

Activation of *RAS* Family Genes in Urothelial Carcinoma

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Abbreviations and Acronyms

BCa = bladder cancer

GTPase = guanosine triphosphatase

PCR = polymerase chain reaction

RFLP = restriction fragment length polymorphism

TCC = transitional cell carcinoma

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Purpose: Bladder cancer is the fifth most common malignancy in men in Western society. We determined *RAS* codon 12 and 13 point mutations and evaluated mRNA expression levels in transitional cell carcinoma cases.

Materials and Methods: Samples from 30 human bladder cancers and 30 normal tissues were analyzed by polymerase chain reaction/restriction fragment length polymorphism and direct sequencing to determine the occurrence of mutations in codons 12 and 13 of *RAS* family genes. Moreover, we used real-time reverse transcriptase-polymerase chain reaction to evaluate the expression profile of *RAS* genes in bladder cancer specimens compared to that in adjacent normal tissues.

Results: Overall H-*RAS* mutations in codon 12 were observed in 9 tumor samples (30%). Two of the 9 patients (22%) had invasive bladder cancer and 7 (77%) had noninvasive bladder cancer. One H-*RAS* mutation (11%) was homozygous and the remaining 89% were heterozygous. All samples were WT for K and N-*RAS* oncogenes. Moreover, 23 of 30 samples (77%) showed over expression in at least 1 *RAS* family gene compared to adjacent normal tissue. K and N-*RAS* had the highest levels of over expression in bladder cancer specimens (50%), whereas 27% of transitional cell carcinomas demonstrated H-*RAS* over expression relative to paired normal tissues.

Conclusions: Our results underline the importance of H-*RAS* activation in human bladder cancer by codon 12 mutations. Moreover, they provide evidence that increased expression of all 3 *RAS* genes is a common event in bladder cancer that is associated with disease development.

Key Words: urinary bladder; carcinoma, transitional cell; genes, ras; mutation; gene expression

BLADDER cancer is the fifth most common cancer in men in Western society with an annual incidence of approximately 18/100,000 population. The peak prevalence of the disease is in patients who are 60 to 70 years old. BCa is curable if diagnosed during the early stages of the disease. Tumors of the bladder develop via 2 distinct but somewhat overlapping pathways, including the papillary and the nonpapillary pathway. Approximately 80% of BCAs consist of superficial exophytic papillary lesions that originate from

urothelial hyperplasia. These typically low grade papillary tumors may recur but rarely invade the bladder wall or metastasize. The remaining 15% to 20% of tumors represent high grade, solid, nonpapillary BCAs that arise from high grade intra-urothelial neoplasia. These tumors aggressively invade the bladder wall and have a high propensity for distant metastasis.¹

The most frequently detected alterations in oncogenes in animal and tumor models of human cancer are mu-

tations in the *RAS* family of oncogenes.² The *RAS* gene family codes for 21 kDa (p21^{RAS}) proteins that are found in the cytoplasm and it is associated with the inner surface of the plasma membrane. The normal function of p21^{RAS} proteins is to interact with tyrosine kinase receptors to activate a signal transduction pathway. Therefore, all *RAS* gene products have GTPase activity and regulate cell growth and differentiation. Mutations in members of the *RAS* gene family are found in a wide variety of human cancers. Most of these mutations are point mutations in codons 12, 13 or 61 that convert the *RAS* gene into a transforming oncogene. Mutated p21^{RAS} has a structure that hinders its ability to bind to GTPase activating protein, thus keeping p21 in a guanosine triphosphate bound activated state. The activation of *RAS* oncogenes also occurs by quantitative changes, eg over expression.³ Spandidos and Lang reported that the over expression of even normal p21^{RAS} is carcinogenic.⁴ We determined *RAS* codon 12 and 13 point mutations status and evaluated mRNA expression levels in TCC.

MATERIALS AND METHODS

Study Design and Clinicopathological Data

Paired tumor and normal tissue samples from a consecutive series of 30 patients with newly diagnosed BCa undergoing transurethral bladder tumor resection at the department of urology at "Asklipieio" General Hospital, Athens were prospectively evaluated for K, H and N-*RAS* gene mutations by RFLP analysis and direct sequencing. The patients studied were of advanced age (mean \pm SD 72.2 \pm 10.6 years). Of the 30 patients 26 (87%) were smokers or former smokers, whereas 19 (63%) were characterized by some level of occupational exposure to agents associated with BCa, such as paints, chemicals etc (table 1).

All tumor specimens were classified and graded by the same pathologist. Histological grading was performed using the 1973 WHO and the 2004 WHO/International Society of Urological Pathology classifications.⁵ Tumor stage was assessed according to the 2002 American Joint Committee on Cancer staging system.⁶ Written informed consent was obtained from all study patients.

The study protocol was approved by the University of Crete ethics committee. Eligibility criteria used were electively resected primary BCa and the availability of DNA from normal and tumor tissue for biomolecular analyses. Study exclusion criteria were a history of neoplasms and chemotherapy or radiation therapy before surgery.

Tissue samples were obtained at surgery from the tumor and using cold cup biopsies from 3 grossly normal selected sites, including the posterior wall, the trigone and the area adjacent to the tumor. Parts of resected normal samples were sent for histopathological analysis. Tumor and normal tissues were frozen immediately in liquid nitrogen, transported and stored at -80°C until DNA extraction.

Patients with nonmuscle invasive BCa were followed with periodic cystoscopic examinations and intravesical

Table 1. Patient clinicopathological characteristics

	No. Pts
Overall*	30
Sex:	
M	27
F	3
Mean age (range)	72.2 (44–86)
Smoking:	
Nonsmoker	4
Former smoker	8
Smoker	18
Occupational exposure:†	
Yes	19
No	11
Stage:	
pTa	1
pT1	12
pT1a	4
pT1b	1
pT2	2
pT2a	1
pT2b	5
pT2+in situ	3
pT3a	1
WHO 1973 grade:	
I	0
II	10
III	20
WHO/International Society of Urological Pathology 2004 grade:	
Low	8
High	22

* Total of 30 TCC and 30 adjacent normal tissue samples.

† To chemicals, paints, pesticides, petroleum, ink etc.

treatment as indicated. Patients with invasive BCa were offered radical cystectomy with or without systemic chemotherapy. At a mean followup of 24 \pm 3 months, 8 patients (26.6%) had recurrent tumors. In those with Ta/T1 vs T2-T3 tumors the frequency of recurrence was 29.4% (5 of 17) vs 23% (3 of 13). In patients with nonmuscle invasive BCa the progression rate was 11.1% and 22.2% for grades II and III disease, respectively. All recurrences were proved by biopsy.

DNA Extraction, Oligonucleotide Primers and PCR Amplification

Genomic DNA was extracted using proteinase K, followed by phenol extraction and ethanol precipitation according to standard procedures. *RAS* genes were amplified using the set of primers and PCR conditions reported by Zarrinos et al.⁷

RFLP Analysis and Direct Sequencing

We performed RFLP analysis and direct sequencing. For K-*RAS* and N-*RAS* 10 to 40 μl aliquots of amplification products were digested for 16 hours with 30 U *Bst*NI. For H-*RAS* 10 to 40 μl aliquots of amplification products were digested for 16 hours with 30 U *Msp*I. RFLP products were analyzed on 3% agarose gel and photographed on an ultraviolet light transilluminator (fig. 1). DNA from the SW480 cell line bearing a homozygous mutation in K-*RAS* codon 12 and EJ, which is mutant at the same codon of the H-*RAS* gene, served as positive controls, respectively.

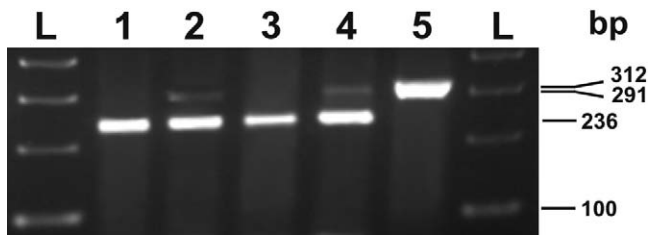


Figure 1. H-RAS 312 bp amplification products were digested with restriction enzyme *Msp* I and electrophoresed on 3% agarose gel. L, 100 bp DNA ladder. Lanes 1 and 3, WT samples. Lanes 2 and 4, heterozygous mutation samples. Lane 5, undigested H-RAS PCR product.

PCR products were purified with a QIAquick® PCR purification kit. The sequencing reaction was performed on a GeneAmp® 9600 thermal cycler using a Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, as previously described.⁷ Sequences were double-checked using forward and reverse primers separately (fig. 2).

RNA Extraction and Reverse Transcription

Tissue specimens were homogenized in TRIzol® reagent using a power homogenizer, followed by chloroform addition and centrifugation. Total RNA was precipitated from supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μ l DEPC treated water. cDNA was synthesized by reverse transcription with the StrataScript® First-Strand Synthesis System, as previously described.⁷

Real-Time PCR

Transcribed products were subjected to real-time PCR assay with SYBR® Green I in an Mx3000P® programmable thermal controller apparatus. All primer pair sequences, amplification conditions and calculation of gene transcription levels were used as previously described (fig. 3).⁷

Statistical Analysis

The nonparametric Spearman rank correlation was used to examine pairwise correlations between RAS mRNA levels and their association with continuous variables, including patient age, smoking, histological type and stage. The Mann-Whitney U test, applied when indicated by analysis, was used to examine the expression status of RAS genes with the various clinicopathological parameters after stratification. Multivariate analysis using a logistic regression model was also performed. All statistical analysis was performed with SPSS® 11.5 with statistical significance set at the 95% level ($p < 0.05$).

RESULTS

Mutation Analysis

Mutation analysis of the K, H and N-RAS genes in codons 12 and 13 was done in 30 patients. No K or N-RAS mutation was detected. However, 9 TCCs bearing mutations in codon 12 were found with amino acid substitutions that could be deduced by direct sequencing. One substitution (2TCC) was ho-

mozygous (11%), while the remainder were heterozygous (89%). Seven of 9 mutant samples (78%) bore a GGC \rightarrow GAC mutation at codon 12, leading to the amino acid substitution of Gly \rightarrow Asp at this site. The 2 remaining samples were GGC \rightarrow GTC codon 12 mutations, resulting in the substitution of Gly \rightarrow Val (fig. 2). No RAS mutation could be detected in codon 13 in any tissues studied. Notably only 2 of the patients bearing H-RAS mutations had invasive urothelial carcinoma, which was stage pT2 plus in situ in 1 and pT3a disease in 1, whereas 7 of 9 (77%) had noninvasive urinary BCa, including stages pT1, pT1a and pT1b.

Expression Analysis

RAS genes were expressed in all TCC and adjacent normal tissues studied. According to the ratio of the level of expression of each RAS gene in TCCs compared to that in adjacent normal tissue we defined 3 groups of expression, including less than 0.5—under expression, 0.5 to 2.0—equal expression and greater than 2.0—over expression (table 2). Of the TCCs 11 (37%) presented with over expression of 1 RAS gene, whereas 7 (23%) were characterized as having 2 over expressed RAS genes. Only 4 TCCs (13%) showed over expression of all 3 RAS genes.

The highest incidence of over expression was detected in K and N-RAS genes (50% each), whereas H-RAS showed a lower rate of over expression (27%). It is clear that RAS oncogenes did not have a similar pattern of over expression in urothelial carcinoma, which is in favor of the hypothesis that this expression has a role in the pathogenesis of bladder tumors. Mean \pm SD levels of under expression of RAS genes were 0.27 ± 0.12 for K and H-RAS, and 0.38 ± 0.04 for N-RAS. The rate of equal expression was similar for the K, H and N-RAS genes (1.03 ± 0.44 , 1.20 ± 0.48 and 0.92 ± 0.45 , respectively). We observed a unique pattern of over expression of all 3 members of the RAS family and each member showed a different TCC-to-normal ratio range (table 2). Specifically while the mean over expression rate of K-RAS was 4.53 ± 2.52 -fold, 4 TCCs showed a significantly increased K-RAS expression level compared to that in adjacent normal tissue (21.67 ± 1.14 -fold) (fig. 4). Six TCCs demonstrated H-RAS over expression (mean 2.64-fold \pm 0.43). However, 2 carcinoma samples were characterized by 18.04 ± 8.24 -fold increased H-RAS expression relative to adjacent normal tissue. N-RAS was found to be over expressed 3.83 ± 1.32 -fold in 11 TCCs. Of note 4 TCCs were characterized by an even more significantly increased level of N-RAS over expression relative to adjacent normal tissue (18.65 ± 5.80 -fold).

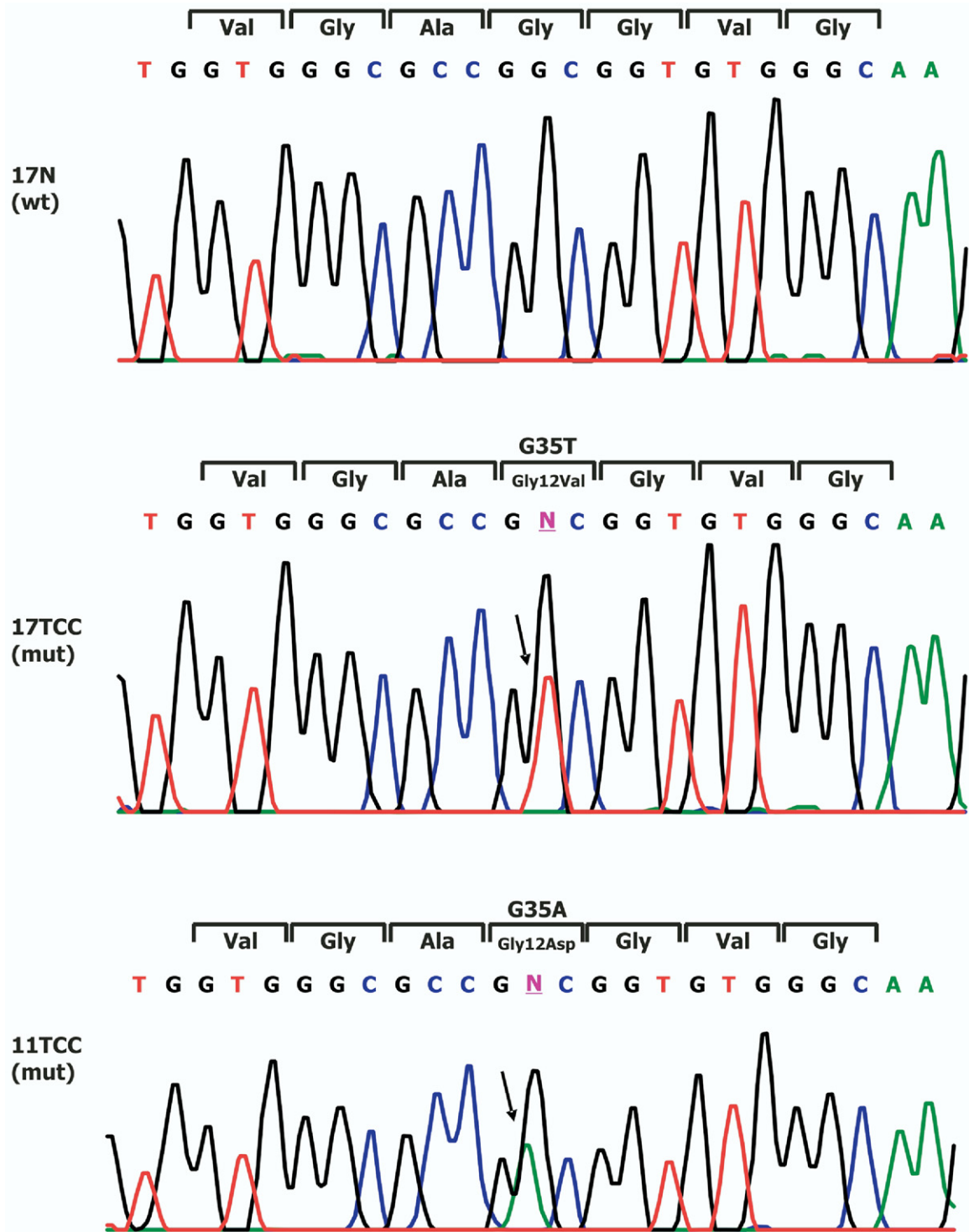


Figure 2. Direct sequencing of RAS PCR products verified mutation (*mut*) in codon 12 in 9 TCCs. A, WT H-RAS sequence of representative normal adjacent tissue from codon 9 to 15 and amino acids encoded by this sequence. B, sequence of representative TCCs bearing codon 12 heterozygous point mutation (GGC → GAC), which leads to Gly12Asp amino-acid change. C, sequence of representative TCCs bearing codon 12 heterozygous point mutation (GGC → GTC), which leads to Gly12Val amino acid.

RAS Analysis vs Clinicopathological Characteristics

Of the patients studied 6 (31.5%) carried a codon 12 H-RAS mutation, while the remaining 13 had tis-

sues that were WT. Three H-RAS mutant samples were found in patients who had no occupational exposure record. In 9 of the 19 patients (47%) with an occupational exposure record only 1 RAS gene

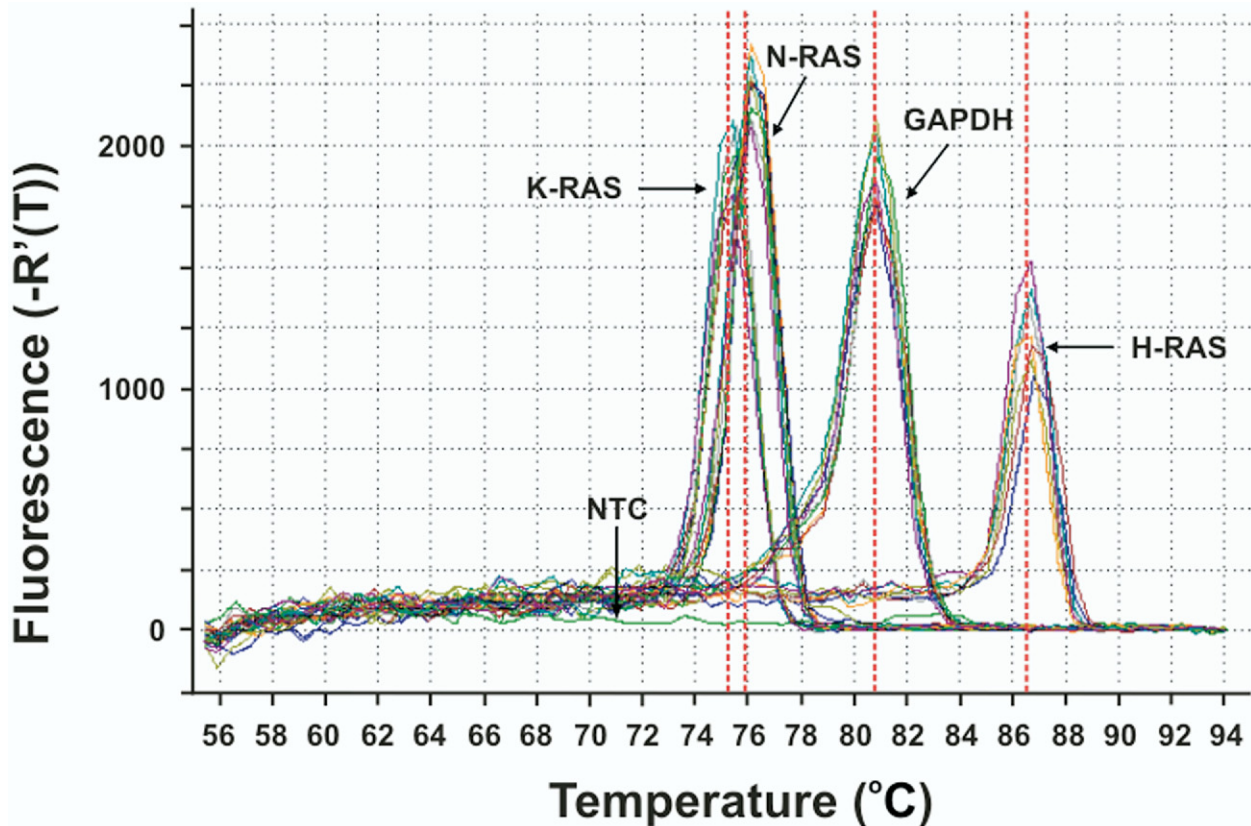


Figure 3. Melt curve analysis of RAS genes and normalizer gene GAPDH shows single peaks for all genes, including 75.8°C for K-RAS, 76°C for N-RAS, 86.2°C for H-RAS and 80.3°C for GAPDH, and absent peaks for nontemplate control (NTC), demonstrating exclusion of any genomic DNA contamination and PCR product specificity.

was over expressed, while 4 and 2 patients had 2 and 3 over expressed genes, respectively. Similarly 3 of these patients did not show any over expression. In contrast, it was not possible to detect any difference in the number of over expressed genes in the 11 patients with no history of occupational exposure. Three of these patients had 1, 2 and no over expressed genes, respectively, while in 2 all 3 RAS genes were over expressed.

Of the 30 patients 26 (87%) were smokers or former smokers, of whom 19% had an H-RAS codon 12 mutation. Interestingly all 4 nonsmokers had the H-RAS codon 12 mutation. Nine of the 26 smokers/former smokers (34%) had 1 RAS gene over expressed, while 27% and 15% had 2 and 3 genes over expressed, respectively. Six smokers/former smokers did not show any RAS gene over expression.

Statistical significance was not attained in the relationship between RAS mutations, expression status and tumor stage or grade. In addition, multivariate analysis using a logistic regression model demonstrated no association between mutation/expression status and clinical outcome (progression and recurrence). However, 78% of H-RAS codon 12 mutations were detected in noninvasive bladder

cancer cases. Moreover, grade III TCC cases showed significantly higher N-RAS mRNA levels than cases of grade II tumors with a statistically significant difference (Mann-Whitney U test $p = 0.041$).

mRNA Expression Pairwise Analysis

Spearman analysis was done to detect possible correlations between RAS co-expression patterns in TCC and adjacent normal tissues. A significant positive correlation was found between H-RAS and N-RAS in TCC samples ($p = 0.001$). No other correlation was present in the remaining gene pairs in the TCC or adjacent normal tissue.

DISCUSSION

The association between the mutated RAS gene family (H, K and N-RAS) and 30% of all human cancers, including bladder cancer, suggests that aberrant RAS function is an important contributor to cancer development.^{3,4} Frequently mutated hot spots are Gly \rightarrow Val in codon 12, Gly \rightarrow Cys at codon 13 and Glu \rightarrow Arg/Lys/Leu at codon 61.^{8,9} Point mutations in RAS genes block intrinsic GTPase activity, thus preventing the normal deactivation of RAS pro-

Table 2. RAS family gene mutation and expression status in patients with TCC

Pt No.	H-RAS Codon 12 Mutations*	TCC/Normal (expression)		
		K-RAS	H-RAS	N-RAS
1	WT	0.27 (under)	0.40 (under)	0.43 (under)
2	Mutation	0.22 (under)	1.88 (equal)	20.50 (over)
3	WT	0.22 (under)	3.38 (over)	3.13 (over)
4	Mutation	0.28 (under)	0.22 (under)	1.64 (equal)
5	WT	0.18 (under)	1.37 (equal)	13.89 (over)
6	WT	0.50 (equal)	0.75 (equal)	2.85 (over)
7	WT	0.10 (under)	0.19 (under)	0.47 (equal)
8	WT	0.43 (under)	2.70 (over)	0.52 (equal)
9	Mutation	0.15 (under)	1.50 (equal)	6.08 (over)
10	Mutation	0.72 (equal)	0.95 (equal)	0.75 (equal)
11	Mutation	21.95 (over)	1.46 (equal)	0.76 (equal)
12	WT	3.56 (over)	0.12 (under)	0.58 (equal)
13	WT	3.00 (over)	2.39 (over)	2.28 (over)
14	WT	1.64 (equal)	0.11 (under)	0.85 (equal)
15	WT	0.43 (under)	24.23 (over)	4.65 (over)
16	Mutation	3.35 (over)	0.70 (equal)	2.37 (over)
17	Mutation	22.31 (over)	1.11 (equal)	0.36 (under)
18	WT	1.07 (equal)	0.35 (under)	5.77 (over)
19	WT	22.41 (over)	2.83 (over)	14.17 (over)
20	WT	9.10 (over)	1.65 (equal)	4.34 (over)
21	WT	19.99 (over)	0.19 (under)	0.35 (under)
22	Mutation	2.24 (over)	0.28 (under)	0.59 (equal)
23	WT	0.41 (under)	0.52 (equal)	1.21 (equal)
24	WT	8.73 (over)	1.84 (equal)	26.05 (over)
25	WT	3.26 (over)	0.43 (under)	1.89 (equal)
26	Mutation	5.47 (over)	2.29 (over)	0.89 (equal)
27	WT	2.70 (over)	12.57 (over)	4.05 (over)
28	WT	6.31 (over)	0.44 (under)	2.61 (over)
29	WT	2.11 (over)	2.25 (over)	4.04 (over)
30	WT	1.24 (equal)	0.71 (equal)	0.89 (equal)

* In all patients K and N-RAS genes, and in all adjacent normal tissues RAS family genes were WT.

teins.³ H-RAS mutations were first detected in the human bladder cancer cell line T24¹⁰ with subsequent studies demonstrating that H-RAS mutations are more frequently detected in urinary tract tumors than K or N-RAS genes.¹¹ Although the initial expectation that H-RAS mutations would have an important role in bladder carcinogenesis has not been upheld and it is now believed that they have a role in only 10% of cases,¹² still more recent reports show a significantly higher frequency of H-RAS mutations in bladder tumors. Fitzgerald et al reported that mutations in H-RAS were detected in urine sediment in 44% of patients with bladder cancer,¹³ Czerniak et al observed H-RAS mutation at codon 12 in 45% of bladder cancers¹⁴ and Haliassos et al reported an even higher incidence of 66%.¹⁵ Furthermore, the most recent report by Przybojewska et al described H-RAS mutation in 84% of patients with bladder cancer.¹⁶ These discrepancies may be due to the methods used to detect RAS mutation.

In the current study we used a sensitive PCR/RFLP assay to test for the presence of RAS mutations in BCa cases. To our knowledge for the first

time results were confirmed by direct sequencing of the PCR product, providing increased accuracy and sensitivity. Our findings support reports indicating that a significant percent of H-RAS gene codon 12 mutations are present in urinary BCa cases. We detected 9 of 30 bladder TCCs (30%) bearing H-RAS mutations (G → A and G → T) in codon 12, which are the most commonly reported point mutations.¹⁷ Of particular note in our study is the fact that 78% of these mutations were found in noninvasive TCCs, whereas only 2 were detected in invasive cancers, including 1 pT3a and 1 pT2 plus in situ. This observation is in agreement with the findings of Ooi et al, who reported that the codon 12 G → T substitution was associated with nonrecurring and recurring primary tumors as well as with initial Ta/T1 lesions in patients with disease progression.¹⁸ Our results substantiate the concept that there is a relationship between H-RAS codon 12 mutations and low stage, well differentiated bladder neoplasms, whereas it seems that advanced TCC lacks such mutations.

Mutations in K-RAS have been detected in more than 80% of human pancreatic tumors, 38% of colon cancer specimens and 31% of small intestine can-

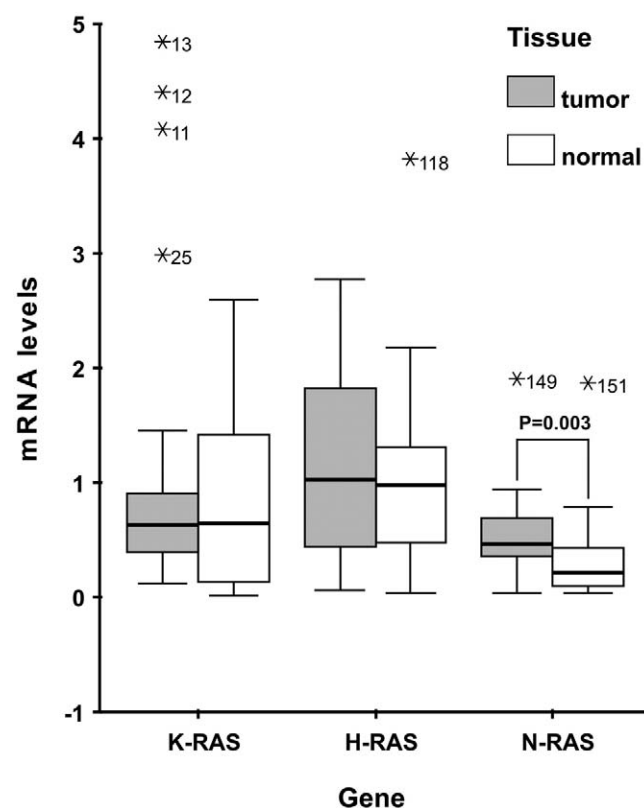


Figure 4. RAS family gene mRNA levels in TCC and adjacent normal tissues. K and N-RAS had higher expression in TCC samples than in normal tissue (N-RAS Mann-Whitney U test $p = 0.003$), whereas H-RAS was equally expressed in 2 tissue types.

cers. To date *N-RAS* gene mutations have been mainly associated with hematopoietic malignancies and melanoma. Our findings concerning the lack of K and *N-RAS* gene mutations in BCa are in accordance with those of other groups, providing further evidence for the tissue specificity of the activation of RAS family genes.

Although point mutations act as an activating mechanism of RAS oncogenes, over expression of RAS transcripts also increases the risk of carcinogenesis and it has been reported for several tumors, including those of the breast, colon, head and neck, and lung. These studies have proved that RAS oncogene expression is increased in premalignant and malignant tumors compared to normal tissues. In bladder cancer specifically Viola et al found increased expression of RAS protein in carcinoma in situ and high grade tumors but not in hyperplasia or low grade tumors on immunohistochemical analysis.¹⁹ Moreover, Vageli et al reported the possible involvement of RAS oncogenes in bladder cancer through over expression.²⁰

However, none of the studies done to date have used advanced technology, such as real-time reverse transcriptase-PCR. To our knowledge the current study is the first to do so using real-time reverse transcriptase-PCR to detect the mRNA levels of RAS genes in TCC and compare them to corresponding levels in adjacent normal tissue. Of 30 samples 23 were found to demonstrate over expression of at least 1 RAS family gene. The highest incidence of over expression was found for K and *N-RAS* genes, which were over expressed in half of TCC samples,

while *H-RAS* showed a lower rate of over expression (27%) compared to paired normal tissue. These rates are in agreement with the results of previous studies²⁰ and indicate that RAS oncogenes do not have a pattern of over expression in bladder cancer that is similar to their pattern in other cancers. The positive correlation detected between H and *N-RAS* expression levels underlines the concept that RAS genes could act synergistically in TCC cases ($p = 0.001$). Moreover, in contrast to the study by Viola et al,¹⁹ statistical significance was not attained between expression levels and tumor stage or grade. Our results are in accordance with studies supporting a lack of correlation between tumor stage or grade and RAS mRNA levels. All samples expressed at least 1 member of the RAS family but 37% of them had over expression of only 1 RAS gene, while 23% had over expression of 2 RAS genes and 13% over-expressed all 3 RAS genes. These results suggest a significant increase in p21^{RAS} in the corresponding tissues. In samples that over expressed all 3 RAS genes the TCC-to-normal ratio of each was not always similar.

CONCLUSIONS

The current study substantiates the concept that activation of the *H-RAS* oncogene by point mutation and the activation of all RAS genes (mainly K and *N-RAS*) by over expression are frequent events in BCa cases. Increased *RAS* mRNA levels may indicate BCa development but they do not appear to be responsible for its progression.

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