A Novel Functional Polymorphism C1797G in the MDM2 Promoter Is Associated with Risk of Bladder Cancer in a Chinese Population

Meilin Wang,1,2 Zhizhong Zhang,2 Haixia Zhu,2 Guangbo Fu,3 Shouyu Wang,2 Dongmei Wu,2 Jianwei Zhou,2 Qingyi Wei,4 and Zhengdong Zhang1,2

Abstract

Purpose: MDM2 is believed to regulate the p53 level in modulating DNA repair, cell cycle control, cell growth, and apoptosis. We hypothesize that genetic variants in the MDM2 gene are associated with risk of bladder cancer.

Experimental Design: We first conducted a case-control study of 234 bladder cancer cases and 253 cancer-free controls, using the haplotype-based tagging single nucleotide polymorphism (SNP) approach involving 13 common SNPs initially identified in 100 control subjects. We then examined the functionality of the important SNP.

Results: We found that the C1797G polymorphism in the MDM2 promoter region is an important SNP because its homozygous variant genotype, but none of the haplotypes, was associated with risk of bladder cancer. Electrophoretic mobility shift assay indicated that the 1797C to 1797G transition within the CAAT/enhancer binding protein α (C/EBPα) core sequence greatly enhanced the C/EBPα binding affinity to the promoter region. The in vitro luciferase assays in various cell lines further showed an increased transcriptional activity of the 1797G allele compared with the 1797C allele. Additional experiments with tumor tissues revealed that the transcriptional activator C/EBPα containing the 1797G allele increased levels of the MDM2 mRNA and protein in bladder tumor tissues.

Conclusions: These data suggested that the novel MDM2 promoter C1797G polymorphism may affect the MDM2 activity by altering the C/EBPα binding affinity to the promoter and, thus, may be a marker for genetic susceptibility to bladder cancer in Chinese populations. Further validation of the functionality of the MDM2 C1797G polymorphism and its association with risk of bladder and other cancers in other ethnic populations is warranted.

The tumor suppressor gene, p53, is known to modulate a number of cellular functions such as DNA repair, cell cycle arrest, and apoptosis through transcriptional regulation (1). Somatic mutations that inactivate the p53 gene have been found in at least half of all human solid tumors, highlighting a crucial role of the p53 protein in carcinogenesis (2). The murine double minute 2 (MDM2) gene encodes a protein that is a key component in the p53 signaling pathway. MDM2 is transcriptionally activated by p53 and regulates the amount of p53 by targeting p53 for the ubiquitin-mediated degradation (3). It has been shown that the MDM2 knockout in mice is embryonic lethal (4); however, this lethal phenotype is rescued by the knockout of both p53 and MDM2 genes, suggesting that the primary function of MDM2 is to regulate the functions of p53 whose functions can be compensated by other proteins such as p63 and p73 (5). In addition, MDM2 regulates p53 to form a negative autoregulatory feedback loop with an important role in regulating cell cycle progression and apoptosis (6).

Human MDM2 gene is located on chromosome 12q14.3 to 15.0 with a genomic length of 34 kb, consisting of 12 exons, and it has three different promoters, of which the second and third ones are responsive to p53 (7–9). In humans, MDM2 expression levels seem to be critical for regulating the functions of p53 whose functions can be compensated by other proteins such as p63 and p73 (5). In addition, MDM2 regulates p53 to form a negative autoregulatory feedback loop with an important role in regulating cell cycle progression and apoptosis (6).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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is well-established that the tobacco smoking and occupational or environmental exposure to chemical carcinogens are the strongest known risk factors for bladder cancer (12). Although many people are exposed to these risk factors, only a fraction of exposed individuals develop bladder cancer in their lifetime, suggesting individual susceptibility to bladder carcinogenesis.

There are at least 253 single nucleotide polymorphisms (SNP) of the MDM2 gene reported in the dbSNP database,5 of which the vast majority are intronic and few in the exons and promoter regions. Recently, the SNP309 (rs2279744) in the intronic p53-responsive promoter of the MDM2 gene was shown to increase the affinity of the transcriptional activator Sp1, resulting in higher levels of MDM2 mRNA and protein (13). This polymorphism has been shown to be associated with an increased risk of bladder cancer (14). Moreover, the SNP309 was found to be related to an earlier onset of superficial disease, TP53 mutations, and poor outcome in invasive bladder cancer (15). Thus, the polymorphisms of the MDM2 gene seem to play an important role in the development and progression of bladder cancer.

Because most of the reported SNPs are in linkage disequilibrium (LD), the haplotype-based association studies are considered more powerful than the single SNP analysis to identify causal genetic variants underlying the etiology of complex diseases such as cancer (16), and the use of tagSNPs that capture most of the haplotypic diversity in association studies has been suggested (17). The international HapMap project provides empirical genome-wide data to explore disease-associated genetic variants by using a whole genome-based tagSNP approach (18).

In the present study, we selected eight tagSNPs from the HapMap database for Chinese subjects to evaluate the associations between the genetic variants in the MDM2 gene and bladder cancer risk in a Chinese population. Among these tagSNPs and other possible haplotypes, we identified a risk-associated MDM2 C1797G polymorphism in the promoter, and our further molecular work suggested that this variant might alter the MDM2 mRNA and protein expression by destroying a binding element for the CCAAT/enhancer binding protein α (C/EBPα).

Materials and Methods

Study subjects. The detailed method of recruiting participants for this study has been described previously (19). Briefly, 234 histologically confirmed transitional cell carcinoma of bladder cases, and 253 cancer-free control subjects were recruited from the First Affiliated Hospital and Huai-An Affiliated Hospital of Nanjing Medical University between January 2003 and May 2006. Those cases that had previous cancer, metastasized cancer from other or unknown origin, and previous radiotherapy or chemotherapy were excluded. All eligible but only Han Chinese patients were included in this study, and which the participation rate was about 95%. The cancer-free control subjects were recruited from those who were seeking health care for conditions other than cancer, including smoking-related conditions, such as ischemic heart disease, cerebrovascular disease, and chronic airway obstruction diseases. The response rate of those control subjects we approached for participation in the study was about 85%. We used a short questionnaire to obtain demographic and risk factor information and frequency matched the controls to the cases by the age (±5 y) and sex. Before recruitment, informed consent was obtained from each of the eligible subjects. We acquired the smoking and alcohol status through face-to-face interviews. Individuals that smoked daily for >1 y were defined as ever smokers. Ever smokers who had quit smoking for >1 y were defined as former smokers, and the other smokers as current smokers. Individuals that consumed three or more alcohol drinks per week for at least 1 y were considered ever drinkers, and the rest were defined as never drinkers. The distributions of selected characteristics of the cases and controls are presented in Supplementary Table S1. The research protocol was approved by the institutional review board of Nanjing Medical University.

SNP selection, haplotype-based tagSNPs identification, and genotyping. The MDM2 gene is ~34 kb in size, consisting of 12 exons, of which the first exon 1 is located in the untranslated region (Supplementary Fig. S1). The HapMap SNP database (public release up to July 2006)6 provides a dense coverage across the MDM2 gene region from exon 2 to 3′-untranslated region but does not include the region upstream exon 2 where the promoter region is. Thus, we selected the SNPs in the promoter region from the dbSNP database that has 253 reported MDM2 SNPs. Because the genotype frequencies and the LD block determination of the SNPs can be influenced by population differences and sample sizes (20, 21), we chose all SNPs with a minor allele frequency (MAF) of >5% from both the HapMap and dbSNPs databases and genotyped them in 100 Chinese control subjects first to reconstruct haplotype/LD blocks for tagSNPs identification. As shown in Table 2, 15 SNPs genotyped by using the PCR-RFLP method, of which 13 SNPs with a MAF of >5% were selected and their LDs were calculated and visually presented by the Haploview 3.2 software (Supplementary Fig. S2). We identified a set of tagSNPs of the MDM2 gene with the following criteria: a minimal set of haplotypes that ensures an R² of at least 0.8 to cover all possible haplotypes that had a frequency of at least 5% as evaluated by the tagSNPs program (22).

The primers and restriction enzymes for genotyping are all listed in Supplementary Table S2. The genotype analysis was done by two persons independently in a blind fashion. To further validate the genotyping results, about 1% of the PCR products were randomly selected and confirmed by direct sequencing, and >10% of the samples were randomly selected for repeated genotyping for confirmation, and the results were 100% concordant.

Construction of reporter plasmids. Because the MDM2 C1797G polymorphism was the only potentially functional SNP that was associated with significantly increased risk of bladder cancer, we then determined whether this polymorphism had an effect on gene expression in vitro. The MDM2 promoter-luciferase reporter plasmids containing either 1797C or 1797G sequence were prepared by amplifying the 400-bp (from 1640-2039 base relative to the first base) MDM2 promoter region by using primers with restriction sites (Supplementary Fig. S3). The primers were 5′-GACGCTAGCTGTCG- CGCAGTTCACGGG-3′ (forward) and 5′-CGCGAAGCTT CTTCCTG- CTCCATCTGTCG-3′ (reverse), including the NheI and HindIII restriction sites (i.e., protective nucleotides marked in bold and restriction sites marked in italic). The amplified fragments were then sequenced to confirm that there were no errors in matched nucleotides and the plasmid encompassed either 1797C or 1797G allele. The amplified fragments and pGL3-basic vector (Promega) were cleaved by restriction enzymes (TaKaRa Biotech Co.), and the plasmid encompassed either 1797C or 1797G allele. The amplified fragments and pGL3-basic vector (Promega) were cleaved by using the NheI and HindIII enzymes (TaKaRa Biotech Co.), and the fragments were then cloned into the pGL3-basic vector. After cloning, the vectors were sequenced to confirm the orientation and integrity of the inserts of each construct.

Transient transfections and luciferase assays. For transfections, HeLa, NIH-3T3, and T24 cells were seeded onto 24-well plates (100,000 cells per well), and each well was transfected with 2.25 µg of the vector DNA with either 1797C or 1797G allele, using polyfectin transfection reagent

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6 http://www.hapmap.org
Fig. 1. Effect of the C1797G polymorphism on the MDM2 promoter activity. A, schematic representation of reporter plasmids containing the 1797C or 1797G allele, which was inserted upstream of the luciferase reporter gene in the pGL3 basic plasmid. B, the two constructs were transiently transfected into the HeLa, NIH-3T3, and T24 cells, respectively. The luciferase activity of each construct was normalized against the internal control of Renilla luciferase. Columns, mean from three independent experiments; bars, SD. *, P < 0.01 compared with the construct counterpart.

To further examine whether the C1797G polymorphism and protein expression levels (Qiagen; Fig. 1). As an internal standard, all plasmids were cotransfected with 10 ng pRL-SV40, which contained the Renilla luciferase gene. The pGL3-basic vector without an insert was used as a negative counterpart.

Independent triplicate experiments were done for each plasmid construct. Differences in the expression levels of different constructs were determined by Student’s t test, and a P value of <0.01 was considered statistically significant.

Electrophoretic mobility shift assay. To further examine whether the C1797G polymorphisms had an effect on protein binding, synthetic double-stranded oligonucleotides 5’-CCCCGCCCTCACAGCCGCCGCGG-3’ and 5’-CCCCGCCCTCACAGCCGCCGCGG-3’ (i.e., bold nucleotide was the polymorphic site) corresponding to the 1797C and 1797G sequence from the MDM2 promoter region were labeled with biotin. Electrophoretic mobility shift assays were done by using the LightShift Chemiluminescent electrophoretic mobility shift assay kit (Pierce Rockford). For each gel shift reaction (10 μL), a total of 20 fmol 3’-end labeled probe were combined with 1 μg nuclear extract prepared from HeLa cells, 1 μg poly(deoxyinosinic-deoxycytidylic acid), and 1X binding buffer. For competition assays, a 100-fold molar excess of unlabeled either 1797C or 1797G probe, an C/EBPα consensus binding site (5’-TGCAGATTGCGCAATCTGCA-3’) was preincubated for 10 min at room temperature with nuclear extracts before addition of the labeled probe. For each supershift reaction (10 μL), 1 μL C/EBPα antibodies (Cell Signaling Technology, Inc.) was incubated with nuclear extracts at 4°C for 30 min, followed by an additional incubation for 30 min at room temperature with a labeled probe or C/EBPα consensus binding sequence. After incubation, samples were separated on a native non-denaturing 4.5% polyacrylamide gel and then transferred to a nylon membrane. The positions of the biotin-labeled probe in the membrane were detected by a chemiluminescent reaction with the stabilized Streptavidin–horseradish peroxidase conjugate according to the manufacturer’s instructions and visualized by autoradiography (Fig. 2).

Detection of MDM2 transcripts by reversed transcript PCR. To further detect the correlation between the MDM2 mRNA levels and C1797G polymorphism in vitro, the 22 bladder tumor tissues of the cases with different genotypes were subjected to extraction of the total RNA isolated from tissues using Trizol Reagent (Invitrogen, Inc.). An aliquot of the total RNA (2 μg) from each tumor tissue was reverse transcribed into single-strand cDNA using oligo (dT)12 primer and Superscript II (Invitrogen, Inc.). Among the 22 tumor samples, 20 were from invasive tumors (stage T3 or higher) and the other two were superficial (stage T1), and two were grade 1, 19 were grade 2, and one grade 3. The total RNA was measured by both reverse transcription-PCR (RT-PCR) and real-time quantitative RT-PCR. Each single strand cDNA was diluted for subsequent PCR amplification of the MDM2 and β-actin, the latter being used as an internal quantitative control. The primers used for amplification were 5’-GGTGGGAGTATCCAAAAAGGA-3’ and 5’-ACACAGAGCCAGCTTTTCAT-3’, which generated 210-bp fragment and, for β-actin, were 5’-CTCAATGAGCCTGTTGCGGC-3’ and 5’-CAGGTCGACAGCCAGATGGCGC-3’, which produced a 271-bp fragment. Fold changes were normalized by the levels of β-actin expression, and each assay was done in triplicate (Fig. 3).

Western blotting analysis. To analyze the correlation between the C1797G polymorphism and protein levels in vitro, the 22 bladder tumor tissues were washed with PBS, and total tissue extracts

Fig. 2. Analysis of transcription factor binding sites in the MDM2 promoter region containing the C1797G polymorphism. A, potential C/EBPα element was identified between the positions from 1792 to 1801 base. B, electrophoretic mobility shift assay (EMSA) with biotin-labeled either 1797C or 1797G probe and HeLa cell nuclear extracts. Lanes 1 and 2, mobility of the labeled probes without nuclear extracts; lanes 3, 7, and 8, mobilities of the polypeptide complexes with nuclear extracts in the absence of competitor. A specific nuclear protein binding can be completely abolished both by 100-fold unlabeled 1797C or C/EBPα probes (lanes 4 and 5). Super shift assays incubating with anti-C/EBPα antibody showed a supershifted protein complex (lanes 2 and 5).
were made using a detergent lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and the protease inhibitor, 1 mmol/L phenylmethanesulfonyl fluoride]. Thirty micrograms of total proteins were run on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Hybond-N+). The membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20 for 2 h at room temperature. The antibodies against MDM2 protein were 1:3, 6, and 3, respectively. The fold change was normalized against β-actin.

Characteristics of the study subjects. The frequency distributions of selected characteristics of the cases and controls are presented in Supplementary Table S1. Besides more ever smokers (56.4%) and ever alcohol users (50.4%) among the cases than among the controls (39.1% and 36.8%, respectively), there was no significant difference in the frequency distributions of other selected variables between the cases and controls (P = 0.915 for age and P = 0.257 for sex). Specifically, former and current smokers had 3.22-fold (95% CI, 1.95-5.31) and 1.49-fold (95% CI, 0.98-2.26) increased risk, respectively, compared with never smokers (data not shown). In addition, the duration of smoking was also associated with an increased risk for bladder cancer (OR, 2.05; 95% CI, 1.28-3.28 for <30 years; OR, 1.99, 95% CI, 1.30-3.04 for ≥30 years), compared with never smokers (data not shown). However, the distributions of the haplotypes between the cases and controls were not significantly different, and the haplotypes had no apparent relationship with risk of bladder cancer (Supplementary Table S3), possibly due to small numbers of observations in each of haplotype strata.

Candidate tagSNPs selection. Using the HapMap and dbSNPs databases, we selected 15 SNPs with a MAF of >5% and regenotyped these SNPs first in our 100 Southern Chinese

were used to infer haplotype frequencies based on the distributions of the haplotypes between the cases and controls. Hardy-Weinberg equilibrium of the genotypes of the controls was tested by a goodness-of-fit \( \chi^2 \) test. Expectation-maximization (EM) algorithm in SAS 9.1 PROC HAPLOTYPE was used to infer haplotype frequencies based on observed MDM2 genotypes. Unconditional univariate and multivariate logistic regression analyses were done to obtain crude and adjusted odds ratios (23) for risk of bladder cancer and their 95% confidence intervals (CIs). The statistical power was calculated by using the PS software. The false-positive report probability test of Wacholder et al. (24) was applied to address the issue of false-positive SNP associations. Kruskal-Wallis one-way ANOVA tests were used for analyzing the results of MDM2 mRNA and protein expression in tumor tissues. Student’s \( t \) test was done to examine the difference in levels of luciferase reporter gene expression between different constructs. All tests were two-sided by using the SAS software (version 9.1; SAS Institute, Inc.).

Results

**Candidate tagSNPs selection.** Using the HapMap and dbSNPs databases, we selected 15 SNPs with a MAF of >5% and regenotyped these SNPs first in our 100 Southern Chinese
control subjects. The SNPs ID, locations, and frequencies are shown in Table 1. We found that two of these SNPs were not common (MAF, <5%) in these control subjects (0.03 for rs2870820/C2326T and 0.04 for rs3730635), which were not consistent with that reported in the databases (0.438 and 0.054, respectively). The inconsistencies may be due to the limited sample size of the databases or sampling errors of Chinese populations. As a result, based on the method of Gabriel et al. (16), the other 13 SNPs were used to reconstruct three major haplotype blocks of the MDM2 SNP clusters (Supplementary Fig. S2). Blocks 1, 2, and 3 extended for 9, 5, and 11 kb, respectively, covering most of the MDM2 gene. However, the LD was low between pairs of SNPs in the promoter region (e.g., \( r^2 = 0.02 \) for rs3730485 versus rs937282, \( r^2 = 0.49 \) for rs937282 versus rs937283, and \( r^2 = 0.20 \) for rs937283 versus rs2279744). For further haplotype analysis, therefore, we used all genotyped SNPs as a single haplotype block to select tagSNPs using the algorithm defined by Stram et al. (22). As presented in Table 2, a set of eight tagSNPs were selected, which could accurately predict the common (>0.05) haplotypes with a minimum Rh^2 of 0.95.

**Table 1.** Primary information for 15 genotyped SNPs of the MDM2 gene in 100 control subjects screened for the TagSNPs

<table>
<thead>
<tr>
<th>Genotyped SNPs</th>
<th>Position</th>
<th>SNPs Location in gene region</th>
<th>MAF for Chinese in database^*</th>
<th>MAF of 100 controls</th>
<th>P for HWE test</th>
<th>Genotyping rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3730485 (-758+)</td>
<td>67487073</td>
<td>-/+ Promoter —</td>
<td>0.280</td>
<td>0.930</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs937282 (C1797G)</td>
<td>67488064</td>
<td>C/G Promoter —</td>
<td>0.457</td>
<td>0.245</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs937283 (A2164G)</td>
<td>67488431</td>
<td>A/G Exon1</td>
<td>0.438</td>
<td>0.330</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs2870820 (C2326T)</td>
<td>67488993</td>
<td>C/T Intron1 —</td>
<td>0.438</td>
<td>0.030</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs2279744 (SNP309)</td>
<td>67488847</td>
<td>T/G Intron1 —</td>
<td>0.438</td>
<td>0.400</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>rs1470383</td>
<td>67493429</td>
<td>A/G Intron3</td>
<td>0.167</td>
<td>0.210</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs1846402</td>
<td>67494845</td>
<td>A/C Intron4</td>
<td>0.167</td>
<td>0.145</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs3730536</td>
<td>67498277</td>
<td>A/G Intron5</td>
<td>0.398</td>
<td>0.388</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs1625525</td>
<td>67499098</td>
<td>T/C Intron5</td>
<td>0.189</td>
<td>0.260</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs3730556</td>
<td>67502788</td>
<td>T/G Intron6</td>
<td>0.189</td>
<td>0.130</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs2291857</td>
<td>67504305</td>
<td>G/T Intron6</td>
<td>0.300</td>
<td>0.288</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs3730581</td>
<td>67505759</td>
<td>G/A Intron8</td>
<td>0.430</td>
<td>0.425</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs3730590</td>
<td>67506903</td>
<td>C/T Intron8</td>
<td>0.167</td>
<td>0.245</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rs3730635</td>
<td>67515390</td>
<td>A/G Intron9</td>
<td>0.054</td>
<td>0.040</td>
<td>100</td>
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<tr>
<td>rs3730646</td>
<td>67518247</td>
<td>G/A Intron11</td>
<td>0.189</td>
<td>0.440</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: HWE, Hardy-Weinberg equilibrium.

^*From both HapMap and dbSNPs databases, the MAF in bold is from the dbSNPs database. The MAFs of the rs3730485 and rs2279744 for Chinese are not available from either HapMap or dbSNPs database.

**Table 2.** Association between the MDM2 TagSNPs and risk of bladder cancer in 234 cases and 253 cancer-free controls

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>SNPs</th>
<th>Frequency of genotype (case/control) %</th>
<th>P *</th>
<th>Adjusted OR (95% CI) ^ for heterozygous genotype</th>
<th>Adjusted OR (95% CI) ^ for rare homozygous genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3730485 (-758+)</td>
<td>-/+</td>
<td>52.1/53.4 38.5/39.1 9.4/7.5</td>
<td>0.754</td>
<td>1.01 (0.69-1.49)</td>
<td>1.34 (0.68-2.63)</td>
</tr>
<tr>
<td>rs937282 (C1797G)</td>
<td>C/G</td>
<td>53.0/58.1 39.7/38.3 7.3/3.6</td>
<td>0.152</td>
<td>1.14 (0.78-1.67)</td>
<td>2.45 (1.02-5.72)</td>
</tr>
<tr>
<td>rs937283 (A2164G)</td>
<td>A/G</td>
<td>43.0/53.0 44.4/39.5 10.3/7.5</td>
<td>0.203</td>
<td>1.34 (0.92-1.97)</td>
<td>1.62 (0.83-3.15)</td>
</tr>
<tr>
<td>rs2279744 (SNP309)</td>
<td>T/G</td>
<td>26.5/25.3 51.7/53.0 21.8/21.7</td>
<td>0.950</td>
<td>0.99 (0.64-1.53)</td>
<td>0.99 (0.58-1.68)</td>
</tr>
<tr>
<td>rs1470383</td>
<td>A/G</td>
<td>68.4/62.4 30.8/34.8 0.8/2.8</td>
<td>0.210</td>
<td>0.83 (0.56-1.22)</td>
<td>0.28 (0.05-1.19)</td>
</tr>
<tr>
<td>rs1846402</td>
<td>A/C</td>
<td>73.5/66.4 26.5/30.8 0.0/2.8</td>
<td>0.161</td>
<td>0.80 (0.53-1.20)</td>
<td>—</td>
</tr>
<tr>
<td>rs2291857</td>
<td>G/T</td>
<td>31.6/41.5 57.3/43.9 11.1/14.6</td>
<td>0.013</td>
<td>1.67 (1.12-2.49)</td>
<td>1.02 (0.56-1.84)</td>
</tr>
<tr>
<td>rs3730590</td>
<td>C/T</td>
<td>49.2/45.4 39.3/36.8 11.5/17.8</td>
<td>0.152</td>
<td>0.99 (0.67-1.47)</td>
<td>0.62 (0.36-1.08)</td>
</tr>
</tbody>
</table>

^*Two-sided \( \chi^2 \) test for the distribution of genotype frequency.

^Adjusted for age, sex, smoking status, and alcohol use in logistic regression model.

^The common genotype was used as the reference.
rs937282 (C1797G) polymorphism within the MDM2 promoter is more likely to be functional because it potentially affects gene expression at the transcriptional level, a possible underlying mechanism for the observed association, and the functionality of this promoter SNP can be tested experimentally.

Effects of the MDM2 C1797G polymorphism on transcriptional activity. To evaluate the native promoter activity associated with the C1797G polymorphism, we constructed luciferase reporter vectors (pGL3), spanning the 1640 to 2039 base from the first base of the MDM2 gene, with either 1797C or 1797G allele (Supplementary Fig. S3) and used them for transient transfections with the HeLa, NIH-3T3, and T24 cells. As shown in Fig. 1B, the vectors with the 1797G allele had a 60% to 188% increase in the relative luciferase activities, compared with that of those with the 1797C allele in all three types of cell lines (P <0.01 for all). These results suggested that the 1797G allele in the promoter region indeed had an increased transcriptional activity of the MDM2 gene.

Identification of the C/EBPα-binding region and allele-specific effects of the MDM2 promoter. We further did bioinformatics analysis of the promoter region surrounding the MDM2 C1797G polymorphism by using a computer algorithm (AliBaba2) and found that the C1797G polymorphism may affect the binding affinity of the core sequence with C/EBPα (Fig. 2A). To determine whether this polymorphism has an effect on binding ability of the transcription factor, we conducted the electrophoretic mobility shift assay to analyze the binding of oligo probes containing either 1797C or 1797G allele to nuclear proteins extracted from the HeLa cell. As shown in Fig. 2B, a specific DNA/nuclear protein complex (a shifted band) was generated by the 1797G but not the 1797C allele probes (lanes 3 and 7). However, the shifted band was abolished both by 100-fold unlabeled the 1797G and C/EBPα probes (lanes 4 and 5). Moreover, anti-C/EBPα antibodies caused a supershift of the biotin-labeled probe/nuclear protein (lane 2), further suggesting that the C/EBPα is indeed the transcription factor that binds the promoter region containing the 1797G allele. In addition, as a positive control, the nuclear extracts incubated with the labeled C/EBPα proteins was also shifted (lane 8) and supershifted by anti-C/EBPα antibodies (lane 9). Taken together, these results suggest that the C1797G polymorphism in the MDM2 promoter sits in the core of the C/EBPα binding motif, and the C to G substitution enhances the affinity of C/EBPα to this region in the MDM2 promoter, possibly leading an increased expression level of the MDM2 mRNA.

Association of the MDM2 C1797G polymorphism with expression levels of the MDM2 mRNA and protein. In this study, we collected 22 tumor samples obtained from the untreated bladder cancer patients with different genotypes of the C1797G polymorphism, and the frequency distribution of the CC, CG, and GG genotypes was 13, 6, and 3, respectively. The effects of these three genotypes on the transcriptional activity or mRNA were evaluated by both the RT-PCR and real-time quantitative RT-PCR (Fig. 3). As expected, the levels of MDM2 mRNA were higher in individuals with the CG or GG genotype than that in those with the CC genotype, and these differences were statistically significant (2.77 ± 0.24 versus 3.73 ± 0.40, P <0.01 for CG versus CC genotype; 3.73 ± 0.40 versus 1.00 ± 0.20, P <0.01 for GG versus CC genotype, in arbitrary unites). The differences in the mRNA levels between individuals with the CG and GG genotypes was also statistically significant (2.77 ± 0.24 versus 3.73 ± 0.40, P = 0.02 for CG versus GG genotype, in arbitrary unites). Furthermore, as shown in Fig. 4, the MDM2 protein levels of individuals with the CG or GG genotype were also significantly higher than that of those with the CC genotype (0.92 ± 0.02 versus 0.45 ± 0.20; P <0.01 for CG versus CC genotype; 0.96 ± 0.14 versus 0.45 ± 0.20; P <0.01 for GG versus CC genotype, in arbitrary unites), which were consistent with the results of the MDM2 mRNA expression levels with different genotypes. In addition, we also found the increased protein levels in individuals with the GG genotype compared with the CG genotype, although the difference did not reach the statistical significance. Meanwhile, no significant association between the genotypes and tumor grade and stage was found in neither mRNA nor protein expression levels, which was possibly due to the small sample size (data not shown). Taken together, the observation of higher levels of the MDM2 mRNA and protein expression in the tumor tissues of the GG genotype than that of other genotypes supports the association between the GG genotype and an increased risk of bladder cancer.

Discussion

Three MDM2 promoters have been identified and numerous genetic polymorphisms in these promoter regions have been reported in the HapMap and dbSNPs databases. Therefore, besides the reported functional SNP309 in the MDM2 promoter (13), we thought that the other genetic variants of the MDM2 gene may also be important and that their roles in human cancer susceptibility should be evaluated. In the present study, we used a haplotype-based approach to identify tagSNPs and evaluated their associations with risk of bladder cancer. Although we did not observe an association between any of the haplotypes and risk of bladder cancer, we observed a statistically significant association between the C1797G polymorphism in the MDM2 promoter and risk of bladder cancer and showed that the C to G substitution of this polymorphism significantly enhanced the binding affinity of the transcriptional activator C/EBPα and increased the transcription activity of the MDM2 gene in vitro. Furthermore, we found that of the MDM2 mRNA and protein were overexpressed in vivo in individuals who carried the 1797G allele in vivo, suggesting that the MDM2 C1797G polymorphism is indeed a functional SNP both in vitro and in vivo as well as a marker for risk of bladder cancer.

MDM2, as a negative regulator of p53, plays an important role in tumor formation and growth. In tumors with the wild-type p53, MDM2 binds to and interacts with p53, negatively regulating its transcriptional functions (25). Bond et al. (13) identified a naturally occurring T to G substitution at the position 309 (SNP309, rs2279744) in the MDM2 promoter that resulted in an 8-fold increase in the MDM2 mRNA expression and seemed to accelerate tumor formation among individuals carrying a germline p53 mutation. Recently, one study on the association between the SNP309 polymorphism and risk of bladder cancer in a case-control study of 75 cases and 103 controls from Turkey showed that the SNP309 might also be potential genetic susceptibility factor for bladder cancer (14). However, in the present study of a Chinese population with a larger sample size, we failed to find any evidence that the
SNP309 polymorphism was associated with risk of bladder cancer. Indeed, in this present study we found that the LD between the SNP309 and C1797G polymorphism was weak ($D’ = 0.41$).

In tumors, the overexpression of the MDM2 mRNA and proteins can substitute for inactivation of p53 in the absence of p53 mutations (26) and is thus often associated with the adverse clinical behaviors of tumors, such as fast progression and poor treatment response (27). In bladder cancer, a positive correlation between the p53 protein accumulation and overexpression of the MDM2 protein was observed; however, the increased MDM2 protein level alone had no effect on disease prognosis (28). Accumulated evidence supported that the increased expression of the MDM2 gene could induce tumorigenesis (29, 30). In the present study, the MDM2 1797G allele was associated with overexpression of the MDM2 gene, which could regulate the degradation of p53 as an E3 ubiquitin ligase, targeting p53 for proteasomal degradation (31). Our molecular epidemiologic findings also suggested that the MDM2 C1797G polymorphism could affect tumorigenesis in bladder cancer, which was consistent with the functional analyses.

Although our initial association study was a hospital-based case-control study that had a relatively small sample size and limited statistical power, we had 80% power at a 0.05 significance level to detect an OR of 1.7 or greater and 0.55 or smaller with an exposure frequency of 30% given our current study sample size (data not shown), and the finding of the positive association between the promoter C1797G polymorphism and risk of bladder cancer had led to the identification of functional relevance, which is biologically significant, and the molecular plausibility revealed by the molecular analysis should not be affected by the limitations of a hospital-based association study. Also, the false-positive report probability value for C1797G polymorphism was notable with a <20% chance of being a false positive (data not shown).

However, this relatively less powered association study may have missed the identification of other important SNPs that had smaller MAFs than that of the C1797G polymorphism. Furthermore, the selection of common tagSNPs was based on the LD in our Chinese population; therefore, some other important SNPs with smaller MAFs and ethnically specific SNPs might have been missed as well. Therefore, given the importance of the MDM2 gene in regulating the p53 pathway, a continuous search for functional variants, particularly the rare ones, by association studies in different ethnic populations with larger sample sizes should be pursued in future. It is well-known that the etiology of cancer is the interplay between both genetic factors and environmental exposure, such as tobacco smoking in bladder cancer. Thus, the significance of this novel functional promoter C1797G polymorphism needs to be further validated in larger studies in which the gene-environment interactions in the etiology of bladder cancer can be verified. Furthermore, there may be some differences in the levels of the MDM2 mRNA and protein between both tumor and normal adjacent tissues, and the changes in gene and protein expression in tumors could be caused by loss of heterozygosity. Therefore, the expression levels of the MDM2 mRNA and protein associated with the promoter C1797G polymorphism should be validated in both microdissected tumor tissues and normal adjacent tissues in future studies.

In conclusions, we have shown the C/EBPα transcriptional factor that binds to the promoter C1797G polymorphism, resulting in altered expression levels of the MDM2 gene, possibly affecting the subsequent attenuation of the p53 pathway and leading to a high risk of bladder cancer. In contrast to the discovery of a transcriptional activator Sp1 resulting in higher levels of the MDM2 mRNA and protein in the promoter SNP309 (13), we found that the C to G change of the novel MDM2 C1797G polymorphism increased the binding ability of transcriptional factor C/EBPα to the MDM2 promoter.

Disclosure of Potential Conflicts of Interest
Conflict of interest statement: None declared.

References


A Novel Functional Polymorphism C1797G in the MDM2 Promoter Is Associated with Risk of Bladder Cancer in a Chinese Population


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