A Polymorphism in HDM2 (SNP309) Associates with Early Onset in Superficial Tumors, TP53 Mutations, and Poor Outcome in Invasive Bladder Cancer

Marta Sanchez-Carbayo,1,2 Nicholas D. Socci,3 Thomas Kirchoff,4 Nadina Erill,5 Keneth Offit,4 Bernard H. Bochner,6 and Carlos Cordon-Cardo1

Abstract Purpose: The HDM2 gene represents one of the central nodes in the p53 pathway. A recent study reported the association of a single nucleotide polymorphism (SNP309) in the HDM2 promoter region with accelerated tumor formation in both hereditary and sporadic cancers. In this study, we aim to evaluate the SNP309 in bladder cancer and to link it to TP53 status. Experimental Design: SNP309 genotyping and TP53 mutation status were done on 141 bladder tumors and 8 bladder cancer cell lines using a RFLP strategy and TP53 genotyping arrays, respectively. Transcript profiling of a subset of cases (n = 41) was done using oligonucleotide arrays to identify genes differentially expressed regarding their SNP309 status. Results: Of 141 bladder tumors analyzed, 36.9% displayed the SNP309 wild-type (WT; T/T) genotype, whereas 11.3% were homozygous (G/G) and 51.8% were heterozygous (T/G) cases. Patients with superficial disease and the G/G genotype had an earlier age on onset than those with the T/G or T/T genotypes (P = 0.029). Tumors with SNP309 WT genotype significantly displayed TP53 mutations when compared with tumors harboring G/G or T/G genotypes (P < 0.05). SNP309 WT cases had a poorer overall survival than cases with G/G and T/G genotypes (P < 0.05). TP53 mutation status provided enhanced prognostic value (P < 0.001). Transcript profiling identified TP53 targets among those differentially expressed between tumors displaying G/G or T/G SNP309 versus WT cases. Conclusions: SNP309 is a frequent event in bladder cancer, related to earlier onset of superficial disease and TP53 mutation status. SNP309 genotypes were found to be associated with clinical outcome.

The HDM2 gene is a transcriptional target of p53. It encodes a negative feedback regulator (p90-Hdm2) that binds to p53 and acts as an ubiquitin-ligase, targeting p53 to proteasomal degradation (1). The association of a single nucleotide polymorphism (SNP309) in the HDM2 promoter region with accelerated tumor formation in both hereditary and sporadic cancers has been studied by several groups (2–24). As Hdm2 expression levels seem to be critical to a well-regulated p53 response, naturally occurring sequence variations in the HDM2 promoter may result in altered expression of the Hdm2, affecting p53 tumor suppression activity (2). In addition, the association of SNP309 to an early age of onset in cancer predisposition syndromes has also been reported (2, 3), although it has also been reported lack of evidence that the HDM2 SNP309 accelerates tumor development in carriers of known pathogenic germ-line mutations, such of BRCA1 (4). Several case-control studies of squamous head and neck tumors, breast, ovarian, lung, and colon carcinomas did not found either such correlation (6–10, 12, 16).

Bladder tumors are known to commonly harbor TP53 mutations and less frequently HDM2 amplifications (25–31). Although the abrogation of normal p53 function may be one of the permissive events promoting proto-oncogene amplification, predisposition to gene amplification of HDM2 in urothelial cancers may not be determined by the presence of p53 alteration alone (32). Advanced urothelial cancers without TP53 mutations may harbor HDM2 amplification, as well as splice variants of HDM2 with lower p53 binding activity (32). The present report aims at evaluating the frequency of SNP309 in primary bladder tumors and bladder cancer cell lines, as well as its association with TP53 mutation status, and its effect on clinical outcome.
Materials and Methods

Tumor samples, cell lines, genomic DNA, and RNA extraction. One hundred and forty-one bladder cancer cases were included for the present study. Specimens were collected under Institutional Review Board–approved tissue procurement protocols at Memorial Sloan-Kettering Cancer Center and BIOPAT Laboratories. Bladder tissues, either embedded in orithine carbamyl transferase or deparaffinized tissue sections, were macrodissected to ensure a minimum of 75% of tumor cells. Bladder cancer cell lines, including RT4, 5637, UIMC3, T24, HT1197, HT1357, TCCSUP, and SCABER, were grown and harvested as reported previously (33). Genomic DNA was extracted using a nonorganic method (Oncor). Total RNA was isolated in two steps using Trizol (Life Technologies) followed by RNeasy purification (Qiagen). DNA and RNA quality was evaluated based on 260:280 ratios (Qiagen). DNA and RNA were analyzed on 2% agarose gels. cDNA was generated from RNA using M-MLV reverse transcriptase (Promega). cDNA was eluted in water and stored at 4°C. cDNA (1 μl) was subjected to PCR in a 10-μl PCR mixture. Images of agarose gels and autoradiographs were obtained on a BioRad Automated Imaging System. A standard Kaplan-Meier methodology. Associations among any molecular targets were analyzed using Kendall’s τ-b test. Statistical analyses were done using the SPSS statistical package (version 8.0).

Results and Discussion

SNP309 is a frequent event in bladder cancer. A WT genotype for SNP309 (T/T) was found for 52 (36.9%) of the cases. The homozygous genotype for SNP309 (G/G) was present in 16 (11.3%) of the cases. The heterozygous genotype for SNP309 (T/G) was present in 25 (18.2%) of the cases. SNP309 was mutated in 34.2% of the cases.

Table 1. Distribution of TP53 mutations among SNP309 genotypes in bladder tumors

<table>
<thead>
<tr>
<th>SNP309 genotype</th>
<th>SNP309 frequency (%)</th>
<th>TP53 mutations (%)</th>
<th>TP53 exon (no. cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>16 (11.3)</td>
<td>6 (37.5)</td>
<td>4 (2), 5 (2), 7 (8)</td>
</tr>
<tr>
<td>T/G</td>
<td>73 (51.8)</td>
<td>25 (34.2)</td>
<td>4 (2), 5 (6), 6 (6), 7 (5), 8 (6)</td>
</tr>
<tr>
<td>T/T</td>
<td>52 (36.9)</td>
<td>22 (42.3)</td>
<td>4 (2), 5 (4), 6 (1), 7 (2), 8 (13)</td>
</tr>
</tbody>
</table>

SNP309 genotypes are associated with TP53 mutation status. DNA obtained from the tumor specimens was submitted for TP53 sequencing using the TP53 GeneChip and/or manual sequencing as reported previously (31, 35). SNP309 genotypes were associated with TP53 mutation status. TP53 was mutated in 34.2% of the cases. SNP309 was mutated in 34.2% of the cases.

SNP309 genotypes were compared with other TP53 genotypes. The comparison of TP53 mutation status and SNP309 genotypes were compared with other TP53 genotypes. The comparison of TP53 mutation status and SNP309 genotypes were compared with other TP53 genotypes.

The Welch’s t statistic was applied to the log of the signal intensity to identify genes differentially expressed between tumors with wild-type (WT) versus SNP309. To deal with the multiple testing problem, the false discovery rate was used (36). Additional studies were designed to obtain insights into the functional pathways, in which these genes are involved. The Ingenuity tool was used to link the most differentially expressed genes in bladder tumors about their SNP309 status with the reported signaling networks of these genes.

Statistical analysis. All cases (n = 141) were used for the analysis of association among SNP309 and p53 mutation status with clinicopathologic variables. Tumor stage analyses compared superficial tumors (pT1 and pT1a) versus invasive tumors (pT2–pT4), whereas grading analyses contrasted low-grade tumors versus medium- and high-grade tumors, as reported previously (25–35). The association of SNP309 genotypes and TP53 mutation status with histopathologic stage and tumor grade was evaluated using the nonparametric Wilcoxon-Mann-Whitney test. Statistical analyses were done using the SPSS statistical package (version 8.0).
number of G allele enrichments among TP53-mutated cases in our series not allowing further statistical analyses, the dominant-negative activity was not assigned and modeled in our experimental design. Further studies are warranted to address this issue in the near future with higher number of tumors.

Transcript analyses support the association of SNP309 genotypes with messenger levels of HDM2. HDM2 transcript levels were obtained from gene expression profiling using U133A GeneChips (Affymetrix) for a subset of the tumors that were genotyped for SNP309 (n = 43). Increased HDM2 transcript levels (probe identification number 205386_s_at) were observed in patients displaying either the homozygous (G/G, n = 5) or the heterozygous (T/G, n = 24) genotype compared with those presenting the WT (T/T, n = 14; Supplementary Fig. S1A; Supplementary Table S1). A nearly significant statistical association was found between mRNA transcript levels of TP53-related genes.

Transcript profiles comparing WT and altered SNP309 genotypes support the effect of SNP309 on the p53 pathway. We searched for genes differentially expressed in the bladder tumors analyzed, focusing on their combined TP53 and SNP309 status. No differentially expressed gene was found between each of the SNP309 genotypes or comparing the WT versus the T/G and G/G forms. However, 245 probes were found to be differentially expressed when comparing cases with mutant TP53 versus those with WT TP53 that had the SNP309 (T/G + G/G). These genes displayed differential expression with P values <0.001, with fold changes >1.41, and at a false discovery rate of 0.05. These genes are reported in Supplementary Table S1. Pathway analyses using the Ingenuity software revealed that one of the nodes associated with these gene profiles included the p53-related network (Supplementary Fig. S2). We also observed that 125 probes were differentially expressed between cases with mutant TP53 compared with those with WT TP53. These genes displayed differential expression with P values <0.001, with fold changes >1.41, and at a false discovery rate of 0.1 (Supplementary Table S2). The limited overlap of these two lists of genes provides specificity to the gene profiles associated with SNP309.

Heterozygous SNP309 is a frequent event in bladder cancer cell lines. Bladder cancer cell lines were also sequenced for both TP53 and SNP309, having the transcript levels evaluated using the U133A GeneChip (Table 2). The purpose of these analyses was to assess the SNP309 status in these cells, which had not been reported, and to relate them to their TP53 status. The majority of these cell lines was heterozygous for SNP309 and associated with TP53 mutations. The bladder cancer cell line with squamous differentiation, SCABER, was found to display a WT SNP309 but had a TP53 mutation in exon 4 (110 CGT → CTT). HDM2 transcript levels of cells were in general low for those cell lines with heterozygous SNP309. None of the bladder cancer cell lines was homozygous for SNP309. Enhanced HDM2 transcript levels in heterozygous (T/G) cell lines were observed compared with the SCABER T/T cells as reported previously (2). The analyses done on bladder cancer cells also served to confirm the association of SNP309 with the tumor transcript profiles shown above. Further analyses will underline functional interaction of the different components of the p53 pathway (39).

SNP309 genotypes are associated with aggressive bladder cancer and clinical outcome. Further analyses were conducted to evaluate the association of SNP309 status with bladder cancer progression and patient outcome. In our series, a great number of those patients displaying the homozygous (G/G) or heterozygous (T/G) SNP309 genotype had invasive bladder tumors. However, no significant association between SNP309 and tumor stage was found when considering each genotype independently or grouping those heterozygous and homozygous for SNP309 versus the WT genotype (Fig. 1). The lack of significant association with cancer progression or the clinicopathologic variables tumor stage and grade could relate to the germ-line nature of this specific polymorphism (2). Similar expression was also found between those presenting the WT (T/T, n = 14; Supplementary Fig. S1C). Nearly significant associations were also found for transcript levels of TP53 (probe identification number 211300_s_at) in homozygous G/G cases compared with heterozygous T/G or WT T/T in SNP309 (P = 0.075; Supplementary Fig. S1D).

The limited overlap of these two lists of genes provides specificity to the gene profiles associated with SNP309.

Figure 1. SNP309 genotypes are not associated with tumor stage. Distribution of each stage in bladder cancer progression in homozygous G/G cases compared with heterozygous T/G or WT T/T in SNP309.
findings were reported for other tumor types, including analyses of uterine leiomyosarcoma, colorectal, and squamous cell carcinoma of the head and neck (3–10).

Clinical outcome associations were done on the invasive tumors under analyses. We found that cases with WT SNP309 had poorer overall survival than those with the SNP309 genotypes \( (P < 0.05; \text{Fig. 2A}) \). This result together with the observation reported above by which patients with WT SNP309 \( (T/T) \) were prone to have mutations in the core domain of TP53 suggests the relevance of p53 mutation status in the aggressiveness of these tumors. The association of TP53 with survival and the high frequency of TP53 mutations among individuals with a T/T genotype are consistent with results reported by Boersma et al. (15). Indeed, TP53 mutation status was shown to provide greater prognostic value than SNP309 alone \( (P < 0.001; \text{Fig. 2B}) \). None of the SNP309 genotypes alone or combined improved the prognosis value of TP53 status. There was no statistical association among any of the combined patterns of mutation status of TP53 together with SNP309. When cases mutated or WT for TP53 were considered separately, none of the SNP309 genotypes alone or combined served to discriminate patients with poor survival. To evaluate the TP53 relevance, further survival analyses were done excluding those tumors with a WT \((TT)\) for the SNP309. In this case, TP53 allowed identification of cases with poor outcome among those cases either homozygous or heterozygous for SNP309 \( (P < 0.05; \text{Fig. 2C}) \). Thus, TP53 status allowed stratification of clinical outcome taking all patients with invasive bladder cancer under study \( (P = 0.07) \). The lack of statistical significance could be related to the fact that the majority of patients analyzed had SNP309 may substitute TP53 inactivation by mutation and they offer additional evidence for the transformation and tumor progression potential of HDM2 in bladder cancer.

**SNP309 was found to be associated with an earlier bladder cancer onset in superficial bladder cancer.** The identification of the SNP309 in the HDM2 promoter has been reported to offer some rationale about the phenotypic variation in cancer susceptibility observed in sets of hereditary and sporadic soft tissue sarcomas (2). In our series, a nearly significant association was found between SNP309 status and age of onset \( (P = 0.07) \). The lack of statistical significance could be related to the fact that the majority of patients analyzed had

**Fig. 3.** SNP309 genotypes are associated with bladder cancer risk. Superficial cases harboring the G/G genotype debut sooner than those with the T/G or T/T genotypes.
advanced disease and the limited number of patients studied. Although lack of significant association was found between the SNP309 and the age of onset when considering together all cases under study, we further addressed the association of SNP309 with bladder cancer risk considering separately superficial and invasive tumors. Interestingly, such analyses revealed that patients with superficial tumors harboring the G/G genotype debut sooner than those with the other genotypes (P = 0.029, Kruskal-Wallis; Fig. 3). This is a relevant finding because superficial early onset cases are associated with an aggressive clinical behavior. Our results indicate that SNP309 genotypes do not associate with tumor stage and that among patients with superficial disease, those with the G/G genotype debut sooner than those with the other genotypes. It is important to indicate that such observations are independent and should not be mixed. In the future, in vitro and mouse modeling analyses would reveal the sequence of SNP309/TP53 molecular alterations along bladder cancer progression. Controversial results have been reported dealing with cancer onset and susceptibility for the SNP309 (2–24). Case-control studies have found enrichment of the G allele (11, 12, 16). Lack of significant contribution to earlier tumor formation was found in series that analyzed uterine leiomyosarcoma, colorectal and head/neck squamous cell carcinomas (7, 10), risk of lung cancer (6, 9), or cancer susceptibility in two independent case-control series of breast and ovarian tumors (3, 5, 8). For ovarian cancer, the lack of association of the HDM2 polymorphism with cancer risk could be consistent with rare HDM2 amplification in these cancers. Lack of association between the arginine/proline polymorphism at codon 72 of TP53 and SNP309 on age of onset has been reported for patients with Lynch syndrome and for patients with sporadic colorectal cancer (4). However, the effect of the HDM2 SNP309 G allele on the age of tumor onset in germ-line TP53 carriers with the Li-Fraumeni syndrome has been reported in other studies (2, 3). The G allele of SNP309 has been shown to accelerate tumor formation in a gender-dependent manner in several solid and hematologic tumors (10–12, 16). Among cases affected with bladder cancer, at least four to one patients are men compared with woman cases. In our series, we did not find any statistical association of the SNP309 with human gender. This finding can be related to the low women to men ratio among patients affected with bladder cancer.

**Conclusions.** In this article, we evaluate the contribution of SNP309 on a tumor type known to have frequently alterations in the p53 pathway. Of relevance, this report evaluated not only SNP309 but also its association to TP53 status in sporadic primary bladder tumors. Moreover, our study reveals the clinical significance of SNP309 at identifying aggressive bladder cancer because superficial cases with G/G genotype had an earlier onset and invasive tumors a shorter survival. It also ratifies the prognostic significance of detecting TP53 mutations in bladder cancer, as it is associated to tumor progression and poor clinical outcome. Together, these data provide novel insights about the roles of TP53 and SNP309 in bladder cancer and suggest that SNP309 may substitute TP53 inactivation by mutation, offering evidence for the transformation and tumor progression potential of HDM2 in this group of neoplasms.

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**References**


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