Original Paper

Pathobiology

Pathobiology 2014;81:53–59
DOI: 10.1159/000355976

Received: August 22, 2013
Accepted after revision: September 24, 2013
Published online: November 9, 2013

Mdm2 SNP309 G-Variant Is Associated with Invasive Growth of Human Urinary Bladder Cancer

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Key Words
Mdm2 SNP309 · Bladder cancer · p53 · Invasion

Abstract
Objective: Human mouse double minute 2 (Mdm2) is essential in degrading p53 by acting as an ubiquitin ligase and therefore plays a vital role in cell cycle and survival. The G-variant of the *Mdm2* SNP309, which is located within the promoter of the *Mdm2* gene, increases expression of Mdm2 and thereby inhibits the p53 pathway. Several studies have investigated the influence of this SNP on disease risk and onset of various malignancies. The impact of *Mdm2* SNP309 on bladder cancer is still to be established due to inconsistent data. ***Methods:*** In a case-control study we determined the distribution of *Mdm2* SNP309 genotypes in 111 patients with an early-onset bladder cancer (diagnosis <45 years of age), in 113 consecutive bladder cancer patients and in a control group consisting of 140 patients without any malignancy. ***Results:*** There was no significant association between the allelic distribution of the *Mdm2* SNP309 and tumor risk, early onset, gender or grade of the tumor. According to tumor stage we found a significant difference in the distribution of the *Mdm2* SNP309 between patients with noninvasive and invasive (≥pT1) tumor growth (p = 0.016). In patients with invasive tumors a significant increase of the G allele was found (T/T vs. T/G + G/G; p = 0.023; OR 2.203, 95% CI 1.111–4.369). ***Conclusion:*** These data indicate that the G-variant of the *Mdm2* SNP309 might influence the development of a more aggressive tumor phenotype in patients with bladder cancer without affecting the overall tumor risk.

Introduction

One major regulator of the p53 network is the human mouse double minute 2 protein (Mdm2). The *Mdm2* gene is located on chromosome 12q14.3-q15 and encodes an E3 ubiquitin ligase that is involved in p53 degradation by negatively regulating p53 stability and transcriptional activity [1]. *Mdm2* gene (amplification) or protein (overexpression) alterations can frequently be found in various

Parts of this study were presented at the 93rd annual meeting of the German Association of Pathology in Freiburg, Germany, June 4–7, 2009.

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1015–2008/14/0812–0053$39.50/0

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cancer types, including bladder cancer [2, 3]. A single nucleotide polymorphism (SNP) in the promoter region of the *Mdm2* gene at position 309 (*Mdm2* SNP309; T<=>G) has been broadly investigated because the G allele binds transcriptional activator Sp1 in a more effective way than the T allele, therefore generating higher levels of Mdm2 RNA and protein [4]. This might accelerate tumorigenesis by degrading p53. An influence of this SNP on cancer risk was shown for various tumor types in different populations [5]. In addition, this SNP might also affect the age of disease onset, especially in patients with Li-Fraumeni syndrome, or even gender-specific cancer risk [4, 6, 7].

Only very inconsistent data are available for the role of the *Mdm2* SNP309 in bladder cancer. To date three case-control studies have been published. Horikawa et al. [8] reported no influence of this SNP on bladder cancer risk. Onat et al. [9] found an increased tumor risk for the homozygous GG-variant, whereas Gangwar and Mittal [10] reported a reduced bladder cancer risk for individuals with the homozygous GG-variant. Regarding disease outcome, ambiguous data have been published too. While Sanchez-Carbayo et al. [11] reported a poorer overall survival of patients with the homozygous TT-variant compared to patients with a least one risk allele, Horikawa et al. [8] found a longer recurrence-free survival in patients with the homozygous wild-type genotype. Recently, a study focusing on stage pT1 bladder tumors found no influence of the SNP on prognosis [12].

Interestingly, the *Mdm2* SNP309 might also have an influence on the survival of bladder cancer patients treated with chemoradiotherapy. In a study by Shinohara et al. [13] patients with at least one risk allele had an improved cancer-specific survival rate after therapy compared to patients carrying the wild-type allele only. However, this positive effect of the risk allele could not be validated in a second, similar study [14]. To advance the still ongoing discussion of the role of the *Mdm2* SNP309 on bladder cancer risk we performed a case-control study including a subset of patients with early disease onset.

Materials and Methods

Patients and Tissue Samples

Two hundred and twenty-four formalin-fixed and paraffin-embedded tissue samples from bladder cancer patients were investigated and compared to 144 samples from a control group of patients without any malignancy acquired at the Department of Urology, University of Regensburg. The tumor group consisted of two subgroups: a consecutive group of bladder cancer patients (n = 113) and a patient cohort with early-onset bladder cancer (less than 45 years of age at diagnosis, n = 111). All patients were Caucasians.

Table 1. Characteristics of study patients

	Control group	Consecutive tumor group	Early-onset tumor group
n	140	113	111
Age, years			
Median	68	69	39
Mean	66.9	68.6	37.6
Range	17–89	37–93	18–45
Stage, n			
Ta		68	49
T1		29	10
≥T2		13	14
Unknown		3	38
Grading, n			
G1		39	24
G2		44	29
G3		30	16
Unknown		0	42
Gender, n			
Male	120	90	64
Female	12	22	24
Unknown	8	1	23

The tumors were diagnosed according to the WHO classification of bladder tumors and staged according the TNM system [15, 16]. All available slides were reinvestigated by one surgical pathologist (A.H.) and all tumors were histologically classified as transitional cell carcinomas. Cases with missing data (e.g. tumor stage, gender) were used for risk evaluation in the case-control study, but were excluded from statistical analyses concerning single parameter tests. All tumors were primary tumors. Characteristics of the study participants are shown in table 1. Institutional review board approval was obtained for the study.

Tissue Microdissection and DNA Isolation

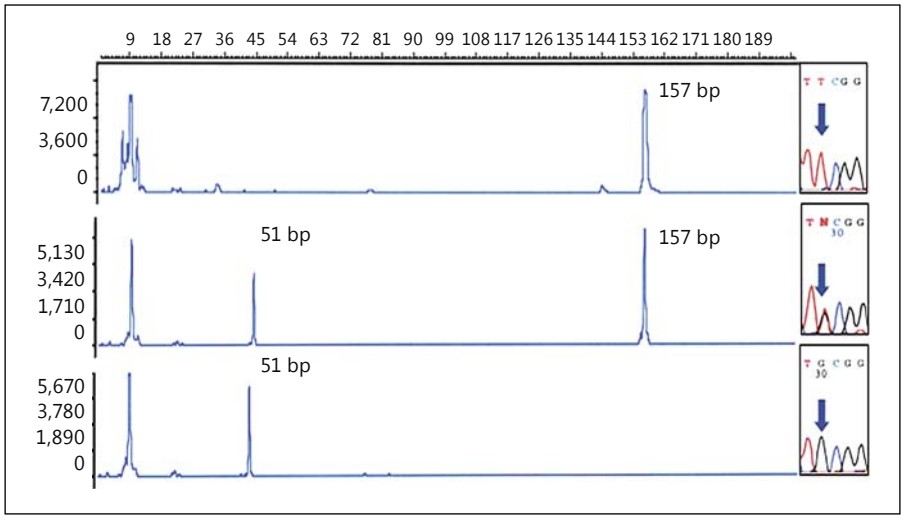
DNA for the study was extracted from normal bladder tissue (microdissection from serial sections) using the High Pure PCR Template Preparation Kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Analysis of Mdm2 SNP309

To analyze the genotype of the *Mdm2* SNP309 restriction fragment length polymorphism (RFLP) analysis was performed. When the G allele was present the region of interest contained an *Msp* A1l site (5′-CMGCKG-3′). The G allele therefore resulted in digestion of the PCR product (157 bp ≥ 106 + 51 bp) whereas PCR products harboring the T allele in SNP309 remained unaffected (157 bp).

Amplification of Promoter Region and RFLP Analysis

The *Mdm2* SNP309 region was amplified by using PCR primers (5-FAM-sense: 5′-CGCGGGAGTTCAGGGTAAAG-3′; antisense: 5′-CTGAGTCAACCTGCCCACTG-3′ [17]) obtained from



Color version available online

Fig. 1. Representative example of the *MDM2* SNP309 RFLP analysis. In the upper lane only the undigested PCR product (157 bp) is visible after the digest resulting from a T/T genotype of the sample. In the middle lane the undigested (157 bp) and the digested (51 bp) products are visible indicating a heterozygous T/G genotype of the sample. The 106 bp product which is also created by the RFLP analysis is not visible due to the 5′ end labeling of the PCR product. In the lower lane only the 51 bp product is visible after the digest suggesting a G/G genotype of the sample. The added results from the Sanger sequencing confirmed the results from RFLP analyses (arrows).

Metabion (Martinsried, Germany) in a total volume of 25 µl containing 100 ng DNA, 0.15 µM primers, 0.2 mM dNTP (Roche Diagnostics) and 0.0025 U/µl GoTaq (Promega, Mannheim, Germany). The thermal cycling conditions were as follows: initial denaturation for 3 min at 95°C, 35 cycles of denaturation at 95°C, annealing at 61°C for 1 min, elongation at 72°C for 1 min and final primer extension at 72°C for 10 min. PCR products were incubated overnight with 5 U *Msp* A1I (New England Biolabs, Frankfurt am Main, Germany) and 100 µg/ml BSA at 37°C in a total volume of 30 µl to ensure complete digestion. Capillary electrophoresis using an ABI PRISM 310 (Applied Biosystems, Foster City, Calif., USA) genetic analyzer was used to separate restriction fragments. Analyses were done using GeneScan® analysis software. Ten randomly selected cases were also sequenced to verify the RFLP results.

Statistical Analysis

Publicly available software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to determine if genotype distribution followed the Hardy-Weinberg equilibrium, whereas χ^2 statistics (two-sided Fisher's exact test or Pearson's where appropriate) were used to evaluate case-control differences in the distribution of genotypes and to analyze associations between clinical or histopathological characteristics and genotype. Statistical analysis was done utilizing the Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Chicago, Ill., USA). p values <0.05 were interpreted as statistically significant.

Results

The genotype distribution followed the Hardy-Weinberg equilibrium in cases (early-onset tumor group, p = 0.342, and consecutive tumor group, p = 0.443) and controls (p = 0.594). Nine cases were randomly selected to verify RFLP analyses: these cases were sequenced and showed a 100% concordance between both methods (fig. 1).

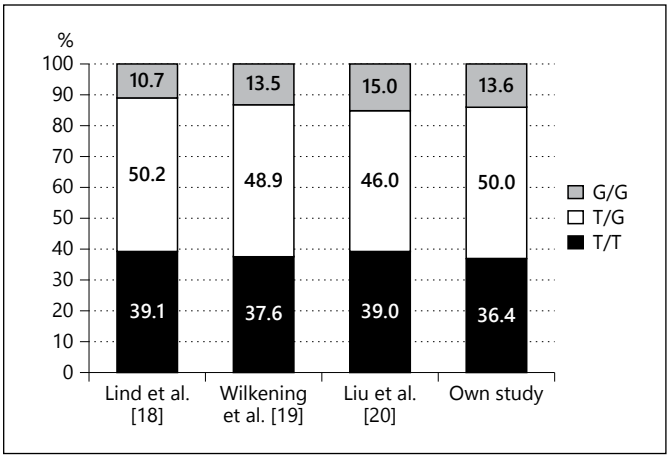


Fig. 2. Distribution of the *MDM2* SNP309 in control cohorts from Caucasian studies. The allele frequencies were very similar to the control cohort from this study.

In order to ensure the usage of our control group as a representative Caucasian cohort we compared the *Mdm2* SNP309 distribution from our cohort with published control cohorts from other studies. A very similar allelic distribution was seen in all 4 studies underlining the use of a nonbiased control group in our study (fig. 2).

There were no significant differences in the distribution of the allelic variants of the *Mdm2* SNP309 between controls and cases (neither analyzed separately nor analyzed as one cohort; table 2; fig. 3a, b). Regarding tumor stage, there was a remarkable difference in genotype dis-

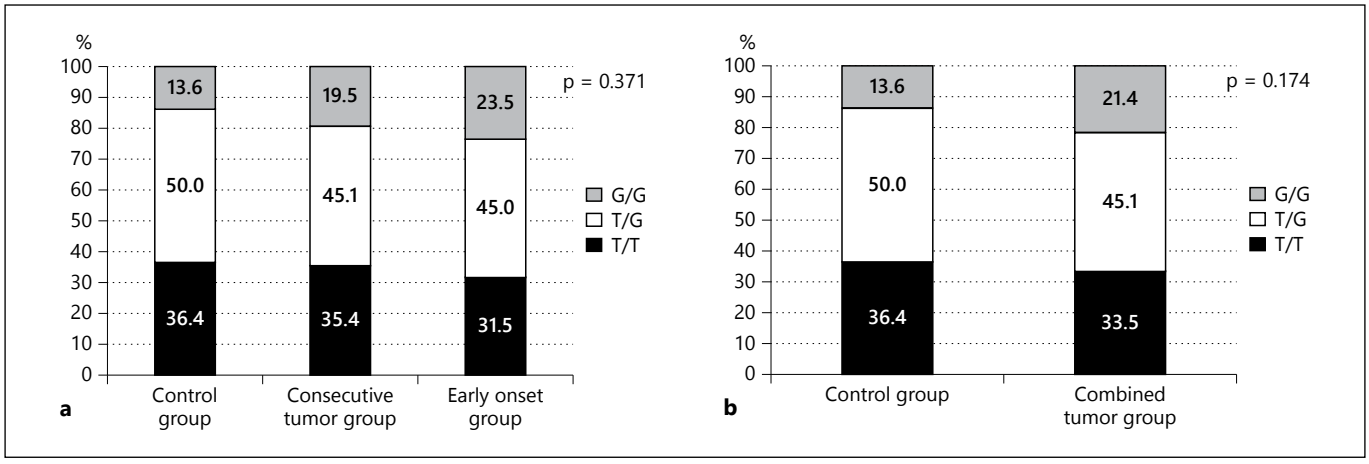


Fig. 3. Distribution of the *MDM2* SNP309 variants in our cohorts. **a** Results for both tumor cohorts (consecutive and early-onset patients). **b** Results for the grouped tumor cohort. No significant differences were found in both analyses.

Table 2. Distribution of allelic variants between the investigated groups

	Control group	Consecutive tumor group	Early-onset tumor group	Comparison p value	OR	95% CI
Genotype						
T/T	51 (36.4)	40 (35.4)	35 (31.5)			
T/G	70 (50.0)	51 (45.1)	50 (45.0)			
G/G	19 (13.6)	22 (19.5)	26 (23.5)	0.371		
		Tumor groups combined[1]				
Genotype						
T/T	51 (36.4)	75 (33.5)				
T/G	70 (50.0)	101 (45.1)				
G/G	19 (13.6)	48 (21.4)		0.173		
T/T	51 (36.4)	75 (33.5)		0.573	1.138	0.732–1.771
T/G + G/G	89 (63.6)	149 (66.5)				

Values are n with percentage in parentheses.
[1] Results of the consecutive and early-onset tumor groups together.

tribution which did not reach statistical significance (table 3). After grouping the cases into the biological relevant categories of noninvasive (stage pTa) and invasive (stage ≥pT1) tumors, there was a significant difference in genotype distribution with a significant increase of the risk allele G in patients with invasive bladder cancer (table 3; fig. 4). No significant differences in genotype distribution were found for tumor grade, gender and age of the patients (data not shown).

In order to enlarge the statistical power of the available analyses on the *MDM2* SNP309 we combined all available data from Caucasian bladder cancer studies to perform a meta-analysis. By doing this SNP data from 243 controls and 581 bladder cancer patients were available [9, 11, 12, this study]. The genotype distribution followed the Hardy-Weinberg equilibrium in controls (p = 0.234) and cases (p = 0.444). The distribution of the allelic variants did not differ significantly between controls and cases (fig. 5). There was also no significant difference in the distribution of the G allele between the two cohorts (p = 0.713; OR 0.942, 95% CI 0.686–1.294).

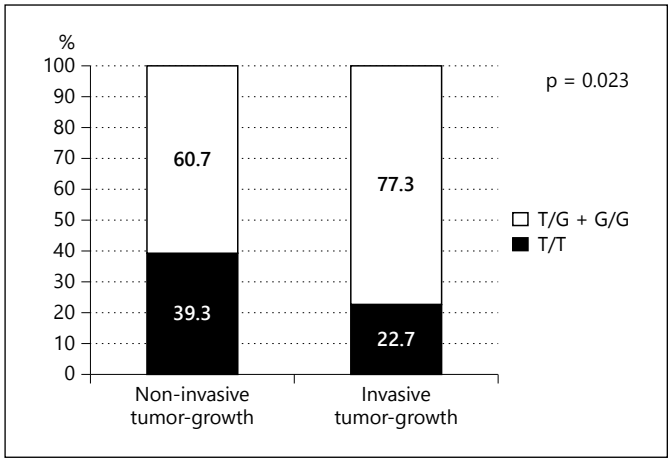


Fig. 4. Distribution of the G allele of the *MDM2* SNP309 in patients with noninvasive and invasive bladder tumors. A significant increase of the G-variant was revealed in patients with invasive bladder cancer.

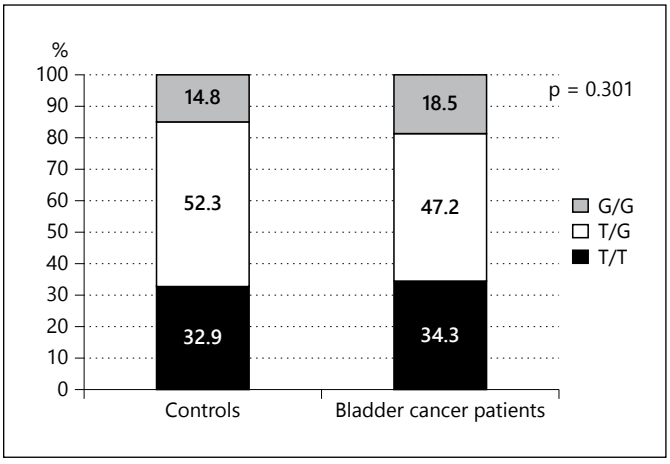


Fig. 5. Meta-analysis of the genotype distribution of the *MDM2* SNP309 in Caucasians. Results from all available studies on bladder cancer were combined. No significant difference in genotype distribution was found.

Table 3. Distribution of *Mdm2* SNP309 genotype according to tumor stage

Tumor group	T/T	T/G	G/G	Comparison p value	OR	95% CI
Stage						
Ta	46 (39.3)	47 (40.2)	24 (20.5)	0.071		
T1	8 (20.5)	25 (64.1)	6 (15.4)			
≥T2	7 (25.9)	16 (59.3)	4 (14.8)			
Ta (noninvasive)	46 (39.3)	47 (40.2)	24 (20.5)	**0.016**		
≥T1 (invasive)	15 (22.7)	41 (62.1)	10 (15.2)			
		T/G + G/G				
Ta (noninvasive)	46 (39.3)	71 (60.7)		**0.023**	2.203	1.111–4.369
≥T1 (invasive)	15 (22.7)	51 (77.3)				

Values are n with percentages in parentheses. Bold values are significant.

Discussion

In the present study we analyzed the distribution of the *Mdm2* SNP309 in a Caucasian cohort of bladder cancer patients and a control group without any malignancy. To our knowledge our study comprised the largest cohorts in a Caucasian case-control study to date. Our data showed that the *Mdm2* SNP309 did not influence the overall risk for bladder cancer. These data are in line with a study by Horikawa et al. [8] that analyzed large cohorts in a Japanese case-control study. In addition, the results from a meta-analysis performed with all available data from studies on Caucasian cohorts further strengthened our data. Contrary to our results, two studies showed contradicting effects of the G-variant of the SNP. These discrepancies to our study might be explained by a small number of cases analyzed (n = 75) [9] or population-based genetic variation [10]. To date no additional case-control studies have been published and a combined analysis of all the available data might be biased by the different ethnical background of the cohorts.

Of great interest was the study by Sanchez-Carbayo et al. [11] that described an influence of the *Mdm2* SNP309

on disease onset in bladder cancer patients, especially in the group of patients with superficial tumors. Our data were contradicting to these findings as we found no association between allelic variants and disease onset, although we analyzed a large subgroup of young bladder cancer patients. These discrepancies might be explained by the fact that the patient cohort from the Sanchez-Carbayo study that showed the age-associated effect in genotype distribution had a mean age of approximately 60 years. As the median age for bladder cancer patients is about 69 years these patients with early disease onset were still within a normal age range [21]. In addition, the study by Sanchez-Carbayo et al. [11] grouped patients with tumor stages pTa and pT1 as patients with superficial disease. This grouping might not reflect the biological background of the tumors, as mRNA expression analyses clearly revealed a very close proximity in the expression profiles of pT1 and muscle-invasive pT2 bladder tumors. The profiles of pTa tumors clearly separated from the invasive tumors [22]. Therefore, a grouping of noninvasive and invasive tumors could bias the results and skew associations between analyzed groups. This grouping should be avoided.

The results from our study suggested a higher risk for individuals carrying the risk allele G for developing an invasive bladder tumor. From these data one might suggest that the higher rate of MDM2 transcription caused by the SNP309 G-variant could be causative for this more aggressive phenotype of bladder cancer. However, high expression of MDM2 in bladder tumors was shown to be associated with a better progression-free and a better overall survival of the patients [23]. Moreover, high expression of MDM2 was also associated with low-stage and low-grade bladder tumors [24]. These data strengthen our findings that the G-variant of the SNP309 was not a general risk factor for bladder cancer as a higher MDM2 expression seemed not to support the development of aggressive bladder cancer per se. Functional studies also showed that MDM2 inhibits the hTR promoter and can therefore regulate telomerase activity in bladder cancer cell lines [25]. This regulatory ability of MDM2 is another hint for a quite protective role of MDM2. Nevertheless, high MDM2 levels in tumor cells are a risk factor for an aggressive disease if p53 accumulation is simultaneously present in the cells. Several studies have shown that concurrent MDM2 and p53 overexpression was correlated with disease progression and shorter survival in bladder cancer patients [26, 27]. As p53 accumulation is mainly caused by mutations it might be speculated that high levels of MDM2 inactivate the remaining wild-type p53 in p53-mutated cells, as was suggested for individuals with Li-Fraumeni syndrome [28]. In bladder cancer, p53 mutations were mainly described in carcinoma in situ and invasive tumors, but these alterations were also found in preneoplastic lesions of the urothelium [29, 30]. Therefore, the G-variant of the *MDM2* SNP309 might trigger the development of a more aggressive bladder cancer disease as soon as p53 alterations occur, and the combination of both events could promote invasive tumor growth. But as the functional proof for this model has not yet been adduced in bladder cancer, this theory remains speculative.

In conclusion, our study showed that the *MDM2* SNP309 has no influence on bladder cancer risk in Caucasians. The G-variant of the SNP was associated with invasive tumor growth and might predispose for the development of a more aggressive phenotype on bladder cancer compared to individuals carrying the T allele only.

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