Interaction of P53 Arg72Pro and MDM2 T309G polymorphisms and their associations with risk of gastric cardia cancer

Ming Yang¹, Yongli Guo¹, Xuemei Zhang, Xiaoping Miao, Wen Tan, Tong Sun, Dan Zhao, Dianke Yu, Junniao Liu and Dongxin Lin

Department of Etiology and Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

¹To whom correspondence should be addressed. Tel: 86 10 87788491; Fax: 86 10 67722460; Email: dlin@public.bta.net.cn

The P53 tumor suppressor pathway plays an important role in cancer development. The auto-regulatory feedback mechanism of the P53 and MDM2 expression is critical in keeping proper tumor suppressor function of this pathway. This study examined the effect of P53 Arg72Pro variants on transactivation of polymorphic MDM2 promoter (T309G) and their associations with risk of developing gastric cardia adenocarcinoma (GCA) in a Chinese population. Luciferase assays consistently showed a significantly higher activity of the MDM2 309G promoter compared with the MDM2 309T promoter. In cells co-transfected with variant P53 cDNAs, P53-72Pro displayed a significantly higher ability to activate the MDM2 promoter than P53-72Arg. Genotype analyses in 500 GCA patients and 1000 controls showed that significantly increased risk for developing GCA was associated with the MDM2 309G and the P53 72Pro allele compared with the MDM2 309T and the P53 72Arg allele in an allele dose-dependent manner. A joint effect between the MDM2 and P53 polymorphisms in intensifying GCA risk was detected, with the odds ratio (OR) for the presence of both MDM2 309G and P53 72Pro/Pro genotypes being 5.05 [95% confidence interval (CI), 2.50–10.20]. These results suggest that the P53 72Pro and MDM2 309G polymorphisms contribute to the risk of developing GCA.

Introduction

Human genome is constantly exposed to various forms of endogenous and exogenous stress that may cause oncogenic transformation and tumorigenesis of the cell. To maintain genomic integrity, a set of complex systems has been evolved for the cell to protect against the genotoxic insults. The P53 tumor suppressor pathway plays a pivotal role in the cellular response to stress by inducing cell growth arrest or apoptosis (1,2). Unusually high frequencies of P53 mutations are observed in many types of human cancer, which is thought to be related to the initiation and progression of malignancies (3). Both mice and humans harboring germline inactivating mutations in one P53 allele are highly susceptible to cancer: they develop cancer very early in life and at very high frequencies (4,5). Moreover, genetic polymorphisms in P53 may also be associated with an increased risk of developing certain cancers (6).

As a key regulator of P53 pathway, MDM2 can directly bind to P53 protein, inhibiting its activity and mediating its location and degradation via the ubiquitination system. On the other hand, P53 can trans-activate MDM2 promoter and elevate the expression of MDM2 (7). In response to cellular stress, such as DNA damage, P53 expression is up-regulated; however, over-expression of MDM2 may inhibit P53 function, which enables damaged cells to escape the cell cycle check-point control and become carcinogenic (8,9). Numerous studies have shown that a variety of human cancers over-express MDM2, and in some cases, MDM2 over-expression but not P53 mutation is observed (10,11), suggesting that it can take the place of inactivating P53 mutations in cancer development.

A single nucleotide polymorphism (309T>G) (dbSNP ID: rs2279744) in the promoter region of MDM2, which increases affinity for binding stimulatory protein (Sp) 1 and results in higher levels of MDM2 expression and the subsequent attenuation of the P53 pathway (12), has been associated with susceptibility to certain types of cancer (12–17). The P53 gene also has a functional polymorphism, the G>C change at codon 72 (dbSNP ID: rs1042522), which results in Arg>Pro amino acid substitution. The Arg/Arg genotype seems to induce apoptosis with faster kinetics and to suppress transformation more efficiently than the Pro/Pro genotype (18,19). The effects of P53-72Arg and P53-72Pro isoforms on regulation of MDM2 transcription have been extensively studied; however, the results are conflicting rather than conclusive (18–21). Therefore, additional studies are needed to address this important issue.

Gastric cardia adenocarcinoma (GCA) is one of the common fatal malignancies in the world, particularly in northern China where esophageal cancer is also prevalent (22). GCA differs from gastric cancer at other sites in epidemiological characteristics, etiology, pathogenesis and clinical behavior (23) but may share common risk factors with esophageal cancer for carcinoma. Although the integrated etiology of esophageal cancer and GCA remains to be fully clarified, several environmental factors such as cigarette smoking, heavy alcohol drinking, micronutrient deficiency, dietary carcinogen exposure and gastroesophageal reflux disease have been linked to these cancers (24–27). All these factors can induce or enhance DNA damage mediated by either oxidative stress or DNA-binding electrophiles, which in turn may initiate and/or promote tumorigenesis. Because of the central role of P53 pathway in the response to DNA damage and preventing cancer pathogenesis, we hypothesized that functional MDM2 309T>G and P53 72Arg>Pro polymorphisms might be a genetic susceptibility factor for the development of GCA.

To test this hypothesis, we conducted a large case–control study of GCA in a Chinese population. We also examined the differential effect of P53 variants on transcriptional activation of MDM2 promoter in P53-null cells to further explore the underlying mechanisms.

Materials and methods

Study population

This study consisted of 500 patients with GCA and 1000 cancer-free controls. Patients were consecutively recruited between January 1997 and July 2004, at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing). GCA was defined as tumors arising at the gastric cardia and/or gastroesophageal junction with or without involvement of other esophageal and/or gastric sub-sites. All eligible patients with histopathologically confirmed GCA were enrolled. Controls were cancer-free individuals selected from a community cancer-screening program for early detection of cancer conducted during the same time period as the patients were collected. Controls were matched in 2:1 to patients on age (±5 years) and sex. The detailed recruitment of patients and controls was described previously (28). Informed consent was obtained from each subject and each participant was then interviewed to collect the information on demographic characteristics and lifetime history of tobacco use.

Genotype analysis

MDM2 309T>G genotypes were analysed using tetra-primer amplification refractory mutation system-polymerase chain reaction (PCR) method and P53 72Arg>Pro genotypes were determined by PCR–restriction fragment length polymorphism (RFLP) technique as described previously (15,16,29,30). Genotyping was performed without knowledge of case/control status of the study subjects. For quality control, a 10% masked, random samples of DNAs from subjects. For quality control, a 10% masked, random samples of DNAs from patients on age (±5 years) and sex. The detailed recruitment of patients and controls was described previously (28). Informed consent was obtained from each subject and each participant was then interviewed to collect the information on demographic characteristics and lifetime history of tobacco use.

Abbreviations: CL, confidence interval; GCA, gastric cardia adenocarcinoma; OR, odds ratio; PCR, polymerase chain reaction.

¹These authors contributed equally to this work.
the patients and controls were tested twice by different people and the results were concordant for all of the masked duplicate sets.

**Plasmid construction**

The MDM2 309T and G allele reporter constructs were prepared by amplifying the 352 bp MDM2 promoter region containing P53-responsive elements (from −461 bp to −110 bp relative to the translation start site) from subjects homozygous for the 309TT or 309GG genotype using the primers 5’-CCCGATTTACCGGAACAGATGGTCTCCGGGTGTAAGG-3’ (forward) and 5’-CCCAAGCTTACGTTGAATGGTGTACCCTGCTG-3’ (reverse), including the KpnI and HindIII restriction sites (underlined sequences). The PCR products were digested with KpnI and HindIII (TaKaRa Biotech Co., Dalian, China) and ligated, respectively, into an appropriately digested pGL3-Basic vector (Promega, Madison, WI) containing the firefly luciferase gene as a reporter. The constructs were designated as pGL3-DM2-309T and pGL3-DM2-309G, respectively. The cDNA encoding P53-72Arg was generated by PCR from a human fetal liver cDNA library (Clontech, Mountain View, CA), with primers 5’-CCGGAACCTTGAAGGAGCCGCGCAGTCGATCTCAGTC-3’ and 5’-CCGGAAATTCCTAGTC GTAGTCAGGCGCCCTCCTGCTGAA-3’, which contain HindIII and EcoRI sites (underlined sequences). To ensure high-fidelity amplification, LA Taq polymerase (TaKaRa) was used in the PCR. The PCR product was subsequently used as a template to generate P53-72Pro cDNA by using site-specific mutagenesis with aforementioned PCR primers and mutagenic primers 5’-CAGAGGCTGCTCCCTCCCCTGCAGACCAC-3’ and 5’- GTGACCAGGGGCAGGGGAGCATCTCGTCTG-3’ (underlined sequences encoded sequences 72Pro and P53-72Arg in the construct, respectively). The constructed plasmids were designated as pcDNA3.1-P53-72Arg or pcDNA3.1-P53-72Pro. Restriction analysis and complete DNA sequencing confirmed the orientation and integrity of each constructs’ inserts.

**Transient transfection and luciferase assays**

P53-null human H1299 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen) in a humidified, 5% CO2 incubator at 37°C. Cells at 10–15% confluence were transfected with a six-well cell culture treatment for 24 h before transfection. Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. All transfection assays were performed, and each was done in triplicate. The resulting constructs were designated as pcDNA3.1-5P372Arg or pcDNA3.1-5P372Pro cDNAs were ligated into pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA), and the resulting constructs were designated as pcDNA3.1-P53-72Arg or pcDNA3.1-P53-72Pro. Restriction analysis and complete DNA sequencing confirmed the orientation and integrity of each constructs’ inserts.

**Western blot analysis**

The co-transfected cells were collected 48 h after transfection and lysed for 15 min at 4°C with 200 μl of radio-immunoprecipitation assay (RIPA) buffer and centrifuged at 10,000g for 15 min. The supernatants were collected, the protein content measured with the BCA (Bio-Rad) assay. Fifty ug of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane at 1000 rpm for 1 h. Membranes were blocked with 5% non-fat milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.4) for 1 h at room temperature and incubated overnight at 4°C with the appropriate primary antibodies (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which were used against P53 and G (Santa Cruz). Proteins were visualized using SuperSignal Chemiluminescence Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Statistical analysis**

The χ2 test was used to examine differences in demographic variables, smoking status and the genotype distribution of MDM2 and P53 polymorphisms between patients and controls. Associations between genotype and risk of development of GCA were estimated by odds ratios (ORs) and their 95% confidence intervals (CIs) computed by using unconditional logistic regression. Those who had smoked less than 100 cigarettes in their lifetimes were defined as non-smokers, otherwise they were considered as smokers. Information was collected on the number of cigarettes smoked per day, the age at which the subjects started smoking, and the age at which the ex-smokers stopped smoking. Light and heavy smokers were categorized by using the 50th percentile pack-year values [pack years = (cigarettes per day/20) × (years smoked)] of the controls as the cut points (i.e. <24 pack years and ≥24 pack years). All ORs were adjusted for age, sex and smoking status or pack years, where it was appropriate. We tested the null hypotheses of multiplicative gene–gene and gene–smoking interaction and evaluated departures from multiplicative joint effect models by including main effect variables and their product terms in the logistic regression model (31). The difference in genotype distributions among subgroups was tested by χ2 test for homogeneity. A P < 0.05 was used as the criterion of statistical significance, and all tests were two sided. All analyses were done with the computer programs of Statistical Analysis System (SAS Institute, Cary, NC, USA).

**Results**

**Subject characteristics and genotype distributions**

As shown in Table I, there were no statistically significant differences in the distributions of sex, age and smoking status between patients and controls. Of the 416 patients who underwent surgical resection and had detailed pathological data, 47 (11.3%) had stage I GCA, 56 (13.5%) had stage II GCA, 154 (37.0%) had stage III GCA, whereas 159 (38.2%) had stage IV disease. Genotyping results are shown in Table I. The allele frequencies for MDM2 309G and P53 72Pro, respectively, were 0.453 and 0.441 in controls and 0.536 and 0.561 in the patients. The observed genotype frequencies of MDM2 and P53 polymorphisms in both controls and patients did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. Distributions of these MDM2 and P53 genotypes were then compared among patients and controls. Frequencies of MDM2 309TT, TG and GG genotypes among patients were significantly different from those expected from the Hardy–Weinberg equilibrium. Numbers of these MDM2 and P53 genotypes were then compared among patients and controls. Frequencies of MDM2 and P53 genotypes in both controls and patients did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. Distributions of these MDM2 and P53 genotypes were then compared among patients and controls. Frequencies of MDM2 and P53 genotypes in both controls and patients did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. Distributions of these MDM2 and P53 genotypes were then compared among patients and controls. Frequencies of MDM2 and P53 genotypes in both controls and patients did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. Distributions of these MDM2 and P53 genotypes were then compared among patients and controls. Frequencies of MDM2 and P53 genotypes in both controls and patients did not deviate significantly from those expected from the Hardy–Weinberg equilibrium.

| Variable | No. of patients (%) | No. of controls (%) | P value
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>500 (100)</td>
<td>1000 (100)</td>
<td>1.000</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>430 (86.0)</td>
<td>860 (86.0)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>70 (14.0)</td>
<td>140 (14.0)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>14 (2.8)</td>
<td>28 (2.8)</td>
<td></td>
</tr>
<tr>
<td>51–60</td>
<td>62 (12.4)</td>
<td>124 (12.4)</td>
<td></td>
</tr>
<tr>
<td>61–70</td>
<td>165 (33.0)</td>
<td>330 (33.0)</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>259 (51.8)</td>
<td>518 (51.8)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>237 (47.4)</td>
<td>434 (43.4)</td>
<td>0.140</td>
</tr>
<tr>
<td>Smoker</td>
<td>263 (52.6)</td>
<td>566 (56.6)</td>
<td></td>
</tr>
<tr>
<td>Smoking level, pack years</td>
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<td></td>
</tr>
<tr>
<td>≤24</td>
<td>128 (24.8)</td>
<td>285 (28.0)</td>
<td>0.652</td>
</tr>
<tr>
<td>&gt;24</td>
<td>135 (26.3)</td>
<td>281 (28.4)</td>
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<tr>
<td>Tumor stage at diagnosis</td>
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<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>47 (11.3)</td>
<td>70 (14.0)</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>56 (13.5)</td>
<td>112 (11.2)</td>
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</tr>
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<td>Stage III</td>
<td>115 (28.7)</td>
<td>230 (23.0)</td>
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</tr>
<tr>
<td>Stage IV</td>
<td>159 (38.2)</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>Two-sided χ2 test.

<sup>b</sup>According to the UICC classification.

<sup>c</sup>Sample size for this analysis was 416 with surgically resected patients only.
Genotype frequencies of MDM2 and P53 polymorphisms among patients and controls and their association with GCA risk

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 1000)</th>
<th>Patients (n = 500)</th>
<th>Genotype</th>
<th>Controls (n = 1000)</th>
<th>Patients (n = 500)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>MDM2 309T&gt;G</td>
<td></td>
<td></td>
<td>P53 72Arg&gt;Pro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>298 (29.8)</td>
<td>107 (21.4)</td>
<td>Arg/Arg</td>
<td>316 (31.6)</td>
<td>123 (24.6)</td>
</tr>
<tr>
<td>TG</td>
<td>498 (49.8)</td>
<td>250 (50.0)</td>
<td>Arg/Pro</td>
<td>486 (48.6)</td>
<td>245 (49.0)</td>
</tr>
<tr>
<td>GG</td>
<td>204 (20.4)</td>
<td>143 (28.6)</td>
<td>Pro/Pro</td>
<td>198 (19.8)</td>
<td>132 (26.4)</td>
</tr>
<tr>
<td>One G, OR (95% CI)*</td>
<td>1.37 (1.05–1.80)</td>
<td></td>
<td>One Pro, OR (95% CI)*</td>
<td>1.32 (1.02–1.72)</td>
<td></td>
</tr>
<tr>
<td>Two G, OR (95% CI)*</td>
<td>1.94 (1.42–2.64)</td>
<td></td>
<td>Two Pro, OR (95% CI)*</td>
<td>1.72 (1.27–2.33)</td>
<td></td>
</tr>
<tr>
<td>*P&lt;0.001</td>
<td></td>
<td></td>
<td>*P&lt;0.001</td>
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</tr>
</tbody>
</table>

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

Association between individual polymorphism and GCA risk

Unconditional logistic regression was used to estimate associations between genotype and risk of GCA (Table II). The MDM2 309G allele was shown to be risk allele. Subjects carrying the 309GG or 309TG genotype had an OR of 1.94 (95% CI, 1.42–2.64) or 1.37 (95% CI, 1.05–1.80) for developing GCA, respectively, compared with subjects carrying the 309TT genotype (trend test, P < 0.001), suggesting a dose-dependent effect of this polymorphism in GCA. Similarly, it was observed that the odds of having the P53 72Pro/Pro genotype in the GCA group was 1.72 (95% CI, 1.27–2.33) compared with the 72Arg/Arg genotype. A significantly increased OR was also associated with the 72Arg/Pro heterozygous genotype (OR, 1.32; 95% CI, 1.02–1.72), suggesting that the effect is also in an allele dose-dependent manner (trend test, P < 0.001). However, we did not find any association between the polymorphism in MDM2 or P53 and disease status among the GCA patients (data not shown). Stratification analyses indicated that there was no gender difference in risk of GCA with the MDM2 309GG genotypes, and there was no difference in age at GCA diagnosis between carriers of the MDM2 309T>G and P53 72Arg>Pro alleles (data not shown).

Effects of P53 variants on transcriptional activation of the MDM2 promoter

MDM2 is precisely regulated by P53 through sequence-specific transcriptional activation. Previous studies have reported conflicting results regarding the effect of P53 variants on transactivation of the MDM2 gene (18–21). To dissect this, we analysed the ability of P53-72Arg and P53-72Pro to transactivate MDM2 intronic P53-dependent promoter including the 309 polymorphic site in P53-null human H1299 cells. When 10–160 ng of P53 expression plasmids were transiently expressed in H1299 cells, P53-72Pro drove 16–23% higher activation of the MDM2 309G promoter compared with P53-72Arg (Figure 1A–D upper). Although a similar trend was observed when 40–160 ng of P53 expression plasmids were used to activate the MDM2 309T promoter (increased by 12–25%), no significant differences were observed when using 10 ng of P53 plasmids (Figure 1A–D upper). At high concentrations of P53 plasmids, both P53 variants squelched transcriptional activation of the MDM2 promoter in some degree, which is consistent with previous reports (32,33). However, when the concentration of P53 plasmids increased to >300 ng, most transfected cells died, probably due to tumor-suppressor function of P53. On the other hand, as measured by luciferase activity, the reporter plasmid containing MDM2 309G allele yielded ~40% higher luciferase level than the reporter plasmid containing MDM2 309T allele (P < 0.01), which is consistent with the observation by Bond et al. (12). We next evaluated the expression of the transfected P53s by western blot analysis and the results showed that the expressions of P53-72Pro and P53-72Arg were identical in our assay system (Figure 1A–D lower). These findings excluded the possibility of differential expressions of P53 isoforms, which may account for the observed differences in MDM2 transactivation. Together, these results indicate that P53-72Pro has a higher ability to activate the MDM2 promoter, which might also be influenced by the MDM2 polymorphism.

Discussion

It has been shown that attenuated P53 pathway resulting from germ-line polymorphisms is associated with increased risk of carcinogenesis (13–17). We have shown previously that both P53 72Arg>Pro and MDM2 309T>G polymorphisms are associated with increased risk for the development of esophageal cancer and lung cancer and significant interaction of them to further increase risk of the cancers were also observed (15,16). In the present study, we examined whether these two polymorphisms have impact on the risk of GCA. On the basis of analysing 500 patients and frequency-matched 1000 controls, we found that about 2-fold increased risk of GCA is associated with the MDM2 309GG genotype compared with the MDM2 309TT genotype. We also detected a moderately increased OR for GCA that was associated with the P53 72Pro allele. Furthermore, a joint effect between P53 72Pro/Pro and MDM2 309GG genotypes and a joint effect between MDM2 309GG genotype and smoking were detected.
Carrying both P53 72Pro/Pro and MDM2 309GG genotypes confers an OR of more than 5 for GCA. These results are consistent with our previous findings from esophageal cancer and lung cancer studies (15,16) and suggest that genetic polymorphisms in the cell cycle regular genes are also involved in the etiology of GCA.

As a tumor suppressor, P53 protein causes growth arrest and/or apoptosis in response to DNA damage and other forms of cellular stress (1,2). The levels and activity of P53 are controlled largely by MDM2, the product of a P53-inducible gene. MDM2 binds to P53 and promotes P53 ubiquitination and subsequent degradation by the proteasome (34,35). On the other hand, the MDM2 expression is regulated by P53, thus, to form an auto-regulatory feedback loop (7). The functional codon 72 Arg.

Pro polymorphism in P53 has been shown to depress the activities of P53 in inducing apoptosis and suppressing transformation (18–20). The difference in sequence-specific transcriptional activation between P53-72Pro and P53-72Arg variants has been extensively studied in many genes, but the results are

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### Table III. Risk of GCA associated with MDM2 genotypes by P53 genotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No. of patients (%)</th>
<th>No. of controls (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>Arg/Arg</td>
<td>19 (3.8)</td>
<td>96 (9.6)</td>
</tr>
<tr>
<td>TT</td>
<td>Arg/Pro</td>
<td>61 (12.2)</td>
<td>150 (15.0)</td>
</tr>
<tr>
<td>TT</td>
<td>Pro/Pro</td>
<td>27 (5.4)</td>
<td>52 (5.2)</td>
</tr>
<tr>
<td>TG</td>
<td>Arg/Arg</td>
<td>59 (11.8)</td>
<td>162 (16.2)</td>
</tr>
<tr>
<td>TG</td>
<td>Arg/Pro</td>
<td>119 (23.8)</td>
<td>222 (22.2)</td>
</tr>
<tr>
<td>TG</td>
<td>Pro/Pro</td>
<td>72 (14.4)</td>
<td>114 (11.4)</td>
</tr>
<tr>
<td>GG</td>
<td>Arg/Arg</td>
<td>45 (9.0)</td>
<td>58 (5.8)</td>
</tr>
<tr>
<td>GG</td>
<td>Arg/Pro</td>
<td>65 (13.0)</td>
<td>114 (11.4)</td>
</tr>
<tr>
<td>GG</td>
<td>Pro/Pro</td>
<td>33 (6.6)</td>
<td>32 (3.2)</td>
</tr>
</tbody>
</table>

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

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Fig. 1. Differential effects of P53 Arg72Pro variants on activation of polymorphic MDM2 promoters. (A–D upper) Luciferase expression of the two MDM2 promoter-luciferase constructs in co-transfected P53-null human H1299 cells. Indicated amounts of pcDNA3.1 vector, pcDNA3.1-P53-72Pro or pcDNA3.1-P53-72Arg expression plasmids were co-transfected with the MDM2 reporter constructs into cells, together with 2.0 ng of pRL-SV40 to standardize transfection efficiency. Luciferase activities were measured in cells 48 h after transfection. All experiments were performed in triplicates at least in three independent transfection experiments and each value represents mean ± standard deviation. (A–D lower) Western blot analysis was performed using half the samples from the transfections to determine the expression status of P53 in all cases.
conflicting in respect with MDM2 (18–21). We therefore compared their abilities to activate MDM2 transcription in a well-controlled in vitro system. Our results in the present study provide evidence for the first time that P53 72Arg>Pro polymorphism influences MDM2 expression in both MDM2 309G and 309T alleles. When there are sufficient P53 levels, the P53-72Pro variant activates MDM2 transcription more strongly than the P53-72Arg variant. Although the exact mechanism for this difference is currently unclear, it has been shown that the P53-72Pro displays more compacted affinity for the TAFI132 and TAFI170 transcription factors (20), which may contribute at least in part to the effect. However, when the levels of P53 are low, the differential activation of MDM2 by different P53 variants turns out to be mainly influenced by MDM2 309 genotypes (Figure 1A upper). This may reflect a kind of adaptation during evolution that in cells with MDM2 309GG genotype, where MDM2 expression is much higher and P53 expression is relatively lower, the transactivation of MDM2 might be more sensitive to P53. This functional relevance of P53 polymorphism is consistent with molecular epidemiological findings, demonstrating that the P53 72Pro allele is a risk allele for GCA.

To the best of our knowledge, this is the first case–control study to investigate the association between the MDM2 polymorphism and GCA risk. MDM2 is considered to be an oncogene and over-expression of this gene has been correlated to carcinogenesis and cancer progression (36–38). The transgenic mice that over-expressed an average of 4-fold more MDM2 in various tissues developed spontaneous tumors in a lifetime (39). It has also been shown that targeted over-expression of MDM2 in the murine mammary epithelium resulted in tumors (40) and targeted expression of MDM2 in the murine epidermis increased papilloma formation induced by chemical carcinogen (41). The latter are coincident with our finding that more than 2-fold increased risk associated with the MDM2 309GG genotype compared with the MDM2 309TT genotype among heavy smokers. Smoking is an established etiologic factor for GCA (24,25), and exposure to smoke causes genotoxic stress including DNA damage (42). It has been reported that after treatment with etoposide to induce DNA damage, which activates the P53 pathway, leading to DNA repair, cell cycle arrest and apoptosis, significant death was observed in cells with the MDM2 309TT genotype but not in cells with the MDM2 309GG genotype (12). In this regard, heavy smokers with both MDM2 309GG genotypes are therefore expected to have the highest risk of GCA.

The P53 72Arg>Pro polymorphism has been extensively studied in many cancer types, including GCA. Shen et al. (43) reported in a case–control study conducted in southern China that the P53 72Arg>Pro polymorphism was not associated with GCA. In contrast, in a Caucasian population, Zhang et al. (44) reported an increased risk of GCA associated with the p53 Arg/Arg genotype. However, both studies recruited too few GCA patients (n = 128 and n = 32, respectively) to detect the moderate effect of this genetic polymorphism. In the present study with 500 GCA patients and 1000 controls, we found that a significantly increased GCA risk was associated with the P53 72Pro allele (72Pro/Pro or 72Arg/Pro genotype).

Interestingly, our results in the present study show that risk for developing GCA associated with the MDM2 309GG genotype was intensified by P53 72Pro/Pro genotype. The joint effect between these two genotypes is biologically plausible because MDM2 and P53 act in the same causal pathway for carcinogenesis (8,9). If a cell carries functional polymorphisms in both of the genes that heighten the expression of MDM2 and diminish the function of P53, a gene–gene joint effect would be expected. The reporter gene assays in the present study suggest that this gene–gene interaction between MDM2 and P53 polymorphisms might be also associated with higher ability of P53-72Pro to transactivate the MDM2 309G allele, which itself has heightened expression compared with the MDM2 309T allele.

In summary, our study provides evidence that P53 72Arg>Pro and MDM2 309T>G polymorphisms are genetic susceptibility factors for the development of GCA in a Chinese population. Furthermore, besides the different apoptotic and transforming activities of the P53 and MDM2 polymorphisms, the difference in transactivating MDM2 of the P53-72Arg and P53-72Pro variants may explain why there is a joint effect between the MDM2 and P53 polymorphisms. These findings support the hypothesis that the P53 tumor suppressor pathway plays an important role in the development of GCA.

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### References

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