The effect of functional MAPKAPK2 copy number variation CNV-30450 on elevating nasopharyngeal carcinoma risk is modulated by EBV infection

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Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) is recognized as oncogenic and simulative role on tumorigenesis by virtue of abnormal expression in cancer including nasopharyngeal carcinoma (NPC). We hypothesized that the copy number variation (CNV)-30450, which duplicates the MAPKAPK2 promoter, may affect MAPKAPK2 expression and be associated with NPC risk. In two independent case–control panels of southern and eastern Chinese with a total of 1590 NPC patients and 1979 controls, we investigated the association between CNV-30450 and NPC risk by genotyping the CNV-30450 with the TaqMan assay, and tested its biological effect. Consistent findings were observed in the two populations, that the increased copy number of CNV-30450 was associated with increased risk of NPC (3/4-copy versus 2-copy: odds ratio = 1.28, 95% confidence interval = 1.10–1.49), in which lies a biological mechanism that the adverse genotypes enhanced the promoter activity of MAPKAPK2 and elevated MAPKAPK2 expression. Moreover, the CNV-30450 adverse genotypes significantly interacted with Epstein–Barr virus (EBV) infection on increasing NPC risk (P = 0.035), and the genotype–phenotype correlation was only significant in EBV-positive cases (P = 0.037) but not in EBV-negative ones (P = 0.366). These data suggest that the functional CNV-30450 in the MAPKAPK2 promoter elevates the NPC risk with a modulation by EBV infection, which may be an indicator of susceptibility to NPC.

Summary: This case–control study suggests that the functional CNV-30450 in the MAPKAPK2 promoter elevates the NPC risk with a modulation by EBV infection, which may be an indicator of susceptibility to NPC.

Materials and methods

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer that is prevalent in south-eastern Asia and other areas like north Africa but rare in most parts of the world (1). In south China, the incidence rate of NPC for males is >20 per 100 000 person–years, which is far above the rest of the world (1,2). The pathogenesis of NPC is complexly associated with Epstein–Barr virus (EBV) infection and genetic as well as environmental factors (3–8). EBV is the most important risk factors of NPC (3). Multiple evidences have demonstrated that EBV causes direct carcinogenesis by triggering various cellular responses, including the activation of the mitogen-activated protein kinase (MAPK) pathway (KEGG PATHWAY: hsa04010), which is known to regulate cell apoptosis, inflammation and tumorigenesis (9–11). Consistently, overactivated MAPK pathway is reported to contribute to NPC development (10,12). Moreover, the genes coding for MAPK pathway are reported to be frequently mutated in cancer (13,14).

MAPK-activated protein kinase 2 (MAPKAPK2, MIM no. 602006) (15) is a downstream substrate of the p38 MAPK pathway. MAPKAPK2 can be directly activated by EBV infection and plays a decisive role in regulating a variety of biological responses (10,16). Previous studies have confirmed the oncogenic role of MAPKAPK2 by directly phosphorylating and activating the oncogene cell division cycle 25B/C (Cdc25B/C) (17) and involving the DNA repair process and cell apoptosis through activation of heat shock protein 27 (HSP27) and HSP25 (18,19). MAPKAPK2 is also a posttranscriptional factor that regulates gene expressions of several oncogenes and proinflammatory genes, such as tristetraprolin (20) and butyrate response factor 1 (21). Meanwhile, the EBV-encoded oncogene product, latent membrane protein 1, can activate the p38/MAPKAPK2 pathway and induce nasopharyngeal carcinogenesis (10). Overexpression of MAPKAPK2 is also reported in various cancers (12,22–24).

The human MAPKAPK2 gene spans over ~49 kb on chromosome 1q32 that contains 10 exons and encodes a 400 amino acid protein. Studies have identified that copy number aberrations in 1q32 were significantly associated with increased cancer risk and poor prognosis (25,26). Recently, one study reported that copy number variations (CNVs) on chromosome 1 are associated with MAPKAPK2 overexpression, which influences prognosis of gastrointestinal stromal tumor (22). Also, we have previously identified that CNV-30450, which is located in the MAPKAPK2 promoter, is associated with the lung cancer risk and prognosis (27). Worth of notice, NPC and lung cancer overlap etiologically through exposure to tobacco and potentially at several genetic susceptibility loci (e.g. TNFRSF19 and TERT) (28–30), we therefore hypothesized that the CNV-30450 in MAPKAPK2 may alter carriers’ susceptibility to NPC.

In the current study, we analyzed the association between CNV-30450 and NPC risk in two independent case–control studies in southern and eastern Chinese with a total of 1590 NPC patients and 1979 controls. We further performed functional assays to assess the biological effect of the CNV-30450.

Materials and methods

Study population

Two independent case–control panels were conducted in southern Chinese and eastern Chinese as described previously (13,31,32). NPC was defined histopathologically confirmed by biopsy. Patients who lacked biopsy and subjects who refused to sign an informed consent or donate blood were excluded. Almost all NPC patients that satisfied the above criteria were consecutively recruited in appointed hospitals. Panel I included 906 NPC cases that were recruited from four urban hospitals (i.e. the First Hospital, the Second Hospital, the Tumor Hospitals affiliated to Guangzhou Medical University and the Guangzhou Chest Hospital) and one suburban hospital of Guangzhou city (i.e. PanYu People’s Hospital) during April 2002 to June 2010 with a response rate of 91%; and 1072 cancer-free controls that were selected with random-stratified sampling and with frequency-matched cases on age (±5 years) and sex from 10 000 participants of healthy check-up programs conducted in two communities of Guangzhou city with a response rate of 84%: Panel II included 684 NPC patients who were consecutively recruited from March 2001 to May 2009 at four urban hospitals of Jiangsu Province (i.e. The First Affiliated Hospital, The Second Affiliated Hospital of Soochow University, The Third Hospital Affiliated to Nantong University and Huaian No. 1 Hospital) with a response rate of 89%; and 907 normal controls.

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; CNV, copy number variation; EBV, Epstein–Barr virus; HSP, heat shock protein; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; mRNA, messenger RNA; NPC, nasopharyngeal carcinoma; OR, odds ratio; SNP, single-nucleotide polymorphism.

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controls who were randomly picked up with random-stratified sampling and with frequency-matched cases on age (±5 years) and sex from a 3500 individual nutritional survey conducted in one community of Suzhou city with an 81% response rate. The response rate is the result of dividing the number of people recruited by the total number of people who were eligible to participate (i.e. individuals recruited and those excluded because of refusal to sign an informed consent or offer necessary materials). All the controls were cancer-free, and no restrictions for recruiting the healthy subjects in neither both healthy check-up programs conducted in two communities of Guangzhou city nor nutritional survey program conducted in one community of Suzhou city. After provision of their written informed consent, each participant was scheduled for an interview to provide data on age, sex, family history of cancer, smoking status and drinking status, all of which are identified to be associated with NPC risk (5–8), and to donate 5 ml peripheral blood. The definition of the smoking status, pack-years smoked, alcohol use and the family history of cancer has been described elsewhere (33,34). The pathological stages of cases were collected from the medical records. Immunoglobulin-A antibodies to EBV capsid antigen and immunoglobulin-A antibodies to EBV early antigen were tested by serologic testing at the time of sample enrollment. All subjects were genetically unrelated ethnic Han Chinese, and those subjects who failed to detect EBV status were excluded. The study was approved by the institutional review boards of Guangzhou Medical University and Soochow University.

**CNV selection**

By searching the Database of Genomic Variants (http://projects.tcgag.ca/variation/?source=hg18), we found that there were three CNVs (i.e. CNV-2091, CNV-30450 and CNV-71062) located on the MAPKAPK2 gene (Supplementary Figure S1A, available at Carcinogenesis Online). Because of the altered copy number frequency of CNV-71062 and CNV-2091 were low (1/39 = 0.026; 15/269 = 0.055, respectively), we only selected CNV-30450 (minor allele frequency: 6/30 = 0.200), which spans over the MAPKAPK2 promoter region with 1.7 kb sequences (−1098−668 nt to initiation transcription code ATG, because of the start site of the CNV with location at −1098 nt and the end site at +668 nt relative to ATG). Moreover, although a recent study has identified eight CNVs on chromosomes, namely 3p14.1, 6p21.33, 7p22.2, 7q11.23, 8q22, 12p13.1, 19p13.3 and 19q13.42, to be associated with NPC susceptibility (35), because human MAPKAPK2 gene is located on chromosome 1q32 and several studies have identified that copy number aberrations in 1q32 were significantly associated with increased cancer risk and poor prognosis (25,26). In this gene-based hypothesis-driven study, we only considered CNVs (i.e. CNV-2091, CNV-30450 and CNV-71062) that are located in the region of MAPKAPK2 gene and genotyped the CNV-30450 of MAPKAPK2 in the current study.

**CNV-30450 genotyping**

The genotyping of CNV-30450 has been described previously (27). In brief, genomic DNA was extracted from 2 ml whole blood and the final concentration of genomic DNA was normalized to 20 ng/μl. We genotyped CNV-30450 by the TaqMan real-time PCR according to the protocol of Applied Biosystems on an ABI 7900HT fast real-time PCR System (Applied Biosystems, Foster City, CA) (36); in 10 μl reaction systems, triplicate 10 ng DNA samples were mixed with TaqMan PCR Master Mix (Applied Biosystems), the control RNase P probe (VIC labeled; Applied Biosystems) and the experimental probe (FAM labeled, cat no. Hs01173160; Applied Biosystems), then the mixture was ran on an ABI 7900HT fast real-time PCR System (Applied Biosystems) and the CNV-30450 genotype was automatically determined by software CopyCaller 2.0 (Applied Biosystems; Supplementary Figure S1B, available at Carcinogenesis Online). The mixture without genomic DNA was defined as zero copy number, and three samples with two copies of CNV-30450 locus found by Affymetrix Genome-Wide Human SNP Array 6.0 were used as a standard sample (27). We also randomly selected 150 samples to validate the above genotyping results with the Accucopy assay (a multiple competitive real-time PCR; Supplementary Figure S2, available at Carcinogenesis Online) by a commercial company (Genesky Bio-Tech Co., Ltd, Shanghai, China). The results were 96.5% concordant with the results from TAQMAN quantitative PCR method. The allele of CNV-30450 was defined as single and dual copy in haplotypes. Therefore, as diploid organism, 2-copy refers to subjects with two copies of CNV-30450, because human MAPKAPK2 has two alleles (Chr1, chr1: 204,923,812 - 204,925,806, referring to the start site and end site of the CNV on human chromosome 1) from subjects with 2-copy genotype. Two primers, including the artificial KpnI and Hind3 enzymes restriction sites: 5′-CGG GGT ACC TCT AAC TGG CCT TAC CAA GAC G3′- (forward) and 5′-CCC AAG CTT ACC GAC CAA CCG CCT CAA TG3′- (reverse), were used. The amplified fragments and pGL3 basic vector (Promega, Madison, WI) were cleaved with the KpnI and Hind3 enzymes (New England BioLabs, Ipswich, MA) and then ligated by T4 DNA ligase (New England Biolabs). The dual copies allelic reporter gene was constructed based on the single copy allelic reporter constructs as follows: the single copy allelic reporter construct was first inserted with MluI and XhoI enzymes restriction sites with two-paired primers; MluI: forward primer 5′-CTG CTA ATT TCA CTC CAT C ACG GGT CC GTC GAG TCA AGG AAG TGA TT3′ and reverse primer 5′-AAT CAC TTC CTT GAC TGC AGG AGC AGT CAT GGA GGT AAA TTA GTA G3′-; XhoI: forward primer 5′-CTG CTA ATT TCA CTC CAT C ACG GGT CC GTC GAG TCA AGG AAG TGA TT3′ and reverse primer 5′-CCA ACA TCA CCT TGG TTA GAG AAG TTA GTA G3′- and two linked single above fragments by using the primers: forward primer 5′- ccg ACG CAG TAC GGA TTA ATT TTT CCA CAT CGG G3′- and reverse primer 5′-CCG CCC CAG GAT GGA GTG AAA TTA GTA GAA G3′-. The amplified fragments were then subcloned into pGL3 basic vector. We sequenced all reporter constructs to confirm the sequence, orientation and integrity of each insert.

**Luciferase reporter assay**

The in vitro luciferase assays were similar as described previously (13,27). Two human NPC cell lines, CNE-1 and CNE-2, were seeded onto 24-well plates and cotransfected with 0.5 μg reporter plasmid and 10 ng pRL-TK plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA) when cells were grown to 80–90% confluence. The pRL-TK plasmid was used as an internal reference. Luciferase activity was then measured with a Dual-Luciferase Reporter Assay System (Promega) in the following 14–16 h. The luciferase activity was calculated by the firefly luciferase activity dividing by the renilla luciferase activity. Three independent transection experiments were performed for each report gene and done in triplicate at one time.

**MAPKAPK2 expression detection**

The messenger RNA (mRNA) levels of MAPKAPK2 and an internal reference β-actin were tested in 22 cases of primary NPC tissues from the tumor hospital affiliated to Guangzhou Medical University (Guangzhou) and in 27 cases of NPC tissues from the First Affiliated Hospital of Soochow University (Suzhou) as described previously (13,27,33) with the SYBR-Green real-time PCR method. The allele of CNV-30450 was defined as single and dual copy in haplotypes. Therefore, as diploid organism, 2-copy refers to subjects with two copies of CNV-30450, because human MAPKAPK2 has two alleles (Chr1, chr1: 204,923,812 - 204,925,806, referring to the start site and end site of the CNV on human chromosome 1) from subjects with 2-copy genotype. Two primers, including the artificial KpnI and Hind3 enzymes restriction sites: 5′-CGG GGT ACC TCT AAC TGG CCT TAC CAA GAC G3′- (forward) and 5′-CCC AAG CTT ACC GAC CAA CCG CCT CAA TG3′- (reverse), were used. The amplified fragments and pGL3 basic vector (Promega, Madison, WI) were cleaved with the KpnI and Hind3 enzymes (New England BioLabs, Ipswich, MA) and then ligated by T4 DNA ligase (New England Biolabs). The dual copies allelic reporter gene was constructed based on the single copy allelic reporter constructs as follows: the single copy allelic reporter construct was first inserted with MluI and XhoI enzymes restriction sites with two-paired primers; MluI: forward primer 5′-CTG CTA ATT TCA CTC CAT C ACG GGT CC GTC GAG TCA AGG AAG TGA TT3′ and reverse primer 5′-AAT CAC TTC CTT GAC TGC AGG AGC AGT CAT GGA GGT AAA TTA GTA G3′-; XhoI: forward primer 5′-CTG CTA ATT TCA CTC CAT C ACG GGT CC GTC GAG TCA AGG AAG TGA TT3′ and reverse primer 5′-CCA ACA TCA CCT TGG TTA GAG AAG TTA GTA G3′- and two linked single above fragments by using the primers: forward primer 5′- ccg ACG CAG TAC GGA TTA ATT TTT CCA CAT CGG G3′- and reverse primer 5′-CCG CCC CAG GAT GGA GTG AAA TTA GTA GAA G3′-. The amplified fragments were then subcloned into pGL3 basic vector. We sequenced all reporter constructs to confirm the sequence, orientation and integrity of each insert.

![Figure 1](https://academic.oup.com/carcin/article-abstract/35/1/46/361631/figure1)
PCR on an ABI Prism 7500 sequence detection system (Applied Biosystems). The primers used for MAPKAPK2 were 5'-CAG CTC CCG CAG TTC-3' (forward) and 5'-CGA ATT TCT CTT GGG TCC TCT-3' (reverse) and for β-actin were 5'-GGC GCC ACC ACC ATG TAC CCT-3' and 5'-AGG GCC CGG ACT CGT CAT ACT-3'. Method of 2^n was used to demonstrate the level of MAPKAPK2 expression. The mean mRNA level of each sample was obtained by triplicate. The CNV-30450 genotypes of all samples, including both blood DNA and tissue DNA, were detected by TAQMAN quantitative PCR method. Moreover, another 54 paraffin sections of primary NPC tissues from Guangzhou city and 34 cases of NPC tissues from Suzhou city were applied for immunohistochemistry to analyze the MAPKAPK2 protein expression in situ by treating with liquid diaminobenzidine reagent, which used 3,3'-diaminobenzidine tetrahydrochloride as the chromogen and hemafoxin as the counterstaining reagent. By using the Image Pro Plus software, the MAPKAPK2 cytoplasmic expression was scored according to the sum of the intensity and percentage by two independent pathologists simultaneously.

Statistical analysis

The chi-square test was used to assess the differences in distributions of demographic characteristics and the CNV-30450 genotypes between cases and controls. Association between the CNV-30450 genotypes and NPC risk was estimated using an unconditional logistic regression model with or without adjustments of age, sex, smoking status, drinking status, family history of cancer and center; the unconditional logistic regression model was also used for the trend test. The Breslow–Day test was used to test the homogeneity among strata odds ratios (ORs). Interactions between MAPKAPK2 adverse genotypes and the selected factors on NPC risk were analyzed with a multiplicative interaction as when OR 11 > OR 10 x OR 01, in which OR 11 = the OR when both factors were present, OR 01 = the OR when only factor 1 was present, OR 10 = the OR when only factor 2 was present (37). The PS Software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize) was used to calculate the statistical power (38). The one-way analysis of variance (ANOVA) test and the cross-validation analysis (39) were used for analyzing the association between CNV-30450 genotypes and MAPKAPK2 expressions. Student’s t-test was applied to examine the difference in activity of different reporter genes. To explore the putative functional single-nucleotide polymorphisms (SNPs), which may affect MAPKAPK2 expression, we queried the gene expression database in all populations at SNPexp (http://app3.titan.uio.no/biotoools/tool.php?apps=snpexp) to test associations between the SNPs and expression levels of MAPKAPK2. All tests were two sided by using the SAS software (version 9.3; SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

Results

Demographics of the study population

The frequency distribution of demographic characteristics of all subjects was shown in Table I. There was no significant deviation in age, sex, family history of cancer between cases and controls. NPC cases were more smokers, drinkers and EBV infestors compared with the controls in both populations (P < 0.05 for all). The Breslow–Day test of homogeneity further showed that the frequency distributions of drinking status and EBV status were not homogeneous between the two populations (P < 0.001), reflecting minorly different lifestyle among two populations. We also enumerated the clinical stages of cases in Table I.

The CNV-30450 genotypes and NPC risk

As presented in Table II, three kinds of CNV-30450 genotypes (i.e. 2-, 3- and 4-copy) were detected. In the southern Chinese, we found significant differences in CNV-30450 genotypes as well as in alleles between cases and controls (P = 0.035; P = 0.002, respectively). Compared with the most common 2-copy, the variant 4-copy was significantly associated with increased risk of NPC (adjusted OR = 1.38, 95% confidence interval (CI) = 1.05–1.83; P = 0.023), and 3-copy was also associated with NPC risk on borderline statistical significance (OR = 1.26, 95% CI = 0.98–1.63; P = 0.076). The dichotomic analysis showed that the 3/4-copy conferred a 1.32-fold increased risk of NPC compared with 2-copy (OR = 1.32, 95% CI = 1.07–1.61; P = 0.009). The findings from the eastern Chinese revealed consistent results with the above findings (Breslow–Day test: P = 0.019), the 3/4-copy of CNV-30450 contributed a 1.25-fold increased cancer risk (OR = 1.25, 95% CI = 1.00–1.57; P = 0.049). We then merged these two populations to increase the study power and found that the 3/4-copy genotype harbored a 1.28-fold increased risk of NPC compared with 2-copy (OR = 1.28, 95% CI = 1.10–1.49; P = 0.001).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Panel I (southern Chinese)</th>
<th>Panel II (eastern Chinese)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case (n = 906), n (%)</td>
<td>Control (n = 1072), n (%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>396 (43.7)</td>
<td>482 (45.0)</td>
</tr>
<tr>
<td>≥50</td>
<td>510 (56.3)</td>
<td>590 (55.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>647 (71.4)</td>
<td>763 (71.2)</td>
</tr>
<tr>
<td>Female</td>
<td>259 (28.6)</td>
<td>309 (28.8)</td>
</tr>
<tr>
<td>Family history of cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>88 (9.7)</td>
<td>83 (7.7)</td>
</tr>
<tr>
<td>No</td>
<td>818 (90.3)</td>
<td>989 (92.3)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>458 (50.6)</td>
<td>481 (44.9)</td>
</tr>
<tr>
<td>Never</td>
<td>448 (49.4)</td>
<td>591 (55.1)</td>
</tr>
<tr>
<td>Drinking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>440 (48.6)</td>
<td>245 (22.9)</td>
</tr>
<tr>
<td>Never</td>
<td>466 (51.4)</td>
<td>827 (77.2)</td>
</tr>
<tr>
<td>EBV infection status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>756 (83.4)</td>
<td>194 (18.1)</td>
</tr>
<tr>
<td>Negative</td>
<td>150 (16.6)</td>
<td>878 (81.9)</td>
</tr>
<tr>
<td>Stages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>51 (5.7)</td>
<td>27 (3.9)</td>
</tr>
<tr>
<td>II</td>
<td>244 (26.9)</td>
<td>177 (25.9)</td>
</tr>
<tr>
<td>III</td>
<td>377 (41.6)</td>
<td>316 (46.2)</td>
</tr>
<tr>
<td>IV</td>
<td>234 (25.8)</td>
<td>164 (24.0)</td>
</tr>
</tbody>
</table>

*P values for a chi-square test.
**Table II. The association between MAPKAPK2 CNV-30450 genotypes and NPC risk**

<table>
<thead>
<tr>
<th>CNV-30450 genotypes</th>
<th>Patients, ( n (%) )</th>
<th>Controls, ( n (%) )</th>
<th>( P )</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)(^a)</th>
</tr>
</thead>
</table>

Panel I (southern Chinese)

<table>
<thead>
<tr>
<th>2</th>
<th>3</th>
<th>4</th>
<th>( n = 906 )</th>
<th>( n = 1072 )</th>
<th>( 2 )</th>
<th>( 3 )</th>
<th>( 4 )</th>
<th>( 2 )</th>
<th>( 3 )</th>
<th>( 4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>617 (68.6)</td>
<td>786 (73.7)</td>
<td>( 0.035 )</td>
<td>1.00 (ref.)</td>
<td>1.00 (ref.)</td>
<td>1.22 (0.95–1.56)</td>
<td>1.37 (1.05–1.79)</td>
<td>1.38 (1.05–1.83)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>152 (16.9)</td>
<td>159 (14.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130 (14.5)</td>
<td>121 (11.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Trend test \( P \) value

<table>
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<tr>
<th>3+4</th>
<th>( n = 684 )</th>
<th>( n = 907 )</th>
<th>( 2 )</th>
<th>( 3 )</th>
<th>( 4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>282 (31.4)</td>
<td>280 (26.3)</td>
<td>( 0.002 )</td>
<td>1.28 (1.06–1.56)</td>
<td>1.32 (1.07–1.61)</td>
<td>1.00 (ref.)</td>
</tr>
<tr>
<td>124 (18.2)</td>
<td>146 (16.3)</td>
<td></td>
<td></td>
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<tr>
<td>81 (11.9)</td>
<td>77 (8.6)</td>
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</tbody>
</table>

Dual copies allele

<table>
<thead>
<tr>
<th>( n = 648 )</th>
<th>( n = 907 )</th>
<th>( 2 )</th>
<th>( 3 )</th>
<th>( 4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.229</td>
<td>0.188</td>
<td>( 0.038 )</td>
<td>1.21 (1.01–1.45)</td>
<td>1.21 (1.00–1.45)</td>
</tr>
<tr>
<td>0.210</td>
<td>0.167</td>
<td></td>
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</tr>
</tbody>
</table>

Merged population

<table>
<thead>
<tr>
<th>( n = 1590 )</th>
<th>( n = 1979 )</th>
<th>( 2 )</th>
<th>( 3 )</th>
<th>( 4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1092 (69.2)</td>
<td>1459 (74.4)</td>
<td>( 0.001 )</td>
<td>1.00 (ref.)</td>
<td>1.00 (ref.)</td>
</tr>
<tr>
<td>276 (17.5)</td>
<td>305 (15.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>211 (13.3)</td>
<td>198 (10.1)</td>
<td></td>
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</tbody>
</table>

**Table III. Stratification analysis of the association between MAPKAPK2 CNV-30450 genotypes and NPC risk**

<table>
<thead>
<tr>
<th>Cases (( n = 1590 ))</th>
<th>Controls (( n = 1979 ))</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)(^a)</th>
<th>( P ) homogeneous</th>
<th>( P ) interaction</th>
</tr>
</thead>
</table>

**Stratification analysis of the association between CNV-30450 and NPC risk**

We further evaluated the possible effect of selected factors on the above association in stratification analysis. As shown in Table III, the association between CNV-30450 and NPC risk was more pronounced in EBV-positive individuals (OR = 1.43, 95% CI = 1.09–1.90) with an approaching significantly higher OR than in EBV-negative individuals (OR = 0.94, 95% CI = 0.72–1.23; Breslow–Day test: \( P = 0.089 \)).

\( a \)ORs were adjusted for age, sex, smoking status, drinking status, and family history of cancer in logistic regression model. "Bold type: statistically significant, \( P < 0.05 \)."

\( b \)Adjusted in a logistic regression model that included age, sex, smoking status, drinking status, family history of cancer and center.

\( c \)The success rates of genotyping were 99.2%.

\( d \)Adjusted for age, sex, smoking status, drinking status, and family history of cancer in logistic regression model. "Bold type: statistically significant, \( Z < 0.05 \)"; above footnote "a".

\( e \)\( P \) value of homogeneity test between strata for the related ORs of MAPKAPK2 (CNV-30450, 3+4-copy versus 2-copy).

\( f \)\( P \) value of test for the multiplicative interaction between CNV-30450 and selected variables on cancer risk in logistic regression models.
were no significant differences in other subgroups (Breslow–Day test: \( P > 0.05 \) for all). Furthermore, we did not observe any significant interaction between the MAPKAPK2 adverse genotypes and other selected factors on increasing cancer risk \( (P > 0.05 \) for all).

**Luciferase activity**

As shown in Figure 1B, the transcription activity of the reporter gene with dual copies drove a 1.70–2.40-fold increased reporter expression in both CNE-1 and CNE-2 compared with the reporter gene with single copy \( (P < 0.001 \) for both), reflecting that the dual copies of CNV-30450 might increase the transcriptional activity of MAPKAPK2.

**Effects of CNV-30450 on MAPKAPK2 expression**

As shown in Figure 2A and B, the MAPKAPK2 expression was significantly increased along with the copy numbers of CNV-30450 increased in mRNA levels (cross validation: \( P = 0.001 \)) and protein expressions (cross validation: \( P = 0.003 \)). The cases with 3- or 4-copy genotype exerted higher mRNA levels as well as protein expressions than those with 2-copy genotype in both samplings (ANOVA test: \( P < 0.05 \) for all), except for the protein expressions in Suzhou samples \( (P = 0.144) \), which may be due to the limited sample size.

Because of the fact that the association between CNV-30450 genotypes and NPC risk was more pronounced in EBV-infected individuals, we evaluated the above genotype–phenotype correlation with regards to EBV infection status. In the tissues with positive EBV infection, higher expression of MAPKAPK2 was observed in cases carrying 3- or 4-copy genotypes than cases carrying 2-copy (cross validation: \( P = 0.011 \), ANOVA test: \( P = 0.037 \); Figure 2C), whereas in the remaining cases without EBV infection, the difference was not significant (cross validation: \( P > 0.05 \), ANOVA test: \( P = 0.366 \)).

**Discussion**

In two independent case–control studies, we found that the CNV-30450 in the MAPKAPK2 promoter was associated with risk of NPC by enhancing the promoter activity and thus promoting MAPKAPK2 expression. Moreover, the association could be modulated by EBV infection because EBV infection had an effect on 3/4-copy genotype causing higher MAPKAPK2 expression. To the best of our knowledge, this is the first study to report a functional CNV that can predict risk for NPC.

Multiple evidences indicated that MAPKAPK2 plays a key role in cell apoptosis (40), cell cycle (41), adhesion (42) and movement (19) in response to oxidative stress (18) and infection (43,44). Through phosphorylation activity, MAPKAPK2 could activate...
several important cancer-related proteins, including Cdc25B/C (17), tuberin (41), Polo-like kinase 1 (45), HSP27 (18), HSP25 (19) and mRNA–AU-rich element-binding proteins (i.e. tristetraprolin, hnRNP A0), all of which could further modulate mRNA stability of multiple genes, including some well-established oncogenes and tumor suppressors like TNFα, CCND1, Plk3, c-Fos, c-Myc and MMP-1 (20,21). Overactivated P38/MAPKAPK2 pathway was observed in various cancers (12,23,24). A recent study reported that MAPKAPK2 overexpression contributed to a poor prognosis of gastrointestinal stromal tumor, and the CNVs in chromosome 1 was associated with MAPKAPK2 expression (22). Meanwhile, we have previously identified the CNV-30450 in MAPKAPK2 to be associated with lung cancer risk and prognosis by influencing MAPKAPK2 expression (27). Consistently, in this current study, we found that the CNV-30450 could also enhance MAPKAPK2 expression in NPC and increase NPC risk. Taken together, the previous and current findings supported that the CNV-30450 in MAPKAPK2 is functional and might be a valuable biomarker to predict risk of NPC.

P38 pathway could be directly activated by EBV infection and subsequently activate MAPKAPK2 (10), and the GEO profiles (http://www.ncbi.nlm.nih.gov/; GEO Profiles, GDS9899/1469) showed that EBV can induce high MAPKAPK2 expression in human cell. Here, we found that the correlation between CNV-30450 genotypes and MAPKAPK2 expression was only significant in cases with positive EBV infection but not in cases without EBV. Otherwise, in contrast to no significant deviation of MAPKAP2 expression shown in cases with 2-copy genotype between EBV-positive cases and EBV-negative ones, EBV-positive cases harboring 3/4-copy genotypes exerted much higher MAPKAP2 expression than EBV-negative tissues harboring 3/4-copy genotypes. All these above suggested that the effect of CNV-30450 was modulated by EBV infection, and that individuals carrying the 3/4-copy of CNV-30450, once infected with EBV, would be at high risk of NPC.

CNV is a prevalent genetic aberration, which covers >1 kb duplication or deletion, and it is assumed that CNV may have great contribution to phenotypic variations by altering gene function or expression (27,46–49). Several CNVs have been reported to be associated with human diseases including cancer (27,49). By now, only one study investigated the CNVs in NPC. This study utilized the genome-wide SNP-based arrays and five CNV prediction algorithms, and identified eight CNVs on chromosomes 3p14.1, 6p21.33, 7p22.2, 7q11.23, 8p22, 12p13.31, 19p13.3 and 19q13.42 to be associated with NPC susceptibility (35). However, the array is with low probe resolution which cannot detect those relatively small CNVs like CNV-30450. In other words, the study had not performed any functional assays. Fortunately, in this study, we could apply functional assays to show the biological effect of CNV-30450 because the CNV is relatively small and located in the promoter of MAPKAPK2. Further functional assays, such as circular chromosome conformation capture (4C) assay, were warranted (48).

The present study has several strengths. We performed two independent case–control panels and reported consistent findings of the association between CNV-30450 and NPC risk, and we had achieved a total of 87.8% study power (two-sided test, α = 0.05) to detect an OR of 1.28 for the 3/4-copy genotypes (which occurred at a frequency of 25.6% in the controls) compared with 2-copy genotype. Furthermore, the functional assays revealed a robust genotype–phenotype correlation. However, there were some limitations. Because this study was restricted to Chinese Han population, it is uncertain whether our findings can be generalized to other populations; and as hospital-based case–control studies, selection biases, such as sampling bias, are unavoidable.

This study suggested that the 3/4-copy of CNV-30450 in MAPKAPK2 promoter is associated with increased NPC risk in Chinese by increasing the expression of MAPKAPK2 and thus promoting tumorigenesis. The variant may be a valuable biomarker for susceptibility to NPC. Validations with larger population-based studies in different ethnic groups are warranted.

Supplementary material
Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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