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

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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.

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

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

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 ornithine carbamyl transferase or deparaffinized tissue sections, were macrodissected to ensure a minimum of 75% of tumor cells. Bladder cancer cell lines, including RT4, 5637, UMUC3, T24, HT1197, HT1376, 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 of absorbances and the integrity was also checked by gel electrophoresis analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies) as reported previously (34).

SNP309 and TP53 genotyping. DNA specimens were genotyped for SNP309 using a RFLP strategy using a PCR amplification using a forward primer 1 (AGTTCAGGGTAAAGGTCA) and a reverse primer 2 (AGCCGTTCACACTAGTTGA) followed by a RFLP sequencing strategy using MspA1 that discriminated the three genotypes for SNP309. DNA obtained from the tumor specimens were submitted for TP53 sequencing using the TP53 GeneChip (Affymetrix) and manual sequencing as reported previously (31, 35).

Transcript profiling. Gene profiling of mRNA belonging to 41 of the 141 analyzed specimens was done using the U133A human GeneChips containing 22,283 probes representing known genes and expressed sequence tags (Affymetrix) as reported previously (34). 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.[7]

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 (pT_a and pT_1) versus invasive tumors (pT_2-pT_4), 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 and Kruskall-Wallis tests (37). The associations of the genotype with overall survival were also evaluated in those cases for which follow-up information was available. Overall survival time was defined as the years elapsed between transurethral resection or cystectomy and death from disease (or the last follow-up date). Patients who were alive at the last follow-up or lost to follow-up were censored. The association of these variables with overall survival was analyzed using the log-rank test (37). Survival curves were plotted using the 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 73 (51.8%) of the bladder tumors under study. No deviation from the Hardy-Weinberg equilibrium was found. In the initial series of DNA extracted from the blood of 50 healthy individuals (2), the SNP309 (a T to G change at the 309th nucleotide in the first intron) was found at relatively high frequency both in the heterozygous state (T/G, 40%) and in the homozygous state (G/G, 12%). In our study, the SNP309 was also analyzed on DNA extracted from the normal urothelium paired counterparts of all bladder tumors under evaluation. Although laser microdissection was not done previous to DNA extraction, all normal specimens provided the same genotype for the SNP309. Comparison of SNP309 in primary tumors versus normal counterparts was not reported to date, to the best of our knowledge. This observation supports the germ-line nature of the SNP309 and excludes somatic association.

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 (31, 35). Interestingly, TP53 mutations were present in 22 of 52 (42.3%) WT SNP309 (T/T) and in 25 of 73 (34.2%) heterozygous (T/G) cases (Table 1). Among the 16 cases homozygous for SNP309 (G/G), 6 of them (37.5%) had TP53 mutations. The SNP309 genotypes were found to be associated to specific TP53 mutations, either when the homozygous and heterozygous genotypes (G/G and T/G) were grouped together (P = 0.016, Mann-Whitney) or when the three genotypes were considered independently (P = 0.043, Kruskall-Wallis). Thus, patients with WT SNP309 (T/T) were prone to display TP53 mutations. The majority of such mutations were in exon 8 (Table 1), affecting the core DNA binding domain. This represents the most frequently reported mutated region in bladder cancer (25, 27–31). Our results differ with those reporting an association of the G/G genotype with TP53 mutations in lung cancer (9) but are consistent with those showing an association of the G/G genotype for the SNP309 with WT TP53 in colorectal cancer (10). These findings support the potential role of SNP309 at substituting TP53 inactivation by mutation in bladder cancer. These results are also consistent with previous reports describing that alterations in HDM2 are usually mutually exclusive with regard to TP53 mutations (38). A recent report describes that the SNP309 interacts with p53 mutational status and finds that the G allele enriches for a non–dominant-negative p53 mutations (17). Due to the low
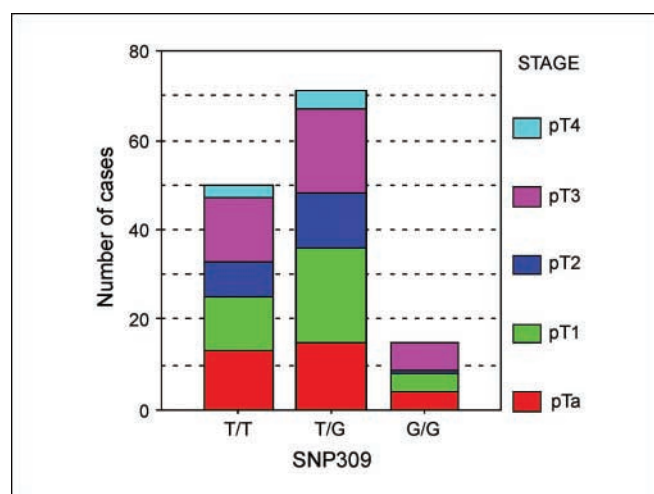
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[7] http://www.ingenuity.com

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 HDM2-A1 (probe identification number 211832_s_at) about the SNP309 versus the WT (P = 0.071; Supplementary Fig. S1B). Interestingly, increased transcript levels for p53-regulated apoptosis-inducing protein 1 (probe identification number 220402_at) were observed in patients displaying either the homozygous genotype (G/G, n = 5) or the heterozygous (T/G, n = 24) compared with those presenting the WT (T/T, n = 14; P = 0.090; 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). Overall, these results suggest an effect of the different SNP309 genotypes on the 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
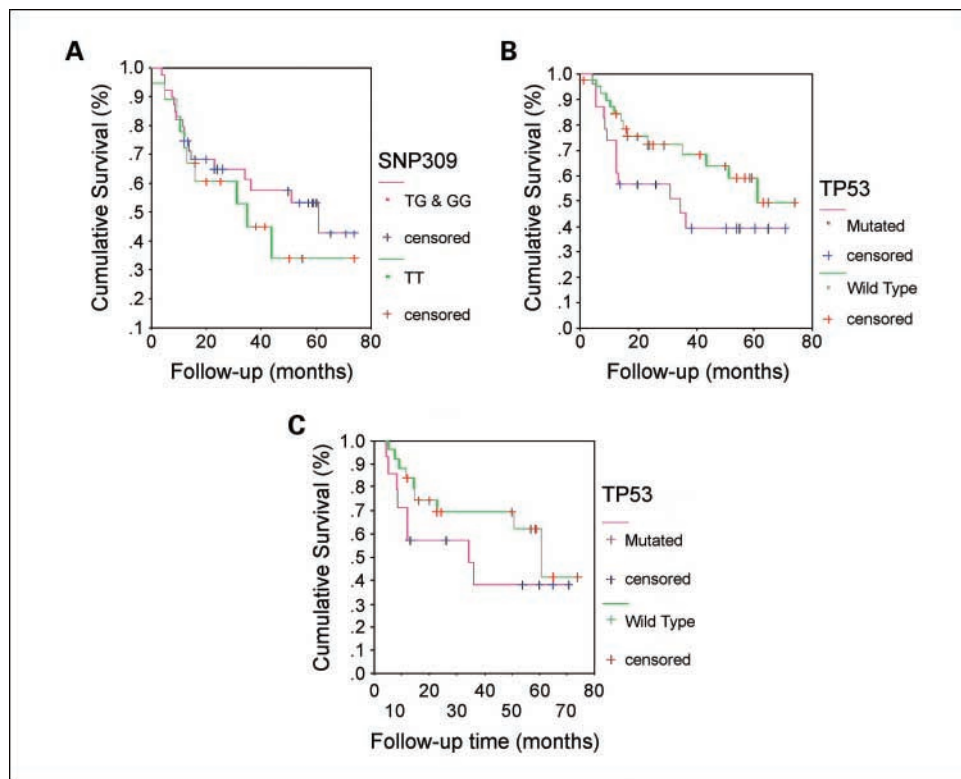

Fig. 1. SNP309 genotypes are not associated with tumor stage. Distribution of each stage in bladder cancer progression in homozygous G/G cases compared with heterozygous T/G or WT T/T in SNP309.

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

Table 2. SNP309 in bladder cancer cell lines: association to transcript expression of HDM2 and TP53 given by U133A oligonucleotide arrays

| Cell line	HDM2 SNP309	TP53 status	HDM2 mRNA	TP53 mRNA
RT4	GT	WT	210.4	589.2
5637	GT	Exon 8	23.5	504.8
T24	GT	Exon 5	5.1	102.1
HT1376	GT	Exon 7	30.1	516.6
HT1197	GT	Exon 11	482.7	425.2
TCCSUP	GT	Exon 10	23.9	162.9
UMUC3	GT	Exon 4	5.9	781.9
SCABER	TT	Exon 4	5.1	889.1

Fig. 2. SNP309 genotypes and TP53 mutation status are associated with aggressive bladder cancer. *A,* SNP309 is associated with poor overall survival in bladder cancer. *B,* TP53 mutation status is associated with poor survival in bladder cancer taking all invasive tumors under analyses. *C,* TP53 mutation status identifies patients with a more aggressive outcome among those cases either homozygous or heterozygous for SNP309, excluding in the analyses those cases with a WT genotype for the 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 ($P < 0.05$; 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 ($P < 0.001$; 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 tumors either homozygous or heterozygous for SNP309 ($P < 0.05$; Fig. 2C). Thus, TP53 status allowed stratification of clinical outcome taking all patients with invasive bladder cancer under study (Fig. 2B) and also excluding those with a WT genotype for the SNP309 (Fig. 2C). Overall, these results are clinically relevant because they show that SNP309 relate to clinical outcome and that TP53 stratify overall survival regardless of the SNP309. Moreover, they suggest that

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
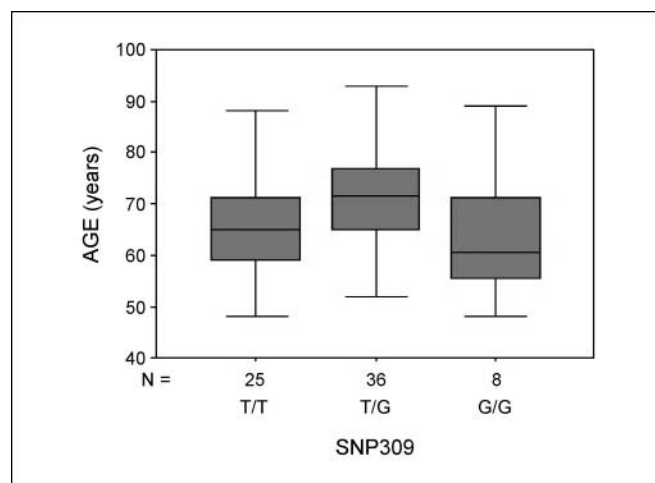
Fig. 3. SNP309 genotypes are associated with bladder cancer risk. Superficial cases harboring the G/G genotype debut sooner than those with the T/G or T/T genotypes.

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, Kruskall-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.

Acknowledgments

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