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Original Article

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Genetic alterations in Ki-*ras* and Ha-*ras* genes in Juvenile Nasopharyngeal Angiofibromas and Head and Neck Cancer

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ABSTRACT

Context: *Ras* gene mutations have been associated to a wide range of human solid tumors. Members of the *ras* gene family (Ki-*ras*, Ha-*ras* and N-*ras*) are structurally related and code for a protein (p21) known to play an important role in the regulation of normal signal transduction and cell growth. The frequency of *ras* mutations is different from one type of tumor to another, suggesting that point mutations might be carcinogen-specific.
Objectives: To study the occurrence of Ki-*ras* and Ha-*ras* mutations. We also studied the relative level of Ha-*ras* mRNA in 32 of the head and neck tumors.
Design: Case series.
Setting: University referral unit.
Participants: 60 head and neck tumors and in 28 Juvenile Nasopharyngeal Angiofibromas (JNA).
Diagnostic test: Using PCR-SSCP we examined the occurrence of Ki-*ras* and Ha-*ras* mutations. The relative level of Ha-*ras* mRNA was examined by Northern blot analysis.
Results: None of the head and neck tumors or JNA samples showed evidence of mutations within codons 12, 13, 59 and 61 of Ki-*ras* or Ha-*ras* genes. However, 17 (53%) of the tumors where gene expression could be examined exhibited increased levels of Ha-*ras* mRNA compared with the normal tissue derived from the same patient.
Conclusions: Our results demonstrate for the first time that mutations of Ki-*ras* and Ha-*ras* genes are not associated with the development of JNA and confirm previous reports indicating that activating *ras* mutations are absent or rarely involved in head and neck tumors from western world patients. Furthermore, our findings suggest that overexpression of Ha-*ras*, rather than mutations, might be an important factor in the development and progression of head and neck tumors.
Key words: *Ras* gene family. Squamous cell carcinoma of the head and neck. Juvenile nasopharyngeal angiofibroma. Gene expression. Mutations.

INTRODUCTION

Head and neck tumors are the sixth commonest tumors in the world[1] and account for approximately 10% of all malignant tumors. In Brazil, metropolitan areas such as São Paulo show one of the highest incidences of head and neck tumors in the world.[2] Only in India is the risk of oral cancer greater than in São Paulo. It has been estimated that in the state of São Paulo in 1990 there were 6800 new cases of tumors from oral cavity, pharynx and larynx.[3]

Juvenile Nasopharyngeal Angiofibroma (JNA) is a benign neoplasm of the nasopharynx and accounts for 0.5% of all head and neck tumors.[4] It is a highly vascular neoplasm that affects almost exclusively adolescent males, although some authors have reported its occurrence in females.[5,6] This preferential occurrence in males suggests a hormonal influence, which has not yet been clearly established as the presence of androgen and/or estrogen receptors in tumoral tissues of JNA patients is controversial.[7,8]

Ras gene mutations have been associated to a wide range of human solid tumors and the overall frequency of mutations has been estimated to be 15%.[9] Members of the *ras* gene family (Ki-

ras, Ha-*ras* and N-*ras*) are structurally related and code for a protein (p21) known to play an important role in the regulation of normal signal transduction and cell growth. Activation of *ras* genes is due to point mutations within codons 12, 13, 59 and 61, which take part in the p21 active site.[10]

The frequency of *ras* mutations is different from one type of tumor to another, suggesting that point mutations might be carcinogen-specific.[11] Mutations of the *ras* gene family are a common event in the development and progression of adenocarcinoma of the pancreas (90%), colon (50%), thyroid (50%), bladder (50%) and lung (30%)[10]. Regarding head and neck cancer, most of the authors have found *ras* mutations in less than 5% of the tumors from western world patients,[12-15] although Nunez *et al.* (1992)[16] and Anderson *et al.* (1994)[30] found *ras* mutations in 36,3% (8/22) and 22% (6/27) of the tumors, respectively. Similar frequencies (35%) of Ha-*ras* mutations have only been reported in oral squamous cell carcinomas from India.[17] A high frequency of *ras* mutations has also been found in carcinogen-induced tumors in animal models.[18] Whereas activating

Table 1 - Associations of Ha-*ras* overexpression and clinicopathological characteristics in patients with head and neck squamous cell carcinomas

			Ha-*ras*[1]		
Characteristics	Categories	n	Negative	Positive	*p*-value[2]
Age	≤ 50 years	12	5	7	
	> 50 years	20	10	10	0.64
Sex	Male	26	11	15	
	Female	6	4	2	0.28
Tumor site	Oral cavity	16	7	9	
	Oropharynx	5	1	4	
	Hypopharynx	5	4	1	
	Larynx	4	2	2	0.26
Lymph node status	Negative	19	10	9	
	Positive	13	5	8	0.42
Histologic grade[3]	I	15	7	8	
	II	9	4	5	
	III	5	2	3	0.96
Tumor stage[4]	II	3	2	1	
	III	9	4	5	
	IV	19	8	11	0.73
Status	Alive, well	23	14	9	
	Recurrence or death	9	1	8	0.01
Tobacco consumption	Smoker	6	4	2	
	Non-smoker	23	10	13	0.58
Alcohol consumption	Yes	10	6	4	
	No	18	8	10	0.43

[1] Negative, patients without Ha-*ras* overexpression; Positive, patients with tumors with Ha-*ras* overexpression.

[2] Chi-square and Fisher's exact tests.

[3] UICC TNM staging system.

[4] WHO classification.

ras mutations appear to be an infrequent event in head and neck tumors from the western world, studies using immunohistological staining and RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) have demonstrated that overexpression is a common event in those tumors.[19,20] However, the exact mechanism accounting for *ras* overexpression is unknown and its association with existing prognostic factors is not clear yet.

When it comes to JNA, little is known about the molecular basis of this disease and only recently Giardiello *et al*. (1993),[21] reported a high incidence of JNA in patients with FAP (Familial Adenomatous Polyposis) suggesting that somatic mutations of APC (Adenomatous Polyposis Coli) gene might be associated to JNA development. This hypothesis led us to investigate whether other genes associated with sporadic colon carcinomas such as Ki-*ras* oncogene are also associated with JNA.

In the present study, we investigated a possible association between Ki-*ras* and Ha-*ras* mutations within codons 12, 13, 59 and 61 and the development of head and neck tumors and JNA, by using PCR-SSCP (Polymerase Chain Reaction-Single Strand Conformation Polymorphism) analysis. The relative levels of Ha-*ras* gene mRNA transcripts were also examined in head and neck tumors by Northern blot analysis.

METHODS

Tissue samples. Tumor samples were obtained from 60 head and neck patients under surgery at Hospital A.C. Camargo and from 28 patients with Juvenile Nasopharyngeal Angiofibroma (JNA) submitted to surgery at Hospital das Clínicas, Faculty of Medicine, University of São Paulo, São Paulo, Brazil. Normal tissue was also obtained from 12 out of the 28 patients with JNA and from all the 60 patients with head and neck cancer. Tumors consisted of squamous cell carcinomas of the head and neck, 28 localized in the oral cavity, 10 in the oropharynx, 8 in the hypopharynx and 14 in the larynx. Ages of the head and neck tumors patients at the time of operation ranged from 27 to 80 years, median 58. The study included a total of 52 males and 8 females. All the JNA patients were males and the age at the time of operation ranged from 11 to 23 years. DNA samples from one colorectal tumor and one endometrial tumor that had already been analyzed for *ras* gene mutations were used as positive controls for the SSCP analysis.

DNA and RNA extraction. Tissue was ground to a powder using a Frozen Tissue Pulverizer (Termovac). For DNA extraction the powder was resuspended in 1ml of lysis buffer (10mM Tris-HCl, pH 7.6, 1mM EDTA (ethylenediaminetetracetic acid), 0.6% SDS

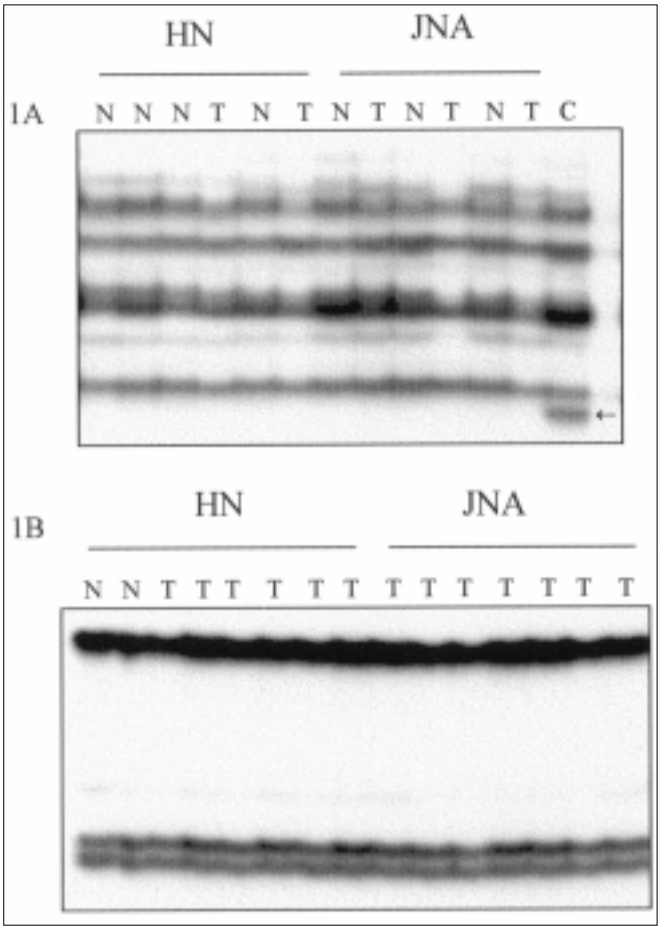

Figure 1 - Representative autoradiographs from PCR-SSCP analysis in head and neck tumors (HN) and Juvenile Nasopharyngeal Angiofibromas (JNA) for detection of Ki-*ras* (1A) and Ha-*ras* (1B) gene mutation within codons 12 and 13. N: DNA from normal tissue; T: DNA from tumoral tissue; C: colorectal tumor used as positive control for Ki-*ras* mutation (the arrow indicates the mobility shift).

(sodium dodecyl sulfate)) and 100 µg/ml proteinase K, and incubated at 37°C overnight. High molecular weight DNA was extracted with phenol-chloroform and precipitated with ethanol. For RNA extraction tissue powder was homogenized in a solution containing guanidine isothiocyanate (4M guanidine isothiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl and 100mM β-mercaptoethanol) and extracted as described by Chomczynski and Sacchi (1987).[22]

Northern blot analysis. Ten micrograms of total RNA from tumors and normal samples were denatured with formaldehyde-formamide, separated by electrophoresis on a formaldehyde 1% agarose gel, and transferred to nylon filters. Northern blot filters were hybridized under stringent conditions with a ^{32}P-labeled Ha-*ras* 6.6Kb *Bam*HI fragment (the probe was labeled using the random oligonucleotide priming technique) for 24 hours. Filters were washed twice at room temperature in 2x SSC (sodium chloride/sodium citrate)/0.1% SDS for 10 minutes and twice at 50°C for 30 minutes in 0.1x SSC/0.1% SDS and then exposed to Kodak X-Omat XAR film with an intensifying screen at -70°C for 2 or 5 days.

PCR-SSCP (Polymerase Chain Reaction Single-Strand Conformation Polymorphism) analysis. DNA sequences containing codons 12-13 and 59-61 of the Ha-*ras* and Ki-*ras* genes were amplified using oligonucleotide primers described by Ichikawa *et al*. (1994).[23] PCR reactions were performed in 25µl volume using 50-100ng of genomic DNA template, 1µM of each primer, 1.5mM $MgCl_2$, 200µM of each deoxynucleotide triphosphate, 0.1µCi of [α^{32}P-dCTP] (Amersham, specific activity, 3000Ci/mmol), 50mM KCl, 10mM Tris-HCl pH 8.0, and 0.5 unit of *Taq* DNA polymerase (Pharmacia, NJ, USA). The reactions were performed with an automated Thermal Cycler - Perkin Elmer 580 as follows: 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C. Amplified products (1µl) were diluted 10-fold in a buffer containing 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, heated at 83°C for 10 minutes and applied (3µl/lane) on 6% polyacrylamide non-denaturing gels containing 2.5, 5.0 and 10% glycerol. Electrophoresis was performed at 13W for 4 - 6 hours at room temperature with two

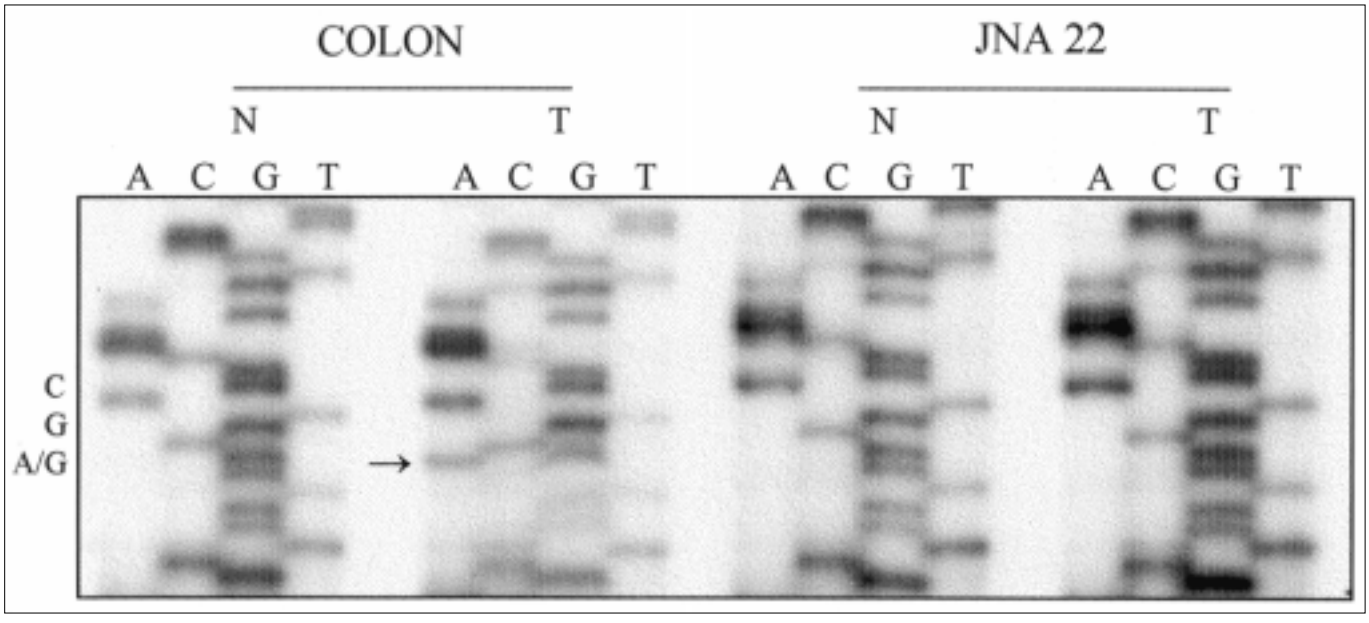

Figure 2 - Representative autoradiographs of direct DNA sequence of 12 and 13 codons of Ki-*ras* gene in Juvenile Nasopharyngeal Angiofibromas (JNA). One mutation in codon 13 was detected in the colorectal tumor sample used as a positive control for PCR-SSCP analysis. The arrow indicates the alteration observed (GGC to AGC; Gly to Ser). N: DNA from normal tissue; T: DNA from tumoral tissue.

cooling fans. Band shift mobility was detected by autoradiography of dried gels using Kodak X-Omat XAR film with an intensifying screen for 5 to 48 hours at -70°C.

Direct DNA sequencing. DNA samples from 1 colorectal tumor with Ki-*ras* mutation detected by SSCP gels, one endometrial tumor with Ha-*ras* mutation, three JNA samples and three head and neck tumor samples were reamplified and direct DNA sequencing was performed. PCR products obtained were purified using Wizard PCR Preps Kit (Promega Corporation, Madison, USA) in accordance with the manufacturer's procedures. Purified DNA was submitted to a dideoxy chain termination reaction using a double strand DNA Cycle Sequencing Kit (Pharmacia, USA) for both sense and antisense primers. Sequencing reaction products were denatured and resolved on 6% denaturing urea/polyacrylamide gels. Gels were fixed for 15 minutes in a 10% methanol/10% acetic acid solution, dried and exposed to X-ray film overnight.

Statistical Methods. Analysis of statistical correlations between Ha-*ras* overexpression and the clinicopathological characteristics of the patients were performed by the χ^2 test and Fisher's Exact test for frequency data in contingency tables.

RESULTS

Tumor DNA from 60 patients with head and neck cancer and from 28 patients with Juvenile Nasopharyngeal Angiofibromas (JNA) were examined for the occurrence of point mutation in Ha-*ras* and Ki-*ras* genes using PCR-SSCP analysis. Representative autoradiographs from SSCP analysis are shown in Fig. 1. No mutations were found in both series of DNA samples analyzed for codons 12, 13, 59 and 61 of the Ha-*ras* and Ki-*ras* genes.

Some JNA and head and neck tumor samples were chosen at random and submitted to direct DNA sequencing in order to confirm the absence of mutation within the specific codons of Ha-*ras* and Ki-*ras* genes. One colorectal and one endometrial tumor sample were also submitted to direct DNA sequencing, as positive controls of Ki-*ras* and Ha-*ras* mutations, respectively. Direct sequencing of both strands of the four amplified PCR products did

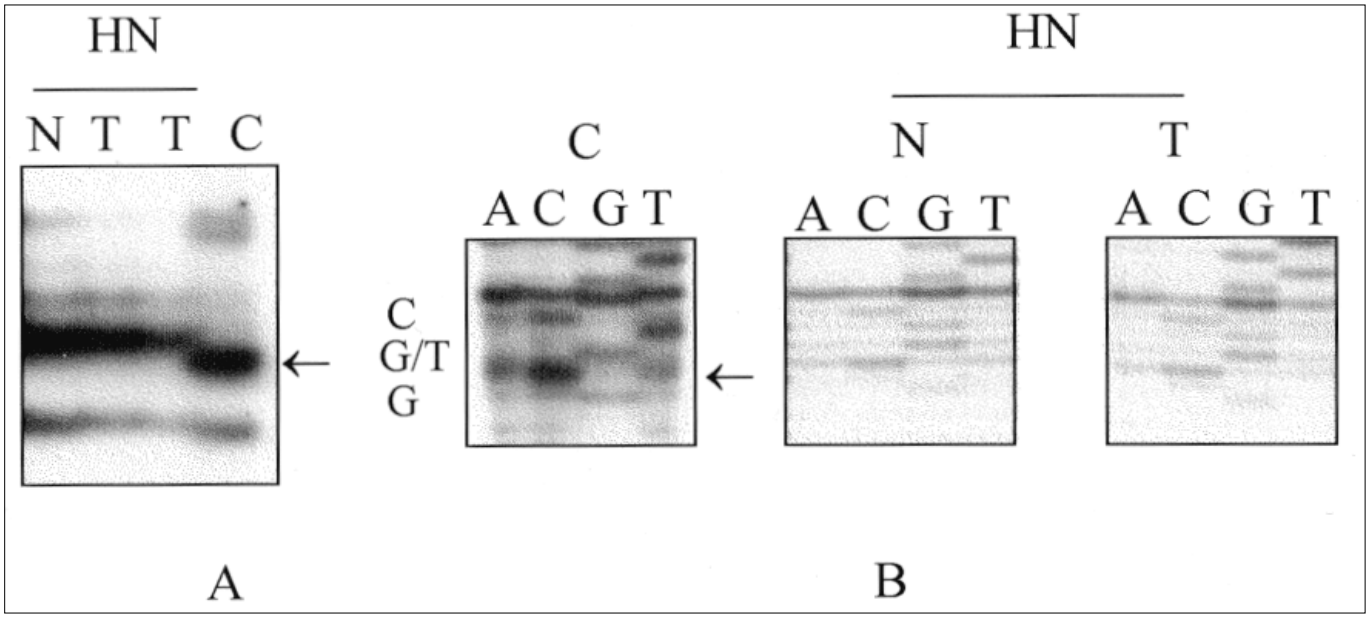

Figure 3 - Representative autoradiographs from PCR-SSCP analysis (A) and direct DNA sequence (B) of codons 12 and 13 of the Ha-*ras* gene in head and neck tumors (HN). One mutation in codon 12 was detected in the endometrial tumor sample used as a positive control for PCR-SSCP analysis. The arrow indicates the alteration observed (GGC to GTC; Gly to Val). N: DNA from normal tissue; T: DNA from tumoral tissue; C: positive control.

not reveal any point mutation in head and neck tumor or JNA samples analyzed. Representative results showing evidence of absence of mutation within codons 12-13 of Ki-*ras* and Ha-*ras* genes in JNA and head and neck tumor samples are shown in Figs. 2 and 3. The presence of point mutations within the positive control samples were confirmed on Ki-*ras* codon 13 (colorectal tumor; GGC to AGC; Gly to Ser) and on Ha-*ras* codon 12 (endometrial tumor; GGC to GTC; Gly to Val).

In 32 of the head and neck cases where total RNA from normal and tumor samples were available, the relative level of Ha-*ras* mRNA expression was examined by Northern blot analysis. Representative results of the Northern blot analysis are shown in Fig. 4. Using densitometric scans, Ha-*ras* mRNA transcripts were found to be overexpressed in 17/32 (53%) of tumor samples relative to the normal sample derived from the same patient. The relative level of Ha-*ras* overexpression in these tumors ranged from 2- to 15-fold. A probe for 18S ribosomal RNA was used to correct the differences between normal and tumor RNA loading.

To evaluate the contributions of Ha-*ras* overexpression to the development and/or progression of the head and neck tumors analyzed, clinicopathological characteristics of the cases with Ha-*ras* overexpression were compared with the characteristics of those cases that showed normal Ha-*ras* expression. No correlation was observed between Ha-*ras* overexpression and clinicopathological characteristics of the patients, such as age, histology, site of the tumor, TNM stage or lymph node status (Table 1). *Ras* overexpression was observed in tumors of patients in all clinical stage but with a trend to be more frequent in stage III tumors. In the present study we found a significant association between *ras* overexpression and clinical outcome. Recurrence or death due to the disease occurred more frequently in patients with tumors showing high levels of Ha-*ras* gene (8/17) than in patients with tumors without Ha-*ras* overexpression (1/15) ($p = 0.01$).

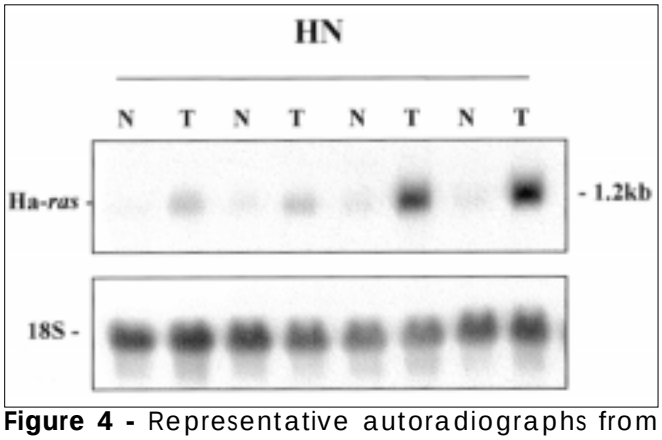

Figure 4 - Representative autoradiographs from Northern blot analysis of Ha-*ras* transcripts in the tissue samples of five patients with head and neck carcinomas (HN). N: DNA from normal tissue; T: DNA from tumoral tissue.

DISCUSSION

Our results show no evidence of mutations within codons 12, 13, 59 and 61 of Ki-*ras* and Ha-*ras* either in Juvenile Nasopharyngeal Angiofibroma (JNA) or in head and neck tumors from a group of Brazilian patients. No data is available about Ki-*ras* and Ha-*ras* mutations related to the development of JNA but when it comes to head and neck tumors, our data are in accordance with other studies that show low rates of *ras* mutations (less than 5%) in head and neck tumors from western populations. Yarbrough *et al.* (1994)[15] analyzed 51 samples from head and neck squamous cell carcinomas and found no evidence of Ki-*ras*, Ha-*ras* and N-*ras* mutations within codons 12, 13 and 61. Irish and Bernstein (1993)[24] obtained similar results, not finding Ki-*ras* mutations within codons 12 and 13. In addition, Kiaris *et al.* (1995)[20] found *ras* gene mutations in only 1.7% of the samples analyzed, when studying a larger panel of squamous cell carcinomas of the head and neck. On the other hand, Nunez *et al.* (1992)[16] and Anderson *et al.* (1994)[30] found *ras* mutations in 36.3% (8/22) and 22% (6/27) of the samples analyzed, respectively, although these discrepancies might be due to geographical differences. Interestingly, this frequency of oral squamous cell carcinomas harboring activating *ras* mutations is similar to that found in India[17] and Taiwan,[25] where betel quid chewing is thought to be the initiating agent.[17,19] A high frequency of *ras* mutations has also been found in carcinogen-

induced tumors in animal models.[18]

Although *ras* mutations do not appear to play a major role in head and neck tumors from Caucasian patients, several studies including ours have revealed that *ras* overexpression is a frequent event in these tumors.[15,19,20] The implication of this finding is still unclear. The same situation is observed in breast cancer in which *ras* gene mutations are rare[26] but overexpression has been reported in about 60% of the tumors analyzed.[27,28] In both types of tumors, *ras* gene amplification is a rare event and the overexpression observed may be due to another activation mechanism of gene expression.[19,27,28] Iwasaka *et al.* (1993)[29] proposed that Ha-*ras* overexpression in HPV transfected cell lines might be due to loss of tumor suppressor gene function or direct integration of HPV DNA sequences in close proximity to cellular oncogenes. In addition, Anderson *et al*. (1994)[30] found Ha-*ras* overexpression associated with HPV infection in 11% of oral squamous cell carcinomas suggesting that viral infection might be associated with *ras* overexpression.

In the present study, no correlations were observed between Ha-*ras* overexpression and clinicopathological characteristics of the patients. However, Ha-*ras* overexpression was associated with poor prognosis, in accordance with the results reported by Azuma *et al*. (1987).[31] On the other hand, using a different technical approach, Field *et al*. (1992)[19] and Kiaris *et al*. (1995)[20] also reported that overexpression is a frequent event in squamous cell carcinomas of the head and neck, but found an association with a favorable prognosis. These differences regarding the prognostic value of *ras* overexpression in head and neck cancer may be mainly due to the use of small series of patients with heterogeneous composition. Further studies examining larger series of patients are required to clarify whether there is an association between Ha-*ras* overexpression and clinical outcome.

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RESUMO

Contexto: Mutações nos genes *ras* têm sido associadas a diversos tumores sólidos humanos. Membros da família de genes *ras (Ki-ras, Ha-ras e N-ras)* são estruturalmente relacionados e codificam para uma proteína (p21) que desempenha papel importante na regulação da transdução de sinal e crescimento celular. **Objetivos:** Estudar a ocorrência de mutações nos genes Ki-ras e Ha-*ras*. Também estudamos a expressão do gene Ha-*ras* em 32 dos tumores de cabeça e pescoço. **Tipo de estudo:** Série de casos. **Participantes:** 60 tumores de cabeça e pescoço e 28 nasoangiofibromas obtidos através de cirurgia. **Local:** Hospital A. C. Camargo e Hospital das Clínicas da Universidade de São Paulo, respectivamente. **Teste diagnóstico:** Pela técnica de PCR-SSCP examinamos a ocorrência de mutações nos genes Ki-*ras* e Ha-*ras*. O nível relativo do mRNA de Ha-*ras* em 32 dos tumores de cabeça e pescoço foi examinado por Northern blot. **Resultados:** Nenhum dos tumores de cabeça e pescoço nem os nasoangiofibromas apresentaram evidência de mutação nos codons 12, 13, 59 e 61 dos genes Ki-*ras* e Ha-*ras*; contudo, 17 (53%) dos tumores, onde a expressão gênica pode ser examinada, demonstraram níveis aumentados do mRNA de Ha-*ras* quando comparados com o tecido normal do mesmo paciente. **Conclusões:** Nossos resultados demonstram, pela primeira vez, que mutações nos genes Ki-*ras* e Ha-*ras* não estão associadas ao desenvolvimento dos nasoangiofibromas e confirmam trabalhos anteriores que demonstram que mutações que ativam o gene *ras* estão ausentes ou raramente associadas aos tumores de cabeça e pescoço de pacientes ocidentais. Além disso, nossos resultados sugerem que o aumento de expressão de Ha-*ras*, e não a mutação desse gene, possa ser um fator importante no desenvolvimento e progressão dos tumores de cabeça e pescoço.