

# Three cases of rare salivary gland tumours: a molecular study of *TP53*, *CDKN2A/ARF*, *RAS*, *BRAF*, *PTEN*, *MAPK2* and *EGFR* genes

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**Abstract.** Salivary gland tumours are rare tumours characterized by histopathologic complexity and a wide variety of morphologic features. Studies on genetic changes in different histological subtypes of salivary gland tumours are important to better understand molecular pathogenetic mechanisms and to identify diagnostic and prognostic markers. Data are even more scanty dealing with unusual subtypes of these tumours. The aim of the present study was to analyse two high grade transformation adenoid cystic carcinomas (hgACC) and one hybrid tumour in order to identify, by mutational and microsatellite analysis, genetic alterations in *TP53*, *CDKN2A/ARF*, *RAS*, *BRAF*, *PTEN*, *MAPK2* and *EGFR* genes. The two hgACCs showed snps missense in *RAS* genes and alterations with allelic instability in *CDKN2A/ARF*; moreover, a double mutation in *TP53* was detected in one case. The hybrid tumour showed alterations in *CDKN2A/ARF* gene and snps missense in *NRAS* genes. Our data suggest that *CDKN2A/ARF* pathway might be involved in pathogenesis of the salivary gland tumours analysed. Further molecular analyses of these very rare tumours are necessary to better understand the role of other genetic alterations detected in our study.

## Introduction

Salivary gland tumours (SGTs) are rare with an incidence lower than 1/100,000 in the World [data reviewed by Ferrario *et al* (1)]; they account for approximately <0.5% of all malignancies and for <10% of head and neck tumours (1). SGTs are remarkable for their histopathologic and biologic

diversity (2) and, according to the 2005 World Health Organization (WHO) classification, there are 24 different histologic subtypes, some of which very rare (3,4).

A large number of SGTs are characterised by double differentiation toward epithelial and myoepithelial/basal elements. SGTs with epithelial and myoepithelial/basal differentiation, even if they share some morphologic features, constituting a wide spectrum of tumours each having a specific biological behaviour (4). Therefore correct histological diagnosis is at the basis of a correct therapeutic approach (5). However, correct histological diagnosis can be tricky due to the overlapping morphology.

Adenoid cystic carcinoma (ACC) is one of the most frequent malignant SGT showing differentiation toward epithelial and myoepithelial/basal elements. ACC is characterized by slow growth, multiple late recurrences and distant metastases. ACC consists of epithelial and myoepithelial cells arranged in tubular, cribriform or solid growth pattern.

Rare variants of ACC with a rapidly aggressive course have been originally described by Nagao *et al* (6) and Cheuk *et al* (7) as tumours characterized histologically by two components: conventional ACC with tubular, cribriform or solid architecture and areas of adenocarcinoma not otherwise specified (NOS) with aggressive features (6). These latter tumours were originally named 'dedifferentiated ACC'; more recently they have been defined on a larger series as 'ACC with high grade transformation' (hgACC) by Sheetala *et al* (8). hgACC should be differentiated from conventional ACC as the former is characterized by a more aggressive course and shorter survival (8).

In addition, ACC can be part of composite tumours characterized by the association of more histologic features, therefore called 'hybrid tumours'. Hybrid tumours account for <0.1% of all salivary gland tumours (9). Recently cases of hybrid tumours reported in literature have been reviewed by Nagao *et al* (10). The identification of molecular markers should help either in the diagnostic process and to better understand the genetic mechanisms of pathogenesis in these very rare histotypes.

In this context, the aim of our study was to evaluate genetic alterations by mutation and microsatellite analysis in genes

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Table I. Clinico-pathologic features of three cases of salivary gland tumours.

Case	Age at time of diagnosis	Gender	Tumour site	Grade	Histopathology	Therapy	Clinical course
1	58	F	Retromolar trigone left mandibular gingiva	G3+	High grade adenoid cystic carcinoma	Surgical excision RT	DOD in 2003 for recurrence after 1 year
2	59	F	Left maxillary gingiva	G3+	High grade adenoid cystic carcinoma	Surgical excision RT	Cerebral metastases after 1 year and DOD after 2 years
3	78	M	Right submandibular gland	-	Hybrid tumour: epithelial-myoepithelial/adenoid cystic carcinoma	Surgical excision	-

RT, radiation therapy; DOD, died of disease.

implicated in oncogenesis, such as *TP53*, *CDKN2A/ARF*, *RAS* oncogenes, *BRAF*, *PTEN*, *MAPK2* and *EGFR*, in three cases of malignant SGTs with unusual histotype.

## Materials and methods

**Samples and genomic DNA extraction.** Three samples of salivary gland tumours, obtained from the Section of Anatomic Pathology of the Department of Hematology and Oncology of the University of Bologna at Bellaria Hospital, constituted the basis of the present study. All tissues were formalin-fixed and routinely processed to paraffin. Histological diagnoses were performed: i) according to the criteria established in the WHO blue book (3) and AFIP atlas (11); ii) according to Seethala *et al* (8) in cases of hgACC. From selected blocks serial sections were cut and stained immunohistochemically using an automated procedure (Ventana, CA, USA). The following antibodies were applied: Ki67 (Ventana, clone 30-9), CD117 (c-Kit, Cell Marque, clone YR145), p63 (Cell Marque, clone 4A4), epithelial membrane antigen (EMA, Cell Marque, clone E29), Bcl-2 (Cell Marque, clone 124), p53 (Ventana, clone DO7). From the same blocks sections were cut to obtain DNA available for molecular analysis.

Cases consisted of two adenoid cystic carcinomas with high grade transformation (hgACC) (cases 1 and 2) and one hybrid tumour (epithelial-myoepithelial carcinoma and adenoid cystic carcinoma; EMC-ACC) (case 3). Clinical information is summarized in Table I. Normal tissue was obtained from surgical margins uninvolved from tumour, available for cases 1 and 2 (hgACC). Genomic DNA (gDNA) was extracted from paraffin-embedded tissue by proteinase K digestion and phenol-chloroform extraction, according to the protocol developed in the laboratory of Thomas Ried (<http://www.riedlab.nci.nih.gov/protocols.asp>). gDNA was amplified by DOP PCR using the DOP PCR Master kit (Roche Diagnostics) following the manufacturer's protocol. Briefly, 50-150 ng of gDNA were amplified twice on a thermocycler GeneAmp System 9700 (Applied Biosystem).

Positive and negative control samples were added to all amplification reactions; PCR products were analysed by agarose gel electrophoresis using a 2% agarose gel.

**Mutational analysis.** Mutational analysis by direct sequencing was performed in the following genes: *TP53* (exons 5-8), *p14<sup>ARF</sup>*, *p16<sup>INK4a</sup>* (exons 1β, 1α, and 2), *HRAS* (exons 2-3), *KRAS* (exons 2-3), *NRAS* (exons 2-3), *PTEN* (exon 7), *BRAF* (exons 11 and 15), *MAP2K2* (exon 2), *EGFR* (exons 19 and 21); selected gene regions are hot-spot for mutations. Templates were prepared by PCR reaction using primers described in Table II. *EGFR* mutations exon 19 E746\_A750del and exon 21 L858R were screened by allele-specific PCR described by Dahse *et al* (12).

PCR reaction was carried out in a 25-μl reaction mixture containing 20-50 ng of genomic DNA or DOP-PCR, and 1X of High Fidelity PCR Master (High Fidelity PCR Master 2x cat. no. 12140314001, Roche). PCR conditions for amplifications were as follows: 3 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at 50°C (changed when necessary), 60 sec at 72°C, and finally, 3 min at 72°C. The purity and specificity of PCR products were monitored by electrophoresis on 2% agarose gel by ethidium bromide staining.

PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and directly sequenced in both directions using the DTCS Quick Start Kit (Beckman Coulter). Cycling conditions were: 96°C for 2 min, followed by 30 cycles of 96°C for 30 sec, 50°C for 20 sec and 60°C for 4 min. Cycle sequencing reactions were performed on a Beckman CEQ 8000 Genetic Analyzer System. Retrieved sequences were subjected to a BLAST analysis (13) and electropherograms were checked manually.

**Microsatellite analysis.** Markers D9S1748, D9S942, D9S974 were investigated for gene locus *CDKN2A/ARF* in cases 1 and 2. Primers sequences were obtained from Genomic Database (<http://www.gdb.org>); D9S1748 (GDB 595589),

Table II. Primer sequences.

Gene, exon and PCR product	Nucleotide sequences and position (forward-reverse)	GenBank accession
<i>TP53</i> exon 5a (139 bp)	F: 5'-CCAGTTGCTTTATCTGTTCA-3' (12991-13010) R: 5'-TGTGGAAATCAACCCACAG-3' (13129-13112)	X54156
<i>TP53</i> exon 5b (191 bp)	F: 5'-CAACTGGCCAAGACCTGC-3' (13085-13102) R: 5'-AACCAGCCCTGTCGCTCT-3' (13275-13257)	
<i>TP53</i> exon 6 (163 bp)	F: 5'-CTCTGATTCTCTCACTGATTGC-3' (13293-13313) R: 5'-GAGACCCAGTTGCAAAACCA-3' (13455-13436)	
<i>TP53</i> exon 7 (190 bp)	F: 5'-TTGCCACAGGTCTCCCCAA-3' (13942-13960) R: 5'-AGGGTGGCAAGTGGCTCC-3' (14131-14114); F: 5'-CCTTACTGCCTCTTGCTT-3' (14413-14430); R: 5'-CGCTTCTTGTCCTGCTTGC-3' (14611-14593)	
<i>TP53</i> exon 8 (199 bp)	R: 5'-GGAGGCGCGAGAACAT-3' (466-482) R: 5'-GGGCCTTTCCTACCTGGTCTT-3' (686-666)	AF527803
<i>CDKN2A/ARF</i> exon 1β (221 bp)	F: 5'-GGGAGCAGCATGGAGCCG-3' (19978-19995) R: 5'-AGTCGCCCGCCATCCCCCT-3' (20180-20163)	
<i>CDKN2A/ARF</i> exon 1α (203 bp)	F: 5'-AGCTTCCTTTCCGTC-3' (23545-23562) R: 5'-GCAGCACCAACAGCGTG-3' (23741-23731)	
<i>CDKN2A/ARF</i> exon 2A (202 bp)	F: 5'-AGCCCAAACCTGCGCCGAC-3' (23661-23677) R: 5'-CCAGGTCCACGGGCAGA-3' (23807-23791)	
<i>CDKN2A/ARF</i> exon 2B (147 bp)	F: 5'-TGGACGTGCGCGATGC-3' (23766-23781) R: 5'-GGAAGCTCTCAGGGTACAAATTC (23954-23932)	
<i>CDKN2A/ARF</i> exon 2C (148 bp)	F: 5'-GGCAGGAGACCCCTGTAGGAG-3' (6180-6199) R: 5'-GGGTCGTATTCTCGTCCACAA-3' (6329-6311)	NG_007666
<i>HRAS</i> exon 2 (150 bp)	F: 5'-GGAAGCAGGTGGTTCATTGAT-3' (6617-6636) R: 5'-TCACGGGGTTCACCTGTACT-3' (6798-6779)	
<i>HRAS</i> exon 3 (182 bp)	F: 5'-GTGTGACATGTTCTAATATAGTCA-3' (5478-5501) R: 5'-GAATGGTCTCGACCCAGTAA-3' (5691-5671)	NG_007524
<i>KRAS</i> exon 2 (214 bp)	F: 5'-TAATAATCCAGACTGTGTTT-3' (23473-23496) R: 5'-ATATTCAATTAAACCCACC-3' (23706-23687)	
<i>KRAS</i> exon 3 (234 bp)		

Table II. Continued.

Gene, exon and PCR product	Nucleotide sequences and position (forward-reverse)	GenBank accession no.
<i>NRAS</i> exon 2 (232 bp)	F: 5'-GATGTGGCTCGCCAAATTAAC-3' (676-695) R: 5'-GGTAAAGATGATCCGACAAAGTG-3' (907-886)	NG_007572
<i>NRAS</i> exon 3 (268 bp)	F: 5'-CCCTTACCCCTCCACACCC-3' (2895-2912) R: 5'-CCTCATTTCCCCATAAAGATTC-3' (3162-3141)	
<i>PTEN</i> exon 7 (229 bp)	F: 5'-CCTGTGAAATAAATACTGGTATG-3' (94415-94581) R: 5'-CTCCCAATGAAAGTAAAGTACA-3' (94606-94585)	NG_007466
<i>BRAF</i> exon 11 (313 bp)	F: 5'-TCCCTCTCAGGCATAAGGTAA-3' (142978-142998) R: 5'-CGAACAGTGAAATTTCCCTTGAT-3' (143290-143267)	NG_007873
<i>BRAF</i> exon 15 (203 bp)	F: 5'-TCATAATGCTTGCTCTGATAGGA (171309-171331) R: 5'-GGCCAAAATTTAATCAGTGGA-3' (171511-171532)	
<i>MAP2K2</i> exon 2 (170 bp)	F: 5'-ACCTGCAGAAAGAGCTGGAG-3' (6514-6530) R: 5'-TCTGTGCTGGACTTTGGTG-3' (6683-6665)	NG_007996

D9S942 (GDB370738), and D9S974 (GDB 434853) are located upstream to exon 1 $\alpha$  at 19, 15.8 and 12.6 kb, respectively (GenBank accession no. AF527803).

PCR reaction was carried out in a 25- $\mu$ l reaction mixture containing 20 ng of DNA, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.5  $\mu$ M/l each primers, and 1.25 U GoTaq DNA polymerase (Promega cat. M3171). The reaction mixtures were denatured at 95°C for 2 min and incubated for 35 cycles (denaturation at 95°C for 40 sec, annealing at 50°C for 1 min and extension 72°C for 1 min); annealing temperature was at 40°C for D9S942. PCR products, after analysis on a 3% agarose gel, were diluted in a loading buffer containing 20 mmol/l EDTA, 96% formamide, 0.05% of both bromophenol blue and xylene cyanol. The products were heated to 95°C for 5 min and chilled on ice. Three microliters of each sample were applied to Gene Excel 12.5/24 Kit and separated for 2 h at 75 W and temperature at 10°C using GenePhor Electrophoresis Unit (Amersham Biosciences). After electrophoresis, gels were stained using DNA silver staining.

## Results

**Histology and immunohistochemistry.** Cases 1 and 2 showed the features of hgACC (6-8). Briefly, both cases presented areas of typical ACC with tubular and cribriform growth pattern intermingled with areas of solid growth, composed of markedly atypical cells with prominent nucleoli and eosinophilic cytoplasm (Fig. 1A). Atypical mitoses and necrosis were present in these latter areas. CD117 staining was strongly positive in both component. Ki67 index was 10% (case 1) and 12% (case 2) in the typical ACC cribriform component, while it raised to 55% (case 1) and 40% (case 2) in the high grade areas (Fig. 1B). Markers of basal and myoepithelial cell differentiation, as p63 and smooth muscle actin, were positive in the myoepithelial cells of the cribriform areas, while they were completely negative in the high grade areas. On the contrary p53 immunostaining stained a few cells in the cribriform areas while it was strongly positive in most neoplastic cells in the high grade areas.

Case 3 areas showed tubular glandular structures, as observed in ACC, intermingled with areas composed of clear myoepithelial cells and eosinophilic epithelial cells, as observed in epithelial-myoepithelial cell carcinoma. Immunohistochemical staining highlighted the dual nature of the neoplastic cells, as the inner eosinophilic layer was positive with low molecular weight keratin and epithelial membrane antigen (EMA) while clear cells of the outer layer reacted with markers of myoepithelial cell differentiation as smooth muscle actin and p63. Areas of ACC stained with CD117 antibody (Fig. 1C and D). Therefore, this tumour was diagnosed as hybrid tumour with EMC and ACC features, closely intermingled.

**Mutational analysis.** Snps synonymous and mutations detected in *TP53*, *CDKN2A*, *RAS* genes and *BRAF* are summarised in Table III.

**TP53.** Case 2 (hgACC) showed two heterozygous missense point mutations in exons 5 and 8: a substitution of valine to isoleucine residue at codon 157 and a substitution of the glutamic acid with alanine at codon 285 (Fig. 2).



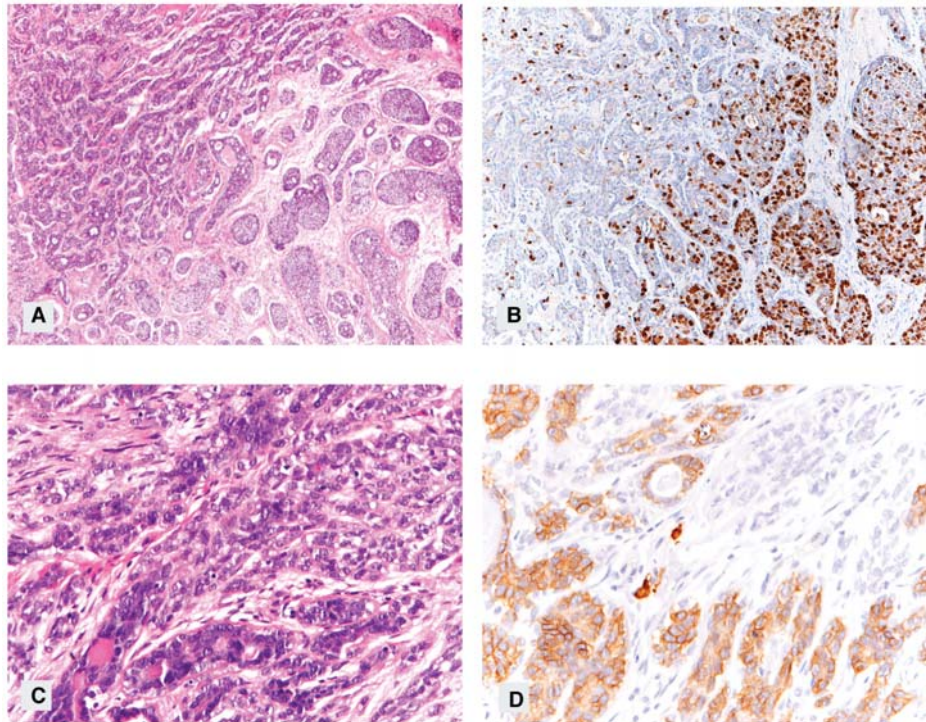
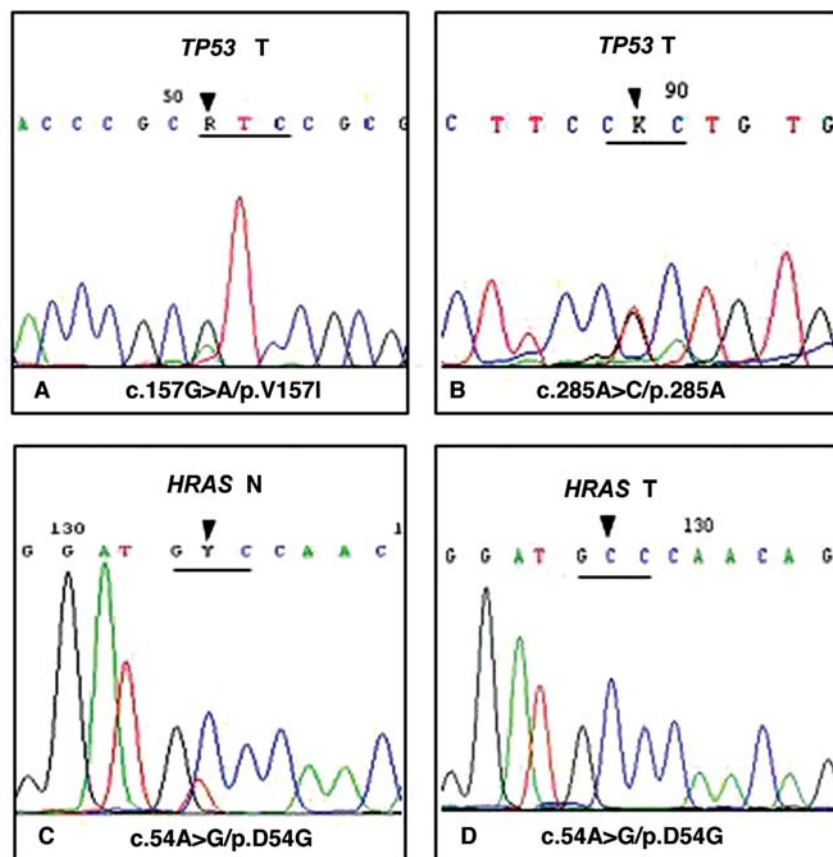


Figure 1. (A) Case 2, hgACC, the upper left part of the image represents typical area of ACC, while the lower right part represents area of high grade transformation (hematoxylin and eosin, H&E, x20); (B) Case 2, hgACC, same case as in (A): Ki67 staining highlights the difference between conventional ACC and high grade areas; (C) Case 3, hybrid tumour, area of transition with EMC-ACC features (H&E, x200); (D) Case 3, hybrid tumour, CD117 stains the neoplastic cells of the ACC area, but is negative in the EMC area (immunohistochemistry, x200).



#### Case 2-hgAcc

Figure 2. *TP53* and *HRAS* sequence electropherograms in case 2-hgACC; (A and B) refer to *TP53*; (C and D) refer to *HRAS* in normal and tumour tissue respectively; N, normal tissue; T, tumour tissue.

Table III. Summary of snps synonymous and mutations detected in *TP53*, *CDKN2A*, *RAS* genes and *BRAF*.

	TP53 <i>SeqRef: NM_000546</i>	<i>p16<sup>INK4a</sup></i> <i>SeqRef: NM_000077</i>	<i>p14<sup>ARF</sup></i> <i>SeqRef: NM_058195</i>	<i>HRAS</i> <i>SeqRef: NM_005343</i>	<i>KRAS</i> <i>SeqRef: NM_004985</i>	<i>NRAS</i> <i>SeqRef: NM_002524</i>	<i>BRAF</i> <i>SeqRef: NM_004333</i>
Case 1 hgACC	-	Exon 1α: no amplification	Exon 1β CAG>TAG c.332C>T/p.Q99X	-	Exon 2 TTT>TCT c.83 T>C/p.F28S	-	-
		Exon 2: GGC>GGT c.333 C>T/p.G111G					
		Exon 2: GCG>GTG c.443C>T/p.A148V					
Case 2 hgACC	Exon 5 GTC>ATC c.469G>A/p.V157I	Exon 1α: no amplification	-	Exon 2: GGC>GGT c.30C>T	-	-	Exon 15 GGT>GGC c.1779T>C/p.G593G
	Exon 8 GAG>GCG c.894A>C/p.E285A			Exon 3: GAC>GGC c.161A>G/p.D54G			
Case 3 Hybrid tumour: ACC/EMC	-	Exon 1α: no amplification	-	-	-	Exon 2: GGA>GAA c.29 G>A/p.G10E	-
		Exon 2: GCG>ACG c.442G>A/p.A148T					
		Exon 2: c.403_404insA p.G135/fs stop at codon 141					

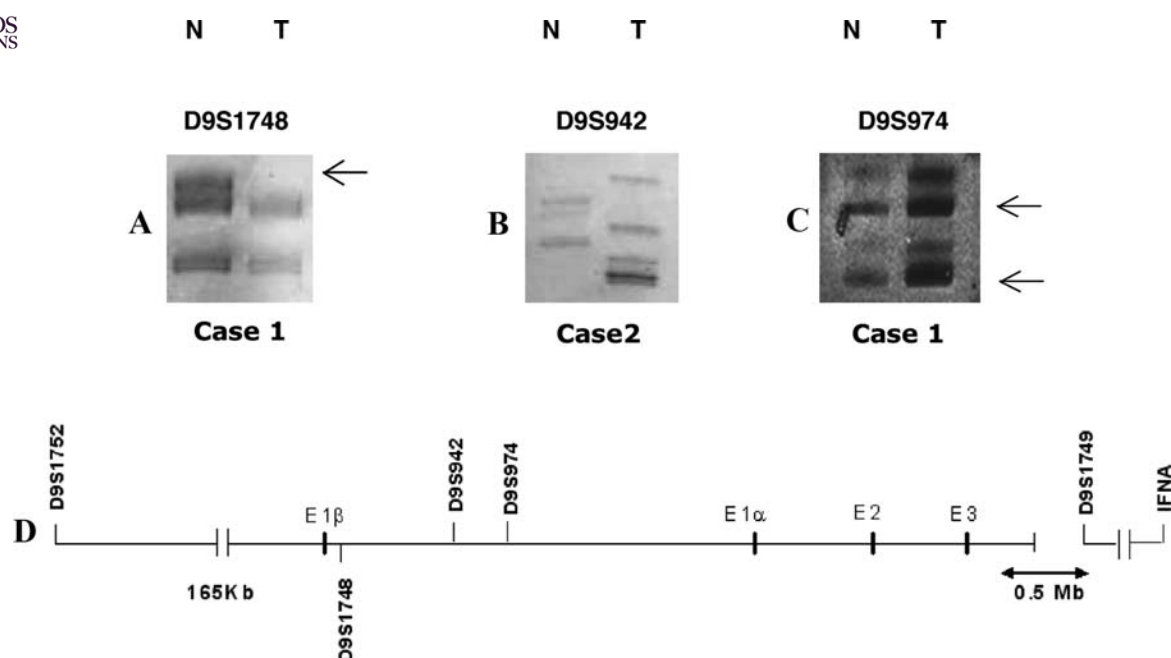


Figure 3. Allelic imbalance by microsatellite analysis (A-C) with markers D9S1748, D9S942 and D9S974 in cases 1 (hgACC) and 2 (hgACC); (D) Schematic representation of microsatellite markers at the *CDKN2A* locus; N, normal tissue; T, tumour tissue. (A) Arrow indicates LOH of upper allele of marker D9S1748 in tumour tissue of case 1; (B) Microsatellite instability of marker D9S942 detected in case 2; (C) Arrows indicate gains in case 1 with marker D9S974.

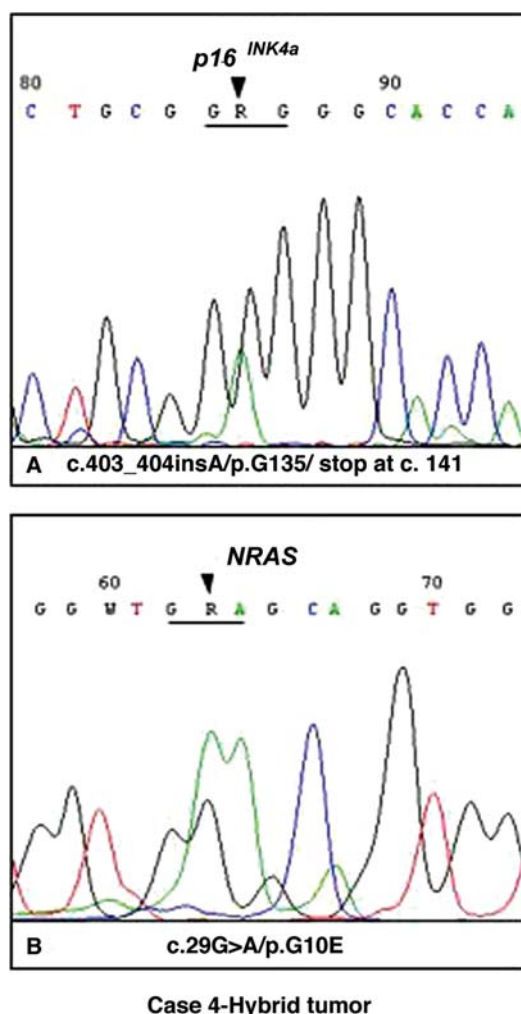


Figure 4. *p16<sup>INK4a</sup>* and *N-RAS* sequence electropherograms in case 3, hybrid tumour.

*p14<sup>ARF</sup>*, *p16<sup>INK4a</sup>*. We did not detect any amplification of exon 1 $\alpha$  in our samples and in normal tissues of cases 1 and 2. Exon 1 $\alpha$  amplification was obtained only in the positive control which consisted of DNA extracted from a healthy blood donor.

Exons 1 $\beta$ , 2 and 3 amplification was detected in all samples. PCR inhibitors were tested by duplex PCR amplification with a 203-bp fragment covering *p16<sup>INK4a</sup>* exon 1 $\alpha$  and a 167-bp fragment of the  $\beta$ -actin gene. We detected  $\beta$ -actin gene amplification in all cases (data not shown). hgACC. Case 1 showed a novel heterozygous stop mutation in exon 1 $\beta$ , a transition at codon 99 (C>T), which alters the codon triplet CAG>TAG.

In addition a snp synonymous substitution in exon 2 at codon 111, GGC>GGT for *p16<sup>INK4a</sup>* protein (UniProtKB/Swiss-Prot accession no. P42771) was detected. This snp synonymous, located within the fourth ankyrin (ANK) repeat domain (110-141), is shared by *p14<sup>ARF</sup>* protein (UniProtKB/Swiss-Prot accession no. Q8N726), consequently altering the codon triplet CCG (Pro)>TCG (Ser) at codon 167, c.536C>T/p.P167S. Case 1 showed also snp missense at codon 148, which lies outside the critical ankyrin repeat domains and has been previously reported as A148T (14).

*Hybrid tumour (case 3)*. The sample showed an insertion of an A between codon 135 and codon 136 (GenBank NM\_000077) in exon 2, causing a frame shift, p.G135fs and a stop at codon 141. Further, a polymorphic homozygous mutation at codon 148, with a substitution of alanine with threonine, was detected. This mutation was identified in other tumours (15), and it is considered as common snp missense in the population without functional significance (14).

*RAS genes*. All samples did not show 'hot-spot' mutations at codons 12, 13 and 61 of *Ras* genes.

*HRAS*. Case 2 (hgACC) showed the following alterations: i) a snp synonymous in exon 2 at codon 10; ii) a novel snp mis-



sense in exon 3 at codon 54 either in the tumour sample and in the normal counterpart; the snp was heterozygous in the normal tissue (Fig. 2).

**KRAS.** Case 1 (hgACC) showed a heterozygous snp missense in exon 2 at codon 28; the snp was not present in the normal counterpart.

**NRAS hybrid tumour.** A rare heterozygous snp missense was detected in exon 2 at codon 10.

**BRAF hgACC.** A heterozygous snp synonymous was found in exon 15 at codon 593 in case 2.

**PTEN, MAP2K2, EGFR** *PTEN* (exon 7), *MAP2K2* (exon 2) and *EGFR* (exons 19 and 21) mutational analysis gave negative results in the three samples.

**Microsatellite analysis.** Microsatellite analysis, performed in hgACC 1 and 2, showed: i) LOH for marker D9S1748 and gains for D9S974 in case 1; ii) microsatellite instability for D9S942 and no amplification for D9S1748 and D9S974 in case 2 (Fig. 3).

## Discussion

In the present study three cases of very rare malignant SGT were analysed by molecular analysis in order to detect alterations in key genes, such as *TP53*, *CDKN2a/ARF* and *RAS* genes, involved in cell proliferation, differentiation and cellular death (15-17).

The two cases of hgACC were very aggressive leading to death of the patients in less than two years; no follow-up data were available for the patient with the hybrid tumour, whose diagnosis was made at the age of 78 years.

In one hgACC (case 1) a novel stop mutation in exon 1β of *p14<sup>ARF</sup>* gene was detected leading to the inactivation of *p14<sup>ARF</sup>* protein, and consequently a defective regulation of the *TP53* pathway. Mutations affecting exon 1β are rare (<http://www.hgmd.cf.ac.uk>) and the first two point mutations were identified in 2001 (18).

The failure to amplify exon 1α of *p16<sup>INK4a</sup>* in all cases suggests a deletion due to an illegitimate V(D)J recombinational mechanism (19); this hypothesis is consistent with the presence of the consensus conserved heptamer V(D)J recognition signal sequence (20), located up- and downstream to exon 1α (GenBank accession no. AF527803).

As regards hgACCs, microsatellite analysis of *p16<sup>INK4a</sup>* exon 1α revealed allelic instability; these data reinforce the hypothesis mentioned above on a deletion event. Moreover, the failure to amplify exon 1α in the normal tissue of hgACC samples may be due to a primary event while other alterations, detected in the tumour counterpart, could be the effect of secondary events, associated with cancer progression.

Both hgACC showed high p53 immunohistochemical expression in the high-grade areas and one of them (case 2) harboured a *TP53* double mutation. The role of p53 in the transformation of ACC, from lower-grade to high grade, has been suggested by several authors since the detection of mutations and immunohistochemical expression in the high-grade component (6-8,21-25).

Mutated p53 was found to be a marker of poor prognosis in a cohort of 70 salivary malignancies and for a prolonged period of 20 years (26). Even if it is difficult to compare

immunohistochemical with molecular results, the present data seem to be consistent with the concept that mutated p53 is a marker of malignant progression also in SGT.

**RAS** mutational analysis detected one mutation in both hgACC cases. To our knowledge there are no functional studies on snp missense F28S and D54G even though these snps lie in the I-domain, 5-63 hydrophobic region β-sheet core, absolutely necessary for Ras transforming function (27).

The molecular analysis of the hybrid tumour showed the absence of exon 1α amplification, a mutation in *p16<sup>INK4a</sup>* and in *NRAS* gene.

The *p16<sup>INK4a</sup>* novel mutation, i.e. G135fs alters the fourth ANK repeat. However, it is very difficult to assess the effect of G135fs on *p16<sup>INK4a</sup>* protein function in connection with EMC-ACC hybrid tumours development and progression, even though it is well known that deletions of the fourth ANK repeat inactivate *p16<sup>INK4a</sup>* (14). G135fs mutation could alter *p16<sup>INK4a</sup>* modifying the folding/unfolding mechanism, a module including the third ANK repeat with functional inactivation of *p16<sup>INK4a</sup>*, that is not a stable protein (28).

G10E mutation, detected in *NRAS*, is highly transforming and tumorigenic since the protein behaves as the wild-type *v-ras* p21, and belongs to a class of mutations whose biological activities do not require GTP-binding (29). The highly conserved glycine at position 10, changed to glutamic acid, is the first residue of a major site of activation (nucleotide phosphate-binding, 10-17) (27) of the p21 protein transforming properties. To our knowledge, among 19,187 mutations identified in *RAS* genes, the mutation here detected has been reported only once in the central nervous system [Forbes *et al* (30), <http://www.sanger.ac.uk/cosmic/>, mutation id 24850).

Incidence of *HRAS*, *KRAS*, *PTEN* and *EGFR* mutations in salivary gland tumours are 17, 3, 38 and 2%, respectively, whereas no mutations of *NRAS*, *BRAF*, and *MAP2K2* are reported (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>) (30).

In conclusion our data suggest that *CDKN2A/ARF* pathway might be involved in the pathogenesis of the salivary gland tumours analysed. Moreover, immunohistochemical p53 staining limited to the dedifferentiated areas of hgACC and the detection of a double mutation in one hgACC suggest that *TP53* could have a role in malignant progression of adenoid cystic carcinoma, as already reported (6-8,21-25).

Further molecular analyses of these rare neoplasms are very important in order to better understand their aetiopathogenesis.

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