Mutation of H-ras Is Infrequent in Bladder Cancer: Confirmation by Single-Strand Conformation Polymorphism Analysis, Designed Restriction Fragment Length Polymorphisms, and Direct Sequencing

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ABSTRACT

A series of 152 human bladder tumors, 14 bladder tumor cell lines, and 1 immortal urothelial cell line were examined by single-strand conformation polymorphism (SSCP) and designed restriction fragment length polymorphism analyses for mutations in exons 1 and 2 of the H-ras gene. Nine tumors (6%) contained mutations. There was complete concordance between SSCP and restriction fragment length polymorphism analyses. Six mutations in exon 1 and three in exon 2 were identified by SSCP analysis. Subsequent restriction fragment length polymorphism analysis showed that of the exon 1 mutations, four were in codon 12 and two in codon 13, and all three exon 2 mutations were in codon 61. Eight mutations were confirmed by direct sequencing. One codon 13 mutation could not be identified by direct sequencing. Distinct strand mobility shifts detected by SSCP analysis identified specific point mutations, and in all cases, strands containing different mutations migrated differently. The base substitutions identified in these bladder tumors were diverse and included four transitions (three G—>T and one A—>T) and four transversions (two G—>A and two A—>G). This pattern of base substitutions is compatible with point mutations at specific hot spots in the ras genes giving rise to transversions (three G—>T and one A—>T) and four transitions (two G—>A and two A—>G). Two strategies have been used to screen for H-ras gene mutations in a large series of tumors of all grades and stages and in human bladder tumor cell lines. As an initial screen, we used SSCP analysis of PCR products (14–16). This method exploits the differential mobility in non-denaturing acrylamide gel electrophoresis of single-stranded DNA fragments differing in sequence by a single base change. To validate findings using SSCP analysis, tumors were then screened using designed RFLPs (17–19), and all mutations detected were directly sequenced.

INTRODUCTION

Mutation of the cellular K-, H-, and N-ras genes has been implicated in the development of many human cancers (1). These genes encode membrane-bound Mr 21,000 proteins with GTPase activity that are involved in cellular signal transduction (2). Activation by point mutations at specific hot spots in the ras genes gives rise to proteins with reduced GTPase activity which under appropriate conditions possess dominant oncogenic potential. Activated ras genes are the most frequently detected oncogenes in human tumors and are present in a very high proportion of certain tumor types. For example, more than 90% of pancreatic tumors contain mutated K-ras (3). A significant specificity of activation has been demonstrated such that carcinomas of the colon, pancreas, and lung and malignant melanoma contain predominantly mutated K-ras (3–6) and leukemias contain predominantly mutated N-ras (7, 8).

In bladder tumors, mutations of H-ras but not of K- and N-ras have been identified. These have been located within codons 12, 13, and 61 (9–12). In initial studies using the NIH-3T3 transfection assay or PCR2 followed by selective oligonucleotide hybridization, it was estimated that 7–16% of bladder tumors had a mutated ras gene. However, two recent studies (12, 13) have reported frequencies of mutations in codon 12 of >30% and 76%, respectively. We have reevaluated the frequency of H-ras mutations in bladder tumors using recently devised strategies for rapid screening for mutants combined with direct sequencing. In addition to obtaining an accurate estimate of the frequency of mutation, we sought to determine whether the presence of H-ras oncogenes is associated with particular clinical parameters. Two strategies have been used to screen for H-ras gene mutations in a large series of tumors of all grades and stages and in human bladder tumor cell lines. As an initial screen, we used SSCP analysis of PCR products (14–16). This method exploits the differential mobility in non-denaturing acrylamide gel electrophoresis of single-stranded DNA fragments differing in sequence by a single base change. To validate findings using SSCP analysis, tumors were then screened using designed RFLPs (17–19), and all mutations detected were directly sequenced.

MATERIALS AND METHODS

Tumors and Cell Lines. Bladder tumor specimens were obtained from 152 patients at cystoscopy. Tissues were cut by diathermy or cold cup biopsy forceps. A representative sample was taken for histopathological assessment, and the remainder was frozen immediately at −20°C or −70°C. Tumors were graded histopathologically (20) and staged using the TNM classification (21). All were transitional cell carcinomas. Fourteen human bladder carcinoma cell lines and one immortal bladder cell line were used: 5637; J.O. 'N.' 4; SCABER (22); VM-CUB-2 (23); RT112; SW1710 (24); HT1376 and HT1197 (25); SD (26); 253J (27); RT4 (28); SW800 (24); and UM-UC-3 (29). The cell line 609 (30) is an immortal urothelial cell line established from normal ureter of a patient with renal carcinoma. The bladder cell line T24 (31) which contains a G—>T substitution at the second position of codon 12 and the melanoma cell line SK2 which contains an A—>T substitution at the second base of codon 61 of H-ras (32, 16) were used as positive controls. An additional control consisted of an NIH-3T3 transfectant clone containing a human H-ras gene with a G—>A substitution at the second position in codon 12 (Gly—>Asp).

DNA Isolation. High-molecular-weight DNA was prepared from cultured cells, tumor specimens, and leukocytes by methods described previously (33). DNA from the cell line SK2 was further purified by centrifugation in a cesium chloride density gradient (16).

PCR-SSCP Analysis. Oligonucleotide primers were synthesised on an Applied Biosystems 381A DNA synthesizer. The primers used in the SSCP analyses are shown in Table 1. Each amplification reaction was carried out in a 10-μl reaction volume containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM MgCl2; 0.001% gelatin; 50 pmol of each primer; deoxynucleotide triphosphates dATP, dGTP, and dTTP at 100 μM each; dCTP at 10 μM; 1 μCi [α-32P]dCTP (3000 Ci/mmol); 100 ng genomic template DNA; and 1 unit of Taq polymerase (Amersham UK). Reaction mixtures were overlaid with a drop of mineral oil and subjected to 30 cycles of amplification on a Technolog programmable Dri-Block PHC-2. Each cycle consisted of denaturation at 96°C for 30 s, annealing at 60°C (exon 1) or 58°C (exon 2) for 1 min, followed by polymerization at 72°C for 1.5 min. Following the last cycle, tubes were incubated for a further 10 min at 72°C. Negative (no template DNA) and positive (T24, NIH-3T3 transfectant containing codon 12 Asp and SK2 DNA) controls were also carried out. Following amplification, 40 μl formamide dye (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were added to each reaction. Four-μl aliquots were denatured at 80°C.

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1 To whom requests for reprints should be addressed.
2 The abbreviations used are: SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; TNM, primary tumor, lymph node, distant metastases.
for 5 min and electrophoresed in 5–8% nondenaturing acrylamide gels with or without 5–10% glycerol at 4°C (35 W) or room temperature (10 W). Dried gels were exposed to FujiRX film (Fuji Photofilm, London) for 1–24 h.

**Designed RFLPs.** Mutations at codon 12 of H-ras were detected by virtue of the fact that all possible mutations eliminate a naturally occurring MspI site. The 308-base pair fragment amplified with primers H12/2S and H12/2A (Table 2) contained an additional MspI site 55 base pairs upstream of codon 12, which provided a positive control for MspI cleavage. Point mutations in codons 12 and 61 were detected by means of designed RFLPs as described by Mitsudomi et al. (19). PCR reactions were carried out using a mismatched primer such that the combination of the wild-type sequence and the mismatch introduced by the primer generate a novel restriction site. PCR products generated from point-mutated templates lack the novel restriction site. Thus, using the primers shown in Table 2, a 250-base pair fragment containing an MspI site is generated from templates with wild-type codon 13, and a 177-base pair fragment containing an AlwNI site is generated using primers H12/1S and H12/1A and H12/2S and H12/2A amplify 63- and 110-base pair fragments, respectively, containing exon 1 (Table 1). The cell line T24 which contains only a single H-ras allele containing a G→T substitution at the second position of codon 12 (Gly→Val) and an NIH-3T3 transfecnt containing a human H-ras gene with a G→A substitution at the second position of codon 12 (Gly→Asp) were used as positive controls, and leukocyte and placental DNA was used as the negative control. In preliminary experiments, various conditions of electrophoresis were assessed. Optimum differential mutation of mutant product from T24, the NIH-3T3 positive control, and normal PCR products was obtained using the primer pair H12/1S and H12/2A and 8% native gels containing no glycerol run at 4°C, or with 5% glycerol run at room temperature. Mutated and wild-type ras genes were distinguished by a mobility shift of the separated strands of the amplified product (Fig. 1a, Lanes 2 and 4). A different single-strand mobility shift was observed in the two positive controls with different mutations at the second position of codon 12 (Fig. 1a, Lanes 3 and 4). Amplified DNA from five of 152 tumors showed altered mobility under these conditions (Fig. 1a, Lanes 5–10). In four cases, strands with mobility similar to those of the amplified product from the normal gene were present in addition to those with altered mobility, indicating the likely presence of one normal and one mutated H-ras allele in these tumors. In one tumor (Fig. 1a, Lane 8, tumor 37)...

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**Table 1 Primers used for SSCP analysis of H-ras**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Amplified fragment (base pairs)</th>
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<tr>
<td>H12/1S</td>
<td>GACGGAGACCTGTTAGGAGGAC</td>
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</tr>
<tr>
<td>H12/2A</td>
<td>GGCTGCTGAGACAGAGGACCTC</td>
<td>110</td>
</tr>
<tr>
<td>H12/2S</td>
<td>CAGGGCCCTGAGAGGGGAGTGGGAAmplicated</td>
<td></td>
</tr>
<tr>
<td>H12/2A</td>
<td>TCCGCTCAACAGTTACTACCG</td>
<td>194</td>
</tr>
</tbody>
</table>

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**Table 2 Primers used for RFLP detection of H-ras point mutations**

<table>
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<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Mismatch (codon)</th>
<th>Restriction enzyme (site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H12/2S</td>
<td>GACGGAGACCTGTTAGGAGGAC</td>
<td>MspI (CCGG)</td>
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</tr>
<tr>
<td>H12/2A</td>
<td>GGCTGCTGAGACAGAGGACCTC</td>
<td>AlwNI (CAGNNNCTG)</td>
<td></td>
</tr>
<tr>
<td>H12/2S</td>
<td>CAGGGCCCTGAGAGGGGAGTGGGAAmplicated</td>
<td></td>
<td></td>
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<tr>
<td>H12/2A</td>
<td>TCCGCTCAACAGTTACTACCG</td>
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<td>H12/2S</td>
<td>GACGGAGACCTGTTAGGAGGAC</td>
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<tr>
<td>H12/2A</td>
<td>GGCTGCTGAGACAGAGGACCTC</td>
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<td>H12/2S</td>
<td>CAGGGCCCTGAGAGGGGAGTGGGAAmplicated</td>
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<td>H12/2A</td>
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**RESULTS**

**Identification of H-ras Gene Mutations by SSCP Analysis.** Initially, all tumors and cell lines were subjected to SSCP analysis to identify point mutations within exons 1 and 2 of H-ras. Primer pairs...

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**Fig. 1. SSCP analysis of H-ras exon 1 mutations.** In a, primers H12/1S and H12/1A were used to amplify DNA from normal leukocytes (Lane 2), T24 (Lane 3), NIH-3T3 cells containing a GOC→GAC substitution in codon 12 (Lane 4), and a series of bladder tumors (Lanes 5–13). The products were run in an 8% acrylamide gel containing 5% glycerol at room temperature. Mobility shifts are present in the products from tumors in Lanes 5, 6, 7, 8, and 10 (tumors 226, 257, 45, 37, and 280). Lane 1, nondenatured product from normal leukocytes (open arrow). Solid arrows, bands with altered mobility. b, products generated with primers H12/2S and H12/2A, run in a 7% acrylamide gel containing 5% glycerol at room temperature. Lanes contain same products as in a. Mobility shifts are present in Lanes 2, 5, 7, 8, and 10 (tumors 222 and 280). Tumor 222 (Lane 9) shows no mobility shift in a (Lane 9). T24 shows a minor mobility shift (Lane 3), and the Asp control (Lane 4) shows no bands with altered mobility.
no normal product was detected, indicating probable loss of the normal allele. In one tumor (Fig. 1a, Lane 7, tumor 85), the mobility pattern of the mutant strand resembled that of the GGC to GAC (Asp) control. No mutants showed a pattern resembling T24. Four tumors were unlike either control (Fig. 1a, Lanes 5, 6, 8, 10, tumors 226, 267, 37, and 280). Two of these had identical mobility shifts. Using primers H12/2S and H12/2A one mutation was detected which had not been identified in the 63-base pair product from primers H12/1S and H12/1A (Fig. 1, a and b, Lane 9, tumor 222). With this primer pair, the H12 Asp positive control showed no mobility shift, T24 only a minor mobility shift (Fig. 1b, Lanes 3 and 4), and mobility shifts were detected in only two tumors (Fig. 1b, Lanes 9 and 10, tumors 222 and 280). No mutations were detected in any bladder carcinoma cell line except T24.

Primers H61/1S and H61/1A (Table 1) amplify a 194-base pair fragment containing exon 2 of H-ras. As a positive control for a position 61 mutation, the cell line SK2 (32), which contains an A—>T substitution at the second base in codon 61 of one H-ras allele (Gln—>Leu), was used. This mutation was resolved under all conditions of electrophoresis tested. Routinely, 6% acrylamide gels containing no glycerol were run at 4°C. Amplified DNA from three tumors (134, 255, and 279) showed altered mobility (Fig. 2). The mobility shift observed in one tumor (tumor 279) resembled that seen in SK2 (Fig. 2a, Lane 1; Fig. 2b, Lane 3). In all three cases, both normal and mutant strands were present. No exon 2 mutations were detected in the bladder carcinoma cell lines analyzed.

Leukocyte DNA was available from all patients except patient 37. SSCP analysis of exons 1 and 2 showed no evidence of constitutional mutations in those patients in whose tumors mutations had been identified.

**Designed RFLP Analysis.** To confirm the utility of SSCP analysis as a method for rapid screening for H-ras mutations at codons 12, 13, and 61, all tumors and cell lines were screened using natural or designed RFLPs to detect mutations at these positions. The effectiveness of this method has been demonstrated previously in several laboratories (17–19). The primers used for the amplification of exon 1 sequences (H12/3S and H12/3A) span two naturally occurring MspI sites. The first, in the intron between exons φ and 1, was used as a

Fig. 2. SSCP analysis of H-ras exon 2 mutations. Primers H61/1S and H61/1A were used to amplify DNA from SK2 (a, Lane 1), normal leukocyte DNA (a, Lane 2), and tumor DNAs (b, c, and e in all lanes). Mobility shifts are present in b (Lane 3, tumor 279), c (Lane 2, tumor 134), and d (Lane 2, tumor 255). a—d, 6% gel containing no glycerol run at 4°C; e, 6% gel containing 5% glycerol run at room temperature. Lane 2, product from the same tumor as in d (Lane 2). Arrows, bands with altered mobility.

positive control for MspI cleavage. The second, located at codon 12, is destroyed by mutations in either of the first two positions. Following digestion of the 308-base pair product with MspI, fragments of 236, 55, and 17 base pairs are generated from wild-type PCR products and fragments of 291 and 17 base pairs from mutant products. Four of
the tumors with altered strand mobility detected by SSCP analysis of exon 1 showed mutant product (Fig. 3a). Mutant product only was detected in one tumor (Fig. 3a, tumor 37), confirming the prediction from SSCP analysis (Fig. 1a, Lane 8) of normal allele loss in this tumor. No further codon 12 mutations were identified in any tumors or cell lines by this assay.

Mutations in codons 13 and 61 were detected by the use of designed RFLPs (Table 2). Primer H13A encompasses codon 13 and contains a single base mismatch in codon 14 such that an Hphl site is generated when wild-type codon 12 is present in the template. With H13S, a 77-base pair sequence is amplified and this can be cut by Hphl into 56- and 21-base pair fragments. Product from codon 13 mutations cannot generate this restriction site. The remaining 2 exon 1 mutants detected by SSCP analysis (tumors 222 and 280) were shown to have mutations in codon 13 (Fig. 3b). No other codon 13 mutations were detected in tumors or cell lines by this technique.

Mutations in codon 61 were detected using primers H61/2S and H61/2A2, which generate an AlwNI site within a 177-base pair fragment from normal but not mutant codon 61. As described by others (19), the efficiency of PCR with these primers was low due to two mismatches in H61/2A2. Therefore, a first PCR reaction was carried out with the perfectly matched primers H61/2S and H61/2A1, and this product was used as a template for PCR with the mismatched primer. All three position 61 mutants detected by SSCP were confirmed by this analysis, and no more mutations were detected (Fig. 3c, tumors 134, 255, and 279).

**Direct Sequence Analysis.** Direct sequencing revealed base substitutions in the expected codons in 8 of the nine tumors in which nucleotide substitutions were detected by SSCP and RFLP analyses. These are shown in Table 3 and Fig. 4. Numerous sequences obtained from tumor 222 revealed only a wild-type sequence. As predicted from the SSCP analysis, none of the four codon 12 mutations were the same as that of T24, and one (tumor 85) was a GGC→AGC substitution (Asp). The two codon 12 mutations which showed similar single-strand mobility shifts (tumors 226 and 280) were confirmed as having identical nucleotide substitutions (GGC→TGC; Cys).

One of three codon 61 mutations was identical to that in the cell line SK2 (CAG→CTG; Leu) as predicted by SSCP analysis, and the other two codon 61 mutations were CAG→CGG (Arg) substitutions. No mutations were identified in the leukocyte DNA of any of these patients.

Since other reports have described higher frequencies of mutation in bladder tumors of high grade and/or high stage (12), sequencing of the mutation hotspots in exons 1 and 2 was carried out on 10 tumors of high grade and stage in which mutations had not been identified by other means. No mutations were found.

**Association of H-ras Mutations with Tumor Grade and Stage.** The grade and stage of the 8 tumors with H-ras mutations which were sequenced is shown in Table 3. The remaining codon 13 mutant (tumor 222) was a grade 2 pTa tumor. Information on tumor grade was available for 135 of the tumors. Some tumors including one with a mutation were not pathologically staged due to insufficient depth of tissue sample in the biopsy. The distribution of mutations according to grade was 3 of 45 grade 1, 3 of 55 grade 2, and 3 of 35 grade 3 tumors. χ² analysis showed no significant association with grade (P > 0.95).

**DISCUSSION**

We have shown that in a large series of bladder tumors which comprises tumors of all grades and stages, the frequency of H-ras mutation is low (6% of all tumors). A similar frequency was reported in the largest series of bladder tumors studied (n = 38) using the NIH-3T3 transfection assay (9). We also studied 14 bladder carcinoma cell lines and failed to identify mutations in any except T24. This is as expected, given the low frequency detected in fresh tissue samples, and suggests that establishment of bladder tumor cell lines in vitro does not select for cells containing ras gene mutations.

In order to make as accurate an estimate of the frequency of mutation as possible, we studied a large number of tumors and used a combination of methods to avoid both false positive and false negative results. Designed RFLPs have been used previously to identify mutations in all three of the ras genes, and results have been confirmed by sequence analysis (19). Similarly, SSCP analysis was shown to be capable of identifying a wide range of ras gene mutations in a series of control cell lines and subsequently was used to identify K-ras mutations in lung tumors, all of which were confirmed by direct sequencing (16). We have found absolute concordance in the results obtained using these two techniques and have confirmed all but one mutation by direct sequencing of PCR products.

SSCP analysis provides a simple and rapid screen for point mutations within a specific region of DNA and is less labor intensive than RFLP analysis or direct sequencing. However, our experience in detecting mutations in exon 1 of H-ras using this technique demonstrated that considerable care must be taken if all mutations are to be identified. Both the conditions of electrophoresis and the choice of DNA fragment amplified had a dramatic effect on the results obtained. Thus, one of the two codon 13 mutations only showed a strand mobility shift when amplified within the 110-base pair fragment generated by primers H12 S/Gly-Asp) showed little or no mobility shift. The base composition of amplified DNA fragments may dramatically influence the nature and stability of the single-strand conformers formed. This region of exon 1 is particularly G:C rich, and this may explain the relative difficulty with which exon 1 mutations were identified by SSCP analysis compared with exon 2 mutations. This illustrates the need for the confirmation of apparently wild-type sequences by other technologies when an accurate estimate of mutation frequency is desired.

It has been suggested that aneuploid bladder tumors have a higher frequency of codon 12 mutations than diploid tumors (12). These tumors represent the more clinically aggressive bladder tumors. Since aneuploidy shows a strong correlation with high tumor grade and stage (34), we looked specifically by direct sequencing at a group of high-grade/stage tumors in which SSCP and RFLP analyses had not identified mutations. None were identified, which confirms that these former techniques do not give false negative results at high frequency and that in this series of tumors, H-ras mutations are infrequent even in clinically aggressive bladder tumors.

Based on previous studies, it was expected that codon 12 mutations would predominate. This was not found to be the case. Three codon 61 and two codon 13 mutations were identified in addition to four.
H ras MUTATIONS IN BLADDER CARCINOMA

Fig. 4. Direct sequences of PCR products from bladder tumors. The primers used for amplification were the sense primers used for SSCP analysis (Table I). Wild-type sequences for codons 12, 13, and 61 are indicated by brackets. Arrows, mutant bands. In tumors 226 and 267, the mutant band (T) was underrepresented compared with the wild-type band (G) at the same position. In all tumors, both wild-type and mutant bands are present.

Codon 12 mutations. It was also predicted that the most frequent substitution in codon 12 would be G → T at the second position (Val), but this mutation was not detected in any tumors. The mutations identified were diverse. Previously sequenced mutations in bladder tumors include H-12 valine (35, 36), H-13 arginine (37), H-61 arginine, and H-61 leucine (9). An H-13 cysteine mutation has also been identified by PCR and oligonucleotide hybridization (11). Two of these mutations, H-61 arginine and leucine, were represented in the present study. In addition, we found four mutations not previously described in bladder tumors (H-12 cysteine, H-12 aspartic acid, H-12 serine, and H-13 valine). H-12 aspartic acid has been identified previously in a breast tumor cell line (38) and in a thyroid follicular carcinoma (39), H-12 serine in an oral carcinoma (40), and H-12 cysteine in a thyroid carcinoma (39). H-13 valine appears to represent a novel mutation.

One tumor (tumor 222) consistently showed only wild-type bands at position 13 on direct sequencing. SSCP and RFLP analysis suggested that the mutant allele was present in tumor 222 in a proportion of cells similar to that in tumor 280 (Fig. 1b). The reason for this is not clear, although direct sequencing is the least sensitive method we have used (see below). All analyses carried out on this tumor utilized the same sample of template DNA. Since the mobility shift detected by SSCP analysis differed from that of tumor 280, we conclude that cross-contamination with DNA from tumor 280 cannot account for false positives on SSCP and RFLP analysis and that the mutation present in tumor 222 tumor is different from that in tumor 280.

It has been demonstrated in experimental models for tumor induction by chemical carcinogens that individual chemical and physical agents have specific mutational spectra which reflect the type of DNA adduct formed (reviewed in Ref. 41). Thus, benzo(a)pyrene induces...
preponderantly G:C→T:A transversions, and N-nitroso compounds induce G:C→A:T transitions. Transitions of G:C→A:T occurring at CpG residues appear to constitute the majority of spontaneous mutations in the experimental systems studied to date and are presumed to result from deamination of 5-methylcytosine to thymine (reviewed in Ref. 42). The mutations detected in this study comprised four transitions (three G:C→T:A and one A:T→T:A) and four transitions (two G:C→A:T and two A:T→G:C). Since none of the transitions are at CpG dinucleotides, it may be suggested that H-ras mutations in bladder tumors are induced by more than one major class of environmental agent. This distribution of base substitutions resembles more closely those identified in ras genes and p53 in human lung tumors and cell lines (16, 19, 43) than in colon tumors (44, 45). A similar spectrum of substitutions is characteristic of p53 mutations in bladder tumors (46).6 This may reflect the known association of increased bladder cancer risk with cigarette smoking (47, 48).

The reason for the discrepancy in the mutation frequencies estimated by NIH-3T3 transfection or SSCP/RFLP analysis and two recent studies (12, 13) which report high frequencies of mutations in codon 12 (36% and 76%, respectively) is not clear. However, differences in the sensitivities of the methods used are unlikely to account for this. In these latter studies, PCR was used to amplify the regions of interest, and mutations were identified by hybridization to specific oligonucleotide probes. Comparison of the frequencies of ras mutations identified in other tumor types using the NIH-3T3 assay and PCR combined with oligonucleotide hybridization does not reveal a consistent discrepancy between these methods (1). PCR amplification and direct sequencing can detect a mutant allele when at least 20% of cells contain the mutation (49). Mismatched primer techniques have been reported to be able to identify ras gene mutations when present at 1:10–1:16 dilution with normal DNA (17, 49), and the sensitivity of oligonucleotide hybridization to PCR products is of a similar order (50).

The possibility exists that low frequencies of mutant alleles (<10%) have not been detected in any of the studies carried out to date. It will be of interest therefore to explore the possible presence of minor populations of mutant alleles using allele-specific PCR (51, 52) or a two-stage PCR assay similar to that described by Levi et al. (49), which has been shown to be capable of detecting mutations of K-ras present in less than 10% of cells. The tumors we have studied have been analyzed for allele loss on a number of chromosome arms, and in most cases where loss of heterozygosity has been detected, this appears to be clonal and minimum residual signal is detected (53).7 This demonstrates that contamination with normal stromal and/or inflammatory cells is low in most of these samples. In a virtually pure population of tumor cells, genetic changes which occurred early in tumor development should be detected in all cells. It appears therefore that H-ras mutations are not an early event in the development of most bladder tumors. If it is subsequently found that a small proportion of cells in a high percentage of tumors contain mutations, this would suggest that H-ras mutation is a very late event in tumor development. However, the possibility would remain that ras activation may be important during the early stages of tumor development but no longer necessary in the later stages.

Uncertainty about the role and timing of ras gene mutation in transformation of human bladder extends to model systems for urothelial cell transformation. Human urothelial cells immortalized by simian virus 40 in vitro and transformed by exposure to a chemical carcinogen do not contain mutated ras genes (54), and it has been shown that transfection of ras oncogenes into immortal urothelial cells does not induce transformation (55). However, under certain conditions, ras genes appear to be able to contribute to the transformation or neoplastic progression of urothelial cells. Thus, overexpression of normal or mutated H-ras in the bladder tumor-derived cell line RT4 induces tumor invasion in vivo (56), and in the in vitro model transformation system of Reznikoff, it has been shown that immortal cells containing specific chromosome deletions (3p-, 11p-, and 13q-) are susceptible to transformation by ras (57). Taken together, these and the present results suggest that although mutation of H-ras can clearly contribute to bladder tumorigenesis, this represents neither an early nor an essential molecular change.

ACKNOWLEDGMENTS

We thank Professor Chris Marshall for supplying the NIH-3T3 transfectant containing a codon 12 Gly-Asp mutation and Dr. T. Sekiya for providing the cell line SK2 used as positive controls in this study. We are also grateful to Dr. Graham Currie for helpful discussions and Jennifer Wood and Elizabeth Collins for help in preparation of the manuscript.

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7 Cairns, Williamson, Elder, Shaw, and Knowles, unpublished results.


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Margaret A. Knowles and Magali Williamson

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