Analysis of H-*ras*, K-*ras* and N-*ras* genes for expression, mutation and amplification in laryngeal tumours

HIPPOKRATIS KIARIS[1,2] and DEMETRIOS A. SPANDIDOS[1,2]

[1]Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave, Athens 116 35; [2]Medical School, University of Crete, Heraklion, Greece

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Abstract. The levels of expression of the *ras* family genes, in 14 tumour specimens from squamous cell carcinomas of the larynx were analysed by reverse transcription-polymerase chain reaction. The H-*ras* was overexpressed in 12 (86%) samples, K-*ras* in 11 (78%) and N-*ras* in 8 (57%) samples. All tumours exhibited overexpression of at least one member of the *ras* family. In addition, each member of the *ras* family was activated independently from the rest of the *ras* family genes. The incidence of amplification in the *ras* family genes was also analysed by differential PCR: K-*ras* was found amplified in 14% (2/14), N-*ras* in 7% (1/14) and H-*ras* in none of the samples tested. Amplification data exhibited no association with the expressional levels of the *ras* genes. Furthermore, we investigated the incidence of codon 12 point mutations in the *ras* family genes but no mutation was found. The present study indicates that overexpression of the *ras* family genes is important for the development of the disease and it is not associated with the amplification status of the genes. In addition, the differential regulation among the members of the *ras* family might play a role in the development of laryngeal tumours.

Introduction

Ras family genes (H-, K- and N-*ras*) encode for a 21 kD ($p21^{ras}$) protein which possesses GTPase activity and participates in a signal transduction pathway (1). Activated *ras* oncogenes have been detected frequently in human cancers and this alteration has been associated with the development of the disease (2). Mutations at codons 12, 13 and 61 are considered the main activating mechanism for the *ras* family genes. However, aberrant expression of the *ras* genes, has been recognised in several human cancers and has been associated with the development of the neoplasia (3), since overexpression of even normal *ras* alleles confer a transforming potential (4).

Most of the investigations concerning *ras* expression in human tumour tissues have been performed by immunoassays, with antibodies that failed to distinguish the products of the three *ras* family genes. However, overexpression of $p21^{ras}$ has been recognised as a common feature of several human tumours, including breast (5), colorectal (6) and squamous cell carcinomas of the head and neck (7).

The levels of expression of the individual *ras* genes has been assessed, but these analyses related mainly to the expression of the H-*ras* (8,9) and sometimes to the K-*ras* (9) as well. However, differential expression of the *ras* genes in human tumours, is probably important in tumorigenesis, although the particular properties of $p21^{ras}$ derived by each *ras* family gene are unknown. A different role for each of the *ras* genes has been postulated by Leon *et al* (10), who found that the *ras* family genes are differentially expressed in the normal tissues of the mouse.

Amplification of the *ras* genes is a detectable disorder which most frequently is accompanied by a mutation in the amplified allele (11). The amplification of specific genes, results in a gene dosage effect which is frequently reflected in the levels of expression of the particular allele which is amplified.

Differential expression analysis of the *ras* genes in human tumour tissues has not been performed, as yet. In the present study we investigated the levels of expression of the *ras* family genes in squamous cell carcinomas of the larynx. Furthermore, we investigated whether the overexpression of the *ras* family genes was caused by specific genetic aberrations that result in the amplification of these genes. In addition we analysed the incidence of codon 12 point mutations for the *ras* family genes. This site has the highest rate of activation of the *ras* family genes in human tumours (3).

Materials and methods

Specimens and RNA extraction. 14 fresh tumour specimens from squamous cell carcinomas of the larynx and two adjacent normal tissues were obtained from the Hippocration General Hospital, Department of Otorhinolaryngology, Athens. The specimens, were stored immediately after the

Correspondence to: Professor D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave, Athens 116 35, Greece

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Table I. Primers and PCR products for the detection of codon 12 point mutations, expression and amplification of H-*ras*, K-*ras* and N-*ras* genes.[a]

gene	sense primer	antisense primer	PCR (bp) product	use
H-*ras*	5-GAGACCCTGTAGGAGGACCC-3	5-GGGTGCTGAGACGAGGGACT-3	312	a, m
H-*ras*	5-GACGGAATATAAGCTGGTGG-3	5-AGGCACGTCTCCCCATCAAT-3	151	e
K-*ras*	5-ACTGAATATAAACTTGTGGTAGTTGGACCT-3	5-TCAAAGAATGGTCCTGGACC-3	157	a, m
K-*ras*	5-ACTGAATATAAACTTGTGGTAGTTGGACCT-3	5-CAAATCACATTTATTTCCTACCAGGACCAT-3	357	e
N-*ras*	5-AACTGGTGGTGGTTGGACCA-3	5-ATATTCATCTACAAAGTGGTCCTGGA-3	83	m
N-*ras*	5-GACATACTGGATACAGCTGGC-3	5-CCTGTCCTCATGTATTGGTC-3	65	a
N-*ras*	5-AATCCAGCTAATCCAGAACC-3	5-TGGTCTCTCATGGCACTGTA-3	150	e
ß-actin	5-GTGGGGCGCCCAGGCACCA-3	5-CTCCTTAATGTCACGCACGATTTC-3	548	e

[a]Primers for the amplification of interferon-γ gene are described in ref. 12. a, amplification; m, mutation; e, expression

dissection at -70°C for RNA extraction. Total RNA was extracted using TRIzol Reagent (Gibco BRL), following the manufacturer's instructions.

Reverse transcription PCR and RNA quantitation. 200 ng of total RNA were reverse transcribed in a 50 µl reaction (10 mM Tris HCl; pH 8.3, 50 mM KCl, 1 mM $MnCl_2$, 200 µM dNTPs, 200 ng antisense primer and 2.5 U Tth polymerase) for 15 min at 70°C. PCR amplification of cDNA was performed by adding 50 µl of buffer containing 75 mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.75 mM EGTA and 200 ng sense primer. The oligonucleotide primers used and the subsequent PCR products are listed in Table I.

The PCR programs consisted of 1-min steps at 95°C, 60°C and 72°C for 28 cycles. Preliminary experiments had revealed the conditions in which the amplification reaction remained in the exponential phase (data not shown) and thus the results could be used for quantitation of the template. 10 µl of the PCR product was electrophoresed through a 2% agarose gel and the intensity of the bands was analysed by a UVP image analysis system.

Differential PCR analysis. The co-amplification (12) of the target gene (H-*ras*, K-*ras* and N-*ras*) and the reference gene interferon-γ was performed in a 50 µl reaction volume containing 300 ng of genomic DNA as a template and 500 µM dNTPs, 30 pM of each forward and reverse primer (Table I), 0.5 U of *Taq* DNA polymerase and 5 µl of 10X buffer (670 mM Tris.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 µM ß-mercapto-ethanol and 1% (w/v) Triton X-100). 20 µl of the PCR product was electrophoresed through a 2% agarose gel.

Detection of ras mutations at codon 12 by restriction fragment length polymorphism (RFLP). PCR amplification of

Table II. Amplification and expression of the *ras* family genes in squamous cell carcinomas of the larynx.

	Gene					
	H-*ras*		K-*ras*		N-*ras*	
Patient number	expression	amplification	expression	amplification	expression	amplification
1	2.4	-	2.6	+	2.7	-
2	1.8	-	1.1	-	1.8	-
3	2.2	-	1.5	-	2.8	-
4	2.3	-	1.9	-	1.7	-
5	1.5	-	1.9	+	1.1	-
6	2.0	-	1.6	-	0.8	-
7	2.5	-	2.2	-	2.0	+
8	2.4	-	2.3	-	1.0	-
9	2.3	-	1.6	-	0.7	-
10	1.3	-	1.8	-	1.2	-
11	2.3	-	1.5	-	1.7	-
12	1.5	-	1.5	-	1.7	-
13	1.3	-	1.1	-	2.4	-
14	1.5	-	1.2	-	1.1	-

the *ras* genes surrounding codon 12 was performed as described above. 30 µl of the PCR product was digested overnight with 20 U of the restriction endonucleases *Msp*I (H-*ras*) and *Bst*NI (K-*ras* and N-*ras*) in conditions recommended by the suppliers, electrophoresed in a 2% agarose gel and stained with ethidium bromide.

Results

Expression of the ras family genes. 14 tumour specimens from patients with squamous cell carcinomas of the larynx were analysed for the levels of expression of H-*ras*, K-*ras* and N-*ras* proto-oncogenes. The expressional levels were interpreted as the ratio of the expression in each tumour specimen versus the average expressional levels of two adjacent normal laryngeal tissues of squamous origin. In addition, prior to the interpretation of the *ras* gene expression, amplification of ß-actin mRNA was performed in order to normalise the quality and the quantity of RNAs. The summary of our results is presented in Table II. RNA levels higher than 1.5 fold versus the levels of the control normal tissues were considered an overexpression. H-*ras* exhibited overexpression in 12 (86%), K-*ras* in 11 (78%) and N-*ras* in 8 (57%) samples. All samples tested exhibited overexpression of at least one member of the *ras* family, indicating that the overproduction of the $p21^{ras}$ is necessary for the development of laryngeal tumours. No association has been found between the expressional levels of the *ras* family genes and the clinicopathological parameters of the patients (i.e. nodes at pathology, histological differentiation and stage).

Amplification of the ras family genes. As interpreted by the differential PCR analysis in which each one of the *ras* family genes where co-amplified with the interferon-γ gene, the incidence of amplification was very low. Our analysis revealed that only two among 14 (14%) samples harboured an amplified K-*ras* allele and only one among 14 (7%) samples was positive for N-*ras* amplification (Table II, Fig. 3). Amplification of the H-*ras* proto-oncogene was not detected in any of the samples tested. In addition, amplification of the *ras* family genes was not associated with the expressional levels of these genes, confirming the suggestion of Sheng *et al* (13) in an analysis of a series of head and neck tumours for H-*ras* expression and amplification. No association has been found between amplification and the clinicopathological parameters of the patients (Table III).

Mutations at codon 12 of H-ras, K-ras and N-ras genes. A combined PCR-RFLP assay was employed for the detection of codon 12 mutations of the *ras* family genes. The analysis failed to detect the presence of any mutations at codon 12 *ras* alleles in the laryngeal tumours tested. This is in agreement with previous investigations that indicated low levels of mutational activation of the *ras* family genes in head and neck tumours in the Western world.

Discussion

In the present study we analysed the levels of expression and the incidence of amplification and codon 12 point mutations of the *ras* family genes in 14 specimens from patients with squamous cell carcinomas of the larynx. We employed for the *ras* expression analysis a reverse transcription-polymerase chain reaction (RT-PCR) assay (14) for the detection of the H-, K- and N-*ras* mRNA levels. Two specimens from adjacent normal tissue were used as control

Table III. Clinicopathological parameters of 14 patients with squamous cell carcinomas of the larynx.

Patient number	Stage	Histological differentiation	Nodes at pathology
1	I	M	-
2	III	-	-
3	III	M	-
4	III	P	-
5	III	P	-
6	I	P	-
7	II	M	-
8	II	M	-
9	I	P	-
10	II	P	-
11	I	P	-
12	II	M	-
13	II	M	-
14	III	W	-

W, well differentiated; M, moderately differentiated; P, poorly differentiated

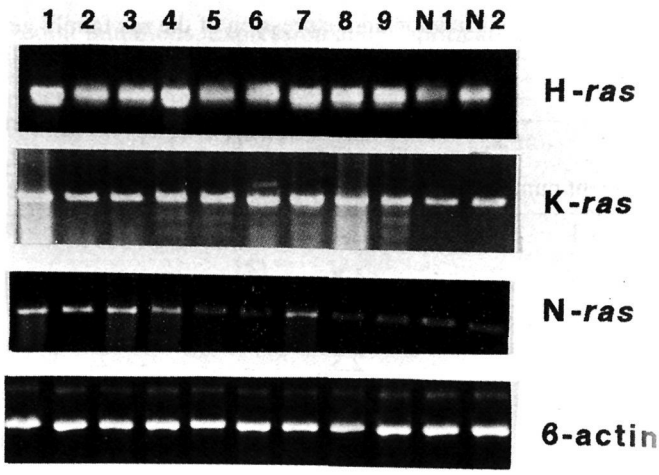

Figure 1. Representative examples of RT-PCR applied to H-, K- and N-*ras*, in 9 tumour specimens of the larynx and two adjacent normal tissues. Amplification of ß-actin was performed in order to normalise the quality and the quantity of RNAs. The intensity of the bands, was analysed with a UVP image analysis system.

in order to evaluate the background levels of expression of the *ras* family genes in the normal mucosa. The quantity and the quality of RNA samples, was normalised after amplification of ß-actin mRNA. The mRNA levels for each member of the *ras* family genes were expressed as the ratio of the intensity of the bands in the tumour tissues versus the average corresponding levels of the normal tissues (Fig. 1). Differential PCR was employed for the detection of amplified *ras* alleles. Co-amplification of the interferon-γ gene served as internal control in this assay. The detection of mutations at codon 12 *ras* gene alleles was performed by a sensitive RFLP assay.

The levels of H-*ras* mRNA overexpression, ranged from 1.4 to 2.6, for K-*ras* from 1.1 to 2.5 and for N-*ras* from 0.9 to 2.6. We arbitrarily considered as overexpression, levels higher than 1.5 fold, versus the expressional levels in the control normal tissues. H-*ras* exhibited overexpression in 12 (86%), K-*ras* in 11 (78%) and N-*ras* in 8 (57%) samples. It is of specific interest, that all samples tested, exhibited overexpression of at least one member of the *ras* family, indicating that overproduction of $p21^{ras}$, regardless of which gene is responsible for the overexpression, is required for the development of a malignant potential (Fig. 2).

In order to investigate possible factors that may result in the overexpression of the *ras* family genes in tumour tissues,

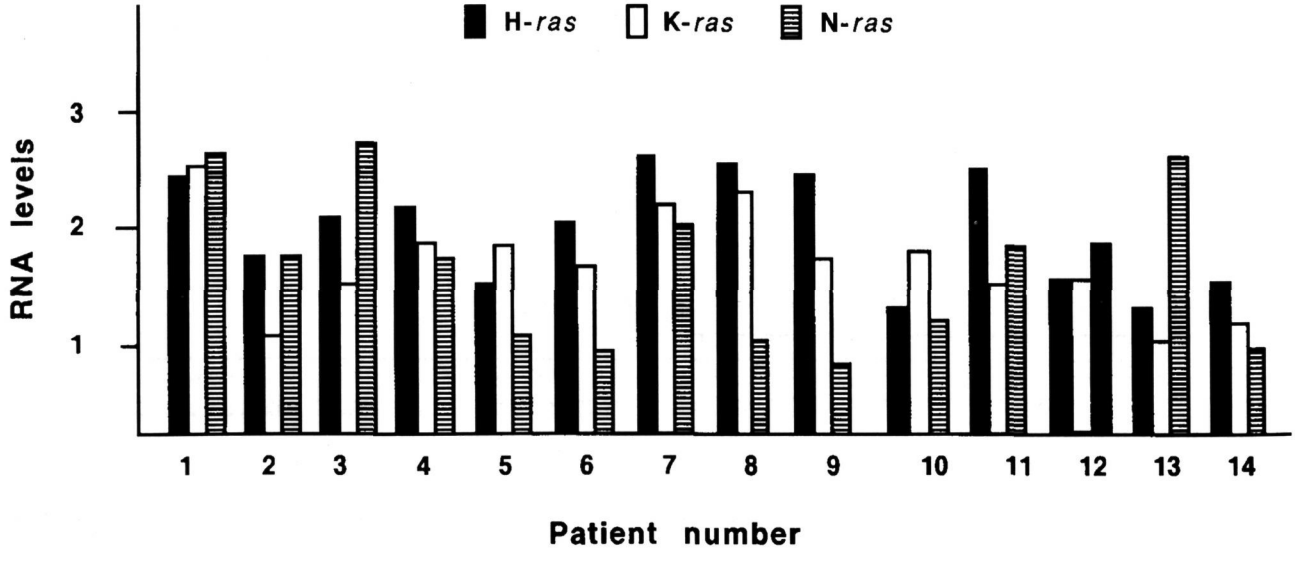

Figure 2. Relative expression of each *ras* family gene, in 14 squamous cell carcinomas of the larynx. The RNA levels for each gene, are expressed as the ratios between the intensity of the bands in the tumour tissues, versus the average intensity of the bands in the normal tissues.

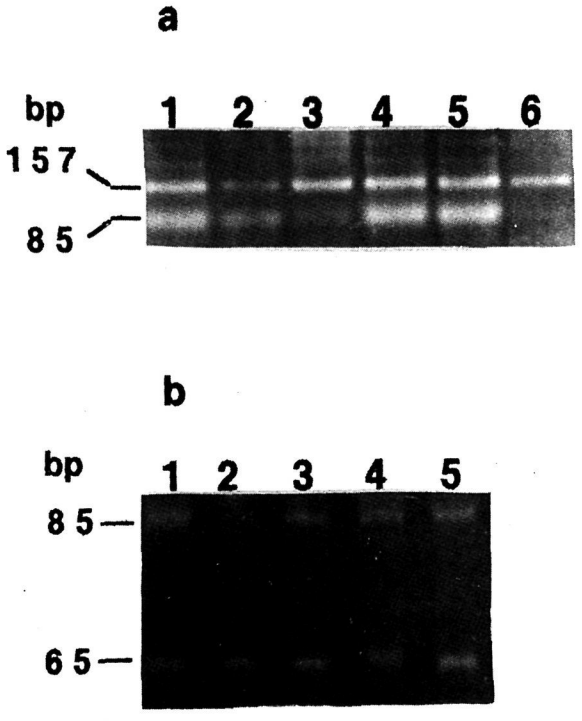

Figure 3. a, Analysis of the K-*ras* gene for amplification. The K-*ras* (157 bp) and the interferon-γ (85 bp) gene products are indicated. Lanes 1, 2, 4 and 5 correspond to negative samples and lanes 3 and 6 to samples positive for K-*ras* amplification. b, Application of the differential PCR to the N-*ras* gene. Lanes 1, 3, 4 and 5 exhibit no evidence of N-*ras* amplification while lane 2 corresponds to a sample with amplification of the N-*ras* gene. 85 bp band corresponds to the interferon-γ gene and 65 bp band corresponds to the N-*ras* gene.

we assessed the incidence of amplification of these genes, by differential PCR. Differential PCR analysis revealed that only two among 14 (14%) samples harboured an amplified K-*ras* allele and only one among 14 (7%) samples was positive for N-*ras* amplification. Moreover, amplification of the H-*ras* proto-oncogene was not detected in any of the samples tested. Although amplification of the *ras* genes is a rare event in laryngeal tumours, overexpression of these genes is frequent and it is not associated with the presence of amplified *ras* alleles. It seems that a gene dosage effect plays a minor role for the development of laryngeal tumours, at least as regards the *ras* family genes. Duesberg and co-workers (15-17) postulated that mutations in the *ras* family genes were overlooked and overexpression of the normal alleles may be more important for the development of the disease. They suggested that mutations within the promoters of these genes or imbalance of chromosomes (resulting in the amplification) may provide further evidence to the understanding of oncogenesis. Although the present study failed to detect amplified *ras* alleles, the lack of *ras* mutations in addition to the overexpression of the normal alleles of the *ras* family, supports the forementioned hypothesis.

The present study provides evidence that all three *ras* family genes are overexpressed in laryngeal tumours. Scambia *et al* (18) reported that $p21^{ras}$ levels in laryngeal tumours were elevated in almost all samples tested. The present investigation indicates that overproduction of $p21^{ras}$ is not due to the activation of only one member of the *ras* family, but all three *ras* family genes may be activated, in all combinations.

The incidence of mutations in *ras* family genes in head and neck cancer is low in the Western world (19). This was confirmed by the present study since no mutations were found in the 14 samples from laryngeal tumours studied. However, overexpression of the *ras* genes possibly plays an important role in the development of the disease. It is now well established that apart from the qualitative changes (i.e. mutations), quantitative alterations in the expression of oncogenes, plays a critical role in carcinogenesis (20). *In vitro* experiments have shown that among transfected cells with similar copy numbers of exogenous proto-oncogenes of the *ras* family, only those that express high levels of *ras* mRNA are morphologically transformed (21).

N-*ras* showed the lowest incidence (8/14; 57%) of overexpression, which may be due to the lower incidence of mutational activation for the N-*ras* in human tumours, among the *ras* family genes. This finding may indicate that N-*ras* possesses a weaker tumorigenic potential, as compared to H-*ras* and K-*ras*, and thus, aberrations of this gene confer less to the malignant transformation.

An additional point of the present analysis is that although some tumours exhibited overexpression of all *ras* family genes (Fig. 2; patients 1, 4, 14), some specimens exhibited activation of different *ras* gene(s), in all possible combinations (i.e. patients 2, 3, 9, 10, 11, 13). We could thus postulate the existence of multiple different genetic events (or factors) that result in the differential activation of the *ras* family genes. The overexpression of each of these factors, in combination with the different affinity this factor possesses with the regulatory regions for each of the *ras* genes, might be responsible for the differential activation they exhibit in the tumours. This is noteworthy because *ras* genes are considered housekeeping genes and inducible regulatory elements have not been recognised, as yet, in the promoter regions of these genes. The factors responsible for the differential regulation of *ras* family genes remain obscure. However, since *ras* family genes may behave independently in the tumours, we could postulate that these genes may be regulated differentially. Genetic events such as instability of the repetitive elements of at least H-*ras*, have been postulated to play a role in the regulation of the gene (8,22). Furthermore, mutations within the regulatory regions of the *ras* family genes may be responsible for their altered expression, which is important for tumorigenesis. It has already been proved that the presence of retroviral promoters is important for the transformation properties of the *ras* family genes, indicating the generation of heterologous promoters (21,23).

Apart from the contribution to the understanding of the basic mechanism of the disease, the studies on the expression of the *ras* family genes in human tumours, may prove to be useful in the development of therapeutic strategies. Several anti-cancer compounds have been developed (i.e. inhibitors to the farnesylation of $p21^{ras}$, anti-sense oligonucleotides, etc.) that target directly the *ras* genes or their product $p21^{ras}$ (24). Assessment of the expressional levels of the *ras* family genes in each tumour may provide useful information as

regards the selection of these compounds and their potential role in the inhibition of the cancer cells of a specific type.

This is the first study to our knowledge, that reports on the levels of expression of the *ras* family genes in human laryngeal tumours. We provided evidence that all three *ras* genes are transcriptionally activated in laryngeal tumours, indicating that overproduction of $p21^{ras}$ is required in the multistage process of the disease. In addition, we found differential activation of the *ras* genes in the tumours tested, and stated the hypothesis that the activation is the result of multiple genetic events. Since the incidence of amplification is very low as compared to that of overexpression, alternative mechanism that result in the transciptional activation of the *ras* family genes should be considered. This includes mutations within the promoters of the *ras* family genes, as already proposed by Duesberg and co-workers (15-17).

More in-depth studies are required, involving a wide range of tumours and a large number of samples, in order to clarify the precise role of the expressional aberrations of the *ras* genes in carcinogenesis. In addition the detection of hot spots for mutations within the non-coding regions of the *ras* family genes may play an important role for the regulation (and disregulation) of these genes and may provide clues for their role in the development of the disease. The role of activation of each individual *ras* gene in the development of the neoplasia also remains to be established.

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