A Single Nucleotide Polymorphism in the Human H-ras Proto-oncogene Determines the Risk of Urinary Bladder Cancer

Andreas Johne, Ivar Roots, and Jürgen Brockmüller

Abstract

Acquired mutations and inherited polymorphisms in the H-ras gene may modulate the risk of urinary bladder cancer. In DNA isolated from bladder cancer tissue, we screened the coding sequence of H-ras, including the exon-intron-junctions, for exon 1 (n = 68 patients), exon 2 (n = 50), and exons 3 and 4 (n = 25). Acquired mutations at codons 12 and 13 (exon 1) and codon 61 (exon 2), which had been described earlier in bladder cancer tissue, were not found in any of the tumors, but we identified a frequent polymorphism at nucleotide 81T→C (exon 1) in a wobble position. The clinical impact of this polymorphism was investigated in a case-control study in which 312 patients with histologically verified bladder cancer were compared with 254 hospital controls; 13.5% of the cases but only 7.1% of controls were homozygous for the 81C-variant of this polymorphism [odds ratio (OR), 2.04; 95% confidence interval (CI), 1.15–3.61; P = 0.014]. The homozygous 81C genotype was overrepresented, particularly in the patient groups with poorly differentiated tumors (n = 145, ≥G3; OR, 2.22; 95% CI, 1.15–4.27; P = 0.017), muscle-invasive tumors (n = 107, ≥T2; OR, 2.65; 95% CI, 1.35–5.23; P = 0.005), and flat transitional cell carcinoma (n = 45; OR, 3.69; 95% CI, 1.60–8.51; P = 0.002). In general, 81CC occurred more frequently in advanced types of bladder cancer. We conclude that individuals harboring the homozygous 81C-genotype of the H-ras proto-oncogene are at an increased risk of bladder cancer.

Introduction

There are four closely related human ras proto-oncogenes (H-, K4A-, K4B-, and N-ras), which encode small guanine nucleotide-binding proteins (p21ras) with intrinsic GTPase activity (1, 2). Mutations in hotspot codons 12, 13 (exon 1), and 61 (exon 2) were identified as the cause of H-ras oncogenic activation in different tumors including bladder neoplasms (3). However, the relevance of these acquired mutations for the development of urinary bladder carcinoma is still controversial. Although one study found an association of somatic H-ras mutations with bladder-cancer grades and stages (4), others failed to confirm this result (5–7). Furthermore, the frequencies of H-ras point mutations in bladder neoplasms varied widely from 0% to 45% between studies (4, 8) with a glycine-to-valine substitution in codon 12, because of a guanine-to-thymine transversion, predominating (9). In addition, apparently inherited polymorphisms in the H-ras gene may modulate the risk of urinary bladder cancer. In DNA isolated from bladder cancer tissue, we screened the coding sequence of H-ras, including the exon-intron-junctions, for exon 1 (n = 68 patients), exon 2 (n = 50), and exons 3 and 4 (n = 25). Acquired mutations at codons 12 and 13 (exon 1) and codon 61 (exon 2), which had been described earlier in bladder cancer tissue, were not found in any of the tumors, but we identified a frequent polymorphism at nucleotide 81T→C (exon 1) in a wobble position. The clinical impact of this polymorphism was investigated in a case-control study in which 312 patients with histologically verified bladder cancer were compared with 254 hospital controls; 13.5% of the cases but only 7.1% of controls were homozygous for the 81C-variant of this polymorphism [odds ratio (OR), 2.04; 95% confidence interval (CI), 1.15–3.61; P = 0.014]. The homozygous 81C genotype was overrepresented, particularly in the patient groups with poorly differentiated tumors (n = 145, ≥G3; OR, 2.22; 95% CI, 1.15–4.27; P = 0.017), muscle-invasive tumors (n = 107, ≥T2; OR, 2.65; 95% CI, 1.35–5.23; P = 0.005), and flat transitional cell carcinoma (n = 45; OR, 3.69; 95% CI, 1.60–8.51; P = 0.002). In general, 81CC occurred more frequently in advanced types of bladder cancer. We conclude that individuals harboring the homozygous 81C-genotype of the H-ras proto-oncogene are at an increased risk of bladder cancer.

Materials and Methods

Patients. DNA from fresh tumor tissue of unselected German patients with histologically verified urinary bladder cancer were screened for mutations in the coding region of H-ras by DNA sequence analysis. Tumor tissue macroscopically selected from the central regions of the tumors but without specific selection of tumor cells was used for this analysis. Sequence analysis was completed in 68, 50, 25, and 25 subjects for H-ras exons 1, 2, 3, and 4, respectively.

The clinical impact of the 81T→C H-ras SNP located in exon 1 was investigated with the help of blood samples from a hospital-based case-control study on urinary bladder cancer, which had been described earlier (14). All of the samples that were still available were analyzed: 312 samples of bladder cancer patients (113 women, 199 men; median age, 73 years; range, 31–94 years) and 254 samples of patient controls not suffering from any malignancy (81 women, 173 men; median age, 67 years; range, 32–91 years). The study had been approved by the Ethics Committee of the University Medical Center Benjamin Franklin. To make sure that no bias was introduced by the hospital control group, we included a second control group of 106 randomly selected healthy volunteers (19 women, 87 men; median age, 28 years; range, 20–62 years).

Sequence Analysis of H-ras. Intronically localized primers for PCR amplification were designed by taking into account the

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3 The abbreviations used are: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; TCC, transitional cell carcinoma.
homologous sequences of K- and N-ras and selecting H-ras-specific sequences (15–17). The biotin-labeled primers for solid-phase binding and the corresponding sequencing primers were the following: biotin-5′-CAGAGGAGCCCTGAGGAGGA-3′, 5′-CACCTGAGCCGGGAGCCTA-3′ (exon 1); biotin-5′-ATGGCCAAGAGAGGAGCGGAGA-3′, 5′-AGAGGTCTGGCTGTGAACT-3′ (exon 2); biotin-5′-CAGGGTCACTAGTG-3′, 5′-CCCTGTGCTCCTGTGCCGCA-3′ (exon 3); and biotin-5′-ACCTCCATGTCCGACTTGGTGT-3′, 5′-CTCTGATCTCCATCTCCTTT-3′ (exon 4). DNA-sequence analysis was performed using the Sequenase 2 single-stranded DNA sequencing kit (United States Biochemical Corp., Cleveland, OH) after 25 cycles of PCR amplification.

**PCR-RFLP Analysis.** For determination of the H-ras SNP 81T→C in DNA from leukocytes, a 200-bp segment was amplified using primers E1-forward (5′-CTTGCTGGTGGGAGAAGA-T-3′) and E1-reverse (5′-CTTGGCAGGTGAGAGG-3′). One unit of Taq-polymerase (Perkin-Elmer, Weiterstadt, Germany), 5 μM each primer, 0.2 mM dNTPs, and 4.5 mM MgCl₂ were contained in a total volume of 25 μL PCR conditions were 35 cycles of 30 s at 94°C, 10 s at 60°C, and 1 min at 72°C. Twenty μL of the amplicons were digested with 5 units of DraIII (New England Biolabs, Schwalbach, Germany). Fragments were separated on a 2% agarose gel stained with ethidium bromide.

**Statistics.** Sample size estimation was based on the results of our exploratory analysis of the H-ras SNP 81T→C in bladder cancer tissue, in which 12 of 68 samples had had a homozygous mutant genotype CC. A minimum sample size of 186/group was calculated with a two-sided χ² test, a type-I error of 0.05, and a type-II error of 0.20, assuming that an OR of 2.0 between cases and controls is medically important. ORs are given with their 95% CIs and two-sided P values calculated with the χ² test. ORs of subgroup analysis were adjusted according to Mantel-Haenszel. Homogeneity of subgroups of each strata was tested with SAS (version 6.12, SAS Institute Inc., Cary, NC).

**Results**
The four coding exons of H-ras and their adjacent intron sequences were screened for mutations in DNA from bladder cancer specimens. Exon 1 was analyzed in 68 patients, exon 2 in 50 patients, and exons 3 and 4 in 25 patients. None of the known mutations in H-ras, either in hotspot codons 12, 13, and 61 nor a previously reported variation at cDNA position —10 upstream of exon 1 (12), were detected in the tissue specimens although we had detected 20 acquired mutations in the tumor suppressor gene p53 with the same method as in the same samples in an earlier study (19).

However, we identified one frequent T→C variation in codon 27 of exon 1 at cDNA position 81, which is located in a wobble base position. The analysis of genomic DNA from tumor tissue and from leukocytes from the same individuals always provided the identical genotype. Because of these findings and because of its high frequency, the H-ras 81T→C variation was considered as an informative SNP in a gene with potential relevance for carcinogenesis. To evaluate the clinical relevance of the H-ras SNP 81T→C, genotype frequencies were analyzed in 312 bladder cancer cases (CC, 42; CT, 119; TT, 151), in 254 patient controls (CC, 18; CT, 130; TT, 106), and in 106 healthy volunteers (CC, 8; CT, 40; TT, 58). The CC genotype occurred more frequently in bladder cancer patients (13.5%) than in patient controls (7.1%), and the difference was statistically significant (crude OR, 2.04; 95% CI, 1.15–3.61; P = 0.014). The comparison of cases with the second control group of healthy volunteers showed the same trend with a crude OR of 1.91 (95% CI, 0.87–4.16). The homozgyous H-ras 81C-variant was significantly more frequent in cases with more advanced types of cancer (Table 1).

Stratification for potentially confounding variables is given in Table 2. ORs adjusted for age, gender, smoking, and risk occupation only marginally differed from the crude OR of the primary comparison, excluding major confounding or effect modification by these variables.

**Discussion**
None of the previously described acquired mutations in the coding sequence of H-ras could be detected in this study. This result conforms to recent studies, in which H-ras mutations in bladder cancer tissue were either not (8), or only infrequently, observed (20). However, we identified a frequent polymorphism in the coding region of H-ras in cDNA position 81T→C, which appeared to be a marker for the risk of bladder cancer development and progression. Our case-control study revealed a 2-fold increased risk of bladder cancer in carriers of the homozgyous mutant C-allele in comparison to heterozygotes (CT) and wild-type carriers (TT). This suggests an autosomal recessive mode of inheritance. The CC-genotype occurred most frequently in the patient groups with invasive (T1) and ≥T2 and less differentiated (≥G3) tumors. Presumably, the H-ras SNP leads to accelerated tumor progression by conferring a growth advantage on tumor cells. Patients with the more malignant flat TCC were more often CC carriers than patients with the comparatively benign papillary TCC. These findings are of particular interest, because the two TCC types differ not only in their clinical outcome but also in their molecular pathways of carcinogenesis (21).

However, this epidemiological data does not reveal the mechanisms by which H-ras SNP 81T→C modifies bladder cancer risk. In contrast to acquired amino acid mutations in the
Table 2: Stratified analysis for H-ras SNP 81T → C as bladder cancer risk factor according to demographic and exposure data

| ORs adjusted for the respective stratification variable, according to Mantel and Haenszel, only marginally differ from the crude OR of the primary comparison, indicating no additional influence of the strata. Heterogeneity between the subgroups of each strata has been rejected by the Breslow-Day test, allowing the use of adjusted OR. |
|---|---|---|
| **Cases** | **Adjusted OR (95% CI)** |
| **n** | **CC (%)** | **n** | **CC (%)** |
| **Total number** | 312 | 13.5 | 254 | 7.1 | 2.04 (1.15–3.61) |
| **Age** | | | | | |
| <65 yr | 84 | 15.5 | 112 | 7.1 |
| ≥65 yr | 228 | 12.7 | 142 | 7.0 | 2.08 (1.17–3.71) |
| **Gender** | | | | | |
| Female | 113 | 14.2 | 81 | 2.5 |
| Male | 199 | 13.1 | 173 | 9.2 | 2.07 (1.17–3.68) |
| Cigarette smokinga | | | | | |
| Nonsmokers | 100 | 13.0 | 58 | 3.4 |
| 1–20 pack-years | 62 | 8.1 | 77 | 7.8 |
| >20 pack-years | 149 | 15.4 | 119 | 8.4 | 2.00 (1.11–3.61) |
| Occupational riskb | | | | | |
| No risk occupation | 183 | 11.5 | 199 | 7.0 |
| Risk occupation | 126 | 15.9 | 54 | 7.4 | 1.90 (1.05–3.43) |

a Missing data in one case. One pack year = consumption of one pack of 20 cigarettes per day for 1 year.
b Working for a period of at least 2 years in an occupation with increased bladder cancer risk as given in Ref. 14. Missing data in three cases and one control.

hotspot codons 12, 13, and 61, which prolong the GTP-bound activated state of the H-ras product (2). SNP 81T → C does not alter p21ras structure. But it is conceivable that SNP 81T → C is linked to other polymorphic sites in functional intron regions of H-ras. An imaginable linkage candidate is a region of a variable number of tandem repeats, about 1 kb downstream of exon 4, with possible transcriptional enhancer activity (22). Rare alleles of these H-ras variable numbers of tandem repeats have been associated with bladder cancer risk (13). Another polymorphic site that could be involved in the development of cancer disease is a hexanucleotide repeat located about 80 bp upstream of the 5’-end of exon 1 (23). In conclusion, a frequent genetic polymorphism in the H-ras proto-oncogene appears to be epidemiologically relevant for bladder cancer risk and progression. Its possible linkage to other polymorphic loci in H-ras could be a promising subject of additional studies.

References

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