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A functional copy number variation in the *WWOX* gene is associated with lung cancer risk in Chinese

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WW domain-containing oxidoreductase (WWOX) is a tumor suppressor that has been reported to lose function due to genetic alterations in several cancers. *WWOX* maps to the common chromosomal fragile site FRA16D and several copy number variations (CNVs) were found within this gene. In this study, we investigated the association between the CNVs of *WWOX* and lung cancer risk in four independent case–control studies, which are on 2942 lung cancer cases and 3074 cancer-free controls of southern, eastern and northern Chinese. A common CNV-67048 was genotyped by the Taqman real-time PCR, and its biological effect was accessed with protein expression and sequencing assays. We found that in comparison with the common 2-copy genotype, the carriers of loss variant genotypes (1-copy or 0-copy) had a significantly increased risk of lung cancer (adjusted OR = 1.39, 95% CI = 1.24–1.55, $P = 9.01 \times 10^{-9}$) in a dose–response manner ($P_{trend} = 1.12 \times 10^{-10}$), and the WWOX protein expressions in lung cancer tissues were significantly lower ($P = 0.036$), accompanying a higher rate of exons absence ($P = 0.021$) in subjects with loss genotypes of CNV-67048. Our data suggest that the loss genotypes of CNV-67048 in *WWOX* predispose their carriers to lung cancer; this might be related with altered WWOX gene expression and exons absence in them.

INTRODUCTION

Although an abundance of association studies, especially the genome-wide association studies (GWAS), has reported that a lot of single-nucleotide polymorphisms (SNPs) are associated with risks of various cancers, only a small proportion of cancer heritability can be explained by those SNPs, reflecting that some 'missing' heritability existed (1). Recently, increasing evidence suggests that the copy number variation (CNV) might hold the key to account for a large proportion of the missing heritability (1–3). CNV is a prevalent genetic aberration that covers >1 kb duplication or deletion, which may contribute to phenotypic variations by altering biological functions or genes expression (4,5). Therefore, if CNVs are located in tumor suppressor genes or oncogenes, corresponding to loss-of-function mutations or gain-of-function mutations would possibly alter the predisposition to cancer.

Human WW domain-containing oxidoreductase (*WWOX*) is an identified tumor suppressor gene. As a pro-apoptotic molecule, WWOX regulates several cancer-related signaling pathways and genes (6–8), and it frequently loses function in the initiation of tumorigenesis as well as neoplastic process (9,10). Not only does WWOX directly suppress the carcinogenic effects of oncogenes such as *c-Jun*, *erbB4*, *MDM2* (11–13), but it also activates tumor suppressor genes such as *p53*, *p73* to induce cell apoptosis (14–16). WWOX knockout mice had increased lung carcinoma susceptibility (10), whereas transfection of WWOX in an animal model caused dramatic suppression of tumorigenesis (17,18). In addition, loss expression of WWOX protein has been found in various tumors (19,20),

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and WWOX is down-regulated by exposure to environmental carcinogens such as Benzo[a]pyrene diol epoxide (9).

The human *WWOX* gene (OMIM: 605131) is located on chromosome 16q23.3–24.1, a region spanning over the common fragile site 16D (FRA16D). Loss of heterozygosis (LOH), homozygous deletions and chromosomal translocations in *WWOX* has been reported in several types of cancer (21–24). In lung cancer, a high incidence of exons absence like the familiar deletion of *WWOX* exons 6–8 was reported (23). However, it is still unclear how these somatic mutations evolve in cancer development. Recently, a germline CNV was found related with LOH in Chr18q22.1 in ileal carcinoid tumor cells (25), implying that the constitutional CNV may induce high frequency of somatic mutations and thus be involved in cancer predisposition. Using bioinformatics analysis, we found that there were several CNVs in *WWOX* (http://projects.tcag.ca/variation/?source=hg18, date last accessed January 6, 2013), and we hypothesized that the CNVs in *WWOX* might be associated with lung cancer risk by disturbing the function of WWOX.

In current study, based on four independent case–control studies, we genotyped one common CNV (CNV-67048) of *WWOX* and assessed its association with lung cancer risk in southern, eastern and northern Chinese populations using a total of 2942 lung cancer cases and 3074 controls. The function of CNV-67048 was further analyzed by protein expression and sequencing assays.

RESULTS

WWOX CNV-67048 genotypes and lung cancer risk

The distribution of demographic characteristics of the discovery set and validation sets was presented in Supplementary Material, Table S1. As expected, there were more smokers in cases than in controls, and case smokers had smoked more pack-years compared with the controls ($P < 0.05$ for all).

The genotype distribution of CNV-67048 was presented in Table 1. Consistent with the genotypes of CNV-67048 reported in DGV databases, we detected three kinds of CNV-67048 (i.e. 2-, 1- and 0-copy) in all samples. We found that there was a significantly higher frequency of loss genotypes (1- and 0-copy) of CNV-67048 among the cases than controls ($P = 5.00 \times 10^{-4}$) in the discovery set, and so was the loss allele ($P = 1.43 \times 10^{-4}$). Compared with the individuals having common 2-copy, those carrying 1-copy harbored a 1.26-fold increased risk of lung cancer (adjusted OR = 1.26, 95%CI = 1.03–1.53; $P = 0.022$), while those with 0-copy had a much higher risk (adjust OR = 2.33, 95%CI = 1.44–3.76; $P = 6.00 \times 10^{-4}$); The effect of the loss allele demonstrated a dose-dependent manner ($P_{trend} = 2.00 \times 10^{-4}$). After the two loss genotypes were combined, they (1- and 0-copy) exerted a 1.35-fold increased risk of lung cancer compared with 2-copy (OR = 1.35, 95% CI = 1.12–1.63; $P = 0.002$). The results in the three validation sets further confirmed the above associations, the loss genotypes of CNV-67048 contributed to a 1.47-fold increased risk of lung cancer (OR = 1.47, 95% CI = 1.12–1.91; $P = 0.005$) in the validation set I, and exerted a 1.40-fold increased risk in the validation set II (OR = 1.40, 95% CI = 1.13–1.74; $P = 0.002$). Similarly, the loss genotypes conferred a 1.32-fold increased risk of lung cancer in the validation set III (OR = 1.32, 95% CI = 1.03–1.70; $P = 0.028$). Because the homogeneity test further showed that the associations in above four sets were homogeneous ($P = 0.915$), we then merged the four populations to increase the study power, and found that the loss genotypes of CNV-67048 harbored a significantly increased risk of lung cancer (OR = 1.39, 95% CI = 1.24–1.55, $P = 9.01 \times 10^{-9}$). The permutation test further confirmed the above association after correction for 10 000 times re-sampling (corrected $P = 1.89 \times 10^{-7}$).

We further analyzed the possible population stratification between cases and controls with the SNP data from the Affymetrix 6.0 (Supplementary Material, Fig. S1), and found that the genomic inflation factor was 1.039; the first three principal components (PCs) were significant with eigenvalues above 1 (i.e. PC1: 1.275; PC2: 1.190; PC3: 1.107). We therefore calculated the correlation between the CNV-67048 and these three PCs, and found that the associations were not significant (Spearman rank correlation analysis: $r = -0.095$, $P = 0.180$ for PC1 and CNV-67048; $r = -0.056$, $P = 0.432$ for PC2 and CNV-67048; $r = -0.003$, $P = 0.963$ for PC3 and CNV-67048). It suggested that the possible confounding effect by population structure on the association between the CNV-67048 and lung cancer risk was not significant.

We also tested whether there were SNPs in linkage disequilibrium (LD) with the CNV-67048 based on the Affymetrix data of 100 controls, but no SNPs that maps to chr16: 76204574-78652476 (covering the *WWOX* gene) showed significant LD with the CNV-67048 (data not shown). At the same time, using our previously published data (26) of five tagSNPs in *WWOX*, we found that this CNV was in a moderate LD with the promoter SNP rs3764340C > G, which had been reported to be associated with lung cancer risk ($D' = 0.581$, $r^2 = 0.138$; Supplementary Material, Fig. S2).

We further assessed possible modifications of the genotype/lung cancer association by surrounding factors in stratification and interaction analysis. Significant associations were observed in most strata except for the subgroups of patients with the histological type of large cell carcinoma ($P = 0.892$), those with small cell lung cancer ($P = 0.123$) and those at stage II of lung cancer ($P = 0.120$) due to a small sample size (Table 2). However, the homogeneity test showed no significant difference between these stratum-ORs ($P > 0.05$ for all). In addition, we did not find any significant interaction between the CNV and surrounding factors on cancer risk ($P > 0.05$ for all, Table 2).

WWOX CNV-67048 genotypes and exons absence

The CNV-67048 is located in the intron 5 of the *WWOX* gene and almost all exons mutations were identified between exon 5 and 9 of *WWOX* (22–24), therefore we tested the transcripts in 37 cases of lung cancer tissues and their adjacent normal tissues. Complete or partial exons absence between exon 5 and exon 9 was identified in lung cancer tissues by qRT–PCR and sequencing assays. As shown in Figure 1A, among 37 lung cancer tissues, we found five cases with abnormal products (case 1, 3, 4, 5 and 8) and one case (case 6) with completely absent *WWOX* transcript, but nothing for other cases or for the

Table 1. The association between *WWOX* CNV-67048 genotypes and lung cancer risk

CNV-67048 Genotypes	Patients *n* (%)	Controls *n* (%)	*P*-value	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
Discovery set					
Total no. of subjects	1056	1056			
2	703 (66.6)	768 (72.7)	5.00×10^{-4}	1.00 (ref.)	1.00 (ref.)
1	299 (28.3)	262 (24.8)		1.25 (1.03–1.52)	1.26 (1.03–1.53)
0	54 (5.1)	26 (2.5)		2.27 (1.41–3.66)	2.33 (1.44–3.76)
Trend test *P*-value				2.00×10^{-4}	2.00×10^{-4}
1 +0	353 (33.4)	288 (27.3)		1.34 (1.11–1.61)	1.35 (1.12–1.63)
loss allele	0.193	0.149	1.43×10^{-4}		
Validation set I					
Total no. of subjects	503	623	0.007		
2	336 (66.8)	465 (74.6)		1.00 (ref.)	1.00 (ref.)
1	146 (29.0)	145 (23.3)		1.39 (1.07–1.82)	1.41 (1.07–1.85)
0	21 (4.2)	13 (2.1)		2.24 (1.10–4.53)	2.11 (1.03–4.31)
Trend test *P*-value				0.002	0.003
1 + 0	167 (33.2)	158 (25.4)		1.46 (1.13–1.90)	1.47 (1.12–1.91)
loss allele	0.187	0.137	0.001		
Validation set II					
Total no. of subjects	773	778			
2	497 (64.3)	561 (72.1)	0.003	1.00 (ref.)	1.00 (ref.)
1	230 (29.7)	188 (24.2)		1.38 (1.10–1.73)	1.35 (1.07–1.70)
0	46 (6.0)	29 (3.7)		1.79 (1.11–2.89)	1.73 (1.07–2.82)
Trend test *P*-value				6.00×10^{-4}	0.002
1 + 0	276 (35.7)	217 (27.9)		1.44 (1.16–1.78)	1.40 (1.13–1.74)
loss allele	0.208	0.158	3.00×10^{-4}		
Validation set III					
Total no. of subjects	610	617			
2	412 (67.5)	452 (73.3)	0.017	1.00 (ref.)	1.00 (ref.)
1	160 (26.2)	145 (23.5)		1.21 (0.93–1.57)	1.23 (0.94–1.60)
0	38 (6.2)	20 (3.2)		2.08 (1.19–3.64)	1.97 (1.12–3.45)
Trend test *P*-value				0.007	0.009
1 + 0	198 (32.5)	165 (26.7)		1.32 (1.03–1.68)	1.32 (1.03–1.70)
loss allele	0.193	0.150	0.004		
Merge set					
Total no. of subjects	2942	3074			
2	1948 (66.2)	2246 (73.0)	2.24×10^{-10}	1.00 (ref.)	1.00 (ref.)
1	835 (28.4)	740 (24.1)		1.30 (1.16–1.46)	1.31 (1.16–1.47)
0	159 (5.4)	88 (2.9)		2.08 (1.59–2.72)	2.05 (1.56–2.68)
Trend test *P*-value				6.38×10^{-11}	1.12×10^{-10}
1 + 0	994 (33.8)	828 (26.9)		1.38 (1.24–1.55)	1.39 (1.24–1.55)
loss allele	0.196	0.149	8.84×10^{-12}		

[a]Adjusted in a logistic regression model that included age, sex, smoking status, drinking status and family history of cancer.

adjacent normal tissues. The aberrant transcripts of *WWOX* were further confirmed by sequencing (Fig. 1B).

The relationship between the CNV-67048 genotypes and WWOX mRNA transcripts was analyzed, as shown in Table 3, lung cancer patients with loss CNV-67048 genotypes (1- or 0-) harbored a significantly higher rate of exons absence in cancer tissues than that of 2-copy genotype (Fisher's exact test: $P = 0.021$). Moreover, those cases with aberrant *WWOX* transcripts showed low or absent protein expression of WWOX (Fig. 1C).

Correlation of WWOX CNV-67048 genotype and with expression phenotype

Because CNVs may be corresponded with exons absence of *WWOX* and thus influence WWOX expression, we used real-time PCR and western blotting to examine the mRNA levels and protein expressions of WWOX. As shown in Figure 2A, the mRNA levels of WWOX in lung cancer tissues were significantly lower than that of their adjacent normal tissues ($P = 0.005$). The loss genotypes of CNV-67048 seemed to have lower WWOX mRNA levels in both cancer tissues and their adjacent normal tissues than 2-copy genotype does, but the difference was not statistically significant (cancer tissues: $P = 0.115$; normal tissues: $P = 0.199$; Fig. 2B). Whereas at protein levels, these cancer tissues with loss genotypes expressed significantly lower WWOX proteins compared with those with 2-copy genotype ($P = 0.036$), while differences in WWOX proteins caused by the genotypes did not occur in normal tissues ($P = 0.104$). In addition, the CNV-67048 genotypes of DNA from tissues were consistent with DNA from their parallel blood samples.

DISCUSSION

In the current four independent case–control studies, we found that the CNV-67048 in *WWOX* was significantly associated

Table 2. Stratification analysis of the *WWOX* CNV-67048 genotypes by selected variables in lung cancer patients and controls

	Patients ($n = 2942$)		Controls ($n = 3074$)		Crude OR (95% CI)	Adjusted OR (95% CI)[a]	$P_{homogeneous}$[b]	$P_{interaction}$[c]
	2 *n* (%)	1 + 0 *n* (%)	2 *n* (%)	1 + 0 *n* (%)	1 + 0 versus 2	1 + 0 versus 2		
Age (years)								
≤60	1049 (66.9)	520 (33.1)	1232 (75.0)	411 (25.0)	1.49 (1.28–1.73)	1.55 (1.30–1.84)	0.191	0.696
>60	899 (65.5)	474 (34.5)	1014 (70.9)	417 (29.1)	1.28 (1.09–1.50)	1.29 (1.09–1.51)		
Sex								
Male	1422 (67.9)	673 (32.1)	1632 (74.4)	561 (25.6)	1.38 (1.21–1.57)	1.38 (1.21–1.58)	0.876	0.792
Female	526 (62.1)	321 (37.9)	614 (69.7)	267 (30.3)	1.40 (1.15–1.71)	1.43 (1.17–1.75)		
Family history of cancer								
Yes	112 (58.0)	81 (42.0)	149 (68.7)	68 (31.3)	1.59 (1.06–2.38)	1.68 (1.11–2.55)	0.501	0.494
No	1836 (66.8)	913 (33.2)	2096 (73.4)	760 (26.6)	1.37 (1.22–1.54)	1.37 (1.22–1.54)		
Smoking status								
Ever	1076 (66.5)	542 (33.5)	1047 (74.7)	355 (25.3)	1.48 (1.27–1.74)	1.49 (1.27–1.74)	0.279	0.239
Never	872 (65.9)	452 (34.1)	1199 (71.7)	473 (28.3)	1.31 (1.13–1.54)	1.33 (1.13–1.55)		
Pack years smoked								
≥20	833 (66.1)	427 (33.9)	691 (74.2)	240 (25.8)	1.48 (1.22–1.78)	1.48 (1.22–1.78)	0. 604	0.304
<20	243 (67.9)	115 (32.1)	356 (75.6)	115 (24.4)	1.47 (1.08–1.99)	1.48 (1.09–2.02)		
0	872 (65.9)	452 (34.1)	1199 (71.7)	473 (28.3)	1.31 (1.13–1.54)	1.33 (1.13–1.55)		
Drinking status								
Ever	301 (67.2)	147 (32.8)	383 (75.5)	124 (24.5)	1.51 (1.14–2.00)	1.61 (1.20–2.17)	0.566	0.519
Never	1235 (65.6)	649 (34.4)	1411 (72.4)	539 (27.6)	1.38 (1.20–1.58)	1.38 (1.20–1.59)		
Histological types								
Adenocarcinoma	624 (66.0)	321 (34.0)	2246 (73.0)	828 (27.0)	1.39 (1.18–1.64)	1.37 (1.16–1.61)	0.234	
Squamous cell carcinoma	496 (64.8)	269 (35.2)			1.47 (1.23–1.75)	1.47 (1.23–1.75)		
Large cell carcinoma	74 (73.3)	27 (26.7)			0.99 (0.63–1.55)	1.01 (0.64–1.59)		
Small cell lung cancer	196 (68.8)	89 (31.2)			1.23 (0.94–1.61)	1.28 (0.98–1.67)		
Other carcinomas[d]	136 (61.3)	86 (38.7)			1.71 (1.29–2.27)	1.76 (1.32–2.34)		
Stages								
I	272 (67.3)	132 (32.7)	2246 (73.1)	828 (26.9)	1.32 (1.05–1.65)	1.33 (1.06–1.66)	0.610	
II	212 (69.5)	93 (30.5)			1.19 (0.92–1.54)	1.22 (0.95–1.58)		
III	581 (65.8)	302 (34.2)			1.41 (1.20–1.66)	1.43 (1.21–1.68)		
IV	707 (65.3)	375 (34.7)			1.44 (1.25–1.65)	1.43 (1.24–1.64)		

[a]ORs were adjusted for age, sex, smoking status, drinking status and family history of cancer in the logistic regression model.
[b]*P*-value of homogeneity test between strata for the related ORs of *WWOX* (CNV-67048, 1 + 0-copy versus 2-copy).
[c]*P*-value of test for the multiplicative interaction between CNV-67048 and selected variables on cancer risk in logistic regression models.
[d]Mixed-cell or undifferentiated carcinoma.

with an increased lung cancer risk. The loss genotypes of CNV-67048 were related with a higher rate of exons absence and lower expression of WWOX than the common 2-copy genotype in lung cancer tissues. To the best of our knowledge, this is the first study conducted to reveal a functional CNV in *WWOX* predisposing development of lung cancer.

The *WWOX* gene is located at the common fragile site 16D (FRA16D), an area with a high frequency of gene deletions or chromosomal alterations. Previous studies have reported that WWOX plays roles in the initiation of tumorigenesis because its expression level decreases in exposure to carcinogens (9,27). WWOX expressions were absent or reduced in 84.9% of lung cancer tissues (20), which was caused by genetic or epigenetic alterations, such as deletion of *WWOX* exons 6–8 in non-small cell lung tissues (23,24,28). In the current study, we showed that WWOX expression was lower in lung cancer tissues compared with their adjacent tissues. Of the 37 lung cancer tissues, we observed five cases with aberrant *WWOX* transcripts containing complete or partial deletion of exons 6–8 and one case with absent *WWOX* transcripts. Germline CNVs is a potential induction factor of these somatic genetic alterations (25), and our findings showed that the loss genotypes of CNV-67048 were associated with a higher rate of exons deletion of *WWOX* than the 2-copy genotype. We also found that the genotypes had significantly lower WWOX protein expressions in tumor tissues but not mRNA levels, indicating a possible mechanism that the CNV might increase the incidence of WWOX exons absence and thus decrease its protein expression, because the aberrant *WWOX* transcripts would result in decreased protein expression as shown in the current study and a previous report (24). However, the above effect was not observed in normal tissues, suggesting that there may be some unrecognized mechanisms on modulating the genetic effect of CNV-67048, such as gene–environment interaction (29). Decreased expression of WWOX is involved in the initiation of tumorigenesis (9,27), and the CNV-67048 could influence the WWOX expression. In summary, it is biologically conceivable that the CNV-67048 was associated with an increased risk of lung cancer. However, the molecular mechanism on how the CNV influences the WWOX exon alteration and gene expression is still unclear.

Previous studies have identified the *WWOX* as a candidate susceptible gene for cancer, in which several SNPs were reported to be associated with multiple cancer risks, including

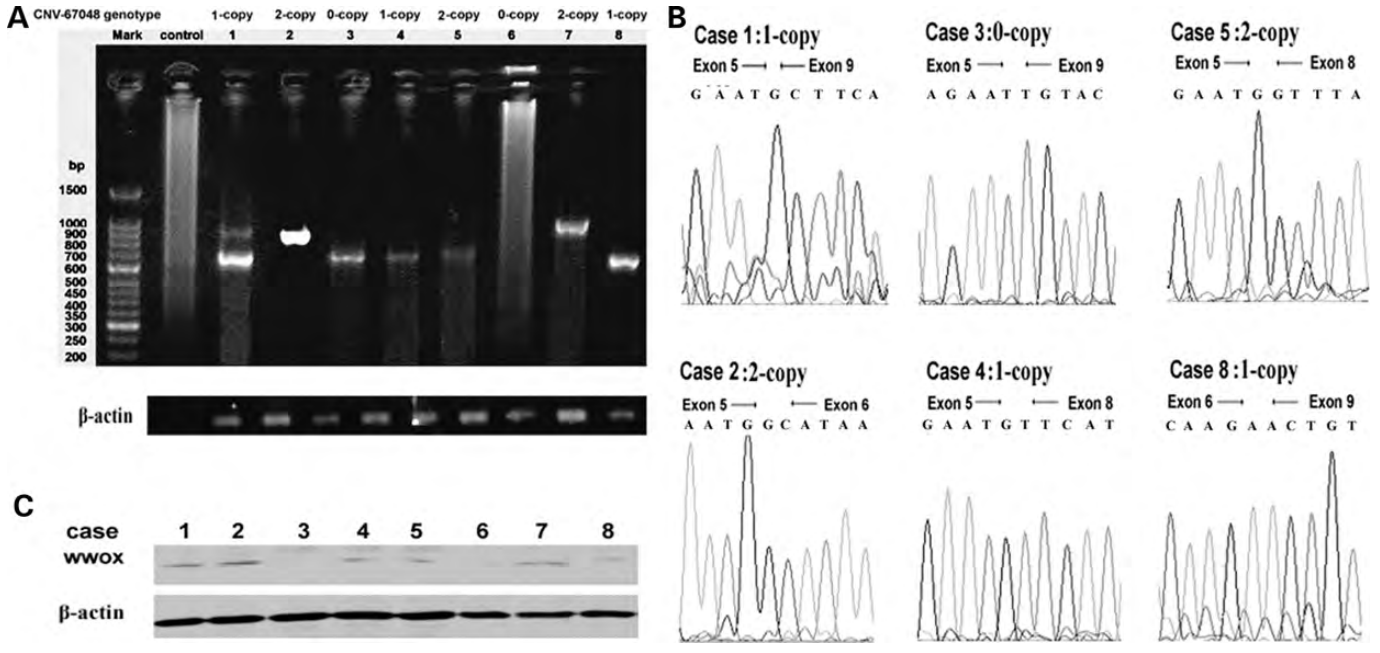

Figure 1. The RT–PCR amplifications of WWOX cDNA in 37 lung cancer tissues. (**A**) Electrophoresis in polyacrylamide gel 2.5% was used to detect wild-type transcript (1284 bp) and alterations in WWOX mRNA transcripts. Five different types of aberrant transcripts were seen in case 1, 3, 4, 5 and 8 as well as one absence of transcript was seen in case 6. (**B**) Sequences showed normal transcript and aberrant transcripts with the loss of exon 6–8. (**C**) The six cases with aberrant transcripts exhibited low protein expressions of WWOX.

lung cancer (26,30–34). Germline CNVs may cause greater adverse effect on cancer risk than SNPs would (35,36). CNVs can directly influence gene expression and phenotypic variation, disrupt gene structure, alter gene dosage and indirectly regulate gene function through position effects, predispose individuals to deleterious genetic changes, or provide substrates for chromosomal change in evolution (37–39), and thus cause disease or confer risk to complex disease traits such as lung cancer (36,40). In the current study, to reveal the association between CNV in *WWOX* and lung cancer risk, we conducted four independent case–control studies with a relatively large data set and found that the common CNV-67048 in *WWOX* harbored an obviously high risk of lung cancer. We also observed that this CNV was in moderate LD with the promoter SNP rs3764340C > G, which was identified to contribute to an increased risk of lung cancer (26). Thus, the CNV-67048 in *WWOX* may be a biomarker for predicting risk of lung cancer.

Our study has several strengths. On the basis of four independent case–control studies, we have got consistent results of the association between the CNV-67048 and lung cancer risk, with a study power of 98.2% (two-sided test, $\alpha = 0.05$) to detect an OR of 1.40 for the loss genotypes (which occurred at a frequency of 27.0% in the controls) compared with the 2-copy genotype, and phenotype analyses further supported the findings that the CNV has significant association with lung cancer risk. However, there are also some limitations. Because this study was restricted to a Chinese Han population, it is uncertain whether our findings can be generalized to other populations; and as hospital-based case–control studies, the selection bias is unavoidable.

In conclusion, this study found that the loss genotypes of CNV-67048 in *WWOX* was associated with an increased risk of lung cancer in Chinese, which was likely related to reduced expression of WWOX accompanying a high rate of WWOX exons deletion. The results suggest that the CNV-67048 in *WWOX* may be a biomarker for susceptibility to lung cancer. Validations with larger population-based studies in different ethnic groups and mechanism studies are warranted.

MATERIALS AND METHODS

Study subjects

A two-stage and four independent case–control studies with a total of 2942 lung cancer cases and 3074 controls were conducted in southern, eastern and northern Chinese. The southern and eastern populations have been described previously (36,41,42). Briefly, in the discovery set, 1056 primary lung cancer patients representing southern Chinese were recruited between March 2007 and March 2009 from five hospitals in Guangzhou city with a response rate of 95%, and 1056 age (± 5 years) and sex frequency-matched healthy controls were randomly selected from a subject pool of >10 000 individuals who participated in the annual healthy checkup programs in the community health stations in Guangzhou city during the same period when the cases were recruited with a response rate of 84%; In the validation set I, 503 lung cancer cases representing eastern Chinese were enrolled from hospitals in Suzhou city between March 2008 and May 2010 with a response rate of 87%, and 623 controls who

Table 3. WWOX transcripts distribution in lung cancer tissues by CNV-67048 genotypes

Genotypes	Normal transcript, *n* (%)	Aberrant transcript,[a] *n* (%)	*P*-value[b]
2	22 (95.6)	1 (4.4)	0.021
1	8 (72.7)	3 (27.3)	
0	1 (33.3)	2 (66.7)	

[a]Including WWOX transcript with exon 6–8 absence and WWOX transcript absence.
[b]*P*-values of Fisher's exact test for 1- and 0-copy versus 2-copy.

were frequency matched to the cases by sex and age (±5 years) were recruited from 3500 individuals responded to a nutritional survey conducted in Suzhou city in the same time period as the cases were recruited with an 81% response rate. In the validation set II, 773 lung cancer patients which were continuously recruited from Guangzhou city between April 2009 and June 2011 with an 89% response rate and 778 sex and age (±5 years) frequency-matched cancer-free controls that were randomly selected from ∼3000 individuals participating in health community programs with an 83% response rate was used. Moreover, a population from Harbin city was used as the validation set III. It included 610 lung cancer cases that were enrolled between December 2009 and March 2012 with an 82% response rate and 617 controls that were randomly selected from 8000 participators in the annual healthy checkup programs with a response rate of 91%. All the participants were genetically unrelated ethnic Han Chinese and none had blood transfusion in the last 6 months. The individuals were scheduled for an interview with a structured inquiring on smoking status, alcohol use and other factors. They were to also donate 5 ml peripheral blood after a written informed consent have obtained. The definitions of smoking status, drinking status, pack year smoking and family history of cancer were described in our previous publications (41–43). The study was approved by the institutional review boards of Guangzhou Medical University, Soochow University and Harbin Medical University.

CNV selection and genotyping

By searching the DGV database (http://projects.tcag.ca/variation/?source=hg18, date last accessed January 6, 2013), we found that 63 CNVs are located in the *WWOX* gene (Supplementary Material, Fig. S3), of which 25 CNVs were reported to exist in Asian or Chinese population. However, among them, the minor allele frequency (MAF) of most CNVs are rare (<1%) except for two CNVs (i.e. CNV-38092: MAF = 0.199 and CNV-67048: MAF = 0.285). The CNV-38092 maps to chr16:76,929,941-76,942,266, whereas the CNV-67048 maps to chr16:76,929,120-76,942,453 which completely comprises the CNV-38092. The two CNVs are both loss of copy number, and by blasting the reported CNV data of individuals with loss of copy number, 98% of individuals that identified to have loss of CNV-38092 actually have loss of CNV-67048. Therefore, we selected the common CNV-67048 in this study.

Genomic DNA was extracted from 2 ml whole blood of each participant. The final concentrations of all DNA samples were 20 ng/μl with good purity (OD_{260}/OD_{280} = 1.8–2.0). According to the protocol of Applied Biosystems (44), the Taqman real-time quantitative PCR (qPCR) method was used to test the copy number of CNV-67048 in all 6016 subjects with TaqMan® gene copy number assays (cat# Hs03922779, Applied Biosystems) and the control RNase P probe (Applied Biosystems; Supplementary Material, Fig. S4A). Furthermore, the ACCUCOPY assay (a multiple competitive real-time PCR) by commercial company (Genesky Bio-Tech Co., Ltd, Shanghai, China; Supplementary Material, Fig. S4B) and the Affymetrix Genome-Wide Human SNP Array 6.0 by commercial company (Biomiao Biological Technology, Beijing, China) were used to validate the above genotyping data in 200 randomly selected samples (Supplementary Material, Fig. S5) (45). The results were 98.6 or 95.0% concordant with the results from the Taqman qPCR method.

WWOX exons absence detection

Thirty-seven primary lung cancer tissues and their adjacent normal tissues from the second hospital affiliated to Guangzhou Medical University (Guangzhou, China) were subjected to extraction of the total RNA and protein. The nest RT–PCR was performed with the cDNA in both lung cancer tissues and their adjacent normal tissues as suggested by Kuroki *et al*. (22). Two primers (Primer1: forward primer, 5′-AGT TCC TGA GCG AGT GGA CC-3′; reverse primer, 5′-TTA CTT TCA AAC AGG CCA CCA C-3′; Primer2: forward primer, 5′-AGG TGC CTC CAC AGT C-3′; reverse primer, 5′- GTG TGT GCC CAT CCG CTC T -3′) were used to amplify the whole length of *WWOX* transcripts, and β-actin amplification served as a control for cDNA quality. The amplified products were analyzed by electrophoresis on a 2.5% agarose gel. DNA bands corresponding to the normal and abnormal size of *WWOX* transcripts were excised and purified from the gel using the QIAquick gel extraction kit (QIAGEN). The amplifications were further applied for direct sequencing by Invitrogen Company (Shanghai, China).

WWOX expression detection

The mRNA levels of WWOX were detected by SYBR-Green real-time PCR in the 37 cases of tumor tissues and adjacent normal tissues with self-designed primers: 5′-TGG GTT TAC TAC GCC AAT C-3′ (forward) and 5′- GTC CGT TCT CAT CAG TTT CT -3′ (reverse) to amplify 124 bp cDNA sequences from exon2 to exon3 of WWOX, the β-actin was used as an internal reference gene with the primers: 5′-GGC GGC ACC ACC ATG TAC CCT-3′ (forward) and 5′-AGG GGC CGG ACT CGT CAT ACT-3′ (reverse). The protein expression of WWOX was detected by western blotting as previously described (42). The expression of β-actin was used as an internal control. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the relative WWOX protein levels normalized to β-actin were semi-quantified with a software Gene Tools (version 4.01, Syngene, Cambridge, UK) assembled in our

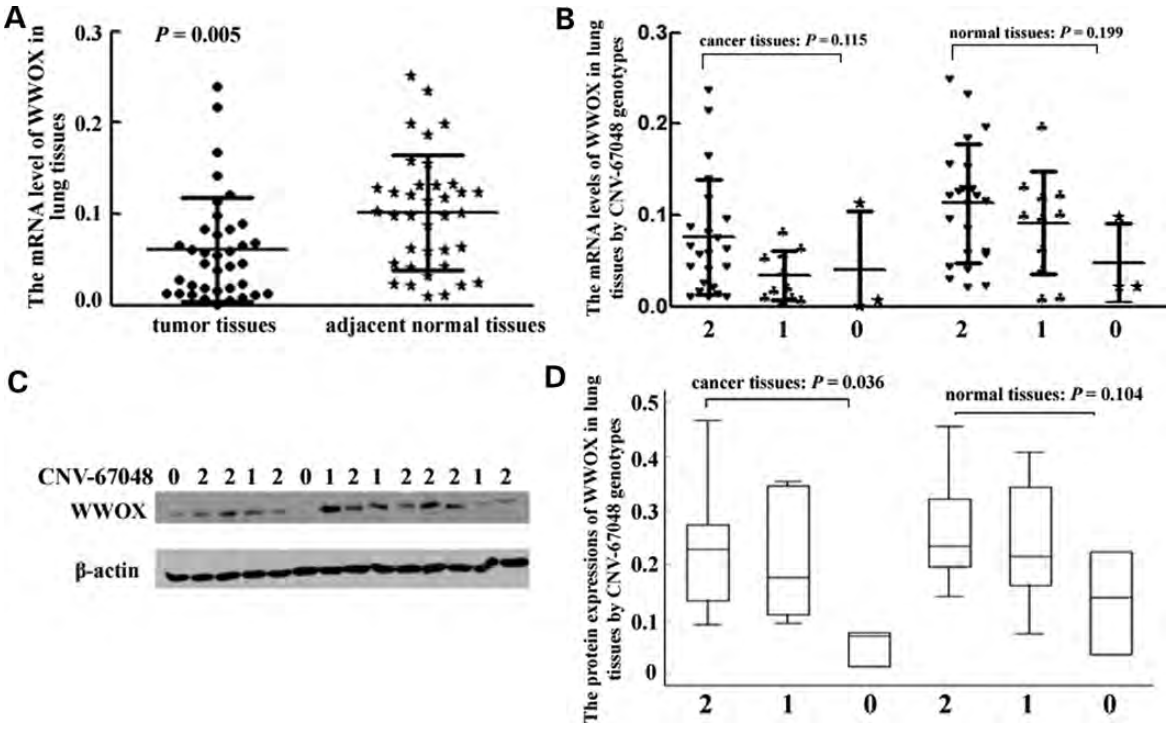

Figure 2. Association between the CNV-67048 and WWOX expression. (**A**) The relative mRNA levels of WWOX in lung cancer tissues compared with adjacent normal lung tissues. (**B**) The relative mRNA levels of WWOX by CNV-67048 genotypes in lung tissues. (**C**) The WWOX protein bands in lung tissues. (D) The protein expressions of WWOX by CNV-67048 genotypes. The WWOX expression was normalized to β-actin. The differences in expression levels of WWOX were analyzed using Student's *t*-test and ANOVA test.

image machine (G:BOX, Syngene, Cambridge, UK). The CNV-67048 genotypes of the samples were detected by the Taqman qPCR method and validated with the ACCUCOPY assay.

Statistical analysis

PCs analysis was performed to use SNP data from the Affymetrix 6.0 to infer population structure between cases and controls using the R statistical software with the GenABEL package and the Spearman rank correlation analysis was used to the association between CNV-67048 and the significant principal components. The Chi-square tests were used to assess differences in the distribution of the CNV-67048 genotypes between cases and controls. Unconditional logistic regression model with and without adjustment for age, sex, smoking status, drinking status and family history of cancer was used to estimate the association between the CNV-67048 and lung cancer risk. The Haploview 4.2 software was used to test the LD between SNPs and CNV-67048. The multiplicative interaction model was used to assess the possible interaction of CNV-67048 and surrounding factors on lung cancer risk (42). The Breslow–Day test was used to test the homogeneity of the results between stratum-ORs, and 10 000 times permutation test was used to estimate *P*-values corrected after re-sampling with the Stata software (version 11.0; StataCorp LP, USA). The statistical power was calculated by using the PS Software (46). Furthermore, One-way ANOVA tests were used for analyzing the association between CNV-67048 genotypes and WWOX expression. The distribution between the CNV-67048 genotypes and WWOX mRNA transcripts was analyzed with Fisher's exact test. All tests were two-sided using the SAS software (version 9.3; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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