Genetic polymorphisms of \textit{MDM2} and \textit{TP53} genes are associated with risk of nasopharyngeal carcinoma in a Chinese population

Mang Xiao$^1$, Lei Zhang$^1$, Xinhua Zhu$^2$, Jun Huang$^3$, Huifen Jiang$^4$, Sunhong Hu$^1$ and Yuehui Liu$^*$

\textbf{Abstract}

\textbf{Background:} The tumor suppressor \textit{TP53} and its negative regulator \textit{MDM2} play crucial roles in carcinogenesis. Previous case-control studies also revealed \textit{TP53} $72\text{Arg} \rightarrow \text{Pro}$ and \textit{MDM2} $309\text{T} \rightarrow \text{G}$ polymorphisms contribute to the risk of common cancers. However, the relationship between these two functional polymorphisms and nasopharyngeal carcinoma (NPC) susceptibility has not been explored.

\textbf{Methods:} In this study, we performed a case-control study between 522 NPC patients and 722 healthy controls in a Chinese population by using PCR-RFLP.

\textbf{Results:} We found an increased NPC risk associated with the \textit{MDM2} GG (odds ratio [OR] = 2.83, 95\% confidence interval [CI] = 2.08-3.96) and TG (OR = 1.49, 95\% CI = 1.16-2.06) genotypes. An increased risk was also associated with the \textit{TP53} Pro/Pro genotype (OR = 2.22, 95\% CI = 1.58-3.10) compared to the Arg/Arg genotype. The gene-gene interaction of \textit{MDM2} and \textit{TP53} polymorphisms increased adult NPC risk in a more than multiplicative manner (OR for the presence of both \textit{MDM2} GG and \textit{TP53} Pro/Pro genotypes = 7.75, 95\% CI = 3.53-17.58).

\textbf{Conclusion:} The findings suggest that polymorphisms of \textit{MDM2} and \textit{TP53} genes may be genetic modifier for developing NPC.

\textbf{Background}

As an important tumor suppressor, \textit{TP53} protein level is low or undetectable in normal cells, but diverse forms of stress may trigger its production, resulting in either cell cycle arrest or apoptotic cell death \cite{1,2}. High frequencies of \textit{TP53} mutation and/or deletion are found in a wide variety of human malignancies, including nasopharyngeal carcinoma (NPC), which is believed to be contributed to tumorigenesis and progression \cite{3-5}.

Recently, Bond \textit{et al.} reported that a $\text{T} \rightarrow \text{G}$ polymorphism at position 309 downstream from \textit{MDM2} intron 1 disrupts an Sp1 regulatory element and the $\text{T}$ allele thus has a strikingly lower promoter activity compared with the $\text{G}$ allele \cite{6}. Moreover, a single nucleotide polymorphism has been identified in the coding region of \textit{TP53}, which causes an Arg72-Pro amino acid substitution \cite{7}.

It has been shown that, compared with \textit{Pro} allele, the \textit{Arg} allele is faster to induce apoptosis and more efficient in suppressing transformation. Many molecular epidemiologic data found that these two polymorphisms are likely candidate genetic markers of certain cancers \cite{8-10}. However, the gene-gene interaction of these two polymorphisms in \textit{MDM2} and \textit{TP53} has not been examined in NPC studies to date. Because of their significant impact in several tumors, these two polymorphisms might also affect the function of \textit{MDM2} and \textit{TP53} and play an important role in NPC development. These two polymorphisms might impact individual susceptibility to carcinogenesis. Based on this hypothesis, we carried out a hospital-based case-control study to investigate the relationship between polymorphisms in \textit{MDM2} 309$\text{T} \rightarrow \text{G}$ and \textit{TP53} Arg72Pro and the risk of NPC in Chinese population.
Methods

Study Subjects
This study included 522 NPC patients and 712 healthy population controls. All subjects were ethnically homogeneous Han Chinese. Patients with newly diagnosed NPC were consecutively recruited from March 2001 to May 2007, at the Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou) and Zhejiang Cancer Hospital (Hangzhou). All eligible patients diagnosed at the hospital during the study period were recruited, with a response rate of 94%. Patients were from Hangzhou city and its surrounding regions and there were no age, stage, and histology restrictions. The tumor, node, metastasis (TNM) classification and tumor staging was evaluated according to the 2002 American Joint Committee on Cancer staging system [11]. The clinical features of the patients are summarized in Table 1. Population controls were cancer-free people living in Hangzhou region; they were selected from a nutritional survey conducted in the same period as the cases were collected. The control subjects were randomly selected from a database consisting of 2500 individuals based on a physical examination. The selection criteria included no history of cancer and frequency matched to cases on age and sex. Median age was 46 years (range 26-81) for case patients and 47 years (range 22-85) for control subjects ($P=0.78$). At recruitment, informed consent was obtained from each subject. This study was approved by the Medical Ethics Committee of Sir Run Run Shaw Hospital and Zhejiang Cancer Hospital.

Polymorphism analysis
Genomic DNA was isolated from the peripheral blood lymphocytes of the study subjects. Genotypes were analyzed using PCR-based methods as described below. Genotyping was performed without knowledge of subjects’ case/control status. A 30% masked, random sample of cases and controls was tested twice by different persons and the results were concordant for all masked duplicate sets.

The genotypes of TP53 Arg72Pro (rs1042522, G>C) were analyzed by PCR-RFLP method on the basis of that reported previously [12]. The primers used were TP53 F, 5’-TTG CCG TCC CAA GCA ATG GAT GA-3’ and TP53 R, 5’-TCT GGG AAG GGA CAG AAG ATG AC-3’, which produce a 199-bp fragment containing the G/C site. Amplification was accomplished with a 25 μl react-

Table 1: Distribution of selected characteristics by case-control status in NPC association analysis.

|                   | Cases |   | Controls |   |
|-------------------|-------|---|----------|---|
|                   | n     | % |          | n | % |
| **Sex**           |       |   |          |   |   |
| Male              | 315   | 60.3 | 404      | 56.7 |
| Female            | 207   | 39.7 | 308      | 42.7 |
| **Age (years)**   |       |   |          |   |   |
| ≤40               | 195   | 37.4 | 242      | 34.0 |
| 41-50             | 134   | 25.7 | 186      | 26.1 |
| 51-60             | 93    | 17.8 | 166      | 23.3 |
| >60               | 100   | 19.1 | 118      | 16.6 |
| **Tumor stage**   |       |   |          |   |   |
| I                 | 126   | 24.2 |          |   |   |
| II                | 208   | 39.8 |          |   |   |
| III               | 177   | 33.9 |          |   |   |
| IV                | 11    | 2.1  |          |   |   |
| **EBV Infection status** |   |   |          |   |   |
| Positive          | 329   | 63.1 |          |   |   |
| Negative          | 162   | 31.0 |          |   |   |
| Data missing      | 31    | 5.9  |          |   |   |
| **Metastasis**    |       |   |          |   |   |
| Present           | 283   | 54.2 |          |   |   |
| Absent            | 239   | 45.8 |          |   |   |
tion mixture containing ~100 ng template DNA, 0.5 μM each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, and 1.2 units of Taq DNA polymerase with 1 x Reaction buffer (Promega, Madison, WI, USA). PCR profile consisted of an initial melting step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and a final elongation step of 7 min at 72°C. The 199-bp PCR products were then subject to the digestion with BstUI (New England Biolabs, UK) and separated on a 3.0% agarose gel. The genotypes identified by BstUI digestion were confirmed by DNA sequencing (Figure 1).

MDM2 SNP309 (rs2279744, T>G) genotypes were analyzed using the tetra-primer amplification refractory mutation system (ARMS)-PCR method [13]. The primers for ARMS-PCR amplification of DNA fragment containing the MDM2 SNP309 T allele were MDM2 F1: 5’-GGG GGC CGG CGG CTG CGG GGC CGT TT-3’ and MDM2 R1: 5’-TGC CCA CTG AAC CGG CCC AAT CCC GCC CAG-3’. For the MDM2 SNP309 G allele they were MDM2 F2: 5’-GGC AGT CGC CGC CAG GGA GGA GGG CGG-3’ and MDM2 R2: 5’-ACC TGC GAT CAT CCG GAC CTC CCG CGC TGC-3’. The amplification was accomplished with a 10-μl reaction mixture containing 10 ng of template DNA, 0.8 μM of primers MDM2 F1 and MDM2 R1, 4.8 μM of primers MDM2 F2 and MDM2 R2, 0.2 mM of dNTPs, 1.5 mM of MgCl₂, and 0.4 U of HotStar TaqTM with 1 × buffer and 1 × Q-solution (Qiagen, Chatsworth, CA). The reaction was carried out with an initial melting step of 15 min at 95°C, followed by 35 cycles of 45 sec at 95°C, 45 sec at 64°C, and 1 min at 72°C, and a final elongation step of 7 min at 72°C. The amplified DNA was visualized on agarose gel containing ethidium bromide. The MDM2 SNP309 T allele generated a 122-bp band, and the G allele generated a 158-bp band. They had a common 224-bp band, which was amplified by primers MDM2 F2 and MDM2 R1. The genotypes identified by PCR-RFLP and ARMS-PCR digestion were confirmed by DNA sequencing (Figure 2).

**Real-time analysis of MDM2 mRNA**

Total RNA was isolated from Seventy-one NPC tissues using the Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH) and converted to cDNA using an oligo (dT)₁₅ primer and Superscript II (Invitrogen, Carlsbad, CA). Relative gene expression quantitation for MDM2, with β-actin as an internal reference gene, was carried out using ABI Prism 7300 sequence detection system (Applied Biosystem, Foster City, CA) in triplicates, based on the SYBR-Green method [8]. The primers used for MDM2 were 5’-TGT AAG TGA ACA TTC AGG TG-3’ and 5’-TTC CAA TAG TCA GCT AAG GA-3’, and for β-actin were 5’-GGC GGC ACC ACC ATG TAC CCT-3’ and 5’-AGG GGC CGG ACT CGT CAT ACT-3’. The PCR reaction mixture consisted of 0.1 μmol/L of each primer, 1 × SYBR Premix EX Taq (Perfect Real Time) premix reagent (TaKaRa, Dalian, China), and 50 ng cDNA to a final volume of 20 μL. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles at 95°C of 15 seconds and 62°C for 1 minute. PCR specificity was confirmed by dissociation curve analysis and gel electrophoresis. All analysis were done in a blinded fashion with the laboratory persons unaware of genotyping data. The expression of individual MDM2 measurements was calculated relative to expression of β-actin using a modification of the method described by Lehmann et al [14].

**Statistical Analysis**

χ² tests were used to examine the differences in the distributions of genotypes between cases and controls. The association between the TP53 and MDM2 polymorphisms and risk of NPC were estimated by ORs and their

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**Figure 1** Representative PCR-RFLP for different genotypes containing the TP53 Arg72Pro polymorphism site and DNA sequencing analysis. (A) M: DNA size markers; lane 1, 2, 4 and 5: TP53 72Pro/Pro; lane 6, 7 and 8: TP53 72Arg/Pro; lane 3: TP53 72Arg/Arg. (B) The PCR products with different PCR-RFLP profiles were sequenced to confirm the genotypes.

**Figure 2** Representative tetraprime ARMS-PCR for different genotypes containing the MDM2 SNP309 T>G polymorphism site and DNA sequencing analysis. (A) M: DNA size markers; lane 1, and 9: MDM2 309TT; lane 2, 3, 4, 6 and 8: MDM2 309TG; lane 5 and 7: MDM2 309TT. (B) The PCR products with different tetraprime ARMS-PCR profiles were sequenced to confirm the genotypes.
95% CIs, which were calculated by unconditional logistic regression models. We tested the null hypotheses of multiplicative gene-gene interactions by evaluated departures from multiplicative joint effect models by including main effect variables and their product terms in the logistic regression model [15]. A more-than-additive interaction was suggested when \( \text{OR}_{11} > \text{OR}_{10} + \text{OR}_{01} - 1 \), for which \( \text{OR}_{11} = \text{OR} \) when both factors were present, \( \text{OR}_{10} = \text{OR} \) when only factor 1 was present and \( \text{OR}_{01} = \text{OR} \) when only factor 2 was present. A more-than-multiplicative interaction was suggested when \( \text{OR}_{11} > \text{OR}_{10} \times \text{OR}_{01} \). The correlation of genotypes and clinical parameters was analyzed via the Fisher’s exact test or \( \chi^2 \) test as appropriate. The normalized expression values of \( MDM2 \) were compared by Kruskal-Wallis one way ANOVA. All \( P \)-values were two-sided with a \( P \)-value < 0.05 considered to be statistically significant. All analysis was carried out with Statistical Analysis System software (Version 9.0; SAS Institute, Cary, NC, USA).

Results

Allele and Genotype Distribution

The genotype results are shown in Table 2. The allele frequencies for \( MDM2 \) \( G \) and \( TP53 \) \( Pro \) were 0.423 and 0.426 in controls, and 0.555 and 0.518 in cases respectively. The observed genotype frequencies of \( MDM2 \) and \( TP53 \) polymorphisms in both controls and cases did not deviated from those expected from the Hardy-Weinberg equilibrium. Distributions of these \( MDM2 \) and \( TP53 \) genotype were then compared among cases and controls. The frequencies of \( MDM2 \) TT, TG and GG genotypes among patients were significantly different compared to controls (\( P_{\text{trend}} < 0.001 \)), with the GG homozygotes being significantly overrepresented among patients compared to controls (\( P < 0.001 \)). Moreover, Logistic regression analysis showed that subjects with \( TP53 \) Pro allele significant increased risk of NPC compared with subjects carrying the Arg allele (OR for the Arg/Pro genotype, 1.43; 95%CI, 1.22-2.13; OR for the Pro/Pro genotype, 2.22; 95%CI, 1.58-3.10; \( P_{\text{trend}} < 0.001 \)), suggesting that the Pro allele is the high-risk allele.

The effects of the \( TP53 \) and \( MDM2 \) polymorphisms were additionally examined with stratification by age, tumor size, metastatic status and Epstein-Barr virus (EBV) infection status. However, no significant association was observed between age and the TNM stage at the time of NPC diagnosis and the polymorphism of this gene, and no interaction was detected between the polymorphism and status of EB virus infection (data not shown).

Gene-Gene Interaction between \( MDM2 \) and \( TP53 \) Polymorphisms

We examined whether there was a statistical interaction between the \( MDM2 \) and \( TP53 \) polymorphisms (Table 3). The data showed that patients who carried the \( MDM2 \) GG genotype were also more likely to carry the \( TP53 \) Pro/Pro genotype than the controls (10.3% vs. 2.8%, \( P < 0.001 \)). The presence of one \( MDM2 \) GG genotype, but not one \( TP53 \) Pro/Pro genotype, were associated with an increased risk of NPC (OR = 2.83, 95% CI = 1.33-5.90), compared to the lack of such a genotype. However, the presence of both \( MDM2 \) GG and \( TP53 \) Pro/Pro genotypes was associated with an even higher risk for NPC.

Table 2: Genotype and allele frequencies of \( TP53 \) and \( MDM2 \) among cases and controls and their association with the risk of NPC.

| Genotype          | Cases (n = 522) | Controls (n = 712) | OR* (95% CI) |
|-------------------|-----------------|--------------------|--------------|
|                   | no. (%)         | no. (%)            |              |
| **TP53 72Arg>Pro**|                 |                    |              |
| Arg/Arg           | 117 (22.4)      | 226 (31.7)         | 1.00 (reference) |
| Arg/Pro           | 270 (51.7)      | 366 (51.4)         | 1.43 (1.22-2.13) |
| Pro/Pro           | 135 (25.9)      | 120 (16.9)         | 2.22 (1.58-3.10) |
| **Pro allele frequency** | 0.518 | 0.426 | |
| **MDM2 309T>G**   |                 |                    |              |
| TT                | 111 (21.2)      | 238 (33.4)         | 1.00 (reference) |
| TG                | 243 (46.6)      | 346 (48.6)         | 1.49 (1.16-2.06) |
| GG                | 168 (32.2)      | 128 (18.0)         | 2.83 (2.08-3.96) |
| **G allele frequency** | 0.555 | 0.423 | |

*OR and 95%CI were calculated by unconditional logistic regression, with TP53 Arg/Arg or MDM2 TT genotype as the reference group and adjusted by sex and age.
increase (OR = 7.75, 95% CI = 3.53-17.58; \( P < 0.05 \), test for homogeneity) compared to those who lacked both genotypes. These results clearly indicate a more than multiplicative interaction [15] between the MDM2 GG and TP53 Pro/Pro genotype in the risk of developing NPC.

**MDM2 RNA Levels in NPC tissues from Different Genotype Carriers**

To examine the effect of the MDM2 309T>G polymorphism on MDM2 expression in the target tissues, the levels of MDM2 mRNA in individual NPC tissues were quantified by real-time PCR (Figure 3). The results showed that the MDM2 GG genotype carriers (n = 19) had significantly higher MDM2 mRNA level than the MDM2 TT genotype carriers [0.050 ± 0.033 (n = 19) versus 0.014 ± 0.009 (n = 18), \( P < 0.001 \)]. However, the MDM2 TG genotype carriers (n = 34) had a MDM2 mRNA level that was very similar to that of the TT genotype carriers (0.015 ± 0.013 versus 0.014 ± 0.009, \( P = 0.610 \)).

### Table 3: Risk of NPC associated with MDM2 genotypes by TP53 genotypes.

| Genotype | Cases | Controls | OR* (95% CI) |
|----------|-------|----------|--------------|
| MDM2 309T>G | TP53 72Arg>Pro | no. (%) | no. (%) | |
| TT Arg/Arg | 24 (4.6) | 68 (9.6) | 1.00 (reference) |
| Arg/Pro | 63 (12.1) | 128 (18.0) | 1.34 (0.75-2.48) |
| Pro/Pro | 24 (4.6) | 42 (5.9) | 1.63 (0.81-3.42) |
| TG Arg/Arg | 54 (10.3) | 120 (16.9) | 1.30 (0.72-2.39) |
| Arg/Pro | 132 (25.3) | 168 (23.6) | 2.12 (1.90-3.86) |
| Pro/Pro | 57 (10.9) | 58 (8.1) | 2.91 (1.53-5.31) |
| GG Arg/Arg | 39 (7.5) | 38 (5.3) | 2.83 (1.33-5.90) |
| Arg/Pro | 75 (14.4) | 70 (9.8) | 3.13 (1.61-5.94) |
| Pro/Pro | 54 (10.3) | 20 (2.8) | 7.75 (3.53-17.58) |

* Data were calculated by unconditional logistic regression, adjusting for sex and age status.

**Figure 3** MDM2 mRNA expression level in NPC tissue as function of MDM2 genotype. Columns, mean; bars, ± SD normalized to β-actin. Expression level among the GG genotype was significantly different from that among the GT or TT genotype (\( P < 0.05 \)).

**Discussion**

In the present study, our group found that MDM2 and TP53 polymorphisms may influence the development of NPC in a Chinese population. On the basis of examining 522 cases and 712 controls, our data showed that MDM2 309GG, which increase MDM2 expression level in NPC tissue, and TP53 Pro/Pro genotype were statistically significantly associated with increased risk of NPC. In addition, the association between these two polymorphisms and the risk of NPC displayed a multiplicative gene-gene interaction, which rendered the subjects carrying both MDM2 309GG and TP53 72Pro/Pro genotypes at much higher risk for developing NPC.

Our results showing an association between risk of NPC and polymorphisms of MDM2 and TP53 are biologically plausible for the following reasons. Firstly, there is broad evidence suggesting that TP53 is a key gene in maintaining genomic integrity and preventing tumorigenesis [16-19]. The association between mutation of TP53 and susceptibility to tumor formations has been tested in several studies with genetically modified animals. It was found that mice lacking the inactivating mutation in one tp53 allele developed fewer tumors than mice harboring it and they developed tumors very early in life and at very high frequencies [20]. Moreover, overexpression of MDM2, which can lead to loss of TP53 activity, was also observed in a variety of tumors with diverse tissue origins [21,22]. Secondly, the investigated polymor-
phisms in the TP53 and MDM2 genes have functional consequences [6,7,23]. Our real-time PCR finding is consistent with recent reports by Bond et al. and Hong et al [6,8] that the MDM2 309GG genotype carriers had significantly higher MDM2 expression in NPC tissues than the TT and TG genotype carriers, suggesting the variant MDM2 genotype may cause attenuated TP53 function.

Several case-control studies have examined the association between these two polymorphisms and many tumor types, but the results are conflicting [8,9,24-27]. A meta-analysis of 21 studies showed that ORs of a variety of cancers associated with the MDM2 GG and TG genotype were 1.17 (95% CI = 1.04-1.33) and 1.15 (95% CI = 1.03-1.28), respectively [28]. Moreover, another meta-analysis study reported that the TP53 Pro/Pro polymorphisms was significantly increase susceptibility to NPC [29]. Phang et al. reported in a study conducted in Singapore Chinese that the MDM2 309SNP was not associated with leukemia [30]. However, Xiong et al. also found an increased risk of acute myeloid leukemia (AML) associated with MDM2 309GG genotype [9]. Zhou et al. shown that MDM2 309SNP may be a risk factor for occurrence of NPC [31]. Moreover, the control frequencies of TP53 Arg72Pro and MDM2 SNP309 in our present study were similar with Asian population in published papers [28,29]. Our study provided strong molecular epidemiologic evidence to support the hypothesis that TP53 72Arg/Pro and MDM2 309T>G polymorphisms also affect the development of NPC.

Although it is generally believed that TP53 pathway also plays a critical role in tumor aggressive course [32,33], we did not find significant correlations between TP53 and MDM2 genotypes and the prognosis status of NPC in the present study. These results suggest that the examined polymorphisms in TP53 and MDM2 might not serve as a sole risk marker of prognosis. Further examinations of larger patient series with prospectively follow-up clinical outcomes especially the survival rates may be required. Moreover, our study may have certain limitations because of the study design. Selection bias and/or systematic error may occur because the cases were from the hospital and the controls were from the community. Some factors which may interact with genotype or act as potential confounders in analysis such as information of nutrition is not available in our case-control study.

Conclusion
The current study demonstrated a significant association between the TP53 72Arg/Pro and MDM2 309T>G polymorphisms and the risk of developing NPC for the first time. The association of MDM2 polymorphism with the risk of NPC displayed a multiplicative gene-gene interaction with the TP53 72Arg/Pro polymorphism. These molecular epidemiology findings are consistent with the results obtained from the functional analysis. Because MDM2 overexpression and high frequencies of TP53 mutation are found in many tumor types, additional studies on other tumor types would be warranted. Moreover, the possible role of these polymorphisms in disease prognosis should also be addressed in the future studies.

Conflicts of interest statement
The authors declare that they have no competing interests.

Abbreviations
MDM2: mouse double minute 2; CI: confidence interval; OR: odds ratio; NPC: nasopharyngeal carcinoma; SNP: single nucleotide polymorphism.

Authors’ contributions
MX participated in molecular genetic studies and drafted the manuscript. LZ performed the genotyping and statistical analysis and participated in the critical revision of the manuscript. JH, HJ and SH participated in collection of data and manuscript preparation. YL conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Author Details
1Department of Otorhinolaryngology-Head and Neck Surgery, Sir Run Run Shaw Hospital, Key Laboratory of Biotherapy of Zhejiang province, Zhejiang University, Hangzhou, China, 2Department of Otolaryngology-Head and Neck Surgery, The second affected hospital to Nanchang University, Nanchang, China, 3Department of Clinical Laboratory, Sir Run Run Shaw Hospital, Key Laboratory of Biotherapy of Zhejiang province, Zhejiang University, Hangzhou, China and 4Department of Clinical Laboratory, Zhejiang cancer hospital, Hangzhou, China

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