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TP53 and P16^{INK4A}, but not H-KI-RAS, are Involved in Tumorigenesis and Progression of Pleomorphic Adenomas

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The putative role of *TP53* and *p16*^{INK4A} tumor suppressor genes and Ras oncogenes in the development and progression of salivary gland neoplasias was studied in 28 cases of pleomorphic adenomas (PA), 4 cases of cystic adenocarcinomas, and 1 case of carcinoma ex-PA. Genetic and epigenetic alterations in the above genes were analyzed by Polymerase Chain Reaction/Single Strand Conformational Polymorphism (PCR/SSCP) and sequencing and by Methylation Specific-PCR (MS-PCR). Mutations in TP53 were found in 14% (4/28) of PAs and in 60% (3/5) of carcinomas. Mutations in H-Ras and K-Ras were identified in 4% (1/28) and 7% (2/28) of PAs, respectively. Only 20% (1/5) of carcinomas screened displayed mutations in K-Ras. p16^{INK4A} promoter hypermethylation was found in 14% (4/28) of PAs and 100% (5/5) carcinomas. All genetic and epigenetic alterations were detected exclusively in the epithelial and transitional tumor components, and were absent in the mesenchymal parts. Our analysis suggests that TP53 mutations and p16^{INK4A} promoter methylation, but not alterations in the *H-Ras* and *K-Ras* genes, might be involved in the malignant progression of PA into carcinoma. J. Cell. Physiol. 207: 654–659, 2006. © 2006 Wiley-Liss, Inc.

Salivary gland (SG) neoplasias are quite rare, accounting for approximately 2–3% of head and neck tumors (De Vita et al., 2001). Most SG tumors are benign and are generally pleomorphic adenomas (PAs). SG tumors are characterized by an extremely varied histomorphological phenotype due to combinations of different epitheliomorphic and mesenchymomorphic features. The molecular mechanisms determining these two histomorphological types and their coexistence in SG tumors are not fully understood, with many aspects of their histogenesis still under discussion. Two relevant theories have been proposed: the "different cell" theory suggests that SG neoplasias originate from two distinct cell populations, that is, mesenchymal and (myo-) epithelial (Soini and Autio-Harmainen, 1993; Su et al., 1993). The "single-cell" theory suggests that SG tumors develop in the process of epithelial–mesenchymal transition (Welsh and Meyer, 1968; Dardick et al., 1983).

Approximately 2–9% of PAs become malignant, evolving into carcinoma ex-pleomorphic adenoma that is characterized by frequent infiltrations of the surrounding, parenchyma and metastatic spreading (Chen, 1978; Gnepp, 1993; Ellis and Auclair, 1995). The most common form of SG malignancy is the cystic adenoid carcinoma, a fairly slow-growing tumor with a tendency for perineural invasion and hematogenous metastasis to the lungs and bones (Bell et al., 2005).

The critical role of different genetic and epigenetic alterations in carcinogenesis has been recognized for some time; some of the most important events in the transformation of normal cells into malignant cells involve mutations in genes encoding the Ras family proteins (Giehl, 2005) and the oncosuppressor TP53 (Papadaki et al., 1996; Steele et al., 1998) as well as the methylation of the p16^{INK4A} gene promoter (Das and Singal, 2004). Little is known about their role in the development of SG tumors (Kishi et al., 2005).

Consequently, the aim of our study was to analyze the frequency of *TP53*, *H-Ras*, and *Ki-Ras* gene alterations and to assess the methylation status of the p16^{INK4A} gene promoter in SG tumors. Furthermore, we examined whether these genetic and epigenetic alterations were associated with epithelial or mesenchymal SG tissue components.

MATERIALS AND METHODS

Study design

A prospective study was performed on tumor samples from a consecutive series of 33 patients who underwent resective surgery for primary operable SG at the Department of Oncology, University of Palermo, Italy. All resection specimens and microscopic slides were examined by two independent pathologists (R.M.T. and V.M.) who were blinded to the original diagnosis and the results of molecular analyses. The complete excision of the primary tumor was histologically proven by examination of the resected margins. The study

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material included 28 PAs, 4 cystic adenocarcinomas, and 1 carcinoma ex-pleomorphic adenoma. Tissues were fixed in 70% ethyl alcohol and paraffin-embedded. At least three single sections were analyzed from each paraffin-embedded tissue. One of the sections was used for microdissection followed by mutational analysis, while the others were used for immunohistochemistry experiments.

Laser-pressure catapulting (LPC) and DNA extraction

Five-micrometer sections of paraffin-embedded tissue specimens, prepared using a microtome, were mounted on the supporting LPC membrane placed on the slide. The samples were pretreated with xylene for 10 min and rehydrated using decreasing grade alcohols (100%, 95% 50% ethanol) and H_2O. Then the slides were stained with hematoxylin and eosin and dehydrated using increasing graded alcohol. LPC was performed using a Zeiss inverted microscope PALM Laser MicroBeam System UV laser at 337 nm. Before performing microdissection, 1 μl of mineral oil was placed on the samples (Bazan et al., 2005b). The areas to be dissected were selected by means of extremely high-precision microcuts (the specimens ranged from as little as 1 μm to 1,000 μm in diameter). LPC dissection was performed using a few shots each of 100 μm in diameter. After catapulting, the material was removed from the cap for genetic analysis. Genomic DNA from tumor and normal specimens was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) following standard protocol.

Detection of *TP53*, *H-Ras* and *Ki-Ras* gene mutations

Normal and tumor samples were used to screen for mutations within the *TP53*, *Ki-Ras*, and *H-Ras* genes using Single Strand Conformational Polymorphism (SSCP) analysis. PCR amplification of the exons 5–8 for TP53 and exon 1 for H-Ras and K-Ras (Table 1) was performed as previously described (La Farina et al., 1998). The abundance and integrity of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining. One hundred nanogram aliquots of the PCR products, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured and used for SSCP analysis. Individual ssDNA fragments exhibiting shifted mobilities relative to normal controls, were electroeluted from polyacrylamide gel, as described previously (Russo et al., 2002), reamplified, and sequenced. The characterization of single mutations was performed by automated sequencing using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the model 3100 DNA sequencer (Perkin-Elmer, Foster City, CA).

Cytokeratin and vimentin immunohistochemistry

Paraffin-embedded sections from alcohol-fixed tissues of all PA cases were immunostained with monoclonal antibodies (Abs) against cytokeratin and vimentin in order to identify the epithelial and mesenchymal components, respectively. Two micrometer sections were pretreated with xylene for 10 min and rehydrated using decreasing graded alcohols (100%, 95%, 50%, ethanol) and H_2O. Endogenous peroxidase activity was blocked by incubating the samples in 3% hydrogen peroxide for 10 min followed by a phosphate-buffered saline (PBS) wash. The sections were then simultaneously immunostained with a mixture of monoclonal Abs against cytokeratin (clone MNF116, dilution 1:50, Dako, Denmark) and vimentin (clone V9, dilution 1:100, BioGenex, Netherlands) for 30 min at room temperature. Applying the indirect peroxidase-antiperoxidase (PAP) method by a standard peroxidase-labeled streptavidin-biotin procedure (LSAB+System-HRP, Dako), the sections were incubated with the biotinylated secondary Ab and then with a mix of streptavidin-horseradish peroxidase-labeled. Detection of the antigens was performed with the use of AEC Substrate-Chromogen kit (Dako-AEC); the slides were then counterstained with Meyer's hematoxylin.

TABLE 1. Oligonucleotide primers for p53, H-ras, K-ras, and p16 genotyping

Exon	Primer sequences	Anneal, °C
ex5 p53	5′-caa cca gcc ctg tcg tct ctc-3′ 5′-ctg ttc act tgt gcc ctg ac-3′	58
ex6 p53	5′-cct cac tga ttg ctc tta gg-3′ 5′-agt tgc aaa cca gac ctc a-3′	50
ex7 p53	5′-caa gtg gct cct gac ctg ga-3′ 5′-tcc tag gtt ggc tct gac-3′	58
ex8 p53	5′-tcc tgc ttg ctt acc tcg-3′ 5′-tcc tat cct gag tag tgg t-3′	54
h-ras	5′-ctg agg agc gat gac gga ata taa gc-3′ 5′-ctc tat agt ggg gtc gta ttc gtc ca-3′	68
k-ras	5′-gtg tga cat gtt cta ata tag tca ca-3′ 5′-gaa tgg tcc tgc acc agt aa-3′	58
p16M	5′-tta tta gag ggt ggg gcg gat cgc-3′ 5′-gac ccc gaa ccg cga ccg taa-3′	61
p16U	5′-tta tta gag ggt ggg gtg gat tgt-3′ 5′-cca ccc caa acc aca acc ata a-3′	66

Methylation status of p16INK4A locus

Normal and tumor samples were analyzed using the CpGenome DNA Modification kit (Intergene Company) following manufacturer's instructions. Peripheral blood leukocytes (PBL) were used as negative controls and universal methylated DNA (ONCOR, Geithersburg, MD) was used as a positive control. DNA was modified with bisulfite treatment. Specifically, 2 μg of DNA were denatured using 0.2 M NaOH for 10 min at 37°C. Then, DNA Modification Reagent I was added and the mixture was incubated for 18 h at 50°C, and subsequently treated with DNA Modification Reagents II and III in 50 μl of water. The modification of DNA was completed with 0.3 M NaOH treatment for 5 min, followed by ethanol precipitation. The modified DNA was amplified by PCR using specific primers (Table 1) to distinguish methylated and unmethylated regions. The size and integrity of the amplification products were verified by 2% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Analysis of genetic and epigenetic alterations in SG tumors

TP53 mutations. The mutational analyses of exons 5 to 8 of the *TP53* gene identified 8 TP53 alterations in 28 (28%) PAs screened. The particular features of these alterations are shown in Table 2. Twenty-five percent (2/8) of the mutations were found in exon 5, 50% (4/8) in exon 6 and 25% (2/8) in exon 7; no mutations were identified in exon 8. All mutations were single-nucleotide substitutions. 50% percent of TP53 mutations were missense (4/8), and 50% (4/8) were silent mutations. Among these alterations, transversions (7/8, 87%) were far more frequent than transitions (1/8, 13%) (Fig. 1) (Table 2). One mutation occurred in highly conserved domains (areas II–V). Considering the specific functional and structural domains of TP53 protein (Cho et al., 1994), one mutation occurred in the L2 loop.

In the cases of carcinoma ex-pleomorphic adenoma and cystic adenoid carcinomas, 4 TP53 mutations were found; 50% (2/4) of those occurred in exon 5, 25% (1/4) in exon 6, and 25% (1/4) in exon 7. All mutations proved to be single-nucleotide substitutions, of which 75% (3/4) were missense mutations, and 25% (1/4) encoded silent mutations (Table 2). All (4/4) mutations were transversions. Twenty-five percent of the mutations (1/4) occurred in highly conserved TP53 domains. Functionally, 25% of mutations (1/4 cases) were in the L2 loop and 25% in the L3 loop.

H-Ras and Ki-Ras mutations. In the analyzed PA cases, 4% (1/28) and 7% (2/28) of the missense mutations were found in codon 13 of H-Ras and Ki-Ras, respectively. A high number of cases 8/28 (28%) displayed a silent mutation in exon 1 (codon 27 His27His CAT>CAC) of H-Ras. In the SG carcinomas analyzed, we identified a missense mutation in codon 13 in one

TABLE 2. TP53, K-H ras, p16INK4 genotyping data[a]

Case	Diagnosis	TP53 mutation	Kras mutation	Hras mutation	p16INK4 alteration
1	Pleomorphic adenoma	WT	WT	Mut Gly13Ala GGT-CGT	M
2	Pleomorphic adenoma	WT	WT	WT	U
3	Pleomorphic adenoma	WT	WT	WT	U
4	Pleomorphic adenoma	WT	Mut Gly13Ala GGC>GCC	WT	M
5	Pleomorphic adenoma	WT	WT	WT	U
6	Pleomorphic adenoma	WT	WT	WT	U
7	Pleomorphic adenoma	WT	WT	WT	U
8	Pleomorphic adenoma	WT	WT	Pol His27His CAT>CAC	U
9	Pleomorphic adenoma	WT	WT	WT	U
10	Pleomorphic adenoma	WT	WT	WT	U
11	Pleomorphic adenoma	Pol ex6 Arg213Arg CGA>CGG	WT	Pol His27His CAT>CAC	U
12	Pleomorphic adenoma	WT	WT	WT	U
13	Pleomorphic adenoma	WT	WT	WT	U
14	Pleomorphic adenoma	WT	WT	WT	U
15	Pleomorphic adenoma	Mut ex5 Val143Met GTG>ATG Mut ex7 His233Pro CAC>CCC	WT	WT	U
16	Pleomorphic adenoma	Mut ex5 Pro128Ala CCT>GCT Pol ex6 Arg213Arg CGA>CGG	WT	WT	U
17	Pleomorphic adenoma	WT	WT	WT	M
18	Pleomorphic adenoma	WT	WT	Pol His27His CAT>CAC	U
19	Pleomorphic adenoma	WT	WT	WT	M
20	Pleomorphic adenoma	Mut ex5 Ser166Pro TCA>CCA	WT	WT	U
21	Pleomorphic adenoma	WT	WT	WT	U
22	Pleomorphic adenoma	WT	WT	Pol His27His CAT>CAC	U
23	Pleomorphic adenoma	WT	WT	WT	U
24	Pleomorphic adenoma	WT	WT	WT	U
25	Pleomorphic adenoma	Pol ex6 Arg213Arg CGA>CGG	WT	WT	U
26	Pleomorphic adenoma	WT	WT	Pol His27His CAT>CAC	U
27	Pleomorphic adenoma	WT	WT	Pol His27His CAT>CAC	U
28	Pleomorphic adenoma	Pol ex6 Ser211Ser CGA>CGG	Mut Gly13Ala GGC>GCC	Pol His27His CAT>CAC	U
29	Adenoid cystic carcinoma	WT	WT	WT	M
30	Adenoid cystic carcinoma	Pol ex6 Ser211Ser CGA>CGG	WT	WT	M
31	Adenoid cystic carcinoma	Mut ex5 Ser166Pro TCA>CCA Mut ex7 Arg249Ser AGG>AGT	WT	Pol His27His CAT>CAC	M
32	Adenoid cystic carcinoma	Mut ex5 Val143Met GTG>ATG	WT	Mut Gly13Ala GGT>CGT	M
33	Adenoid pleomorphic carcinoma	WT	WT	Pol His27His CAT>CAC	M

[a]WT, wild-type; Mut, mutation; Pol, polymorhism; ex, exon; M, methylated; U, unmethylated.

case (25%), and silent mutations in codon 27 of H-Ras in two cases (50%). No mutations in Ki-Ras were observed (Table 2).

Methylation status of the *p16*^{INK4a} gene. The analysis of the methylation status of the p16^{INK4a} gene promoter revealed that 14% (4/28) of the PAs examined showed aberrant methylation within the CpG island. Furthermore, in all the cases (5/5) of carcinoma examined, we found hypermethylation of the p16^{INK4a} promoter (Fig. 2) (Table 2).

Multiple genetic and epigenetic alterations. Multiple alterations were found in 10% (3/28) of the analyzed PA cases; one case presented a double mutation in exons 5 and 7 of TP53 (codon 143 Val-Met, GTG>ATG and codon 233 Ist-Pro CAC>CCC), while the remaining cases showed methylation of the p16^{INK4a} promoter as well as alterations in exon 1 of both H-Ras (codon 13 Gly-Ala GGT>CGT) and Ki-Ras (codon 13 Gly-Ala GGC> GCC).

It is interesting to note that two triple alterations were observed in the five cases of carcinoma examined. Specifically, in addition to the methylation of p16^{INK4a}, our study showed either a double mutation of *TP53* gene (codon 166 Ser-Pro TCA>CCA and codon 249 Arg-Ser AGG>AGT) or single mutation of *TP53* gene (codon 143 Val-Met GTG>ATG) and H-Ras (codon 13 Gly-Ala GGT>CGT) (Table 2).

Analysis of genetic and epigenetic alterations in the epithelial and mesenchimal components

In the examined PA cases, only the epithelial component exhibited H-Ki-Ras missense mutations, TP53 mutation, and methylation of the p16^{INK4a} promoter, whereas the mesenchymal and transitional cells were not mutated. All H-Ki Ras and TP53 mutations, and p16^{INK4a} methylations detected occurred in the epithelial component of the tumor, as defined by immunohistochemistry prior to microdissection. In the mesenchymal areas, which were negative for cytokeratin, and positive for mesenchymal markers, no genetic alterations in H-Ki-Ras, TP53, and p16^{INK4a} were detected (Figs. 2 and 3).

DISCUSSION

Substantial clinical and experimental evidence supports the claim that alterations in the *TP53* and *p16*^{INK4A} tumor suppressor genes and in Ras oncogenes play a critical role in tumorigenesis (Serrano et al., 1996; Bazan et al., 2002; Iacopetta, 2003). The importance of *TP53* and *Ras* gene mutations has been particularly well defined in the model of colorectal cancer (CRC) (Fearon and Vogelstein, 1990; Hsieh et al., 2005) where both of these alterations are now accepted prognostic markers (Russo et al., 2005). Furthermore, specific TP53 mutations, more than any mutations, are biological indicators of CRC progression and outcome (Bazan et al., 2005a). The alteration in the gene encoding p16^{INK4A} has been well studied in different types of tumors, especially in melanoma and pancreatic cancer where it plays an important role during tumorigenesis or tumor progression (Bartsch et al., 1995; Bahuau et al., 1998).

Until now, only few studies examined the above genetic alterations in SG tumors. These limited data have not explained the role that TP53, Ras and p16^{INK4A}

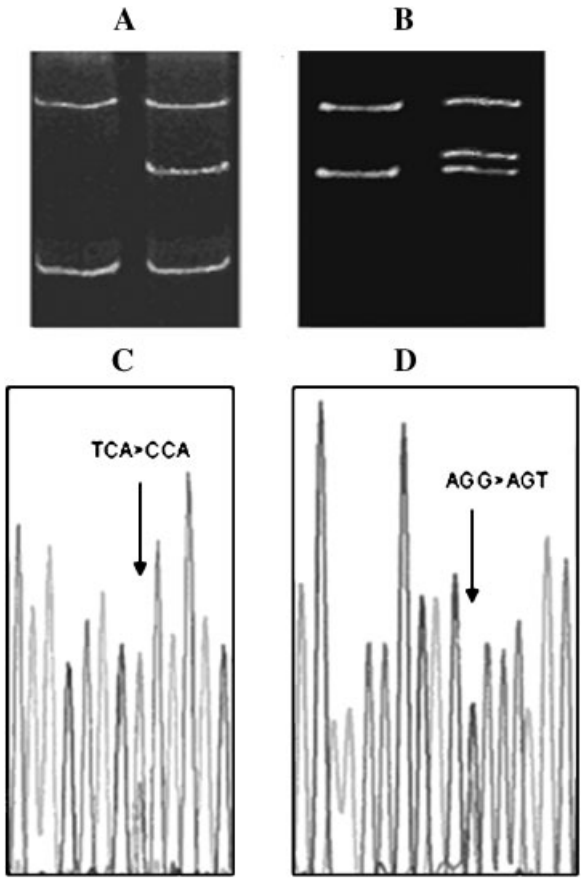

Fig. 1. SSCP analysis of exons 5 and 7 (**A** and **B**) of *TP53* gene amplified from adenoid cystic carcinoma (Case 31) and genomic DNA from normal tissue from one patient (Case 29). In A and B, tumor DNA is on the right (WT) and normal tissue DNA is on the left (N). The extra bands visualized by SSCP correspond to ssDNA molecules haboring mutations in exon5 Ser166Pro TCA>CCA and in exon7 Arg249Ser AGG>AGT, as confirmed by sequencing (**C** and **D**).

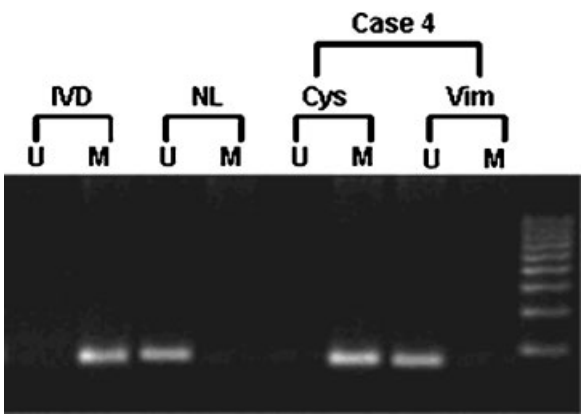

Fig. 2. $p16^{INK4a}$ analysis using MSP in one pleomorphic adenoma (Case 4). Bisulfite-treated DNA (which changes the unmethylated but not the methylated cytosines into uracil) is subjected to PCR amplification using primers designed to anneal specifically to the methylated bisulfite-modified DNA. MSP results are expressed as unmethylated $p16^{INK4a}$-specific bands (U) or methylated $p16^{INK4a}$-specific bands (M). Normal lymphocytes (NL) and in vitro methylated DNA (IVD) are used as negative and positive controls, respectively. Case 4 with methylated p16INK4a within the epithelial tumor (Cyt, cytokeratin), but not in the mesenchymal component (Vim, vimentin). The last lane contains 100 bp DNA ladder.

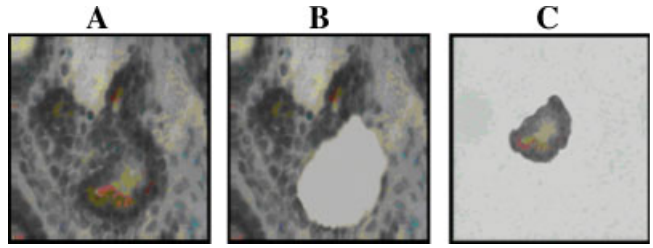

Fig. 3. Typical image of the LPC process involving epithelial tissue immunostained with mAbs against cytokeratin placed on a slide after the addition of a 1 μl of mineral oil. **A** and **B**: Cutting and catapulting of the gland. The images are at 40× magnification; (**C**) catapulted gland on the eppendorf cap; 5× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

might play in the etiology and progression of SG tumors. In addition, our study is the first one addressing the possible cooperation of the studied genetic and epigenetic alterations in SG cancer progression.

Point mutations in the *TP53* tumor suppressor gene are the most common cancer-related genetic abnormality in human malignancy. Previous analyses of different types of tumors have shown that most of TP53 mutations affect exons 5–8, especially residues 130–286 encoding TP53 DNA-binding domain and the region controlling folding and stabilization of tertiary structure. Only rarely have mutations outside exons 5–8 been identified, including those in the introns of the splicing sites, which probably give rise to an aberrant RNA splicing (18). We found a fairly high rate (14%) of mutations in TP53 DNA, which is higher than the rate of mutations (10%) reported in the single published study on the subject (Weber et al., 2002a). The higher incidence of TP53 mutations observed by us could be related to a more sensitive and accurate methodology (SSCP and then sequencing) (15), when compared with methodology (direct sequencing) used by other authors. Also, the phenotype of SG tumors examined could reflect different environmental and dietary factors, which might contribute to the cancerogenesis (Zheng et al., 1996).

Normal growth factor-dependent Ras signaling can be bypassed by mutations in codons 12, 13, and 61, leading to functional redundancy of the intrinsic activity of GTPase and constitutive activation of downstream pathways and, in consequence, increased mitogenesis (Milasin et al., 1993; Okutsu et al., 1993). The role of Ras oncogenes in SG tumors, and particularly in PAs, is still not fully understood. In fact, no published reports addressed the frequency of mutations in exon 1–2 in the *K-Ras* gene in such tissue. Our present study, which shows a mutation rate of 7%, is therefore the first relevant work. By analyzing exon 1 of H-Ras, we identified a missense mutation rate of 4% and a silent mutation rate of 28%; the latter type of alteration is often found in the healthy population (Perrone et al., 2003). Notably, the H-Ras alterations in PA were reported in only one previous study that employed hybridization with synthetic oligonucleotide probes (Milasin et al., 1993).

The *p16*^{INK4a} tumor suppressor gene is inactivated by mutation, homozygous deletion, or gene methylation. It has been shown that hypermethylation of the $p16^{INK4a}$ promoter is frequent in some primary tumors such as lung, pancreatic cancer (Sherr and McCormick, 2002).

A recent study of 42 cases of PA of SG has shown that $p16^{INK4a}$ does not present microdeletions or specific mutations in any exon, but in 28% of cases there was

methylation of the $p16^{INK4a}$ promoter, which correlated with loss of mRNA transcription (Weber et al., 2002b). Our present analyses showed a hypermethylation rate of 14% of the promoter of the tumor oncosuppressor gene $p16^{INK4a}$. This difference between our study and the other report is probably due to dietary habits (Davis and Uthus, 2004).

The population of adenoid cystic carcinomas and carcinoma ex-pleomorphic adenoma analyzed by us included several tumors that probably originated from preceding pleomorphic adenomas. In accordance with previously published reports, these tumors were characterized by a high rate of TP53 missense mutations (three of five cases) a fairly high rate of H-Ras missense mutations (one of five cases), and a low rate of K-Ras mutations (one of five cases). In fact, some authors described a fairly high percentage of *TP53* gene mutations in salivary gland carcinomas, (66–22%) (Papadaki et al., 1996; Zhang et al., 2004), while K-Ras and H-Ras mutations were found in 5–35% and 0–8% of salivary gland carcinoma cases (Yamamoto et al., 1996; Yoo and Robinson, 2000).

In our study, we described methylation of the $p16^{INK4a}$ promoter in five out of five cases. Other reports described the different rates of $p16^{INK4a}$ promoter methylation in SG tumors, from 11% to 47% (Nishimine et al., 2003; Li et al., 2005). It is possible that these data differ from our own because their studies were performed without the use of microdissection techniques and also because they refer to analyses regarding Oriental populations.

Very little is known about the molecular mechanisms of carcinogenesis in SG tumors developing from PAs. We carried out separate analysis of mesenchymal and epithelial components obtained by immunohistochemistry followed by microdissection, not only in order to distinguish the epithelial, mesenchymal, and transitional areas, but also to avoid false-positive or false-negative results, which may often be brought about by contamination with inflammatory cells or fibrotic areas. As Weber et al. (2002b) have already reported, in PAs, methylation of $p16^{INK4A}$ and TP53 mutations occur only in the epithelial component, which suggests that it is these areas of the adenoma which potentially evolve into a carcinoma.

In conclusion, our analysis found a low rate of H-Ras and Ki-Ras missense mutations in all types of SG tumors studied, suggesting that the Ras oncogens might be not involved in the malignant evolution of SG PA into SG carcinoma. On the other hand, the frequency of TP53 mutations and methylation of the $p16^{INK4A}$ was significantly greater compared to that of Ras mutations. Moreover, we observed the accumulation of alterations in these genes in SG carcinomas. Thus, future analyses of TP53 mutations and $p16^{INK4A}$ methylation might be useful for discriminating between benign and malignant SG tumors and to evaluate the prognostic value of these mutations.

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