**Introduction**: Identification of serous acinar differentiation is the basic step for the diagnosis of acinic cell carcinoma (ACC). α-amylase was described to be specific for normal acinar cell but its affinity to malignant counterpart is being contentious. Recently discovered marker anoctamin (DOG1) specific for diagnosis of GISTs has been reported for its efficacy to stain the malignant acinar cells and so, it is being of value in ACC diagnosis. On the other hand, negativity of acinar cells to basal cell marker as p63 will give more pronouncement to ACC diagnosis.

**Objectives**: The study were designed to scrutinize the expression of recent marker DOG1 versus α-amylase in ACC diagnosis. Also to correlate between clinical, radiological, histopathological and immunohistochemical findings in ACC cases.

**Material and methods**: Fourteen cases of ACC were obtained and stained immunohistochemically for DOG1, α-amylase and p63. Clinical data as well as radiological findings were obtained from the patients’ file to form correlation with immunostaining results.

**Results**: All ACCs (100%) revealed positivity toward DOG1 staining, 6 (44%) cases out of 14 showed α-amylase positivity, and 1 case (7%) showed focal positivity for p63. No significant correlation was found between clinical, radiological and immunostaining results. Significant correlation was obtained between results of DOG1 and α-amylase with p-value=0.034, also significant correlation was obtained between DOG1 and p63 with p-value=0.02. On the other hand, no significant correlation was obtained between results of both α-amylase and p63 with p-value=0.546.

**Conclusion**: DOG1 has a higher efficacy in the diagnosis of ACC than α-amylase and must be used especially for cases with some conflicts as poor tissue sampling or suspicious cases with other carcinomas especially with carcinomas of clear cell features or having a vacuolated cytoplasm.

**Keywords**: DOG1, α-amylase, p63, acinic cell carcinomas, salivary gland tumors

**Introduction**

Acinic cell carcinoma (ACC) is a low grade malignant tumor and represents about 17% of all salivary tumors. It has more predilections to parotid gland with lesser extent to minor salivary gland as well as parotid lymph node [1]. Women are commonly affected and many factors as genetic, familial and radiation are considered the main risk factors for its development [2,3].

Histopathological examination revealed differentiation towards acinar cells. Variable microscopic variants and patterns are seen as solid, papillary, cystic, microcystic, follicular and lymphoid. In addition to these variants, cells in ACC may be granular, clear or vacuolated. Overall, irrespective of cell type, scant mitoses are generally seen [4,5].

Recognition of acinar cell differentiation is the basic step of ACC diagnosis [6]. For this purpose, α-amylase was widely used to highlight these acinar cells. Conversely, a positivity of acinar cells for α-amylase appeared to be restricted to normal acinar cells with low affinity to its malignant counterpart. This motivated the researches to investigate about a novel marker that is being more specific and expressed by malignant acinar cells until one of researchers found positivity of malignant acinar cells to DOG1 that was applied initially for diagnosis of
gastrointestinal stromal tumors (GISTs) [6].

Anoctamin-1 (DOG1, TMEM16a) or (ANO1) is a calcium-activated chloride channel protein that resides on the chromosome 11q13. It was initially described in GISTs [7,8] but now known to be expressed in a variety of normal and tumor tissues including salivary tissue [9]. Chromosomal studies revealed amplification in the region 11q13 that the ANO1 harbor. This yield in DOG1 over-expression that was seen in the varieties of tumor, but its amplification in salivary gland tumors, especially those originating from acinic cell has not been proved [10,11]. Several studies revealed that DOG1 has an essential role in the secretory function of salivary gland rather than its presence [12-14].

p63 protein (p63) is a nuclear protein, a member of the TP53 family and act as a transcription factor that plays a critical role in the growth and development of many epithelial organs [15,16]. It is confined to basal cells of squamous epithelia as well as basal cells/myoepithelial cells in breast, sweat glands, prostate and salivary glands [17,18]. However, the differential role and function of the main p63 gene in the development and biological features of salivary gland tumors remain unknown [19,20].

Our presenting study was aimed to in-vestigate the expression of a recent marker DOG1 versus α-amylase in association with p63 in ACC. The goals of this investigation were to find a more sensitive marker that help in reaching the accurate diagnosis, especially for cases with some conflicts as poor tissue sampling or suspicious cases with other carcinomas as carcinomas with clear cell features, and finally was to correlate between clinical, radiological and immunohistochemical findings in ACC cases.

Material and methods
Fourteen formalin-fixed, paraffin-embedded specimens that had been diagnosed previously as ACC were collected from archival material from Al-Baha province’ hospitals (King Fahd and Bilgrorashi) Hospitals, Saudia Arabia. These cases were obtained from period between 2000-2014. In each case, clinical data including radiographic findings were obtained from the patients’ file as well as from reference sheet. The clinical data obtained included the age and clinical presentation. All specimens were collected after taking written approval from both health manager of both hospital under number 235/2014 and 196/2014 respectively.

For H&E staining
Tissue samples were routinely fixed in 10% formalin, embedded in paraffin, cut into 4 µm thick sections and stained with hematoxylin and eosin stain (H&E). All H&E stained sections were reexamined by two histopathologists before immunohistochemical staining.

Immunohistochemical staining
Immunohistochemical procedures were run on consistently practiced, formalin- locked, paraffin rooted tissue.

For DOG1: Immunohistochemical staining was done using DOG1 (IgG, rabbit monoclonal antibody SP31, Ventana, USA, with dilution 1:50). For α–amylase: Immunohistochemical staining was performed using Anti-α Amylase antibody (Abcam, USA) (Mouse monoclonal to α-amylase ab54765, concentration 100 µg at 0.5 mg/ml, isotype IgG2a, monoclonal, of kappa light chain) the antibody was diluted 1/50 and Labeling was performed using the DAB detection kit [21]. For P63: immunohistochemical procedures were done by using the 4A4 anti-p63 antibody (Dako, Denmark, Code M7247). This antibody has the ability to identify all p63 isotypes.

For all markers, the following procedures were done; tissue specimens were cut into 4 µm, build up on poly-L-lysine-immersed slides, were depareffinized, rehydrated, and put in microwave in 10 mmol/L citrate buffer at pH 6.0 in a 750 W oven for 15 minutes. Slides were permitted to cool at room temperature for 30 minutes. The diluted antibody was preserved at room temperature for 2 hours in an automated Stainer (manufactured by BioGenex, San Ramon, CA, USA). Detection steps were completed by the instrument using the MultiLink-HRP kit (BioGenex). Peroxidase activity was localized using 3,3-diaminobenzidine or 3,3-diaminobenzidine-nickel chloride. Standardized development time periods allowed accurate comparison of all samples.

Evaluation of staining was considered as a regard cell type and percentage of stained cells. For DOG1 and α-amylase a positive staining was considered as cytoplasmic (dark for DOG1 and reddish brown for α-amylase) diffuse or granular staining for p63, distinct nuclear staining was only considered. The staining intensity was graded as negative, weak (focal apical cytoplasmic staining), or strong (diffuse strong cytoplasmic staining). Percent of positive cell less than 5 was considered negative and focal if more than 5 and less than 10%, moderate between 10 and 50% and diffuse if more than 50% of cells showing positivity [6].

Statistical analysis
Chi-square test and Fisher’s exact tests were used to compare the DOG1, α-amylase and p63 percentage and staining intensity data. p-values less than or equal to 0.05 were considered significant. The sensitivity of DOG1, α-amylase and p63 were calculated by calculation of percentage of positive cases to total ACC cases. Harvard Graphics was used for drawing figures. Computer software Statistical Package for the social science (SPSS) version 17 was used in the analysis of the presenting study.

Results
Analysis of clinical data
The age of the presented cases was ranged from 16-62 years, with mean±SD: 46±2.1. All cases were presented by infra-auricular mass. 3 out of 14 cases were presented with pain, 1 case was presented by facial nerve palsy. 2 cases showed cervical
lymph node metastasis. Staging of ACC was done according to AJCC [22] tumor stage and was as follows; 80%, 18%, and 2% in stages I, II, and IV, respectively. 12 out of 14 cases were treated by surgical excision and two cases were treated by total excision followed by radiotherapy.

Radiographic findings
From patients’ file, CT scan was done in 12 cases out of 14 and obtained information appeared that most of lesions were seen in the superficial lobe of the parotid. 8 cases revealed solid component, 2 cases were cystic and 2 cases showed mixtures of solid and cystic areas. All solid masses in the parotid gland showed nonspecific appearance, hypoeptinnouating regions of central necrosis, irregular enhancing solid component, which appeared as microcyst, hemorrhage, or necrosis on pathologic examination. No intratumoral calcifications were seen, two cases showed metastatic lymphadenopathy on imaging and histology study (Figure 1A and Table 2).

An MRI was done in all cases and revealed that 10 cases were solid lesions, 2 cases were cystic and 2 cases were mixed solid/cystic lesions. All cases showed nonspecific appearance, 2 cases with cystic lesions were T2 hyperintense, while 10 cases with solid and 2 cases of mixed lesions were mildly T2 hyperintense, well-marginated and showed enhancement on the post-contrast sequence (Figure 1B and Table 2).

Histopathological results
All histopathological slides were re-examined by two pathologists and all cases were diagnosed as ACC of different histologic patterns mainly papillary (Figure 2A) and solid pattern (Figure 2B).

Results of DOG1, α-amylase and p63 immunostaining

Results of DOG1 revealed that all cases of ACC showed DOG1 staining. Of the positive cases; 6 cases (43%) showed diffuse staining as more than 50% of cells showed positivity, 7 cases (50%) showed moderate positivity and 1 case (7%) revealed weak positivity for DOG1. The weak positivity in this case was due to the presence of more vacuolated cells. The staining was membranous and cytoplasmic, more intense in acinar cell types (Figure 3A) than intercalated duct (Figures 3B and 6), more intense in compact than vacuolated cells and more localized in an apical part of serous acini (Tables 1,3).

Alpha-amylase revealed positivity in 6 out of 14 ACC cases (43%) and negativity in 8 cases (57%). Of the positive cases, 3 cases showed diffuse staining, 2 cases with moderate positivity and one case was weak (Figures 4A-4C and 6) (Table 1,3).

Results of p63 revealed that 13 out of 14 (93%) of ACC cases were completely negative for p63 staining, while one case (7%) showed focal positivity (Figures 5A,5B and 6) (Tables 1,3).
As regards results obtained for 3 markers, we found one case showed positivity for all markers, 5 cases were DOG1+/α-amylase+/p63- and 8 cases showed DOG1+/α-amylase-/p63- (Table 3).

Sensitivity of DOG 1 staining for ACC is 100%, of α-amylase 44%, while the sensitivity of p63 immunostaining for ACC cases is 7%.

Table 1. Correlation between DOG 1, α–amylase and p63 expression in ACC cases.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Number of positive cases</th>
<th>Differential positivity count and its percentages</th>
<th>Negative</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>DOG 1</td>
<td>14</td>
<td>Focal (+) Moderate (+++) Strong (+++)</td>
<td>0</td>
<td>Correlation between results of DOG1 and α-amylase=0.034 (significant), between DOG1 and p63=0.021 (significant), between alpha-amylase and p63 p-value= 0.546 (insignificant)</td>
</tr>
<tr>
<td>α-amylase</td>
<td>6 (44%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P63</td>
<td>1</td>
<td></td>
<td></td>
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</table>

Table 2. Reactivity of cases to immunostains in relation to solid/cystic appearance in MRI findings.

<table>
<thead>
<tr>
<th>MRI findings</th>
<th>DOG1</th>
<th>α–amylase</th>
<th>P63</th>
</tr>
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<tbody>
<tr>
<td>Solid</td>
<td>-ve</td>
<td>Focal</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cystic</td>
<td>-ve</td>
<td>Focal</td>
<td>Moderate</td>
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<tr>
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<td>0</td>
<td>0</td>
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<td></td>
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<tr>
<td>Mixed</td>
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Table 3. Results of studied cases according to the three markers used.

<table>
<thead>
<tr>
<th>Immunostain</th>
<th>Number and Percent</th>
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<tbody>
<tr>
<td>DOG1+/α-amylase+/p63+</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>DOG1+/α-amylase+/p63-</td>
<td>5 (36%)</td>
</tr>
<tr>
<td>DOG1+/α-amylase-/p63-</td>
<td>8 (57%)</td>
</tr>
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</table>

Figure 4. (A) A case of acinic cell carcinoma showing moderate positivity of acinar cells for α-amylase antibody immunostain (less than 50% of tumor cells were positive for anti α-amylase antibody) for anti α-amylase mouse monoclonal antibody 3,3′-diaminobenzidine (DAB) (x200). (B) A case of acinic cell carcinoma showing strong positivity of intercalated duct cells for α-amylase antibody (more than 50% of tumor cells were positive for anti α-amylase antibody) (DAB) (x200). (C) A case of acinic cell carcinoma granular cytoplasm showing focal positivity for α-amylase antibody immunostain (DAB) (x1000).
We noticed that the intensity of staining for DOG1 was marked in 2 cystic lesions than solid lesions, while for α-amylase, one case was moderate staining and another case was negative (Table 2). No significant correlations were obtained between intensity of staining of all markers and staging of tumor.

There was no significant correlation obtained between clinical findings (symptoms and signs) as well as radiological findings, (p-value=0.564). Also no significant correlations were obtained between clinical findings with all of the results of immunostaining markers; clinical findings with DOG1; (p-value= 0.547), with α-amylase (p-value=0.643), with p63=0.512). Finally, no significant correlations were obtained between radiological findings and results of immunostaining markers; correlation of radiological findings with DOG1; (p-value=0.574), with α-amylase p-value=0.659), with p63=0.571).

Discussion
In the presenting study, we found the mean age of ACC cases was 46 years and the main presenting symptoms was painless infra-auricular mass except in three cases in whom pain was present. Also one case was presented by facial palsy and 2 cases had cervical lymph node metastases. According to the AJCC [22], 80% of cases were in stage I while in 20% of cases were distributed among stage II and IV respectively. As regards these clinical findings, there was no unusual presentation of ACC cases in the presenting study and all these symptoms are in agreement with many previous studies [5,23,24]. There was no significant correlation obtained between intensity of staining of all markers and staging of tumor. No previous studies did correlation between reactivity results of these markers and ACC staging. The intensity of DOG1 in addition to other markers with the staging of the tumor.

MRI findings revealed solid lesions of non specific appearance in 10 cases, cystic lesions in 2 cases and mixed lesions in 2 cases. Also, there was increased intensity to DOG 1 in cystic lesions than in solid lesions. This might be explained by the presence of tissue necrosis that reflect high grade malignancy, but this is not enough to say that ‘the higher the grade, the higher the DOG1 intensity’ due to the low number of cases subjected to the study. There was no significant correlation obtained between clinical, radiological findings and results of immunostaining markers. No previous studies did correlation between these parameters and results of immunostaining.

Diagnosis of ACC depends on the morphological appearance of acinar cells. For this reason, α-amylase was being used but its expression in ACC is not frank as reported previously, hence DOG1 has been evoked [25].

In the presenting study, we used DOG1, α-amylase and p63 as a panel to diagnose ACC. We found that DOG1+/α-amylase+/p63- was present in 5 cases, DOG1+/α-amylase+/p63+ in one case and DOG1+/α-amylase-/p63- in 8 cases. These results clarify that DOG1 is superior to α-amylase in the diagnosis of ACC. However α-amylase gave positivity in 6 cases out of 14 (43%) cases, it appears to be less sensitive for ACC. On the other hand using these 2 markers together may yield in synergistic effects on ACC diagnosis. In the presenting study 1 case was focally positive for p63 and 13 cases were negative, close observation of this case revealed that this focal staining was attributed to the presence of scattered myoepithelial cells among acinar cells as a result of extensive destruction of tissue obtained by malignant tumor cells.

The high positivity of malignant acinar cells to DOG1 immunostaining in the present study is in accordance with previous studies [6,10,11]. The explanation of this high positivity is simplified by a symbol of an overstated acinar phenotype to a certain extent rather than gene amplification. Carles et al. [10] found that DOG1 expression in ACC were more localized in an apical serous acini and explained this observation by the fact of role of apical part of serous acini in the processing of anion channel across the cell membrane [10].

In the presenting study, we found one case showed focal weak staining for DOG1. Close observation of this case revealed
presence of many vacuolated cells as evidenced in the H&E stained section. This observation was also noticed by Ousin-gsawat et al., [14] who accredited this observation by the presence of several factors as dispensation in the Golgi apparatus and endoplasmic reticulum, a disparity distribution of isoforms, differences in post-translational adjustment and the presence of lipid microvacuoles.

In the presenting study, we found 6 out of 14 ACC cases positive for α-amylase, this coincides with studies done by Sumitomo et al., [26] and Ihrler et al. [27]. Sumitomo et al., [26] studied the ex-pression of α-amylase in various salivary gland lesions either inflammatory or neoplastic as well as in normal salivary glands and found that the positivity of α-amylase was confined to irregularly staining serous acinar cells in normal parotid, submandibular glands, and to demilunes in sublingual glands, negative or scattered focal among neoplastic lesions and of irregular from high to low in inflammatory lesions.

Ihrler et al., [27] studied the expression of several markers beside α-amylase in ACC versus adenocarcinoma not otherwise specified and found that α-amylase was expressed weakly in ACC. Childers et al., [26] studied the reactivity of anti-amylase antibody in ACC versus cystadenocarcinoma and found that only 4 of 27 cases (13%) of ACC cases showed reactivity and concluded that anti-amylase antibody is of limited value in the recognition of ACC when light morphological features are insufficient for diagnosis.

In the presenting study, we found 13 out of 14 ACC cases negative for p63 antibody this coincides with studies done by Weiler et al., [28], Sams and Gnep [29] and Mitani et al. [30]. Weiler et al., [28] reported that all cases of ACC subjected to his study showed 100% p63 negativity. Sams and Gnep [29] found complete negativity in all ACC cases with p63 and demonstrated a complete lack of basal cell component manifested by negative p63 expression. All these results give more support to our presenting study.

Mitani et al., [30] studied the reciprocal p63 TA and ΔN isofrm expression in certain benign and malignant salivary gland tumors, of these ACC and found that salivary duct and ACC, tumors that lack myoepithelial and/or basal cells, were deficient for both isoforms and hence, all cases of ACC were negative for p63 immunostaining in contrast to tumors with myoepithelial and/or basal cell participation.

Conclusion
Application of panels composed of DOG1, α-amylase and p63 in the diagnosis of ACC is of diagnostic value, especially in cases with conflicts with other salivary gland carcinoma especially those of clear cell features. DOG1+/α-amylase+/p63- or even DOG1+/α-amylase-/p63- will support the diagnosis of ACC. Not all cases of ACC positive for α-amylase but most cases of ACC give positive staining for DOG1. So DOG1 is more superior in the diagnosis of ACC than α-amylase. Negativity of p63 in ACC cases is the rule and its positivity is the exception, hence DOG1+/α-amylase+/p63- is superior than DOG1+/α-amylase-/p63- in ACC diagnosis.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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