




Diagnostic Value of Nuclear Receptor Subfamily 4 Group A Member 3 in Salivary Gland Carcinomas

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Abstract

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BACKGROUND: Salivary gland carcinomas (SGCs) are diagnostically challenging lesions in heterogeneous groups. However, recently described genomic alterations may be helpful and have future therapeutic implications. Diagnosis of acinic cell carcinoma (AcCC) is challenging due to its rarity and similarity with other SGCs or normal acinar cells.

AIM: Herein, the diagnostic performance of transcription factor nuclear receptor subfamily 4 Group A member 3 (NR4A3) was analyzed immunohistochemically.

METHODS: This study was done on 68 cases of SGCs, including 32 of AcCC, 14 of adenoid cystic carcinoma (AdCC), eight of mucoepidermoid carcinoma (MEC), seven of epithelial myoepithelial carcinoma (EMC), four of salivary duct carcinoma (SDC), and only one case from each of carcinoma ex pleomorphic adenoma (CXPA), polymorphous adenocarcinoma (PAC), and secretory carcinoma (SC) for detecting NR4A3.

RESULTS: All stained samples of AcCC (100%) responded positively to the NR4A3 antibody. However, only one case of AdCC and MEC demonstrated NR4A3 immunostaining in <10% of cells. On the other hand, the different types of SGCs, including EMC, SDC, CXPA, PAC, and SC, revealed negative expressions.

CONCLUSION: Therefore, we can conclude that NR4A3 can be used as a potential marker for AcCC rather than other SGCs.

Introduction

Salivary gland carcinomas (SGCs) are crucial in diagnostic pathology, with significant morphological diversity and overlapping histopathological features [1], [2].

In recent years, several characteristic molecular alternations have been described in certain salivary gland neoplasms, resulting in abnormal fusion oncogenes [3]. Several techniques such as fluorescence *in situ* hybridization (FISH), polymerase chain reaction, and immunohistochemistry can detect these fusion oncogenes and their oncoprotein products [3].

Acinic cell carcinoma (AcCC) is a low-grade malignant salivary gland neoplasm that comprises approximately 17% of primary salivary gland malignancies or about 6% of all salivary gland neoplasms. AcCC is the second most common epithelial malignancy in the pediatric age group following mucoepidermoid carcinoma [4]. AcCC histologic features are often characteristic, with serous acinar cells containing basophilic zymogen granules

mixed with intercalated duct-type cells with cytoplasm variably clear, vacuolated, or oncocytoid. It has different patterns of proliferation may be solid, microcystic, follicular, or papillary [5].

Although AcCC has been considered for a long time as a neoplasm with a good prognosis, new researchers point it out as a malignancy with the unpredictable clinical course due to the propensity of this tumor to recur, metastasize, and even lead to death, especially regarding a subgroup of AcCC described as “dedifferentiated” or “AcCC with the high-grade transformation” (HGT-AcCC) [6], which can overlap with other salivary tumors, particularly when serous acinar differentiation is subtle [7].

Secretory carcinoma (SC), demonstrates a recurrent ETV6–NTRK3 fusion, is the most histologic mimic of, especially in zymogen-poor AcCC [7]. At present, it can be defined by a specific chromosomal translocation t (12;15) (p13; q25), leading to the formation of an ETV6-NTRK3 fusion oncogene [7], [8]. On the other hand, the genetic landscape of AcCC is still a conflict.

Mucoepidermoid carcinoma (MEC) is the most common salivary gland cancer that has a unique chromosomal translocation t (11;19) (q14–21; p12–13), involving MECT1 (mucoepidermoid carcinoma translocated-1 or CRCT1) gene at 19p13 and MAML2 (mastermind-like 2) gene at 11q21, which have been reported in approximately 60–70% of MEC [9], [10], [11]. These translocations are a reliable diagnostic and prognostic biomarker for MEC [12], [13]. MEC may mimic AcCC when its mucinous component is not identified.

Adenoid cystic carcinoma (AdCC) is the second most common malignant salivary neoplasm, characterized by the specific translocation t (6; 9), involving MYB-NFIB fusion oncogene, which presents in approximately 80–90% of tumors [14], [15], [16]. Studies by Weinreb *et al.*, 2014 [17], have demonstrated that polymorphous adenocarcinoma (PAC) has consistent abnormalities of the PRKD1 gene. AdCC can be differentiated from AcCC clinically and histopathologically unless dedifferentiation and conventional tumor precursor are not found.

The transcription factor nuclear receptor Group A family of orphan receptors (NR4A1-3) is superfamily of nuclear receptors. They regulate genes involved in proliferation, cell migration, and apoptosis [18]. This family consists of three members, NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (NOR-1) [19].

Firstly Haller *et al.*, study at 2019 found NR4A3 as a highly sensitive (98%) and specific (100%) marker for AcCC by quantitative real-time reverse transcription- polymerase chain reaction [20].

Depending on the above, the present study was aimed to throw a beam on NR4A3 (NOR-1) as a powerful diagnostic marker for AcCC using immunohistochemistry.

Material and Methods

Tissue Samples

Sixty-eight cases of malignant salivary gland tissue samples from patients were investigated in this retrospective study from the archival paraffin blocks, which were re-diagnosed to confirm the previously made diagnosis according to the World Health Organization (WHO) histological typing of SGCs (2017), including 32 cases of AcCC, 14 of AdCC, eight cases of MEC, seven cases of EMC, four cases of SDC, and only one case from each of CXPA, PAC, and SC.

The specimens were retrieved from the archival paraffin blocks from the Oral and Maxillofacial Pathology Department files, Faculty of Dentistry, Mansoura University, and General Pathology Department, Faculty

of Medicine, Ain Shams University.

This retrospective study was approved by Faculty of Medicine, Ain Shams University ethics committee No. FWA000017585. Patients had approved to use their tissue biopsies in research work which was taken.

Immunohistochemical staining

For immunohistochemical examination, the primary antibody used was mouse monoclonal anti-human NR4A3 antibody in the form of pre-diluted antibody (1:150), ready for staining procedure, Cat. TA804855 (100 ML), Thermo Fisher Scientific, U.S.A.

Serial sections (4 microns thick) were cut from each paraffin block and were placed on the positively charged slides for the staining procedure. Sections were prepared to receive primary antibody through passing the steps of deparaffinized and rehydrated in graded concentration of alcohol and incubated the cells with 0.3% H₂O₂ in methanol for 10 min. Then, the sections were ready to interact with primary antibody (anti-NR4A3).

Streptavidin peroxidase solution labels were placed on each slide similarly, and the incubation condition was repeated. Hematoxylin was used to counterstain cell nuclei, and sections were mounted.

Immunohistochemical evaluation of NR4A3

For each positive section, four microscopic fields were selected, and photomicrographs were captured at a magnification of 40×. This was performed using a digital camera (C5060, Olympus, Japan) which was mounted a light microscope (BX60, Olympus, Japan). Images were then transferred to computer system for analysis.

All the steps for immunohistochemical evaluation were carried out using the ImageJ software's image analyzer computer system (Image J, 1.41a, NIH, USA). Images were first manually corrected for brightness and contrast, then images converted into 8 bit types gray scale. The images were masked by red binary color which could be measured by the computer system after color thresholding was performed automatically (Figure 1). Mean values were obtained for the whole specimens in each group.

Assessment of NR4A3 (NOR-1) results

The collected data were tabulated using Microsoft Excel (Microsoft Office 2010). For each case, the area fraction of immunopositivity for at least four different microscopic fields was measured. The mean area fraction for each case was then calculated and used for statistical analysis.

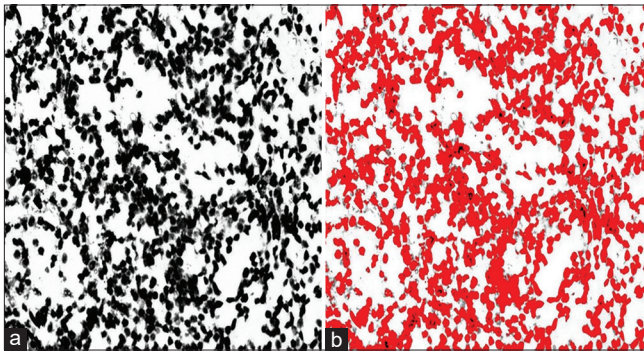


Figure 1: A captured photomicrograph of acinic cell carcinoma showing the measurement of nuclear receptor subfamily 4 Group A member 3 immunoreactivity. The brown immunostain colour converted into grey (a), then masked by a red binary colour (b)

The mean of area fraction was scored from 1 to 3 depending on the distribution of NR4A3 immunostaining into: Score (0) = 0%, score (1) ≤10%, score (2) = 10–25%, and score (3) ≥25%.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS Corp. Released 2013, IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Monte Carlo test for comparison of two or more groups. The significance of the obtained results was judged at $p \leq 0.05$ level. Spearman’s rank-order correlation is used to determine the strength and direction of a linear relationship between two non-normally distributed continuous variables and ordinal variables, and a one-way ANOVA test was used to compare more than 2 independent groups.

Results

Clinical characteristics of studied SGCs

Regarding TNM clinical staging system, the present study revealed four various tumor sizes include; T1, T2, T3, and T4. However, 54.5% of samples were evaluated as T2, while the other was distributed as T1 (22.1%), T3 (19.1%), and T4 (4.4%) (Table 1).

Table 1: Tumor size of the studied cases

Studied SGCs	Tumor size	n	Diagnosis							Total	
			AcCC	AdCC	CXPA	EMC	MEC	PAC	SDC		SC
T1	n	10	3	0	0	1	0	1	0	0	15
	%		31.3%	21.4%	0.0%	14.3%	0.0%	100.0%	0.0%	0.0%	22.1%
T2	n	16	9	0	0	3	5	0	3	1	37
	%		50.0%	64.3%	0.0%	42.9%	62.5%	0.0%	75.0%	100.0%	54.4%
T3	n	6	1	1	1	3	1	0	1	0	13
	%		18.8%	7.1%	100.0%	42.9%	12.5%	0.0%	25.0%	0.0%	19.1%
T4	n	0	1	0	0	2	0	0	0	0	3
	%		0.0%	7.1%	0.0%	0.0%	25.0%	0.0%	0.0%	0.0%	4.4%
Total											68
											100.0%

Monte Carlo test $p = 0.177$

Monte Carlo test. *Statistically significant if $P < 0.05$, n: number. SGCs: Salivary gland carcinomas, AcCC: Acinic cell carcinoma, AdCC: Adenoid cystic carcinoma, CXPA: Carcinoma ex pleomorphic adenoma, EMC: Epithelial myoepithelial carcinoma, MEC: Mucoepidermoid carcinoma, PAC: Polymorphous adenocarcinoma, SDC: Salivary duct carcinoma, SC: Secretory carcinoma.

On the other hand, most of the current SGCs expressed negative regional lymph node involvement (72.1%). Meanwhile, 18 cases exhibited nodal involvement was divided equally between N1 and N2. Notably, only one case of SDC was more significant than 6 cm without extranodal extension (N3a), (Table 2). No cases with distant metastasis were recorded.

Statistically, no significant difference was detected between the studied SGCs and clinical data in terms of gender, age, tumor size, and regional lymph node involvement.

Regarding NR4A3 immunoreactivity and clinical parameters, there was no statistically significant difference observed and no correlations. In contrast, inverse relation was obtained between NR4A3 expression and MEC and age and lymph node involvement of AdCC (Table 3).

Histopathological findings of studied SGCs

The present study was carried out on 68 cases of SGCs from which 32 (47%) of cases are AcCC. Mostly all studied AcCC cases composed predominately of serous acinar cells in solid proliferation pattern with abundant granular eosinophilic cytoplasm, and round darkly stained eccentric nucleus. Smaller vacuolated intercalated duct like cells also found, clear cells noted in minimal amount. Connective tissue stroma was scanty fibrovascular. Lymphoid tissue was predominant in 10 cases (Figure 2).

AdCC is 14 (20.5%) cases which mainly formed of solid groups of basaloid, cuboidal epithelial cell nests with little number of ducts and cyst formation.

MEC is 8 (11.8%) cases composed of mucous secreting cells (various shapes have abundant, pale, and foamy cytoplasm), epidermoid cells (polygonal shape), and intermediate cells (smaller basal cells) in varied proportions lining cystic spaces and present in solid masses. Clear cells predominately proliferate in two cases.

EMC is 7 (10.3%) cases composed of small cuboidal cells form ducts with granular, eosinophilic cytoplasm, and central nucleus, clear cells also noted in a fibrous connective tissue (Figure 2).

SDC is 4 (5.9%) cases which formed of polygonal cells proliferate in tubular pattern with

Table 2: Regional lymph node involvement (N) of the studied cases

Studied SGCs			Diagnosis							Total	
			AcCC	AdCC	CXPA	EMC	MEC	PAC	SDC		SC
Lymph node involvement (N)	N0	n	22	12	1	5	6	1	1	1	49
		%	68.8%	85.7%	100.0%	71.4%	75.0%	100.0%	25.0%	100.0%	72.1%
	N1	n	6	2	0	0	1	0	0	0	9
		%	18.8%	14.3%	0.0%	0.0%	12.5%	0.0%	0.0%	0.0%	13.2%
	N2	n	4	0	0	2	1	0	2	0	9
		%	12.5%	0.0%	0.0%	28.6%	12.5%	0.0%	50.0%	0.0%	13.2%
	N3a	n	0	0	0	0	0	0	1	0	1
		%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	25.0%	0.0%	1.5%
	Total										68
											100.0%
	Monte Carlo test		p = 0.135								

Monte Carlo test. *Statistically significant if $p < 0.05$, n: number. SGCs: Salivary gland carcinomas, AcCC: Acinic cell carcinoma, AdCC: Adenoid cystic carcinoma, CXPA: Carcinoma ex pleomorphic adenoma, EMC: Epithelial myoepithelial carcinoma, MEC: Mucoepidermoid carcinoma, PAC: Polymorphous adenocarcinoma, SDC: Salivary duct carcinoma, SC: Secretory carcinoma.

eosinophilic cytoplasm in a dense fibrous connective tissue stroma (Figure 2).

CXPA is 1.5% only one case found epithelioid cells proliferation with increased cellular and nuclear pleomorphism, normal and abnormal mitosis detected hyperchromatism. Furthermore, plasmacytoid group of cells noted. Connective tissue stroma had areas with chondromyxoid stroma (Figure 2).

Table 3: Correlation between NR4A3 (NOR-1) immunoexpression and clinical parameters of studied SGCs

Diagnosis	Clinical data		NR4A3
AcCC	Tumor size lymph node involvement (N)	R	0.069
		P	0.709
		R	0.051
		P	0.780
AdCC	Tumor size lymph node involvement (N)	R	0.040
		P	0.891–0.113
		R	0.700
		P	
MEC	Tumor size lymph node involvement (N)	R	-0.286
		P	0.493–0.216
		R	0.607
		P	

r: Spearman correlation coefficient. *Statistically significant if $P < 0.05$. SGCs: Salivary gland carcinomas, AcCC: Acinic cell carcinoma, AdCC: Adenoid cystic carcinoma, MEC: Mucoepidermoid carcinoma, NR4A3: Nuclear receptor subfamily 4 Group A member 3.

PAC is 1.5% composed of cuboidal cells that have uniform ovoid nuclei and eosinophilic cytoplasm in a fibrovascular stroma, hyaline in some areas (Figure 2).

SC is 1.5% formed of small basaloid cells with vacuolated granular cytoplasm arranged in tubular pattern and vesicular central nucleus. Connective tissue was scanty fibrous with secretions (Figure 2).

Immunohistochemical findings with NR4A3 (NOR-1)

All stained samples of AcCC (100%) responded positively to the NR4A3 antibody. In addition, in 20 AcCC cases (62.5%) were evaluated as (score +2), whereas, 12 AcCC cases (37.5%) were recorded as score +3, (Table 4 and Figure 3). However, only one case of AdCC (7.1%) out of 13 cases and MEC (12.5%) out of seven cases demonstrated as score +1. On the other hand, the different types of SGCs, including EMC, SDC, CXPA, PAC, and SC, were revealed with negative expressions (Table 4 and Figure 2).

Based on statistical analysis, a statistically significant positive correlation was discovered between

NR4A3 (NOR-1) immunoreactivity and studied SGCs cases (Table 4).

Discussion

Correct and early diagnosis is necessary step for effective treatment plan and preventing metastasis process. As, metastasis considers the main result of mortality in cancer patients. Therefore, noticeable progress in diagnosis and clarifying the molecular sequencing are important to eradicate this fatal process in cancer [21], [22]. Diagnosis of SGCs is made primarily on their characteristic morphological appearances [2]. The molecular investigations are becoming helpful tools to help establish a correct diagnosis and provide prognostically important information, but they are expensive and less sensitive in some cases. However, recently described genomic alterations may help challenge cases and have future therapeutic implications [2], [3].

AcCC is the third most common epithelial malignancy of the salivary glands in adults, following mucoepidermoid carcinoma and adenoid cystic carcinoma. Its diagnosis is on the basis of the characteristic histomorphologic appearance in low-grade cases, but it is challenging in cases with high-grade transformation “zymogen granule” [4], [7]. At 2019, Haller *et al.* study discovered a novel genomic rearrangement (t [4; 9] [q13; q31]), leading to the upregulation of nuclear receptor subfamily 4 Group A member 3 (NR4A3) at 9q31 through molecular analysis of several AcCCs [20]. Their study was first studying the clinical utility of NR4A3 IMH in diagnosing AcCC and distinguishing precisely from SC [20].

Interchange genomic rearrangements involving conversely driver events can happen in a mutually exclusive way inside the same translocation-driven salivary gland tumors. For illustration, oncogenic actuation of closely related qualities sharing fundamental structural likenesses (e.g., gene family individuals) can apply the same functional impact [20]. As, there are exclusive genomic alterations occurring which can be considered as a characteristic diagnostic feature of each lesion. For example, AdCC could be diagnosed

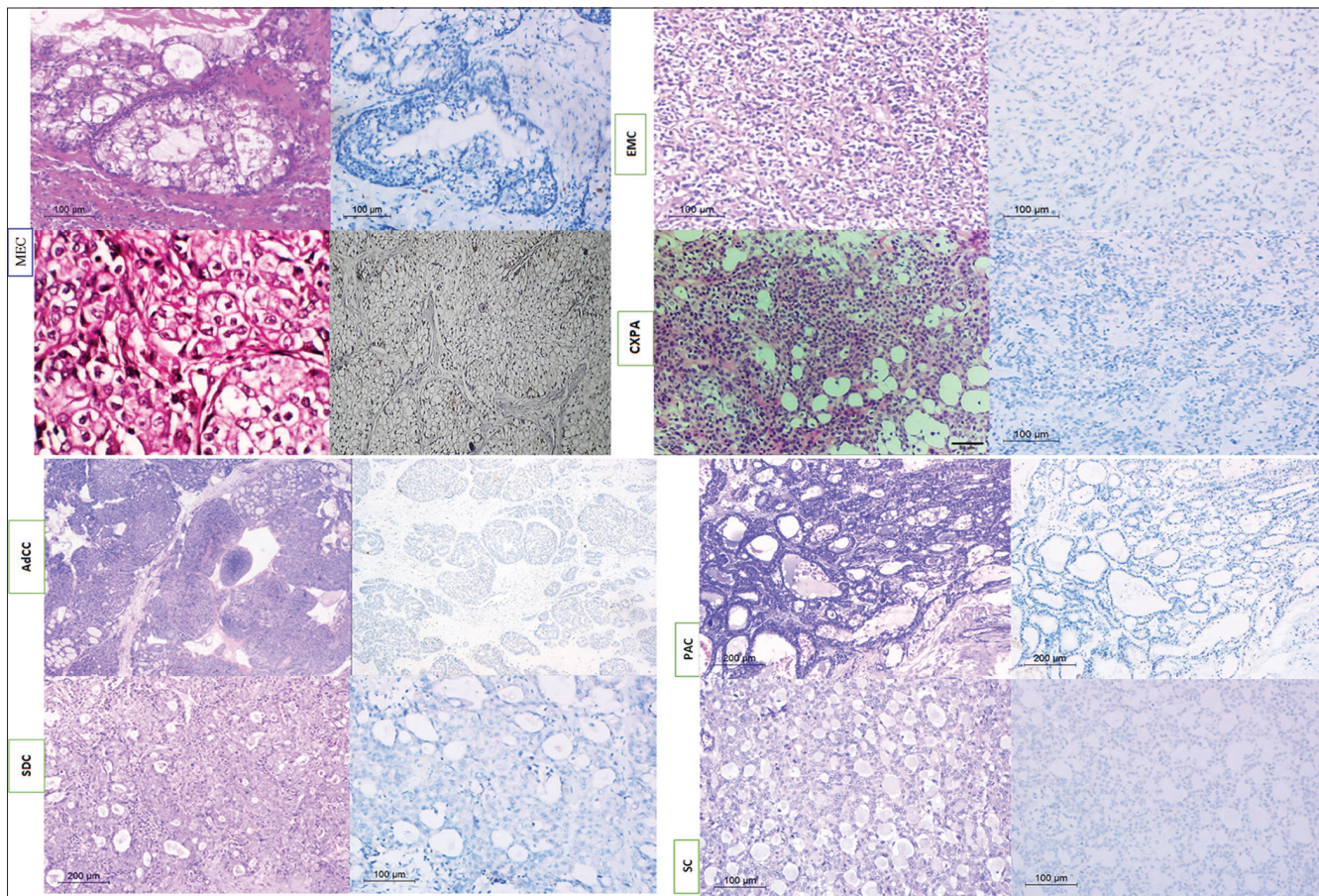


Figure 2: Photomicrographs of studied malignant salivary gland tumors revealing negative nuclear receptor subfamily 4 Group A member 3 immunoreactivity

by detection of MYB oncoprotein or through presence of related gene MYBL1. They observed through oncogenic activation and genomic rearrangement of the gene loci, which presents in approximately 80-90% of tumors [14], [15], [16], [20]. As well, in MEC detection of MECT1 gene and MAML2 gene, might be considered as a characteristic diagnostic feature, which have been reported in approximately 60–70% of MEC [9], [10], [11].

Detection of NR4A3 oncoprotein paving the way for the pathologists to differentiate AcCC from other salivary tumors [6], [20], [23], [24]. The NR4A family of orphan receptors (NR4A1-3) belongs to the superfamily of nuclear receptors. They regulate genes involved in proliferation, cell migration, and apoptosis [18].

By screening, the result of the present study

NR4A3 (NOR-1) immunoreactivity was showed in all studied AcCC cases, which was consistent with the previous immunohistochemical investigations of Cheng *et al.*, 2020 [24], and Haller *et al.*, 2020 [25], who detected a positive reaction of NR4A3 among their samples of salivary AcCC.

In the present work cases with N2, they were shown with a score +3 intense NR4A3 immunoexpression. This finding was consistent with Wong *et al.*, 2021 [23], who analyzed that NR4A3 overexpression represents an effective biological detector, as an increased expression insignificant, aggressive cases.

Furthermore, the main observation of the current work on the other malignant cases of salivary gland involving; EMC, SDC, CXP, PAC, and SC were

Table 4: Correlation between NR4A3 (NOR-1) immunoreactivity and studied cases of SGC

NR4A3 scores		Diagnosis									
		AcCC									
		AdCC									
		CXP									
		EMC									
		MEC									
		PAC									
		SDC									
		SC									
NR4A3	0	n	0	13	1	7	8	1	4	1	
	%		0.0%	92.9%	100.0%	100.0%	87.5%	100.0%	100.0%	100.0%	
+1	n	0	1	0	0	1	0	0	0	0	
	%		0.0%	7.1%	0.0%	0.0%	12.5%	0.0%	0.0%	0.0%	
+2	n	20	0	0	0	0	0	0	0	0	
	%		62.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	
+3	n	12	0	0	0	0	0	0	0	0	
	%		37.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	
Total = 68	n	32	14	1	7	8	1	4	1		
	%		100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
Monte Carlo test		p < 0.001*									

Monte Carlo test. *Statistically significant if p < 0.05, n: Number. SGCs: Salivary gland carcinomas, AcCC: Acinic cell carcinoma, AdCC: Adenoid cystic carcinoma, CXP: Carcinoma ex pleomorphic adenoma, EMC: Epithelial myoepithelial carcinoma, MEC: Mucoepidermoid carcinoma, PAC: Polymorphous adenocarcinoma, SDC: Salivary duct carcinoma, SC: Secretory carcinoma, NR4A3: Nuclear receptor subfamily 4 Group A member 3.

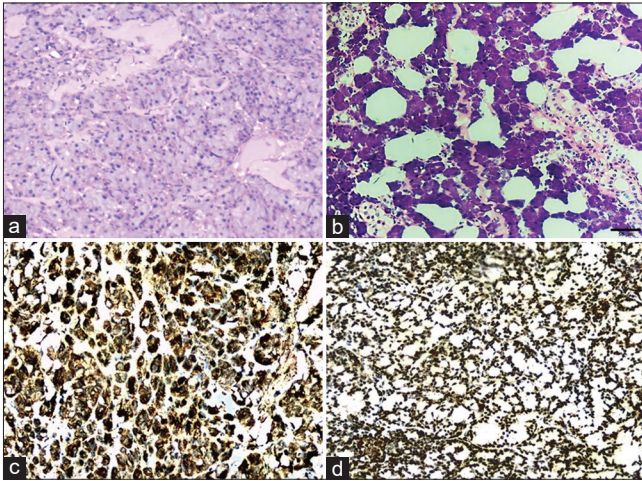


Figure 3: Photomicrographs of acinic cell carcinoma (AcCC) showing sheet of polygonal cells with hyper chromatic eccentric nuclei, (a and b, H and E $\times 200$). c and d photomicrographs demonstrating positive nuclear nuclear receptor subfamily 4 Group A member 3 immunoreactivity among AcCC cells (C and D anti NR4A3 (C $\times 200$) (D, $\times 100$))

negative for NR4A3 biomarker, which was compatible with Wong *et al.*, 2021 [23]. However, only one case of AdCC and MEC revealed NR4A3 immunopositivity in negligible percent between neoplastic cells. This finding was in disagreement with Cheng *et al.*, 2020 [24], and Wong *et al.*, 2021 [23]. Technical mistakes may contribute to this result.

Conclusion

Eventually, from the present and the previous findings, it can be stated that NR4A3 (NOR-1) can be used as a potential marker for distinguishing AcCC from the other malignant salivary gland neoplasm. Furthermore, NR4A3 (NOR-1) can be considered an available, most sensitive, and cost useful diagnostic tool for excluding AcCC from other SGCs, even being dedifferentiated or with high-grade transformation. Meanwhile, molecular testing is expensive and less sensitive than IMH testing.

Recommendations

As a result of few research work on this NR4A3 oncoprotein, so further studies are needed to identify clinicopathologic differences on abroad number of cases.

Cases with distant metastasis were not presented in our work so we recommend further work on them.

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Authors' Contributions

Everyone in our teamwork contribute in this study. ALgharyani A.S, Maghrabi M. M, Ezzat S.K., Shakweer M.M, and Esmaeil D.AM were contributed in conception and design of the study. Data analysis and acquisition were done by ALgharyani A.S, Maghrabi M. M. Laboratory work, staining evaluation, and figures taking had been done by Ezzat S.K. and Esmaeil D.AM. Manuscript writing and adding Shakweer M.M and Esmaeil D.AM. Statistical analysis of study data was done by ALgharyani A.S, Maghrabi M. M, and Ezzat S.K. Tables and charts selected and formatted by Esmaeil D.AM.

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