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Evaluation of the Role Of H-Ras in Tumorigenesis of Salivary Glands Tumors at Immunohistochemical and Molecular Biological Levels

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Abstract

This study evaluates immunohistochemical (IHC) expression of H-Ras proteins in cases of pleomorphic adenoma (PA) and Warthin tumors (WT) as benign salivary tumors and mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (AdCC) as malignant salivary tumors. The study was conducted on 37 formalin-fixed, paraffin-embedded (FFPE) tissue blocks of salivary gland tumors (17 MEC cases, 14 AdCC cases, 4 cases of PA, 2 cases of WT). IHC markers Antibodies for H-Ras and DNA were extracted from FFPE tissues belonging to 9 patients using polymerase chain reaction (PCR) with minor modifications. The result was that 76% of cases (28/37) had H-ras mutations by IHC. High-grade MEC and AdCC cribriform patterns significantly correlated with H-ras mutation incidence. Age, gender, and tumor site did not significantly correlate with H-ras mutation. Nine cases tested for H-ras gene mutations by DNA sequencing showed no detection of genetic mutation despite being positive for IHC.

Key words: Salivary Gland Tumors, Salivary Gland Neoplasm, Mucoepidermoid Carcinoma, Adenoid Cystic Carcinoma, Immunohistochemical Expression.

Introduction

Salivary glands are divided into major and minor categories, with the parotid, submandibular, and sublingual glands being the major ones. Salivary gland tumors are abnormal cells growing in the ducts that drain the glands, representing 2-4% of head and neck neoplasms [1,2]. The parotid gland is the largest major salivary gland producing serous saliva carried to the oral vestibule through the parotid duct [3]. The structure of the major salivary glands is essentially the same, with the acinar apparatus producing saliva and a secretory ductal system carrying saliva to the oral cavity [4–6]. Our study included pleomorphic adenoma (PA), and Warthin tumor (WT) as representatives of benign tumors, and mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (AdCC) as representatives of malignancy [7]. Despite advances in immunohistochemistry (IHC) and

molecular pathology, the World Health Organization (WHO) still uses histomorphology as the primary basis for classification [8]. H-Ras protein, a GTPase, is associated with cell membranes and activates proteins necessary for the propagation of the receptor's signal. Mutated Ras genes, such as N-Ras and K-Ras, can lead to increased cell proliferation. H-Ras mutations have been previously studied in various reports [9,10] . MEC was studied by 50 DNA samples to evaluate the frequency and pattern of alterations of the H-Ras gene and to discover a possible correlation between the mutation and clinical or morphologic parameters. The authors found that H-Ras mutations occur at codons 12 and 13 and codon 61. The expression of the Ras protein and gene alteration in twelve cases of PA was also performed. The researchers indicated that tumor cells in all 12 cases were strongly stained for Ras protein, suggesting the enhanced expression of the Ras protein in PA.

This study aims to evaluate the immunohistochemical expression of H-Ras proteins in studied cases of salivary gland tumors and explore any mutations of H-ras genes. Determination of any correlation between H-Ras protein expression or gene mutations and clinicopathological parameters of studied cases of salivary gland tumors was also conducted.

Materials and Methods

1- Cases: The present study was carried out on Thirty-seven formalin-fixed, paraffin-embedded (FFPE) tissue blocks of salivary gland tumors (seventeen MEC cases, fourteen AdCC cases, four cases of PA, two cases of WT) collected from the National Cancer Institute at Cairo University, the Pathology Department at the Oncology Center of the Faculty of Medicine of Mansoura University, and the Pathology Department at the Faculty of Medicine of Cairo University.

2- Immunohistochemical markers: Antibodies for: **H-Ras**: which is a mouse polyclonal IgG1 κ, cited in 11 publications, provided at 200 µg/ml raised against recombinant H-Ras protein of human origin was obtained from Chongqing Biopsies Co., Ltd (China) (concentrated) [11]. The findings in the study cases were compared to a control group of normal hepatocytes stained utilizing the same technique.

The study assessed the immunostaining of tumor cells using Computer Assisted Digital Image Analysis (CADIA). The specimens were evaluated based on the percentage area of positive cells staining for H-Ras and/or cytoplasmic or membranous pattern for H-Ras. Positive specimens had brown cytoplasm/membrane or nuclei, while negative specimens showed only blue nuclei or cytoplasm/membrane. The software routine for area measurement and stain quantification involved image acquisition, enhancement of color tones, automatic extraction of target stain area, and conversion of the matrix to greyscale. A 3D histogram was constructed based on pixel data, and integrated density was calculated presenting both stain area and stain intensity. DNA was extracted from FFPE tissues belonging to 9 randomly selected patients (who had positive IHC stain for H-ras mutation) using a polymerase chain reaction (PCR) with minor modifications. The samples were deparaffinized, suspended in DNA extraction buffer, and incubated with shaking at 55˚C overnight. The DNA in the top layer was collected and precipitated with ammonium acetate and 100% ethanol, and the DNA was pelleted by centrifugation at 14,000 rpm for 20 minutes. The DNA was dissolved in TE buffer, and the concentration and quality were determined using a Nano Drop1000 thermo scientific spectrophotometer polymerase chain reaction (PCR) [12]. The amplification process involved a thermocycler programmed with various temperature changes, allowing for multiple copies of the target region to be produced. The primers used were designed specifically for the DNA region of interest. A 2% agarose gel electrophoresis was performed on the PCR products, which were then separated by electrophoresis in 1.5 % (w/v) agarose gels in 1× TAE-buffer containing 0.5 µg/ml ethidium bromide. The PCR fragments were excised from the gels and placed in 1.5 ml tubes. A binding buffer was added to the gel slice, and the gel mixture was incubated at 60°C for 10 minutes. The PCR products were then purified using the GeneJET Gel Extraction Kit according to the manufacturer's protocol. The PCR fragments were excised from the gels and placed in 1.5 ml tubes. The binding buffer was added to the gel slice, and the gel mixture was incubated at 60°C for 10 minutes. The PCR products were then analyzed using a gel documentation system. The results showed that the PCR products were highly specific to the target region of interest, with the H-ras gene being the most sensitive. The PCR products were then sequenced using a qPCR kit [13–20].

3- Statistical analysis was performed with SPSS 20, graph pad prism, and Microsoft excel 2016. All qualitative data were presented as frequency & percentages, all comparisons were performed by using chi square test, and presented in tables and figures.

4- Ehics: This study was approved by the ethical committees of all participating institutions. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Results and Discussion

This study included 37 cases of salivary glands tumors. The H-Ras proteins showed positive IHC expression in 28 cases (76%). Thirty-one cases were of parotid origin while 6 cases were of submandibular origin. The histological categories included the following: 4 PA, 2 WT, 6 low-grade MEC, 1 intermediate-grade MEC, 10 high-grade MEC, 4 AdCC cribriform pattern, 3 AdCC tubular pattern and 7 AdCC solid pattern cases. There were 28 males and 9 females in the study samples. Five patients aged younger than 40, 14 patients aged between 40 – 60, and 18 patients aged older than 18.

Patients aged older than 60 years were significantly higher (48.6%) in this study (p= 0.001). Male patients (75.7%) were significantly higher than females (24.3%) in our report (p < 0.0001). The incidence of tumors originating from parotid gland (83.8%) was significantly higher in our study (p < 0.0001). Histological variants in our study showed that high-grade MEC significantly had the highest incidence in our report (27%) while intermediate-grade MEC had the lowest (2.7%) with p= 0.002. There was a statistically significant association between histopathological types and gender in this study. Comparison between different pathological types in different age ranges was performed and showed significant differences. Accordingly, there was statistically significant association between histopathological type and age with p < 0.05. In patients younger than 40, WT (40%) was significantly the highest. In patients aged between 40 and 60, high-grade MEC and low-grade MEC (27.8%) were significantly the highest with insignificant difference between them. In patients older than 60, AdCC solid pattern (42.9%) was significantly the highest. High-grade MEC had the highest incidence in the parotid gland tumors (32.3%) while AdCC cribriform pattern was significantly the highest tumor in the submandibular gland (50%) with p < 0.05.

Table 1: Association between H-Ras and gender

Gender	Total		H-Ras				Chi-square test	
			Negative		Positive			
			No.	%	No.	%	x2	p-value
Male	28	75.7%	7	77.8%	21	75.0%	0.02	0.86 ns
Female	9	24.3%	2	22.2%	7	25.0%		
Total	37	100.0%	9	100.0%	28	100.0%		

N: count %: percentage

**Significant difference as P<0.05*

Ns: non-significant difference as P>0.0

Table 2: Association between H-Ras and age "years"

Age Group	Total		H-Ras				Chi-square test	
			Negative		Positive			
	No.	%	No.	%	No.	%	x2	p-value
<40 years	5	13.5	3	33.3%	2	7.1%	5.81	0.06 ns
>60 years	18	48.6	5	55.6%	13	46.4%		
40-60 years	14	37.8	1	11.1%	13	46.4%		
Total	37	100.0%	9	100.0%	28	100.0%		

N: count %: percentage

**Significant difference as P<0.05*

Ns: non-significant difference as P>0.05

Table 3: Association between H-Ras and tumor site

Site	Total		H-Ras				Chi-square test	
			Negative		Positive			
	No.	%	No.	%	No.	%	x2	p-value
Parotid	31	83.8	9	100.0%	22	78.6%	2.31	0.12
Submandibular	6	16.2	0	0.0%	6	21.4%		
Total	37	100.0%	9	100.0%	28	100.0%		

N: count %: percentage

*Significant difference as P<0.05

Table 4: Association between H-Ras and histological type

Histological type	Total		H-Ras				Chi-square test	
			Negative		Positive			
	No	%	No	%	No	%	x2	p-value
PA	4	10.8	4	44.4%	0	0.0%	25.1	0.001*
WT	2	5.4	2	22.2%	0	0.0%		
MEC low	6	16.2	2	22.2%	4	14.3%		
MEC Int	1	2.7	0	0.0%	1	3.6%		
MEC High	10	27.0	0	0.0%	10	35.7%		
ADCC cribriform	4	10.8	0	0.0%	4	14.3%		
ADCC Tubular	3	8.1	0	0.0%	3	10.7%		
ADCC Solid	7	18.9	1	11.1%	6	21.4%		
Total	37	100	9	100%	28	100.0%		

N: count %: percentage

*Significant difference as P<0.05

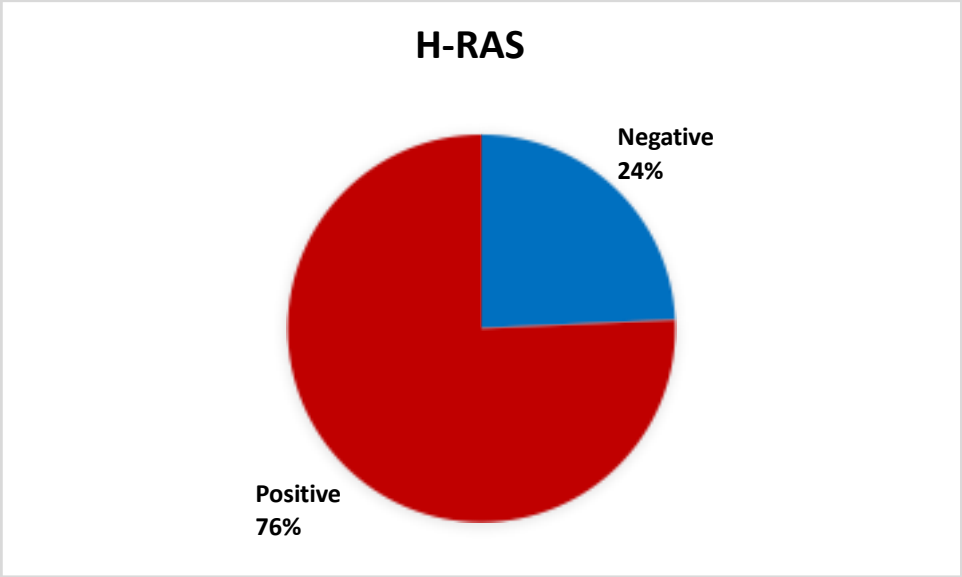

Figure 1: Pie chart H-Ras distribution among the study participants

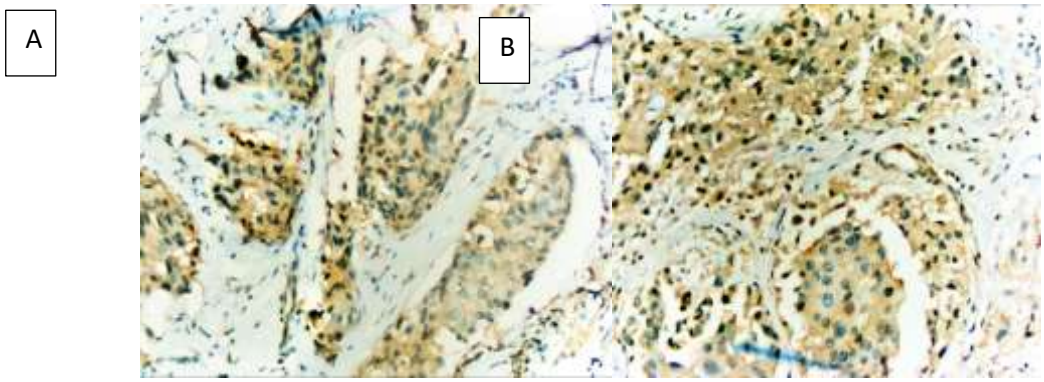

Figure 2A – 2B: Photomicrograph immunohistochemical staining of H-Ras proteins in mucoepidermoid carcinoma showing intermediate cell reaction.

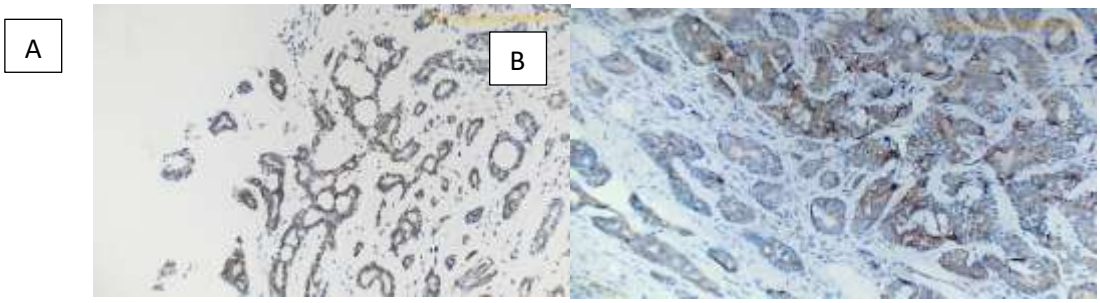

Figure 3A – 3B: Photomicropraph immunohistochemical stain of H-Ras proteins in the cribriform pattern Adenoid cystic carcinoma showing cytoplasmic reaction in the whole of the tumor nest and nuclear reaction in some cells.

Figure 4A – 4B: Sequencing results of the polymerase chain reaction (PCR) products amplified the full-length exon 3 of the human H-Ras gene. **Figure 4A:** A part of the DNA sequencing chromatogram of a PCR product that amplified exon 3 of the human H-Ras gene. **Figure 4B:** The nucleotide sequence and features of the PCR products that contain exon 3 of the human H-Ras gene.

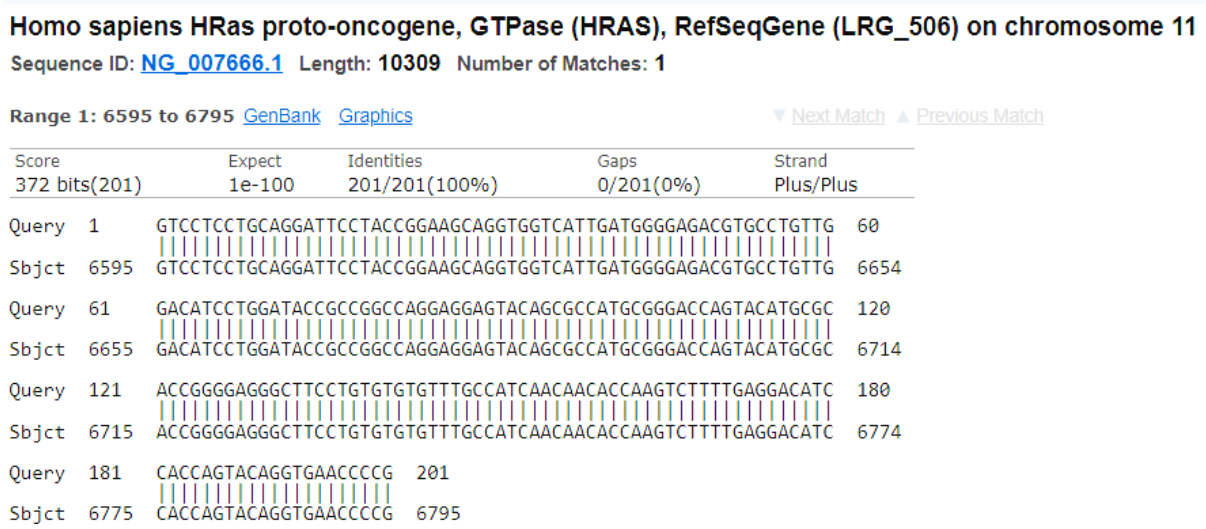

Figure 5: Alignment of the nucleotide sequence of exon 3 of mucoepidermoid carcinoma and adenoid cystic carcinoma H-Ras gene amplifications to the GenBank reference sequence NG_007666.

The incidence of positive IHC expression of H-Ras proteins in this study (28/37; 75.7%) was significantly higher than the negative (9/37; 24.3%) expression ($p < 0.0001$) **(Figure 1)**. Gender, age, and tumor site were insignificantly associated with the incidence of H-Ras positive IHC expression in our report ($p > 0.05$) **(Tables 1 – 3)**.

High-grade MEC was significantly the highest tumor to show positive IHC expression of H-ras proteins while PA was significantly the lowest to show negative expression with $p < 0.05$. All cases of high-grade MEC showed positive stain (10 cases) while all cases of PA showed negative stain (4 cases). Benign salivary gland neoplasms were found to have negative H-ras immunostaining in both the epithelial element and mesenchymal-like tissues. All two cases (100%) of WT showed negative H-ras immunostaining in the bilayer epithelium and lymphoid tissue. In low-grade MEC, 14.3% of cases showed positive immunoreactivity, while 35.7% of high-grade cases showed membranous positive reactions of H-Ras and cytoplasmic reactions **(Figure 2A – 2B)**. Malignant salivary gland neoplasms were found to have positive H-ras expression in the cytoplasm and nucleus of tumor cells arranged in AdCC cribriform, tubular, and solid patterns **(Figure 3A - 3B) (Table 4)**.

Exon 3 of the Mucoepidermoid carcinoma and adenoid cystic carcinoma H-Ras gene

A sequence from the mucoepidermoid carcinoma and adenoid cystic carcinoma of the HRAS gene containing the full-length exon 3 was amplified by PCR using a specific primer pair which produced 201 bp bands. The PCR products were sequenced from both directions. Sequencing of the amplified fragment produced a nucleotide sequence of 201 bp that covers a part of intron 2 (1-12 bp), full-length exon 3 (13-191 bp), and a part of intron 11 (192-201 bp) of HRAS gene **(Figure 4A – 4B)**.

All the PCR products of the 9 samples had the same nucleotide sequence and no mutations were identified among them. Moreover, the alignment of the nucleotide sequence to the GenBank reference sequence NG_007666.1 showed that no genetic variations could be detected between the 2 sequences **(Figure 5)**.

Discussion

This is a cross-sectional study that included 37 patients with variable salivary gland tumors. The aim of the study was to evaluate the expression of H-Ras proteins and mutations in salivary gland tumors of the studied population. The associations between different clinicopathological variables and H-Ras mutations were also studied. H-Ras mutations proved to be positive in 76% of the cases in this report. As per our study, high-grade MEC and cribriform pattern of AdCC correlated significantly with the incidence of H-Ras mutation by IHC. On the other hand, age, gender, and site of the tumor did not correlate significantly with the incidence of H-Ras mutation. Nine cases of the total number of the cohort who tested positive for H-Ras mutation by IHC were randomly selected and subsequently tested by DNA sequencing for evidence of H-Ras gene

mutation. All the nine tested cases did not show evidence of H-Ras gene mutation by DNA sequencing despite being positive by IHC.

Our study agreed with the reported literature in the male dominance among patients with salivary gland tumors. This may be explained by the effect of sex hormones on this entity of neoplasms [21]. The fact that WT mainly affects the parotid gland is attributed to the presence of lymph nodes within the parenchyma of the parotid gland. This unique feature is lacking in other salivary glands as they acquire their fibrous capsule earlier than the parotid gland does leading to infiltration of the parotid gland by lymphoid tissue which contributes in the pathogenesis of WT, also known as papillary cystadenoma lymphomatosum [22].

A study conducted by Yoo and Robinson in 2000 showed that H-Ras mutations correlated with increasing severity of the histological type in MEC [10]. This comes in agreement with what was found in our current study. The nucleus and the cytoplasm react positively in most lesional cells throughout the tumor. Histologically, MEC is composed of dual population of cells: mucous and squamous. The reaction is usually strongly found in the cytoplasm of the tumor cells in duct-like structures and that of myoepithelial cells especially plasmacytoid cells. High expression rates of K-Ras and H-Ras mutations were found to be associated with the invasiveness and metastatic ability of salivary gland malignant neoplasms.

In terms of AdCC, the cribrifom pattern showed positive H-Ras reactivity in the acinar and myoepithelial cells. The tubular pattern showed positive H-Ras immunoreactivity in the solid component of the tumor and the peripheral cells of the perineural invasion component of the tumor. RAS mutations are known to promote cell proliferation subsequently paving the way to malignant transformation [23]. High RAS expression was observed in many head and neck cancers including oral mucosal melanoma, maxillary sinus squamous cell carcinoma and laryngeal carcinoma. Overexpression of RAS has been a culprit in late tumorigenesis of the head and neck cancer [24]. Moreover, H-Ras expression can participate in AdCC pulmonary metastasis [25].

The notion of targeting H-Ras gene and proteins has been evolving over the last four decades. Current hope stems from the perspective of involving signal transduction therapies. The main hurdles against the development of such therapies are the ultrastructure and the microbiological features of the H-Ras itself. These are smooth proteins with no distinct binding site for a potential drug. The high GTP-binding affinity of the molecule makes it difficult for a molecule – small enough to pass through the cell membrane – to cause any significant effect on the intracellular signaling pathway. Some drugs are already being tested for their effectiveness against H-Ras pathway with promising results [26].

Our report disagreed with another report that found a significant between H-Ras mutations and male pheochromocytoma (PCC) [27]. Another contradiction was that for PCC, H-Ras mutations were associated with benign and sporadic disease. It is worth noting that the pathology in both studies is completely different on histological and anatomical backgrounds. This major difference can explain the contradiction between the findings in both studies.

Limitations to this study include the relatively small sample size. Unfortunately, our report lacks significant data regarding the cure and recurrence rates. Other epidemiological factors are deficient including smoking status of the patients. The cross-sectional design of this study makes it vulnerable to the inherited recall bias of the demographic data. All patients included in the DNA sequencing failed to show H-Ras mutations despite being positive by IHC. This can be explained by the small number of cases who were randomly selected for DNA sequencing among the study population. The reason for the that is the relatively high cost of the procedure in contrast to the limited available resources.

Conclusion

The IHC expression of H-Ras is strongly associated with high-grade MEC and cribriform pattern of AdCC. Overexpression of H-Ras usually reflects more aggressive tumor behavior. Age, gender, and tumor site did not correlate significantly with the H-Ras expression. Targeting H-Ras genes can be a future perspective for individualized targeted therapy. DNA sequencing of a sample of tumors that showed positive IHC expression of H-Ras failed to identify RAS genetic mutations or variations. This negative finding should encourage researchers into conducting more research with larger sample sizes and better resources.

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