Search for mutations in signaling pathways in head and neck squamous cell carcinoma

THAIS GULIM DE CARVALHO[1], ANA CAROLINA DE CARVALHO[1], DANIELLE CALHEIROS CAMPELO MAIA[1], JULIANA KAORI OGAWA[1], ANDRE LOPES CARVALHO[2] and ANDRE LUIZ VETTORE[1]

[1]Cancer Molecular Biology Laboratory, Department of Science Biology, Federal University of São Paulo (UNIFESP), 04039-032, São Paulo, SP; [2]Department of Head and Neck Surgery, Barretos Cancer Hospital, 14784-400, Barretos, SP, Brazil

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Abstract. Mutations in JAK-STAT signaling pathway genes have been associated with the development of various hematological tumors, but have not been investigated in head and neck tumors, and the *PIK3CA*, *BRAF* and *KRAS* genes have been described in a few cases of head and neck squamous cell carcinoma (HNSCC). In the present study, we determined the mutation status in members of the MAPK, PI3K-AKT and JAK-STAT pathways in HNSCC. Mutations in the *KRAS*, *BRAF*, *PIK3CA*, *JAK1* and *JAK2* genes were evaluated in 94 HNSCCs by direct DNA sequencing analysis using cDNA synthesized from RNA extracted from patient tumor cells. All patients evaluated had wild-type *KRAS*, *BRAF* and *PIK3CA* genes. Furthermore, although some known polymorphisms have been found in *JAK1* genes (rs45598436, rs17127063, rs2230587, rs3737139, rs2230588 and rs12129819) and *JAK2* (rs10429491, rs2230723, rs2230724 and rs41316003), no mutation could be detected. Our data indicate that mutations in these kinase genes seem to be rare events in HNSCC.

Introduction

Head and neck cancer is among the 10 most common types of cancer, with more than 600,000 new cases diagnosed annually worldwide (1); the squamous cell carcinoma subtype is the most frequent, corresponding to 90% of all cases (2). This disease originates at distinct head and neck topologies including the oral cavity, the pharynx and the larynx. Due to the critical location in the upper aerodigestive tract, these types of cancer and their treatment significantly impair patient quality of life as they affect breathing, swallowing, speech and even appearance. In the past decades, a reduction in the mortality rates has been observed in carcinomas of different anatomical sites; however, this trend was not observed in the head and neck squamous cell carcinoma (HNSCC) (3). Despite different strategies used in the current treatment of HNSCC, a high percentage of these patients progresses with locoregional and distant recurrences (4). Therefore, identifying molecular aberrations in HNSCC may improve our understanding of head and neck carcinogenesis, allowing for the identification of new strategies for subdividing patients into biologically and clinically relevant subgroups, and highlighting novel therapeutic options.

Cancer is a progressive genetic disease characterized by the accumulation of genetic and epigenetic modifications. Among the most common genetic alterations are the mutations in coding and non-coding sequences (5) and the presence of mutations in the phosphorylation domain of protein kinases that have been linked to the development of various tumors. Therefore, protein kinase inhibitors have demonstrated a significant efficacy in the treatment of different types of cancer (6).

An important cellular signaling pathway is the RAF/MEK/ERK (MAPK) pathway, which plays a role in mediating cellular response to cell growth (7). Several studies have reported the presence of hotspot mutations in *KRAS* (codons 12 and 13) and *BRAF* (codons 469 and 600) which produce perpetually active proteins resulting in increased proliferation and survival signaling (8-13). There are few reports on the frequency of mutations in *KRAS* and *BRAF* genes in HNSCC cases (7,14-16).

KRAS downstream effector pathways also include the PI3K-AKT molecular signaling cascade. The presence of mutations in *PIK3CA* hotspots (codons 542, 545 and 1047) was linked to increased cell survival by inhibiting apoptosis (17). Mutations in *PIK3CA* have been found in different types of human cancer, including lung, gastric, breast, ovarian, hepatocellular, pancreatic, esophageal and head and neck (18-22).

Aside from the MAPK pathway, the JAK-STAT signaling pathway also controls cell proliferation, differentiation and survival by transmitting signals from the plasma membrane to the cell nucleus (23). In cells, cytokine receptors are associated through its cytosolic domain to one of the four members of

Correspondence to: Professor Andre Luiz Vettore, Cancer Molecular Biology Laboratory, Department of Science Biology, Federal University of São Paulo (UNIFESP), Pedro de Toledo, 04039-032, São Paulo, SP, Brazil
E-mail: andre.vettore@gmail.com

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the JAK family of tyrosine kinases (JAK1, JAK2, JAK3 and TYK2). Conformational changes in the receptor enable JAKs to transphosphorylate and also phosphorylate the receptor itself, activating them. Both are capable of recruiting active substrates such as STAT proteins (signal transducer and activator of transcription proteins), which when phosphorylated, become active, translocate to the nucleus and regulate expression of different genes (23). In recent years, several studies have correlated the presence of mutations in proteins of the JAK-STAT pathway with hematopoietic disorders. High frequency of JAK2 V617F mutation is observed in myeloproliferative cases, whereas *JAK1* and *JAK3* sequence alterations have been found in cases of myeloid leukemia megakaryoblastic, acute lymphoblastic leukemia and lymphomas (24-26). In solid tumors, mutations in *JAK1* have been detected in lung and breast cancer and changes in the *JAK3* gene were identified in breast and gastric carcinomas (27).

Therapies targeting inhibition of multiple points along the signal transduction pathways are potential new approaches in the treatment of cancer. In non-small cell lung cancer (NSCLC), the presence of activating mutations within the kinase domain of epidermal growth factor receptor (EGFR) is common (28). Therapeutic approaches based on drugs that inhibit the tyrosine kinase activity of this receptor (gefitinib and erlotinib) have improved survival rates for NSCLC patients (29).

Cetuximab, a chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), is used in treatment of colorectal cancer (CRC). Recently, the presence of mutations in *KRAS* was described as a marker for the resistance to therapy with cetuximab in CRC (30). In other words, CRC patients carrying mutations in this oncogene cannot benefit from treatment with this drug, unlike patients whose tumors have wild-type *KRAS*.

Cetuximab is the only kinase-targeted therapy approved by the Food and Drug Administration (FDA) for the treatment of locally and regionally advanced HNSCC in combination with radiation. However, this agent generally presents only modest efficacy, and efforts to identify the subset of responsive patients who could benefit from this therapy have not been successful (31).

The aim of the present study was to examine the mutation status of *KRAS*, *BRAF*, *PIK3CA*, *JAK1* and *JAK2* in primary head and neck cancer and to verify if the mutation status could identify specific molecular subgroups of HNSCC with clinical or therapeutic implications and if *KRAS* mutations could be useful as a marker for the resistance to therapy with cetuximab in HNSCC.

Materials and methods

Patients. This retrospective study involved 94 tumor specimens obtained from patients diagnosed with HNSCC, who were treated at the Department of Head and Neck Surgery of Barretos Cancer Hospital (São Paulo, Brazil) between 2007 and 2010. These samples, available at the tumor bank of the Hospital, were collected during surgery for treatment of HNSCC. Tissue samples were snap-frozen in liquid nitrogen within 30 min after resection and stored at -80˚C. Only patients diagnosed with primary HNSCC, not previously treated, that were over 18 years of age, treated surgically with curative intent and presenting with tumors at the oral cavity, the pharynx, or the larynx were included in the study. The HNSCC diagnoses were confirmed by a pathologist and all samples were examined microscopically for the presence of neoplastic tissue and the absence of contaminating normal mucosa.

The study protocol was reviewed and approved by the Ethics Committee of Barretos Cancer Hospital and was performed in accordance with the ethics guidelines of the 1975 Declaration of Helsinki.

RNA extraction and cDNA synthesis. Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. The concentration of the resulting RNA was measured using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Fischer Scientific, Waltham, MA, USA) and the quality of the RNAs was checked by electrophoresis on 1% agarose gel stained with SYBR® Safe (Invitrogen).

Two micrograms of total RNA from each sample were used to synthesize cDNA molecules using SuperScript® III First-Strand Synthesis system (Invitrogen) according to the conditions provided by the manufacturer. The cDNA obtained was diluted 10x prior to use.

Sequencing analysis. PCR amplification of cDNA was performed and analyzed for mutations at hotspots of *KRAS* (codons 12 and 13), *BRAF* (codons 469 and 600) and *PIK3CA* (codons 542, 545 and 1047), as previously reported (32-34). To search for mutations in *JAK1* and *JAK2*, primers were designed to generate seven fragments covering the entire coding region of each gene. All primers used in this study are presented in Table I.

Amplification reactions contained 1 μl of cDNA as template in a 25 μl reaction mixture consisting of 1X reaction buffer, 1 mmol/l deoxynucleotide triphosphate, 2 mmol/l magnesium, 200 nmol/l of each primer, and 1.2 unit Platinun Taq (Invitrogen). Following purification using QIAquick® Multiwell PCR Purification Kit (Qiagen, Hilden, Germany) and Gel Band Purification (GE Healthcare, Little Chalfont, Buckinghamshire, UK), the PCR products were subjected to sequencing (both directions) using BigDye® Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA), ethanol precipitation, and an automated sequencer (ABI PRISM 3130XL Genetic Analyzer; Applied Biosystems).

The sequences obtained were analyzed with the BioEdit Sequence Alignment Editor software (35) and the Phred/Phrap/Consed Package (36). The sequences were compared to a human reference sequence, available at UCSC Genome Bioinformatics (http://genome.ucsc.edu/). The annotation of the sequence variations identified was performed using the Ensembl database (http://www.ensembl.org/) and the NCBI database (http://www.ncbi.nlm.nih.gov/).

Results

Patient characteristics. Table II summarizes the clinical and histological characteristics of the patients enrolled in the study. Ninety four primary tumor samples were collected from untreated HNSCC patients (80 male and 14 female). Median age was 57.8 years (range, 32-82 years). Tobacco or alcohol

Table I. Primers used for amplification of fragments from *BRAF*, *KRAS*, *PIK3CA*, *JAK1* and *JAK2* genes.

Fragment	Sense	Sequence 5'-3'	Annealing temperature (°C)	Size (bp)
BRAF-11[b]	F	GACGGGACTCGAGTGATGAT	62	155
	R	CTGCTGAGGTGTAGGTGCTG		
BRAF-15[b]	F	GCACAGGGCATGGATTACTT	62	195
	R	GATGACTTCTGGTGCCATCC		
KRAS[c]	F	GGCCTGCTGAAAATGACTGAA	62	253
	R	CACAAAGAAAGCCCTCCCCA		
PI3K-9[d]	F[a]	AATTGGTCTGTATCCCGAG	60	199
	R	CGGGGATAGTTACACAATAGT		
PI3K-20[d]	F[a]	TGGAATGCCAGAACTACAATC	60	175
	R	ATGCTGTTTAATTGTGTGGAAG		
JAK1-1	F	GCCCAGGCGCACACGGA	62	680
	R	GACAGCCATCCCTAGCACTCGTTC		
JAK1-2	F	TTTGGTGAAATGCCTGGCTCCTAT	62	594
	R	CCACTCTTCCCGGATCTTGTTTTTC		
JAK1-3	F	CTGGAAAATAAACACAAGAAGGATGAGGAG	62	610
	R	CGGGGCTTGGGCTGGC		
JAK1-4	F	AGCCACCTCAAGAAGCAGATCCTG	62	642
	R	TGAGCTTGATGAATGGGCCACA		
JAK1-5	F	GAAATGTGTGTACTAAAAACCTCCTCCTGG	62	636
	R	TCCTTTTTCAGATCAGCTATGTGGTTACC		
JAK1-6	F	GGGGACAATACAGGGGAGCAGG	62	408
	R	GTGTAATACTCCTTATCGGTTTCAATTGCTTT		
JAK1-7	F	TTCACCGGGACTTGGCAGCA	62	525
	R	CATTTGTTGCAGGAGAAGGACTTGATAA		
JAK2-1	F	AGAAGCAGGCAACAGGAACAAGATG	62	529
	R	ACGTTCAGCACCTCGAGATATTCCAT		
JAK2-2	F	TGAGTCAACCAGGCATAATGTACTCTACAG	62	588
	R	CCTCTTGACCACTGAATTCCACCG		
JAK2-3	F	GGAAACTCTGCAGTCTGCCTTCTACAC	62	638
	R	AGTTCTTCTTTGTCCCACTGAGGTTGTAC		
JAK2-4	F	TTTTTGACTTTTGCTGTCGAGCGA	62	693
	R	TTCTTCTAGAAAATGCATGGCCCA		
JAK2-5	F	TGGAGTATGTGTCTGTGGAGACGAGAA	62	693
	R	AACTGTGTAGGATCCCGGTCTTCAAA		
JAK2-6	F	GCCTTCTTTCAGAGCCATCATACGA	62	703
	R	CTCTCTGTCAGTGATTCTGGAGCATACC		
JAK2-7	F	CGAGAAATATATTGGTGGAGAACGAGAAC	62	659
	R	AATGTCTTTTACTGGTGGCCTCATGAA		

[a]Minor modifications in the primer sequences with respect to the sequences previously described. Described references: [b](32), [c](33), [d](34).

consumption (current or former) was reported by 91.2 and 74.7% patients, respectively. Primary tumor sites included: oral cavity (70.2%), larynx (14.9%), oropharynx (12.8%) and hypopharynx (2.1%). Regarding tumor stage, the majority was T3/T4 (81.7%). Forty two patients presented positive lymph nodes, among those 15 (19.0%) presented extracapsular spread. Perineural invasion was detected in 24 cases (27.0%) among 89 patients with data available, while lymphovascular invasion was observed in 15 cases (17.4%) among 86 patients with data available.

Table II. Demographic and clinical characteristics of the HNSCC patients included in this study (n=94).

Parameter	n	%
Age (years)		
<55	33	35.1
≥55	61	64.9
Mean	57.8	
Range	32-82	
Gender		
Male	80	85.1
Female	14	14.9
Tobacco consumption		
Yes	83	91.2
No	8	8.8
Alcohol consumption		
Yes	65	74.7
No	22	25.3
Tumor site		
Oral cavity	66	70.2
Larynx	14	14.9
Oropharynx	12	12.8
Hypopharynx	2	2.1
T stage[a]		
T1/T2	17	18.3
T3/T4	76	81.7
N stage		
N0	41	49.4
N+	42	50.6
Extracapsular spread		
Yes	15	19.0
No	64	81.0
Perineural invasion		
Yes	24	27.0
No	65	73.0
Lymphovascular invasion		
Yes	15	17.4
No	71	82.6

[a]Stage according to clinical TNM classification of malignant tumors (TNM Classification of Malignant Tumours, 6th edition).

Mutation analysis. The presence of sequence variations in the hotspots previously described in the coding regions of *KRAS* (codons 12 and 13), *BRAF* (codons 469 and 600) and *PIK3CA* (codons 542, 545 and 1047) was examined in cDNA molecules from 94 HNSCC specimens. The assembly of the consensus sequences, as well as the evaluation of base qualities, were conducted with Phred/Phrap/Consed Package (36). No somatic mutations were detected in any of these genes in all cases examined. Representative cases are shown in Fig. 1.

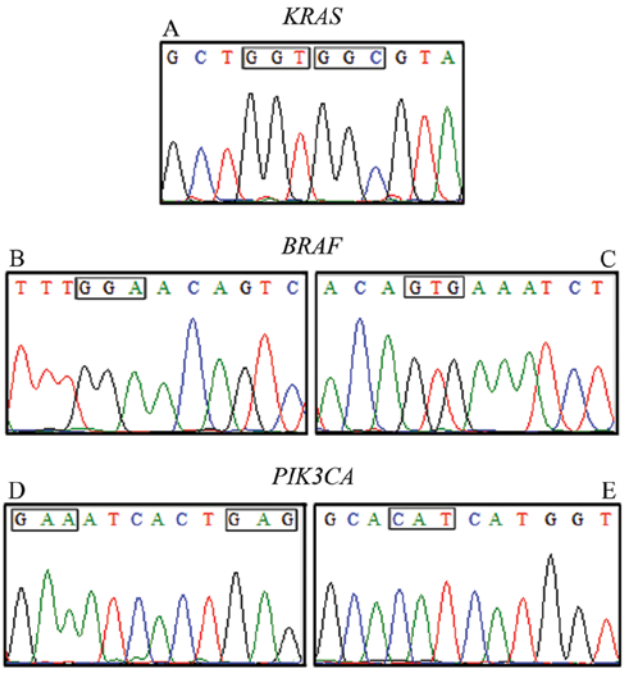

Figure 1. DNA sequencing performed on tumor samples of 94 patients with HNSCC. Representative cases of wild-type *KRAS* codons 12 and 13 (A), *BRAF* codons 469 (B) and 600 (C), and *PIK3CA* codons 542, 545 (D) and 1047 (E).

Mutations in *JAK1* and *JAK2* genes have been reported in different types of cancer, mainly in hematological malignances; however, no hotspots were identified in the sequence of these genes. Therefore, primers were designed to generate overlapping fragments covering the entire coding region of *JAK1* (seven fragments, size 408 to 680 bp) and *JAK2* (seven fragments, size 529 to 703 bp). We searched for sequence variations in 20 HNSCC samples, but were not able to detect any mutations within the *JAK1* and *JAK2* coding sequences.

The analysis of the complete sequences from *JAK1* and *JAK2* genes allowed the identification of some sequence variations reported as polymorphisms in the Ensembl database. *JAK1* presented 6 different polymorphisms at positions 579 T>C (codon 193), 1977 C>T (codon 659), 2049 C>T (codon 683), 2097 C>G (codon 699), 2199 A>G (codon 733) and 3096 G>A (codon 1032). Of note, none of these alterations resulted in amino acid changes (Table III). For *JAK2*, we found four different polymorphisms at positions 489 C>T (codon 163), 1177 C>G (codon 393), 2490 G>A (codon 830) and 3188 G>A (codon 1063) (Table IV). The alteration of the C nucleotide by a G at position 1177 promotes a substitution of a Leucine residue by a Valine (codon 393), whereas the replacement of G at position 3188 by an A induces the change of an Arginine by a Histidine (codon 1063) (Table IV).

Discussion

The management of HNSCC patients has improved in recent years with developments in surgical techniques, the introduction of altered fractionation radiation, new chemotherapeutic agents, and combined use of chemoradiotherapy; however, long-term survival rates have improved only marginally, and the 5-year overall survival rate remains approximately 50% (3).

Table III. Single nucleotide polymorphisms (SNPs) of the *JAK1* gene found in 20 HNSCC samples analyzed.

SNP	Codon	Exon	Amino acid	No. of cases	Frequency observed (%)
rs 45598436	193 GCT>GCC	6	Ala>Ala	4	20
rs 17127063	659 CGC>CGT	14	Arg>Arg	1	5
rs 2230587	683 AGC>AGT	15	Ser>Ser	6	30
rs 3737139	699 GCC>GCG	15	Ala>Ala	1	5
rs 2230588	733 CCA>CCG	16	Pro>Pro	5	25
rs 12129819	1032 AAG>AAA	22	Lys>Lys	4	20

Table IV. Single nucleotide polymorphisms (SNPs) of the *JAK2* gene found in 20 HNSCC samples analyzed.

SNP	Codon	Exon	Amino acid	No. of cases	Frequency observed (%)
rs 10429491	163 CAC>CAT	7	His>His	13	65
rs 2230723	393 CTT>GTT	9	Leu>Val	1	5
rs 2230724	830 CTG>CTA	20	Leu>Leu	17	85
rs 41316003	1063 CGT>CAT	25	Arg>His	2	10

To date, two promising strategies tested for the treatment of HNSCC are blocking growth factors signaling pathways and interfering in pathways related to angiogenesis. EGFR has been studied extensively as a therapeutic target due to its high expression in different tumors and its influence in the regulation of proliferation, apoptosis, metastasis, angiogenesis, and cell differentiation. The extracellular portion of EGFR can be inhibited by monoclonal antibodies and intracellular part by tyrosine kinase inhibitors. The monoclonal antibody cetuximab, an EGFR extracellular inhibitor, has been used in the treatment of colorectal tumors (37). Therapies targeting epidermal growth factor receptor (EGFR/HER-1/ERBB1) pathways have also been used in HNSCC, but only a minority of patients have benefited from these treatments, since small-molecule tyrosine kinase inhibitors (gefitinib and erlotinib) and monoclonal antibodies against EGFR (cetuximab) have shown limited efficacy (38-40).

It is well known that cetuximab prolongs overall survival of locally advanced HNSCC patients when delivered in combination with radiation (41). This drug has also shown activity in the first-line treatment of recurrent and/or metastatic HNSCC in combination with cisplatin or cisplatin/fluorouracil (42,43). However, the response rates of cetuximab as a single agent are approximately 13% (40). Thus, the identification of clinically effective markers for selection of HNSCC patients responsive to cetuximab therapy is of paramount importance.

In colorectal cancer, there is a well-established association between the presence of mutations in *KRAS* and the response to cetuximab treatment (30). Considering all types of human cancer, *KRAS* mutations are the most common type of Ras mutation, with *HRAS* being the least common; however, in head and neck cancer, *Ras* mutations appear to be exclusively *HRAS* mutations (44). To date, few studies have attempted to assess the *KRAS* mutation status in HNSCC and their results are contradictory. In a recent study, Smilek *et al* (14) examined the *KRAS* mutation status of 27 HNSCC patients and found alterations in 14.8% (4/27) of the cases. Weber *et al* (7) found *KRAS* mutations in 6% (3/89) of HNSCC samples examined, while Van Damme *et al* (15) evaluated 22 HNSCC specimens and identified a mutation in one case (4.5%). On the other hand, no *KRAS* mutation was detected among 183 HNSCC samples investigated from a Japanese cohort (45). Mutations in the *KRAS* gene were also infrequent in oral squamous cell carcinomas (OSCCs) (16,46,47). In the present study, wild-type *KRAS* was detected in all 94 HNSCC samples evaluated. In light of these findings and in contrast to colorectal cancer, our results together with others indicate that *KRAS* mutations may not be considered a predictor of sensitivity to cetuximab in HNSCC.

As well as the RAS family, another member of the MAPK pathway is *BRAF*. Somatic point mutations of *BRAF*, such as those that occur at hotspot V600E of its kinase domain, can result in elevated BRAF kinase function (48). Mutations in this gene have been associated with various types of cancer, including non-Hodgkin lymphoma, colorectal cancer, thyroid cancer and lung carcinoma (10,11,49,50). A German study found *BRAF* mutated in 3% (3/89) of HNSCC cases evaluated (7), while a study evaluating American HNSCC patients reported *BRAF* mutations in 2.4% (1/42) of the cases (16). In the present study, none of the 94 samples analyzed presented alterations in *BRAF* sequence.

Mutations in PIK3CA, the catalytic subunit of PI3-kinase, are reported to have higher oncogenic potential. Recent exome sequencing analyses revealed *PIK3CA* activating mutations in 6-8% of HNSCC (51,52). Two independent studies, evaluating 38 HNSCC samples and 37 OSCC specimens, identified *PIK3CA* mutations in approximately 11% of the cases (47,53). Kozaki *et al* (54) detected mutations in *PIK3CA* in 7.4% (8/108) of OSCC, whereas Fenic *et al* (55) and Bruckman *et al* (16) found a lower frequency of mutations in HNSCC (2.9 and 3%,

respectively). A study analyzing South-Asian HNSCC patients found *PIK3CA* mutations in 10.5% (2/19) of the Indian cases, while only wild-type *PIK3CA* was detected in Vietnamese tumors (0/18) (56). We did not identify any *PIK3CA* mutations in all 94 HNSCC patients evaluated. It should be noted that we are unable to discuss the presence of mutations in other gene regions not within hotspots previously described in *KRAS*, *BRAF* and *PIK3CA*.

The MAPK and PI3K-AKT signaling pathways are involved in HNSCC tumorigenesis, but the oncogenic activation of these pathways may include additional mechanisms other than point mutations, such as chromosomal amplification or gene overexpression. Consistent with this, *PIK3CA* was found frequently amplified in OSCC (57-59) and Hoa *et al* (60) detected increased expression of *KRAS* in HNSCC cell lines.

The JAK-STAT signaling pathway is essential for the physiology of healthy individuals as it mediates cytokine responses in hematopoietic cells. This pathway is also responsible for regulating the hormone receptor signaling, including various growth hormones and prolactin receptors. In recent years, several mutations in *JAK* genes associated with constitutive activation were identified and it is possible that mutations in this pathway component are present in various types of cancer (23). It is important to define the role of this pathway in solid tumors, as new therapeutic agents that have JAK as target are being developed and some JAK kinase inhibitors are already being tested in clinical trials (61).

Mutations in *JAK* genes are commonly found in hematological tumors (62-64); however, few mutations have been described in solid tumors, such as breast, lung and gastric tumors (27). To the best of our knowledge, our study is the first to search for *JAK* mutations in HNSCC. We analyzed the complete cDNA sequence of *JAK1* and *JAK2* genes in 20 HNSCC samples and, although some known polymorphisms have been evidenced, no mutation could be detected.

In conclusion, the present study investigated the presence of mutations in *JAK1* and *JAK2* genes in HNSCC patients examined and also evaluated the presence of sequence alterations in *PIK3CA*, *BRAF* and *KRAS* genes. Overall, no mutation in these genes could be detected in the HNSCC specimens examined. Also, *KRAS* mutation suggests that alterations in this gene do not seem to be useful as a cetuximab predictor of sensitivity in HNSCC.

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