0.457-4.547)	0.648	(0.528-6.104)	0.184	8	32	25	(0.412-1.816)	0.536	(0.264-1.046)	0.074			(0.904-3.408)	0.086

*Compraed with control group,*P<0.05; Odds Ratio adjusted by age, gender, smoking status, alcohol intake, but not stratified factor; [a]Calculated for c1/c2 against c2/c2; [b]Calculated for c1/c1 against c2/c2; [c]Calculated for CD against CC; [d]Calculated for DD against CC; [e]Calculated for slow acetylation against rapid acetylation

distribution disequilibrium did not be found in CYP2E1 genotypes (Table 2). Therefore, with regard to this slightly weak equilibrium of the distribution of NAT2 polymorphism, further stratification analysis was performed according to nationality (Table 6).

Association between CYP2E1 Rsa I/Pst I, Dra I, NAT2 polymorphisms and lung cancer susceptibility

The genotype frequencies of CYP2E1 Rsa I/Pst I, Dra I and NAT2 polymorphisms among controls and cases are shown in Table 3. Significant linkages were simultaneously found in the three polymorphic locus. Lung cancer risk was higher in individuals with c1 allele than individuals with c2 allele (c1/c2 compared with c2/c2, OR=1.417, 95%CI:0.925-2.018, p=0.076; c1/c1 compared with c2/c2, OR=1.893, 95%CI:1.237-2.941, p=0.003; c1 compared with c2, OR=1.382, 95%CI:1.178-1.587, p<0.001) Individuals with D allele (DC compared with CC, OR=1.472, 95%CI:1.068-2.185, p=0.028; DD compared with CC, OR=1.678, 95%CI:1.192-2.453, p=0.002; D compared with C, OR= 1.241, 95%CI: 1.053-1.419, p<0.001) and slow acetylation (slow acetylation compared with rapid acetylation, OR=1.359, 95%CI:1.042-1.768, p=0.056; Mx compared with Wt, OR=1.145, 95%CI:1.004-1.304, p=0.018) also have greater lung cancer susceptibility.

Gene-gene interaction

As shown in Table 4, when c1/c1 and slow acetylation were synergistically taken into action, drastically increased risk of lung cancer was observed. (c1/c1 and slow acetylation compared with c2/c2 and rapid acetylation, OR=2.831, 95%CI=1.085-7.387, P=0.030).

Stratified analysis

In addition to the overall association analysis, we did a stratified analysis by potential risk factors among cases and controls, including smoking status, alcohol intake and histological type. Results of analysis stratified by potential risk factors are summarized in Table 5. Strong associations were simultaneously observed in smokers with CYP2E1 c1/c1, DD, and NAT2 slow acetylation (OR=2.365, 95%CI:1.325-4.148, p=0.002; OR=1.916, 95%CI:1.106-3.268, P=0.013; OR=1.841, 95%CI: 1.233-2.759, p=0.002; respectively) and lung cancer risk. However, as for the subset of alcohol intake, there was no statistical linkage between the three above gene polymorphisms and lung cancer. In the subset of histological type,

CYP2E1 DD genotype significantly increase the risk of Squamous cell carcinoma (OR=1.967, 95%CI: 1.161-3.212, *p*=0.013).

Stratified analysis also performed among Han and Mongolian population to explore whether there exists variance in the two nationalities with NAT2 polymorphism (shown in Table 6). Smoking statue were also considered as subsets on account of that previous result in our research showed that smoking is one of the main trigger factors to lung cancer. We found that Han ex- or current smokers with NAT2 slow acetylation (OR=1.974, 95%=1.251-3.121, P=0.004) have higher lung cancer risk than other Han smokers with rapid acetylation. But, this difference did not be found in Mongolian population (OR=1.712, 95%=0.959-3.094, *p*=0.101).

Discussion

It is well established that the distribution of CYP2E1 and NAT2 genotypes varies much in different races or nationalities (Lakkakula et al., 2013; Zabost et al., 2012). Previous studies were not observed significant disequilibrium in the genotype distribution of CYP2E1 among Han and Mongolian population in Inner Mongolian region, which, may due to small sample size (Su et al., 2011). In our research, we enlarged the sample size (930 lung cancer patients and 1000 healthy controls) to re-estimate the genotype distribution in the two different nationalities. As a result, NAT2 slow acetylation accounted for 17.4% among Han lung cancer patients, while, among Mongolian lung patients the percentage reduced to 11.6%, which indicate that the significant disequilibrium distribution of NAT2 genotypes. However, we disregarded the nationality when exploring the association between gene polymorphism and lung cancer, but further stratification was performed above NAT2 according to nationality. In the stratified analysis of NAT2 with the lung cancer susceptibility, we found that Han population with slow acetylation of NAT2 have higher risk than Mongolian population with that, especially those Han smokers. This variance may derive from the different living circumstance or habits of the two nationalities.

The frequency of genotype and allele of CYP2E1 and NAT2 genes were compared to calculate odds ratio (OR) which was used to estimate its association with lung cancer. We finally found that carriers with c1/c1, DD and slow acetylation of CYP2E1 Rsa I/Pst I, Dra I and NAT2 Wt/Mx polymorphisms have higher risk of lung cancer. The result was in conformity with several trials conducted in Inner Mongolian region (Guo et al., 2010; Su et al., 2011). Furthermore, an increased risk of lung cancer was observed in association with c1/c1, DD and slow acetylation among smokers, but not non-smokers. The stratified result indicated that genetic variance, especially when synergetically take functions with smoking, largely increase the risk of lung cancer.

The association of CYP2E1 Rsa I/Pst I, Dra I and NAT2 Wt/Mx polymorphisms and lung cancer risk was somehow biologically plausible. Marchand et al. evaluated the association between Rsa I/Pst I polymorphism and activity of CYP2E1, as a result, decreased activity of CYP2E1 was found with variant C allele. Hayashi S et al. found that 10-fold enhanced transcriptional activity may result from Rsa I/Pst I polymorphism in the 5' flanking region by altering its binding to HNF1(a transcription factor) (Hayashi et al., 1991). In terms of DraI polymorphism, *in vitro* expression studies also indicated that it is associated with increased transcriptional activity (Uematsu et al., 1994). On the other hand, it is of interpretability that why CYP2E1 gene polymorphism and smoking takes significantly synergistic effect to increase the risk of lung cancer: N-nitrosamines are a carcinogenic tobacco-specific substance which can be metabolized by NAT2 enzyme (Hecht et al., 2014). To date, several meta-analyses have showed smokers with NAT2 slow acetylation have increased risk of bladder cancer (Deng et al., 2014), prostate cancer (Gong et al., 2011), breast cancer (Zhang et al., 2010) and hepatocellular carcinoma (Zhang et al., 2012). But, there are no latest meta-analysis or GWAS studies within 5 years about the overall association of NAT2 genotypes and lung cancer risk.

In the subset of alcohol intake, CYP2E1 polymorphism showed none elevated or decreased risk of lung cancer. Prior studies have already provided evidence that protein stabilization and altered degradation represent a mechanism for controlling CYP2E1 expression in response to ethanol (Roberts et al., 1995). However, according to existing published literatures, alcohol intake seems have more influence on liver injury (Zhang et al., 2014), since CYP2E1 is an ethanol-induce enzyme and liver is indicated as the main alcohol-metabolizing organ. Tian Z et al. (2012) conducted a huge review and indicated that the c2 allele of CYP2E1 RsaI/PstI (c1/c2) polymorphism may be a protective factor for HCC among East Asians, especially among China populations.

When stratification was taken into histological type, results showed connection between CYP2E1 Rsa I/Pst I, Dra I polymorphism and Squamous cell carcinoma (OR=1.704, 95%CI:(1.042-2.814), *p*=0.059; OR=1.967, 95%CI:1.161-3.212, *p*=0.013; respectively). Lei Cao et al. concluded that C allele of CYP2E1 Rsa I/Pst I polymorphism was associated with increased lung squamous cell carcinoma (Cao et al., 2014). Wang J et al. (2003) concluded that CYP2E1 Rsa1 wild type has a promoting effect on susceptibility to lung adenocarcinoma. By far, researches targeting study the association between specific type of lung tumor and the aforementioned three genetic polymorphisms are limited.

Except for nationality, smoking status, alcohol intake and histological type, we also estimated gene-gene interaction between CYP2E1 and NAT2 gene polymorphisms. c1/c1, and slow acetylation are suspicious risk factors for lung cancer, and have strong synergetic function in increasing the risk of lung cancer. Although, several pertinent "at-risk" genotypes of phase I and II metabolic enzyme genes, such as CYP1A1 and GSTM1 (Kiyohara et al., 2012, Jiang et al., 2014), have combined function in increasing the risk of lung cancer, but in our research we did not find connection between CYP2E1 Dra I and NAT2 polymorphisms. At present, researches targeting gene-gene interaction between CYP2E1 and NAT2 are also limited.

In conclusion, the pathogenesis of lung cancer is extremely complex. Genetic factors and environmental factors have been associated with lung cancer and also related to, gender, age, nationality and other factors (Wang et al., 2014; Kim et al., 2014; Groot et al., 2012). In order to eliminate potential bias, in our research, comparatively particular stratifications were set based on selected risk factors. We found that c1/c1, DD, and slow acetylation of CYP2E1 Rsa I/Pst I, Dra I and NAT2 Wt/Mx polymorphisms contributes to the development of lung cancer, especially when c1/c1 and slow acetylation take a combination of act. Besides, the slow acetylation of NAT2 may be a susceptible factor to lung cancer, but may plays more functional role on Han population, especially smokers, in Inner Mongolian region. Among potential risk environmental factors, smoking is a main cancerogen which is significantly elevating the risk of lung cancer when synergetically functions with risk genotypes.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81160406, 81260499), Natural Science Foundation of Inner Mongolia Autonomous region (2011MS1113).

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