IDENTIFICATION OF GLANDULAR DIFFERENTIATION IN APPENDICEAL CARCINOID TUMOR- A STUDY IN SULAIMANIA CITY

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ABSTRACT

Background

Carcinoid tumor of appendix is the most common appendiceal tumor. Both the classical and mixed carcinoid tumor could be identified using the routine H&E stain. Using additional stains such as Periodic Schiff stain, Alcian blue and cytokeratin 20 (CK20) stain have been used to detect these appendiceal mixed (glandular) carcinoid tumors.

Objective

To identify glandular differentiation and mucin production in appendiceal carcinoid tumors by using special stains and immunohistochemistry.

Patients and Methods

This retrospective case-control study was carried out in research laboratory of Pathology Department/School of Medicine. All pathological reports of patients with appendiceal carcinoid tumor were collected from the laboratory of pathology-Sulaimani Teaching Hospital over the period of six years (from the 1st of January 2006 to the 31st of December 2011), patient's age, sex and histopathological diagnosis were documented. Fifteen cases (out of 10533 appendectomies) were divided into 2 groups: classical (insular) and mixed (insular with mucinous differentiation). Formalin-fixed, paraffin-embedded, 4- micrometer- thick sections were immunostained with CK20 using HIER-LSAB method. All cases were stained with Alcian blue stain for acidic mucin detection, and PAS stain for neutral mucin detection within the carcinoid tumor. Then the results of H&E stain, immunostain for epithelial differentiation and the special stains for mucin production were evaluated together and compared with similar studies.

Results

The carcinoid tumor is more common in females with a peak age range of 21-30 years, mean incidence 0.14% for fifteen cases of carcinoid tumors. Carcinoid tumor in patients above 50 years is usually mixed type. PAS and Alcian blue stains are of no statistically significant correlation with glandular differentiation, while CK20 has statistically significant correlation with glandular differentiation but staining intensity is variable.

Conclusion

H&E is the main method for the identification of glandular differentiation of carcinoid tumor, however CK20 has important value to assist the diagnosis, while mucin stains have limited value in the identification of glandular differentiation of carcinoid tumor.

Keywords: *Appendix, mixed carcinoid Tumor, PAS stain, Alcian blue, CK20*.

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INTRODUCTION

Appendiceal carcinoid tumor is the most common appendiceal tumor, making up 51% of all malignant tumours of appendix [1-3] with the most favourable prognosis [4]. These tumours originate in cells of diffuse neuroendocrine system of GIT which are also called APUD (**A**mine **P**recursor **U**ptake and **D**ecarboxylation) [2-3]. Seventy four percent of the carcinoids occur in the GIT, 25% in broncho-pulmonary system while the remaining 1% is reported in ovary, thymus, testis, liver, spleen, and breast [5-8]. In the GIT, 26% are found in the appendix. The incidence of appendiceal carcinoid in several studies ranges from 0.02-1.5% of surgically removed appendices [5, 9] . A large female predilection is reported in all series (2-3:1) with incidence peaks at ages 20-39 years, and the tumours are mainly located at the tip of the appendix [3,9]. Goblet cell carcinoid (GCC) has been recognized as a distinct, rare tumor of the appendix since its first description by Gagne et al. in 1969 [10]. It has been referred to by a wide variety of names, including mucinous carcinoid, adenocarcinoid, amphicrine cell carcinoma, and crypt cell carcinoma [11-13]. GCC has uncertain biologic behaviour. Recent studies indicate aggressive behaviour with tendency to metastasize to intra-abdominal organs and peritoneum in 20% of cases especially ovaries in female patients [14-16]. These tumors are mucin-rich which is predominantly acidic in type, and are strongly positive for PAS and Alcian blue [17]. Goblet cell carcinoid shares histologic features of both classical (typical) appendiceal carcinoid and colonic signet ring cell adenocarcinoma [18] .The molecular events in the pathogenesis of GCCs of the appendix are similar to typical appendiceal carcinoids and differ from those in colonic adenocarcinomas suggesting that they are variants of carcinoid tumors [19].

Periodic acid-Schiff (PAS) is a stain used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans), typically found in connective tissue, mucus, glycocalyx, basal lamina and tumors secreting neutral mucins [20]. **Alcian blues** are a family of polyvalent basic dyes, of which, the Alcian blue 8G (formerly called Alcian blue 8GX) has been historically the most common and the most reliable member. It is used to stain acidic polysaccharides such as glycosaminoglycans in cartilages and other body structures, some types of mucopolysaccharides, sialylated glycocalyx of cells etc [21]. At pH 1.0 it stains only sulfated polysaccharides. Uronic acids intensifies the stain of hyaluronic acids which would also be stained relatively weakly by their half sulfate esters at pH 1.0 [22]. In 3% acetic acid solution (pH2.5), it stains carboxyl group containing sugars such as sialic acid. The blue colour of the stain is due to the presence of copper in the molecule [23]. Because GCC is considered to be derived from crypt cells [16], there are other immunohistochemical techniques for their diagnosis including Cytokeratin 20 and CK7 [24,25].

Cytokeratins are intermediate filament cytoskeletal proteins essential for development and differentiation of epithelial cells. Approximately 20 different cytokeratins have been identified, classified and numbered according to molecular weight and isoelectric points. CK20 (Clone KS20.8) is to identify epithelial cells in normal and neoplastic tissues using immunohistochemical methods. CK20 is available as histodiagnostic tool for the subtyping of various carcinomas, including adenocarcinomas. It is an aid for a more precise classification of many epithelial tumors whose differential diagnosis is otherwise difficult. This antibody reacts with the 46 kDa cytokeratin intermediate filament protein [26].

Aim of the study

To detect glandular differentiation and mucin production in appendiceal carcinoid tumors by using special stains and immunohistochemistry.

MATERIALS AND METHODS

This retrospective case-control study was carried in Sulaimani Teaching Hospital. All pathological reports of patients with appendectomies were reviewed over the period of six years (from 1st of January 2006 to 31st of December 2011). Fifteen cases (out of 10533 appendectomies) were collected and reviewed for patient's age, sex and histopathological diagnosis then they were subclassified into two groups: **classical** insular carcinoids and **mixed** carcinoids i.e. insular with microacinar formation or goblet cell clusters.

Formalin-fixed, paraffin-embedded, 4 micrometer-thick sections were immunostained with CK20 (monoclonal mouse antihuman cytokeratin 20, clone Ks 20.8), purchased from DAKO (DK-2600, Glostrup, Denmark), using HIER-LSAB method [27]

after dewaxing with xylene and rehydration. Antigen retrieval (pH 9) was done by immersing the slides in antigen retrieval solution in electric pressure cocker (GEEPAS) for 10 minutes and rinsed with phosphate buffer saline (PBS) (two changes, 15 minutes each). Adding peroxidase block and incubation in humidity chamber for one hour. Diluted primary antibody (1/100 dilution) was added and incubated for 30 minutes, then washing with PBS (two changes, 15 minutes each)-BS. Adding secondary antibody and incubation for 15 minutes, washing with PBS (two changes, 15 minutes each). Adding enough drops of streptavidin (Dako LSABTM/HRP KIT, Code K0679) to cover specimen and incubation for 15 minutes, wash with PBS. Substrate-chromogen (diaminobenzidine–DAB) solution were added to cover specimen for 30 minutes and washed with distilled water. Counterstain with haematoxylin, dehydrate in ascending concentrations of ethanol, clear in xylene and mount. Normal mucosal lining of the appendix was used as internal positive control, while sections without primary antibody staining were used as negative control. Staining intensity was classified as strong (3+) in comparison to positive control cases, moderate (2+), weak (1+) and absent (0) [28,29].

All cases were stained with Alcian blue 8GX 3% at pH 2.5 special stain for acidic mucin detection, and PAS special stain for neutral mucin detection within the carcinoid tumor. The two mucin stains results were re-evluated and any positive case for any one of the two stains was regarded as mucin positive and regarded as negative if both stains were negative. Then the results of H&E stain, immunostain for epithelial differentiation and the special stains for mucin production were campared together and to similar studies. All data (mean, standard deviation, percentile) and graphical presentation were inferred and analyzed using SPSS v 21, Chi square were used to compare between different groups, p value <0.05 was considered significant in difference between two groups.

RESULTS

Among the 10533 appendectomies during 6 years fifteen cases were diagnosed as carcinoid tumor of the appendix. The mean incidence of carcinoid tumor in these 6 years was (0.14%) as shown in Table 1. Out of the 15 cases there were 5 (33.3%) male cases and 10 (66.7%) female cases. Patients with a mean age of 30.8 years (range of 19 - 62 years) and highest incidence (20.4%) was between 21-30 years of age as shown in Table 2.

The majority of the cases were ≤50 year of age (86.7%) in which 60% were females, while 13.3% were >50 years of age with equal male to female ratio as shown in (Fig. 1).

The relation of H&E morphology of carcinoid tumor cases distributed in different age group showed that two cases of carcinoid tumors were >50 year and of mixed morphology while 7 cases were ≤50 year and of mixed morphology as shown in Table- 3. Morphology of classical and mixed carcinoid tumors of the appendix stained with H&E stain is shown in (Fig.2).

The comparison between H&E morphology of carcinoid tumors cases and PAS, Alcian blue special stains with CK20 marker revealed that 6 (85.7%) out of nine cases of mixed carcinoid tumor were PAS positive with no statistically significant correlation between this stain and H&E morphology (P-value = 0.057) as shown in Table-4. Morphology of mixed carcinoid tumors stained with PAS stain is shown in (Fig.3).

Four cases of mixed carcinoid tumors (66.7%) were Alcian blue positive with no statistically significant correlation between this stain and H&E stain (P-value = 0.66) as shown in Table-4. Morphology of internal positive control and mixed carcinoid tumors stained with Alcian blue is shown in (Fig.4). It was found that 8 cases of mixed carcinoid tumors (80%) cases were positive for CK 20 and there was statistically significant correlation between H&E morphology and CK20 expression positivity (P-value = 0.025) as shown in Table-4. Morphology of carcinoid tumors stained with CK20 with variable intensities is shown in (Fig.5). At the same time P. value was also calculated between both mucin stains and mixed carcinoid tumors stained by H&E stain and found that there was statistically significant correlation between them (P-value=0.02).

The two mucin stains results were combined together and cases were regarded as negative only if they were negative for both mucin stains. It showed that 3 cases out of 7 of mixed carcinoid were mucin negative with no statistically significant correlation (P. Value=0.20) between mucin stain and H&E morphology, as shown in Table- 5. There were 2 cases out of 8 were mucin positive in classical

carcinoid. P-value was also calculated between mucin stains and mixed carcinoid and still there was no statistically significant correlation between the two variables (P- value=0.31).

There was statistically significant correlation between mucin stains and CK 20 expression (P. value= 0.06) as shown in Table 6.

Comparison between the variable intensities of CK20 and H&E staining found that out of 9 cases of mixed carcinoids, one case was negative for CK20, while 3 other cases were stained weakly, 2 of them were moderately stained and the remaining 3 cases were strongly stained with no statistically significant correlation between H&E morphology and CK staining intensity (P-value = 0.12) as shown in Table 7. In this study the stained areas were ranging from focal to diffuse.

Table 1. Carcinoid tumor incidence during 6 years.

Years	Total Appendicectomy	Cases of Carcinoid	Incidence of Carcinoid
2006	1431	0	0
2007	1895	3	0.15 %
2008	1650	1	0.06 %
2009	2052	4	0.19 %
2010	1755	6	0.34 %
2011	1750	1	0.05 %
Total	10533	15	0.14 %

Table 2. Age group and sex distribution among patients.

Age group of patients	Sex of patients			
	Male		Female	
	Count	%	Count	%
11-20	1	6.8%	2	13.3%
21-30	0	.0%	3	20.4%
31-40	0	.0%	2	13.3%
41-50	2	13.3%	1	6.8%
51-60	1	6.8%	1	6.8%
61-70	1	6.8%	1	6.8%
Total	5	33.3%	10	66.7

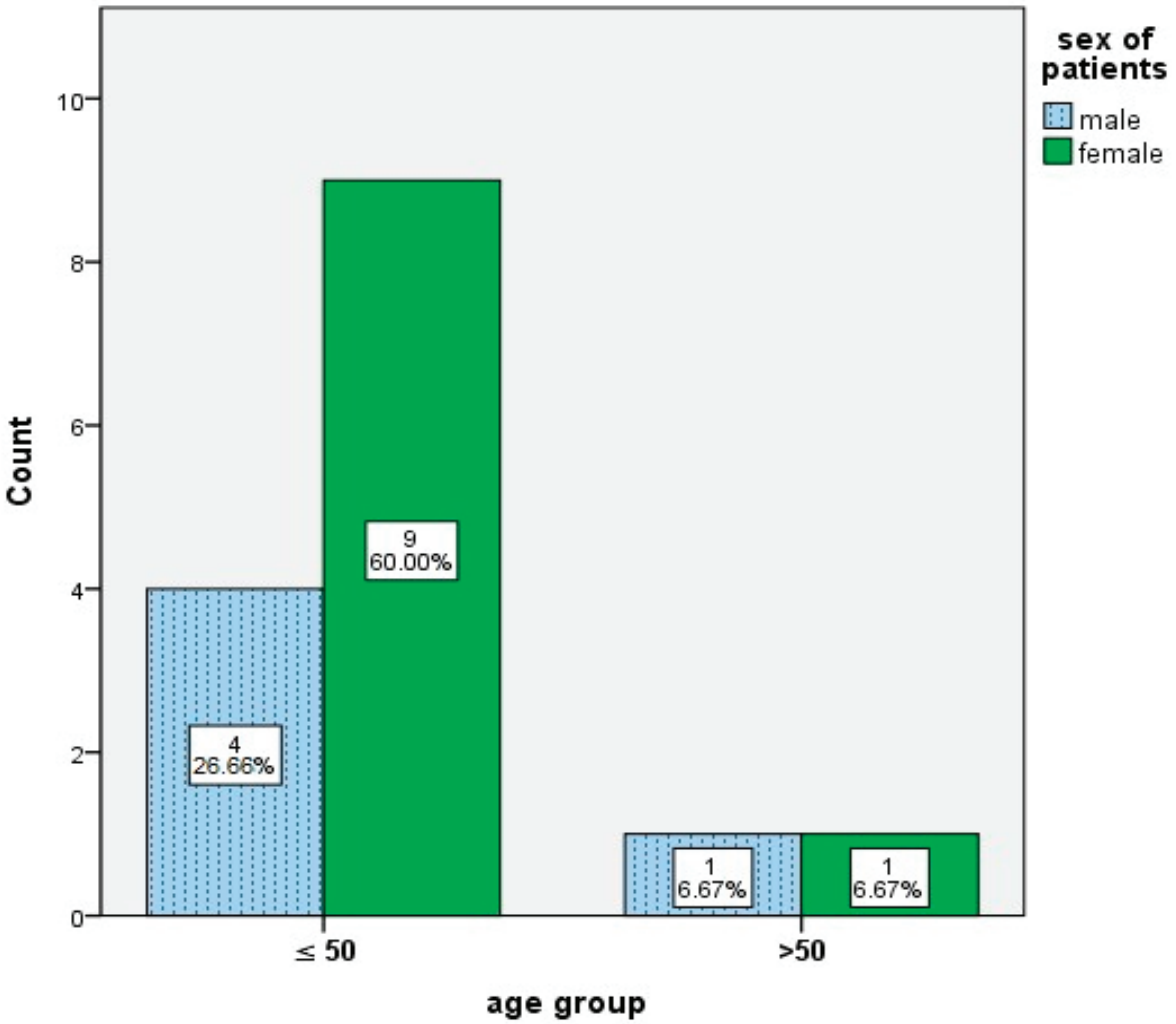

Figure 1. Number of cases ≤ 50 year and those >50 year in both sexes.

Table 3. Relation between H&E morphology and age groups.

H&E morphology	Age groups		Total	P. value
	≤50	>50		
Classical	6	0	6	
Mixed	7	2	9	0.21
Total	13	2	15	

Table 4. Comparison between H&E morphology of carcinoid tumor with special stains and CK20 marker.

H&E morphology	Alcian Blue stain		PAS stain		CK20 expression	
	negative	positive	negative	positive	negative	positive
Classical	4 (44.4%)	2 (33.3%)	5 (62.5%)	1 (14.2%)	4 (80%)	2 (20%)
Mixed	5 (55.5%)	4 (66.7%)	3 (37.5%)	6 (85.7%)	1 (20%)	8 (80%)
P. value	0.66		0.057		0.025	

Table 5. Comparison between H&E morphology and mucin stains.

H&E morphology	Mucin stain		Total	P value
	negative	positive		
Classical	4	2	6	
Mixed	3	6	9	0.20
Total	7	8	15	

Table 6. Comparison between CK20 expression positivity and mucin stains.

CK20 positivity	Mucin stains		Total	P value
	negative	positive		
Negative	4	1	5	
Positive	3	7	10	0.06
Total	7	8	15	

Table 7. CK20 expression intensities in comparison to H&E morphology.

CK20 intensity	H&E morphology		Total	P value
	Classical	Mixed		
Negative	4	1	5	
Weak	1	3	4	0.12
Medium	1	2	3	
Strong	0	3	3	
Total	6	9	15	

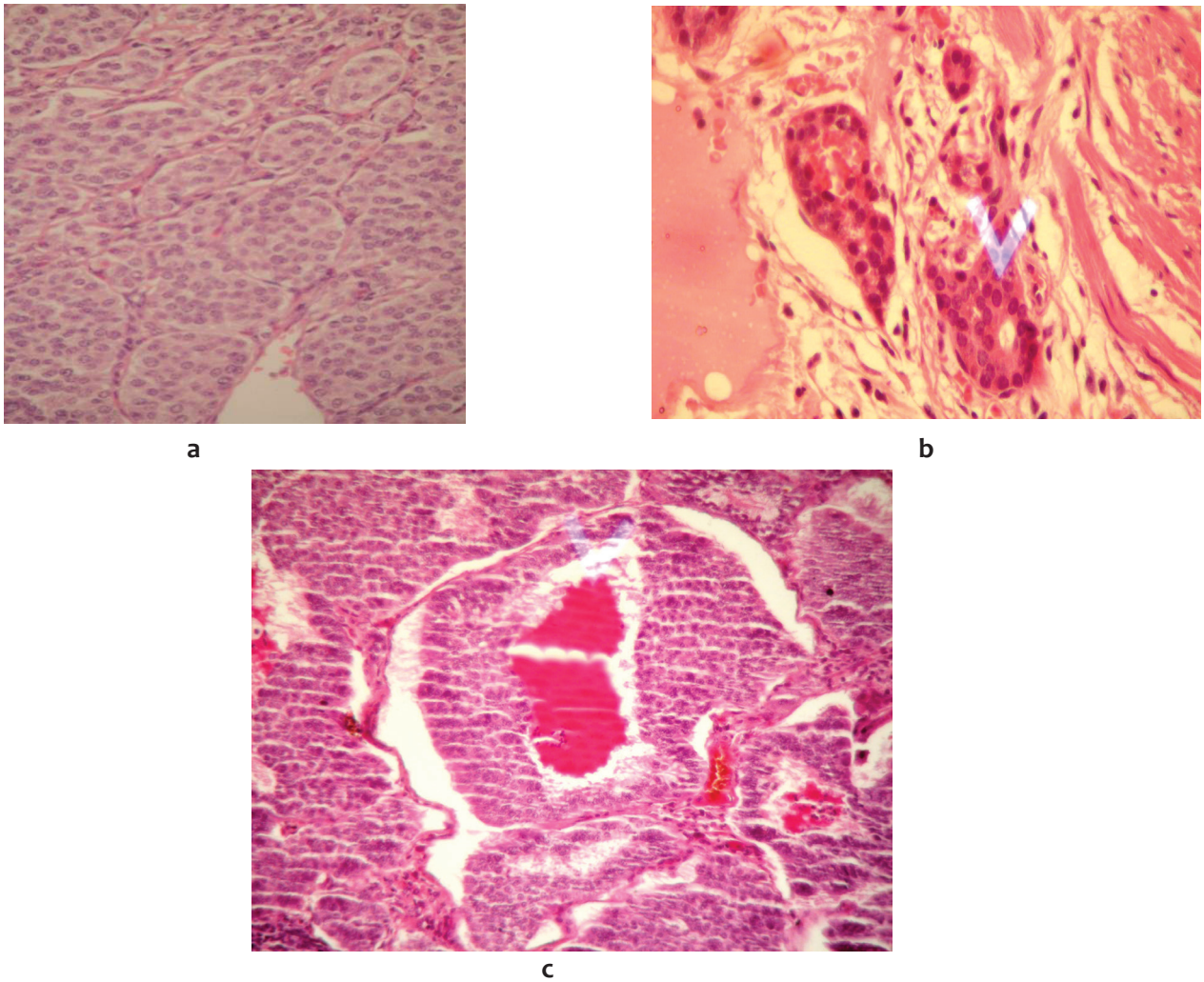

Figure 2. (a) Classical (insular) carcinoid tumor (H&E, X200), (b&c) mixed carcinoid tumor (H&E, X400, X200).

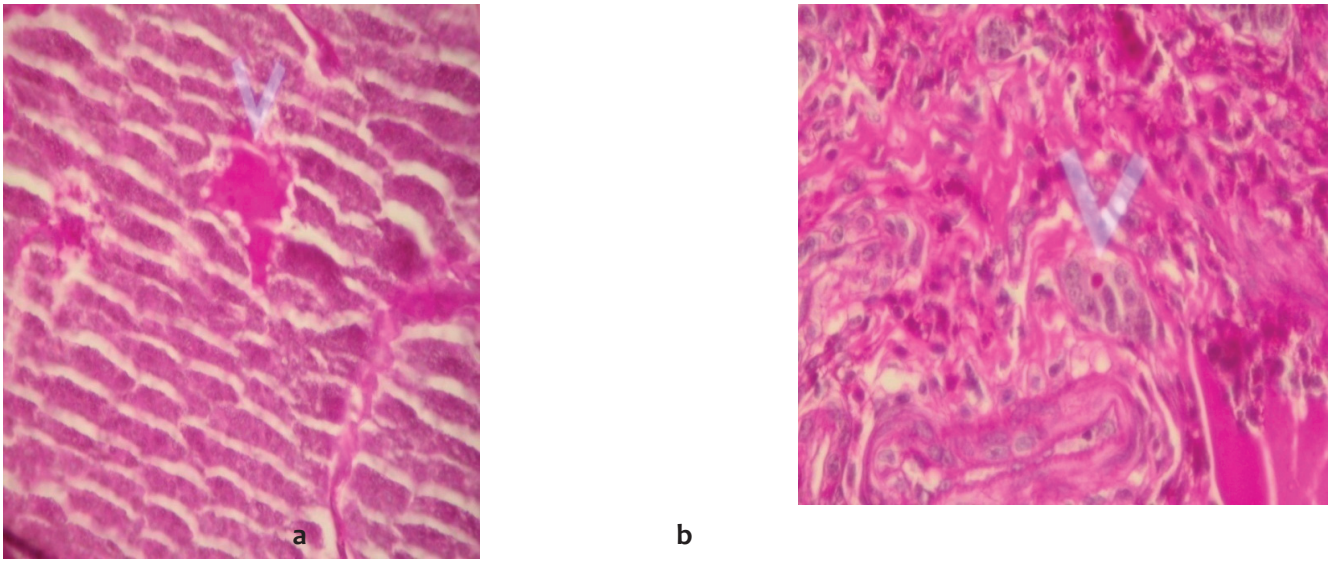

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Figure 3. (a) and (b). Mixed carcinoid tumor (PAS stain, X400)

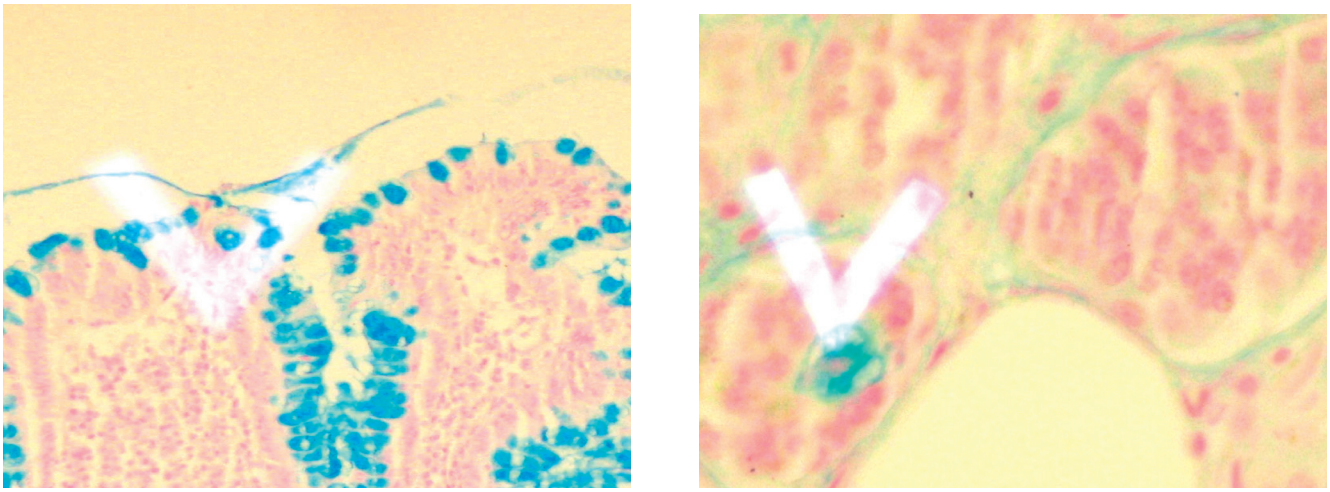

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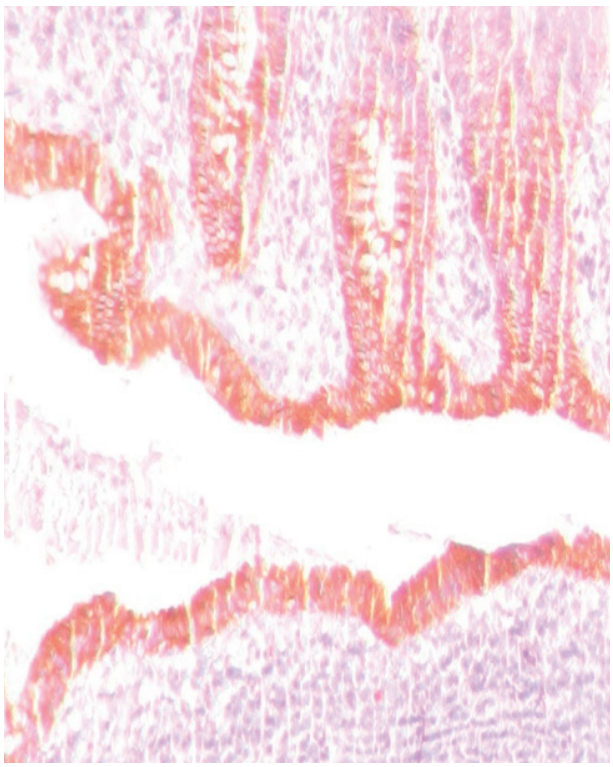
Figure 4. (a) Internal positive control stain of goblet cells which lining normal appendiceal mucosa (Alcian blue stain, X200), (b) mixed carcinoid tumor with glandular differentiation (Alcian blue stain, X400)



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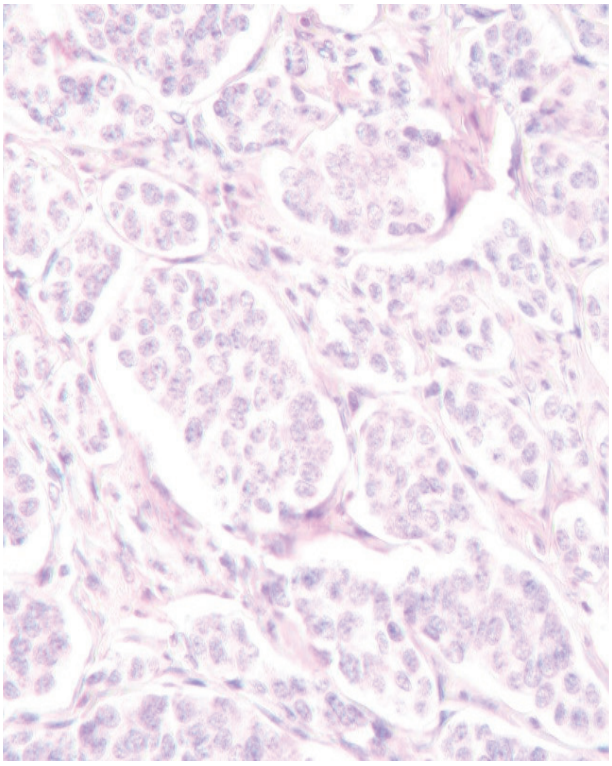

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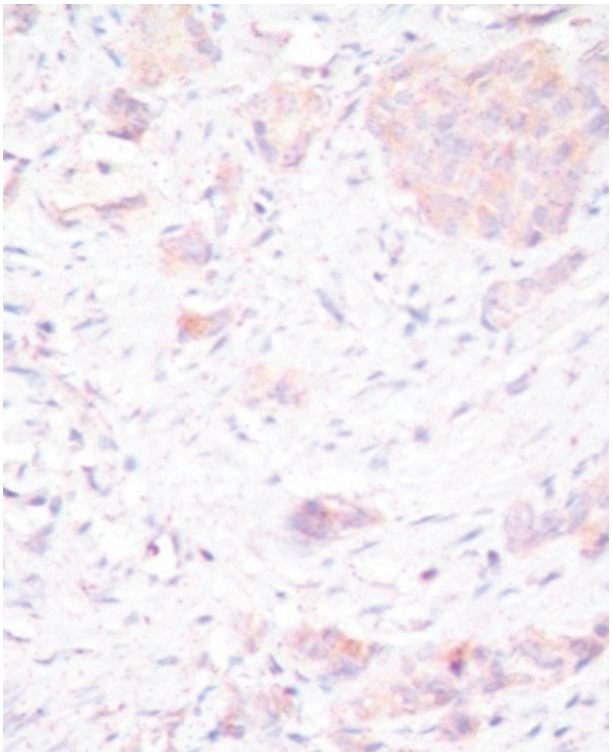

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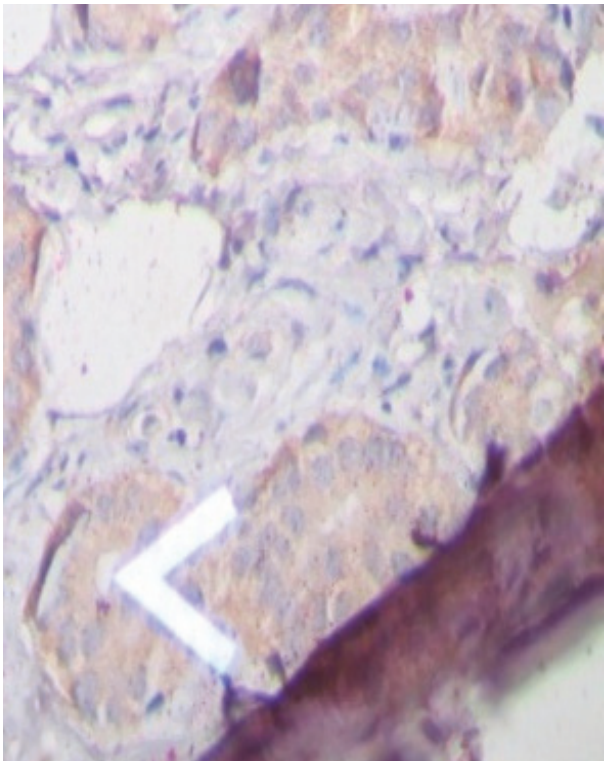

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Figure 5. (a) internal positive control staining of mucosal lining of the appendix, (b) negatively stained classical carcinoid tumor score (0), (c) weak intensity staining score (+1), (d) strong intensity staining score (3+), (CK20, X200).

DISCUSSION

The present study showed that incidence of carcinoid tumor during 6 years of study ranged from 0 to 0.34% with a mean incidence of 0.14%. A female predominance was noted (66.7%) with a peak incidence in 21-30 years. This is consistent with other studies that showed the incidence of appendiceal carcinoid ranged from 0.02 to 1.5% of all surgically removed appendices with large female preponderance (female: male ratio is 2-3:1) and peak incidence at ages 20-39 years [30-32].

In this study there were 2 cases above the age of 50 years (one male and one female) and both of them had mixed goblet cell carcinoid. Peter H. Watson and Antonio Alguacil-Garcia in 1987 in Canada studied the clinicopathological features of six cases of mixed crypt cell carcinoma of the appendix and they found that 5 cases were males and 1 case was female with the average of 62 years [33]. Other studies stated that the age of presentation of GCC ranges from 18 to 89 years with the majority of patients presenting in their fifth or sixth decades [34-36], the reported male to female ratio of GCC has varied in literature between 1.4:1 to 1:2.2 [33,37].

This study showed that there was variable positivity of PAS and Alcian blue stains in the two types of carcinoid tumors with no statistically significant difference between these stains and H&E stains. While other studies showed that all vacuolated cells were positive for mucicarmine, PAS, PAS with diastase and Alcian blue [38].This can be attributed to the type of mucin secreted by the tumor whether acidic or neutral. Negativity of both stains was related to the absence of both intracellular and intraluminal mucin secretion in well defined microacinar formation. Positivity of these stains in classical carcinoid could be attributed to focal microacinar formation and/or single goblet cells formation that were not easily identified by H&E stain.

In the present study 2 out of 6 cases of classical carcinoid tumor showed weak and medium staining intensity of CK20, while 8 out of nine cases of mixed carcinoid had variable staining intensities. This is comparable with the WHO 2010 criteria which state that (well differentiated appendiceal carcinoid had variable expression of CK 20, while carcinoma Ex-GCC had 100% positive for CK20) [39].

Alsaad KO,Serra S,Schmitt A,Perren A, Chetty R. in 2007 in Canada found that 4 cases (16%) out of 25 of classical carcinoid tumors showed immunolabeling for CK20 in focal distribution of the tumor cells [40]. This is highly consistent with the current study in which CK 20 was focally expressed in tumor cells. According to t-test there was no statistically significant difference between the positive cases in classical type of this study and that of Alsaad KO result (p= 0.221).

While Lin BT and Grown AM from Trazana in USA found that mixed carcinoid and adenocarcinomas were cytokeratin (CK) 20 positive, while classical carcinoid tumors were CK20 negative [41].

Peiguo Chu et al. did an immunohistochemical survey on 435 cases of different epithelial tumors and found that among 15 cases of GIT carcinoid only 1 case (7%) was CK 20 positive [42] . This could explain the negative staining result seen in this study and the variability in the CK20 intensity expression.

In 2012 in Baltimore in USA, Matsukuma and Montgomery stained 8 cases of tubular carcinoid with CK 7 and CK20 and found that staining was variable, ranging from none to focal staining for either or both CK7 and CK20, to diffuse expression of CK7 or CK20. So they concluded that CK 7 and CK 20 have limited value when the differential diagnosis includes primary tubular carcinoid and well-differentiated metastatic adenocarcinoma (43). This finding is consistent with the current study that showed variable staining from focal to diffuse pattern.

In conclusion, H&E is the main method for the identification of glandular differentiation of carcinoid tumor. CK20 has important value to assist the diagnosis of glandular differentiation within mixed carcinoid tumor. PAS stain and Alcian blue stain have poor correlation with carcinoid morphology.

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