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Activating mutations of *ras* family genes in prostatic cancer

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Abstract. *ras* family genes (H-, K- and N-*ras*) encode for a 21 kD membrane protein which possesses GTPase activity and participates in a signal transduction pathway. Activating mutations of the *ras* family genes occur at codons 12, 13 and 61 and have been detected in a variety of human tumours, including colonic, bladder and pancreatic cancers. Prostatic cancer is among the most common malignancies throughout the world and a major cause of death from cancer in males. Data reported on the implication of the *ras* family genes in the development of the disease are conflicting. The aim of this study was to determine the incidence of mutations at codon 12 of H-*ras*, codon 12 of K-*ras* and codon 61 of N-*ras* proto-oncogenes, in a Greek population with prostatic cancer. Our analysis revealed that 4 out of 20 (20%) samples harboured a K-*ras* codon 12 point mutation, 1 out of 20 (5%) specimens contained mutations at codon 12 of the H-*ras* and 1 out of 20 (5%) at codon 61 of the N-*ras*, indicating a role for the *ras* genes in the development of the disease.

Introduction

Multistage carcinogenesis proceeds through the activation of proto-oncogenes and the inactivation of tumour suppressor genes (1). Among oncogenes, *ras* family genes (H-, K- and N-*ras*) have a critical role in the development of the disease. Activated *ras* genes have been detected in several human tumours, at various frequencies. Mutational activation represents the major activating mechanism for the *ras* family genes. Hot spots for mutations are codons 12, 13 and 61 (2). Apart from mutations, overexpression of the *ras* family genes, without any obvious genetic alteration, is often observed and could be a potential contributor to the initiation or development of the malignancy (3).

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Mutations in at least one member of the *ras* family genes, have been detected in almost every human tumour, including colorectal, endometrial, pancreatic and lung carcinomas (4). Generally, *ras* mutations are considered as early events in carcinogenesis and have also been described in benign tumours and cancer predisposing diseases (5), leading to the proposal that they may serve as tumour markers.

Prostatic cancer is one of the most common malignancies worldwide and a major cause of death from cancer in males in the Western world. The implication of the *ras* family genes in the development of prostatic cancer has not been studied in depth and the available data are still conflicting. Several investigators have proposed a minor role for the *ras* family genes in the development of prostatic cancer, with the majority of the sparse mutations, affecting mainly codon 12 of the H-*ras* gene (6-8). However, a Japanese group detected activating mutations at codon 12 of the K-*ras* gene, in approximately 25% of the samples tested (9). This finding could be interpreted as a reflection of the different environmental factors between the Western world and Japan, providing further evidence to the suggestion that *ras* family genes behave in a carcinogen specific manner. In addition, prostatic tumours induced by N-methyl-N-nitrosourea in rats, frequently develop mutations at codon 12 of K-*ras* gene (10).

The aim of our study was to determine the incidence of point mutations of the H-*ras* at codon 12, K-*ras* at codon 12 and N-*ras* at codon 61 in adenocarcinomas of the prostate in Greek patients.

Materials and methods

Specimens. 20 formalin fixed paraffin-embedded tissue sections from adenocarcinomas of the prostate, were obtained by the Medical School, University of Thrace, Alexandroupolis, Greece. The clinicopathological parameters of the patients are given in Table I.

DNA extraction. Paraffin sections were digested overnight at 50°C in a solution containing 100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% SDS and 100 μg/ml proteinase K, followed by phenol/chloroform extraction. DNA was ethanol precipitated. DNA from the cell lines SW480 (positive control for codon 12 K-*ras* mutation) and HL60 (positive control for codon 61 N-*ras* mutation) was isolated in a similar manner.

Table I. Clinicopathological parameters of 20 adenocarcinomas of the prostate.

Patients number	H-*ras* codon 12 point mutation	K-*ras* codon 12 point mutation	N-*ras* codon 61 point mutation	Histological differentiation	Stage
1	-	+	-	Poor	C2
2	-	-	-	Poor	C2
3	-	-	-	Well	A
4	+	-	-	Well	A
5	-	-	-	Poor	C2
6	-	-	-	Well	A
7	-	+	-	Poor	B2
8	-	-	-	Poor	B2
9	-	-	-	Poor	A
10	-	-	-	Well	A
11	-	-	-	Well	A
12	-	-	-	Moderate	B2
13	-	-	-	Well	A
14	-	-	-	Well	A
15	-	-	-	Well	B1
16	-	+	-	Poor	A
17	-	-	-	Well	A
18	-	-	-	Moderate	A
19	-	-	-	Moderate	B2
20	-	+	+	Well	A

Table II. PCR primers, PCR products and digestion products for the detection of mutations at codon 12 of H-*ras*, 12 of K-*ras* and codon 61 of N-*ras*.

	H-*ras* codon 12	K-*ras* codon 12	N-*ras* codon 61
set of primers used	5'-GAGACCCTGTAGGAGGACCC-3'	5'-ACTGAATATAAACTTGTGGTA GTTGGACCT-3'	5'-GACATACTGGATACAGCTGGC-3'
	5'-TCAGGGAGCAGAGTCGTGGG-3'	5'-CCAGGTCCTGGTAAGAACT-3'	5'-CTGGTTATGTACTCCTGTCC-3'
PCR product	312 bp	157 bp	65 bp
Digestion product	normal, 236 bp mutant, 291 bp	normal, 113 bp mutant, 144 bp	normal, 44 bp mutant, 65 bp

PCR amplification. The amplification reactions, were performed in a 50 µl reaction volume containing 200 ng of genomic DNA, 500 µM dNTPs, 10 pM of each forward and reverse primer (Table II), 0.5 U of Taq DNA polymerase and 5 µl of 10X buffer (670 mM Tris HCl, pH 8.5; 166 mM $(NH_4)_3SO_4$; 67 mM $MnCl_2$; 1.7 mg/ml BSA; 100 µM, ß-mercaptoethanol and 1% (w/v) Triton X-100). DNA was initially denatured for 5 min at 95°C and amplified for 35 cycles of 95°C for 30 sec (denaturation), 58°C for 30 sec (annealing) and 45 sec at 72°C (extension).

RFLP analysis. H-*ras* codon 12: 10-20 µl of the amplification product were digested overnight with 20 U MspI, at 37°C. K-*ras* codon 12: 10-20 µl of the amplification product were digested for 3 h with 20 U BstNI, at 60°C. N-*ras* codon 61: 10-20 µl of the amplification product were digested overnight with 20 U MscI, at 37°C. Enzymes were purchased from New England Biolabs.

Digestion products were electrophoresed through a 2% agarose gel for K-*ras* and H-*ras* or an 8% native polyacrylamide gel for N-*ras*. Gels were stained with ethidium bromide and visualised on a UV transilluminator.

Results and Discussion

In the present study we analysed 20 adenocarcinomas of the prostate for mutations of the H-*ras*, K-*ras* and N-*ras* proto-oncogenes. Tumour specimens were obtained from patients with various stages and histological differentiation of the disease. The analysis revealed the presence of 4 out of 20 (20%) mutant samples at K-*ras* codon 12 (Fig. 1), 1 out of 20 (5%) at H-*ras* codon 12 (Fig. 2) and 1 out of 20 (5%) N-*ras*

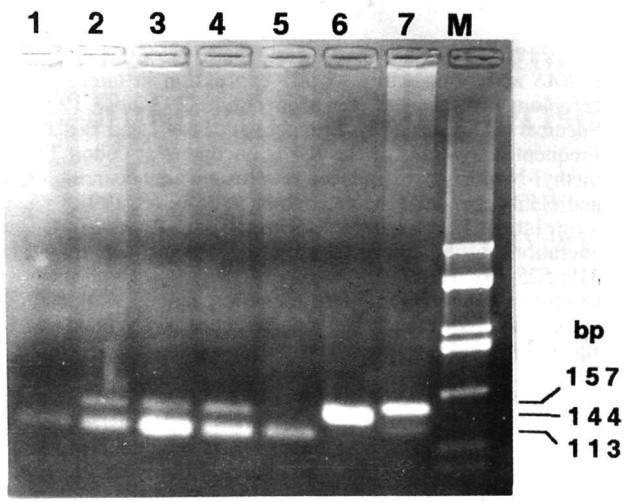

Figure 1. Mutations at K-*ras* codon 12 detected by a combined PCR-RFLP assay. Lanes 1 and 5: normal samples, lanes 2, 3 and 4: mutant samples, lane 6: DNA from the cell line SW480 harbouring only a mutant allele, lane 7: undigested PCR product, M; molecular weight DNA marker.

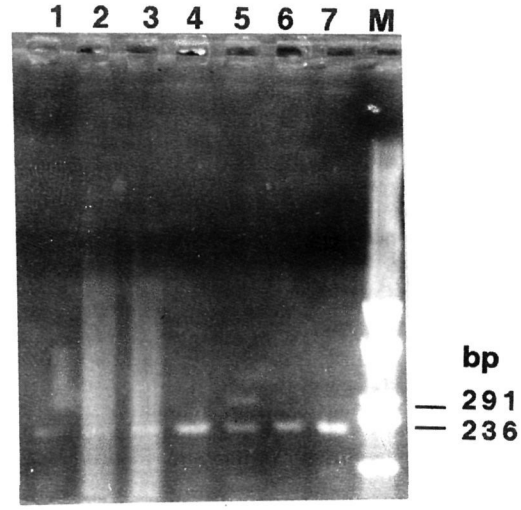

Figure 3. Mutations at codon 12 of H-*ras* detected by PCR-RFLP. Lanes 1-7: PCR products digested with MspI. Resistance to MspI digestion (lane 5) indicates the presence of a mutation. M; molecular weight DNA marker.

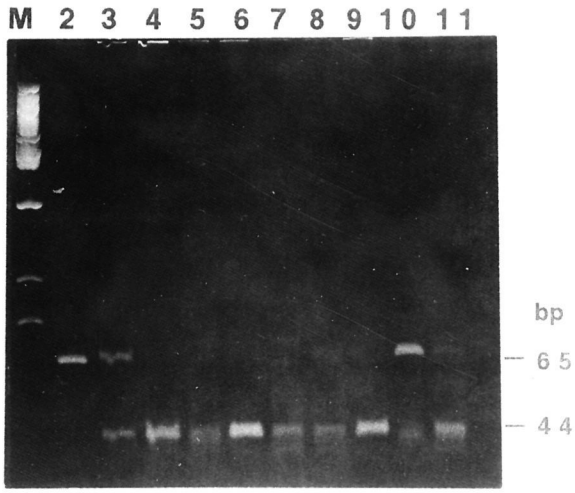

Figure 2. Mutations in codon 61 of the N-*ras* gene were detected by PCR-RFLP analysis. M: pBR322/HaeIII DNA marker. Lane 2: Undigested positive control (HL60 cell line) (65 bp), lane 3: positive control digested with MscI, lanes 4-11: PCR products digested with MscI. Resistance to MscI digestion (lane 10) indicates a mutated N-*ras* gene in codon 61.

codon 61 (Fig. 3). All but one of the K-*ras* mutant samples were poorly differentiated tumours (Table I), associating the activating mutation with low level of differentiation. The stage of the disease for the mutant samples has been classified as A for two of them and as B and C for the rest of the samples, indicating no association with the aforementioned clinicopathological parameter. Patients with H-*ras* and N-*ras* mutations (patients number 4 and 20 respectively) had well differentiated stage A tumours. Patient number 20 had a concurrent K-*ras* codon 12 and N-*ras* codon 61 point mutation.

In all cases with a mutation, the normal allele has been retained. In models of colorectal (11) and skin carcinogenesis (12), the deletion of the normal allele has been proposed to follow the *ras* mutation, in a later stage of the tumour progression. It might be argued that if the mutations in the *ras* family genes play a significant role in the development of prostatic cancer, as suggested by this and other studies (6,9,10), heterozygosity is sufficient for the development of a malignant change and a gene dosage effect may play a minor role.

Several investigators have explored the incidence of *ras* mutations in prostatic cancer. In some cases the reported incidence of mutations was very low (1-10%) and almost exclusively restricted to the H-*ras* proto-oncogene (6-8). Mutations in the K-*ras* and N-*ras* have rarely been detected. However, the forementioned results are contrary to the results of a Japanese group (9) which reported a relatively high (25%) frequency of K-*ras* mutations in prostatic cancer. This finding possibly reflects the influence of environmental factor(s) in Japan that specifically mutate the K-*ras* proto-oncogene. Specific mutations in the K-*ras* proto-oncogene are also caused by N-methyl-N-nitrosourea (10) in rats. Such a behaviour is similar to that reported for *ras* family gene mutations in cancer of the head and neck. The incidence of mutations is very low in the Western world, while it is almost 30% in India and it is associated with tobacco chewing (13).

The relatively high incidence of K-*ras* mutations described in our study also suggests that some environmental factor(s) are responsible for the presence of these genetic alterations. Identification of the precise nature of the mutations could give us more information.

The preference exhibited by the K-*ras* point mutations to the poorly differentiated tumours argues in favour of a role for the *ras* genes and the K-*ras* in particular in the pathogenesis of prostatic cancer. However, overexpression of the *ras* family genes should also be considered as a contributing factor in the development of prostatic cancer.

This study indicates that the *ras* genes may have a role in the development and progression of prostatic cancer. A more detailed analysis involving a large number of patients is required in order to establish the precise role of the *ras* family genes in prostatic cancer.

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