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, \( P = 9.01 \times 10^{-2} \)) in a dose–response manner (\( P_{\text{trend}} = 1.12 \times 10^{-10} \)), 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 CNVs may cover 

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 reported in DGV databases, we detected three kinds of CNVs, we 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 (adjusted OR = 2.33, 95% CI = 1.44–3.76; P = 6.00 × 10^{-4}); The effect of the loss allele demonstrated a dose-dependent manner (P_trend = 2.00 × 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 = 0.892), those at stage II of lung cancer (P = 0.892), those with small cell 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
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).

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

<table>
<thead>
<tr>
<th>Patients</th>
<th>Controls</th>
<th>P-value</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
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</table>

**Discovery set**
- Total no. of subjects: 1056
- 2: 703 (66.6)
- 1: 299 (28.3)
- 0: 54 (5.1)
- Trend test P-value: \( 5.00 \times 10^{-4} \)
- 1 + 0 loss allele: 0.193
- 1.43 \times 10^{-4}

**Validation set I**
- Total no. of subjects: 503
- 2: 336 (66.8)
- 1: 146 (29.0)
- 0: 21 (4.2)
- Trend test P-value: 0.002

**Validation set II**
- Total no. of subjects: 773
- 2: 497 (64.3)
- 1: 230 (29.7)
- 0: 46 (6.0)
- Trend test P-value: 6.00 \times 10^{-4}

**Validation set III**
- Total no. of subjects: 610
- 2: 412 (67.5)
- 1: 160 (26.2)
- 0: 38 (6.2)
- Trend test P-value: 3.00 \times 10^{-4}

**Merge set**
- Total no. of subjects: 2942
- 2: 1948 (66.2)
- 1: 835 (28.4)
- 0: 159 (5.4)
- Trend test P-value: 8.84 \times 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
genetic or epigenetic alterations, such as deletion of WWOX transcripts containing complete or partial deletion of 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. 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 with an increased lung cancer risk. 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).

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 might influence the WWOX expression. 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 might influence the WWOX expression.
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 ($\pm 5$ 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
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 89% 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. The institutional review boards of Guangzhou Medical University, Soochow University and Harbin Medical University. The study was approved by one having reviewed 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 (Biomia 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).

**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.

**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 CAT C-3′ (forward) and 5′-GTC GTG 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 GCC ACC ACC ATG TAC CCT C-3′ (forward) and 5′-AGG GCC CGG ACT CGT CAT ACT C-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, Synegene, Cambridge, UK) assembled in our lab.

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

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Normal transcript, n (%)</th>
<th>Aberrant transcript, a n (%)</th>
<th>P-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22 (95.6)</td>
<td>1 (4.4)</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>8 (72.7)</td>
<td>3 (27.3)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
<td></td>
</tr>
</tbody>
</table>

aIncluding WWOX transcript with exon 6–8 absence and WWOX transcript absence.
bP-values of Fisher’s exact test for 1- and 0-copy versus 2-copy.

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 (OD260/OD280 = 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 (Biomia 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.

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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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