# Role of Myofibroblasts and Collagen Quality in Elaboration of Debatable Nature of Odontogenic Keratocyst

**Original Article**

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## ABSTRACT

**Background:** Odontogenic keratocyst (OKC) is one of the debatable odontogenic lesions. The need for more researches in order to determine the true nature of this lesion was one of the WHO's recommendations in 2016. Stromal reactions to epithelial neoplasms are marked by the appearance of alpha smooth muscle actin (α-SMA) positive myofibroblasts (MFs). Stromal collagen has a vital role in pathogenesis of odontogenic lesions.

**Objectives:** To compare the count of α-SMA positive MFs, collagen quality and packing in OKC, in relation to dentigerous cyst and ameloblastoma.

**Material and Methods:** α-SMA immunohistochemically positive MFs count, as well as the fraction of yellow-green fibers to detect the collagen quality and packing using picrosirius red staining with polarized light microscope, were compared in 11 samples of each of ameloblastoma, OKC and dentigerous cyst. Statistical analysis was performed.

**Results:** The average number of MFs were 53.7, 41.9 and 11.3 in ameloblastoma, OKC and dentigerous cysts respectively with statistically significant differences (*P-value* <0.001, Effect size = 2.619). The mean area fraction of yellow-green fibers were 65.5%, 47.72% and 6.1% in ameloblastoma, OKC and dentigerous cyst respectively. Differences were statistically significant (*P-value* <0.001, Effect size = 2.645). Statistically significant direct correlation between MFs cell counts and area fraction of yellow-green fibers was found (ρ = 0.614, *P-value* <0.001).

**Conclusions:** The immunohistochemical profile of OKC as regard to α-SMA positive MFs as well as histochemical profile as regard to collagen packing and quality were much closer to odontogenic tumors (ameloblastoma) than odontogenic cysts (dentigerous cyst) suggesting the neoplastic nature of this debatable lesion.

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**Key Words:** α-SMA, ameloblastoma, dentigerous cyst, odontogenic keratocyst, picrosirius red.

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## INTRODUCTION

Oral and maxillofacial surgeons face different pathological lesions during their clinical practice. The controversy regarding the surgical approach of certain pathological lesions might be a major obstacle in order to achieve success. This controversy is attributed to the genetic and histopathological features of some lesions that are reflected on its clinical behavior. One of those lesions is odontogenic keratocyst (OKC), which in some occasions has been referred to as a tumor[1,2].

Beside the evident histological variations between odontogenic cysts and tumors, their clinical behavior is also of principal value for differentiation between the two categories. In general, odontogenic cysts exhibit a benign clinical behavior, considering bone disfigurement, tooth displacement and jaw fracture to be the major problems encountered with them[2]. On the other hand, odontogenic tumors show a diverse clinical behavior, ranging from benign hamartomatous lesions to malignant conditions with its associated complications[3].

Pogrel[4] stated that OKC was first described by Philipsen in 1956, but was well recognized in 1970 by Browne, who clearly described the lesion both clinically and histologically and referred to it as primordial cyst, a name which is not used anymore. OKC has been renamed as keratocystic odontogenic tumor (KCOT) in some occasions. This was attributed to its special features, having both cyst and benign tumor-like characters. For instance, the presence of mural growth, with the epithelial lining proliferating into cancellous bone allows the lesion to reach a significant size before notable jaw expansion. This supports the categorization of OKC as a tumor. Moreover, the lesion has high growth potential and an aggressive behavior reflected by its high recurrence rate. Another finding that supports the tumor-like nature of the lesion is that it could be associated with nevoid basal cell carcinoma (NBCC) syndrome. This syndrome is associated with patched homolog (PTCH) gene mutations which is also found in BCC cases, suggesting a neoplastic nature[5]. Accordingly, in 2005 the WHO reformed the nomenclature of OKC to KCOT and classified it as a benign odontogenic

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neoplasm[6]. However, in cases of sporadic OKCs not associated with NBCC syndrome, the PTCH gene mutation was found only in 30% of the cases. This reduces the possibility of OKC being dealt with as a neoplasm, and favors a cystic nature[2]. Furthermore, treatment of the lesion, even extensive ones, with marsupialization may cause complete resolving and replacement of the epithelial lining of the lesion by epithelium similar to that of normal oral mucosa. This supplements the classification of the lesion as OKC rather than KCOT[4]. These findings further proof the need to do more research in order determine the true nature of OKC as recommended by WHO in 2016, which will help when planning the treatment strategy[7].

In our study, immunohistochemical and histochemical methods were used to elaborate the nature of OKC and whether it can be considered as an odontogenic cyst or tumor. This was attained by comparing OKC to both an odontogenic cyst and a tumor. The dentigerous cyst will be used as an example of odontogenic cysts, with its benign behavior causing only bone expansion[8]. On the other hand, ameloblastoma will be the representative of the odontogenic tumors. Despite being benign in nature, still it is locally aggressive, may reach very large size and can turn into malignant ameloblastoma[9]. Comparing the previous lesions with OKC might help in better understanding its nature.

Despite the fact that epithelial cells proliferation is considered as a primary element for cyst formation, connective tissue can also be considered to play a major role in cyst behavior, and not just a structural support for the epithelium. The interplay between epithelium and connective tissue is assumed to play a significant role in the pathological process of odontogenic cysts development[10].

Myofibroblasts (MFs), by simple definition, are specialized fibroblasts, having features like those of smooth muscles, being characterized by the presence of contractile apparatus[11]. These cells play a central role in wound healing, which makes them very important for the mammalian body in terms of integrity. On the other hand, MFs can also be threatening by its ability to promote tumor development. In general, stromal reactions to epithelial neoplasms is marked by the appearance of MFs[12-16].

Previously, it was thought that the presence of MFs at the invasive front of the malignant tumors was a host reaction to prevent invasion of malignant cells. Later, it was proved with plenty of evidence that their presence at the invasion front is not considered as a defensive mechanism by the host against invasion, but actually promotes it, by the production of angiogenic factors and extracellular matrix (ECM) components[14].

On the other hand, collagen fibers are the most abundant protein in the body, constituting 34% of the total ECM proteins. They play a vital role in maintaining the functional integrity of tissues, including the odontogenic apparatus. In pathological conditions, collagen can show variations in the way the individual fibrils are organized into fibers[17,18].

The aim of this work was to compare immunohistochemical expression of α-SMA and histochemical quality and packing of collagen fibers in OKC, in relation to a cystic odontogenic lesion (dentigerous cyst) and a neoplastic odontogenic lesion (ameloblastoma) to help in nature determination of this query lesion.

## MATERIAL AND METHODS

### *Cases selection*

Thirty-three formalin fixed, paraffin-embedded archival blocks of ameloblastoma, odontogenic keratocyst and dentigerous cyst (eleven of each lesion) were obtained from the archives of the Oral Pathology Department, Faculty of Dentistry, Ain Shams University and Misr International University, Egypt.

### *Immunohistochemical procedures*

Four µm sections were cut from all specimens, then mounted on positively charged glass slides. Xylene was used to deparaffinize the sections, that were then rehydrated in graded ethyl alcohol. Sections were then immersed in citrate buffer solution of pH 4.8, then placed in the microwave oven before staining procedures. For immunostaining, a universal kit (Lab Vision) was used, peroxidase anti-peroxidase method of immunostaining using the streptavidinbiotin system was carried out, and 3% hydrogen peroxide was added to the sections to block the endogenous peroxidase activity. Sections were immunostained using the concentrated primary monoclonal antibody against α-SMA, and then incubated overnight at room temperature after rinsing with PBS (phosphate buffered saline) solution. Link antibody was then used to cover the sections, followed by the streptavidin biotin labeling antibody; after rinsing with PBS, DAB chromogen was applied to the sections followed by counter stain, then sections dehydration was performed in graded alcohol, followed by the use of xylene for the sections to be cleared. Finally, the sections were mounted. Immunohistochemical staining was carried out in Ain Shams University Specialized Hospital.

### *Assessment of immunohistochemical procedures*

Four microscopic fields viewing highest immunopositive areas were chosen from each positive section. Each field was photomicrographed at a magnification of 40X using a digital camera (LEICA DFC295, Wetzlar, Germany) which was mounted on a light microscope (LEICA DM LS2, Wetzlar, Germany). Images analysis was then performed after they were transferred to the computer. The manual count of immunopositive MFs were recorded excluding those lining blood vessels. Imaging and image analysis were performed in the research unit, Misr International University, Egypt.

### *Picrosirius red staining procedure*

Paraffin-embedded tissue blocks were sectioned at five µm thickness; the sections were floated onto the glass slides and incubated at 60° C on the slide warmer

for the proper adhesion of sections to the slides. Sections were then deparaffinized, hydrated and stained with picrosirius red stain (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer protocol. The stained slides were then viewed using polarized light microscopy in the Precision Measurements Unit, Faculty of Dentistry, Ain Shams University. Four fields of each slide were photomicrographed at a magnification of 20X. Collagen fibers showed polarizing colors varying from red-orange, yellow-green. The percentage of yellow-green fibers were assessed in relation to the total area of the fibers using image analysis software (Image J, 1.41a, NIH, USA).

### *Statistical analysis*

In order to explore numerical data for normality, data distribution was checked, and using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). All data showed non-parametric distribution. Data were presented as median and range values. Kruskal-Wallis test was used to compare between the three groups. Dunn's test was used for pair-wise comparisons. Spearman's correlation coefficient was used to determine significant correlation between area fraction of yellow-green and MFs cell count. $P \leq 0.05$ was the significance level set. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

## RESULTS

### *Immunohistochemical results*

α-SMA positive MFs were detected in the three groups (Figure 1). The average number (median and range values) was shown in table 1. Statistically significant difference was found between the groups (*P-value* <0.001, Effect size = 2.619) (Table 1,Figure 3a).

### *Picrosirius red staining results*

Different color birefraction of collagen fibers was observed in the three groups (Figure 2). The median and range values of area fraction of yellow-green fibers in the three groups was shown in table 1. Statistically, there was a significant difference between the groups (*P-value* <0.001, Effect size = 2.645) (Figure 3b,Table 1).

There was a statistically significant direct correlation between MFs cell counts and area fraction of yellow-green fibers (ρ = 0.614, *P-value* <0.001). An increase in MFs cell count is associated with an increase in area fraction of yellow-green fiber (Figure 3c).

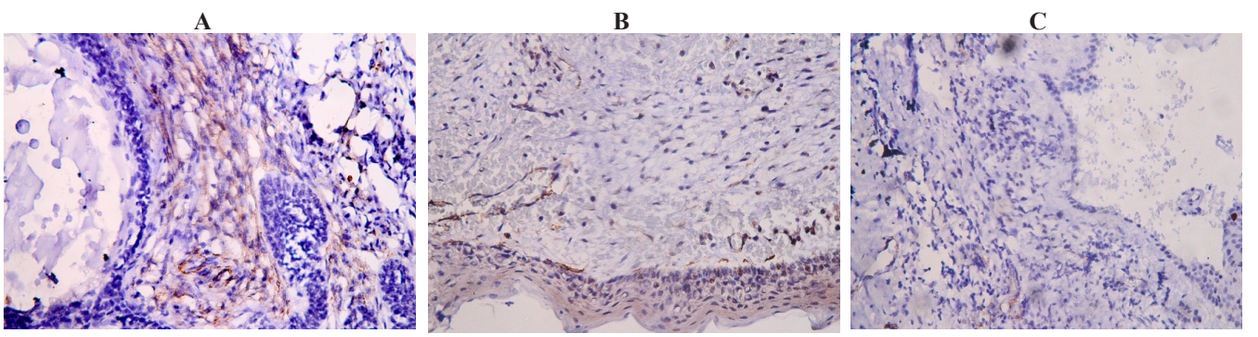


**Fig. 1:** Immunohistochemical expression of α-SMA showing MFs in (a) ameloblastoma, (b) OKC, (c) dentigerous cyst (original magnification x40).

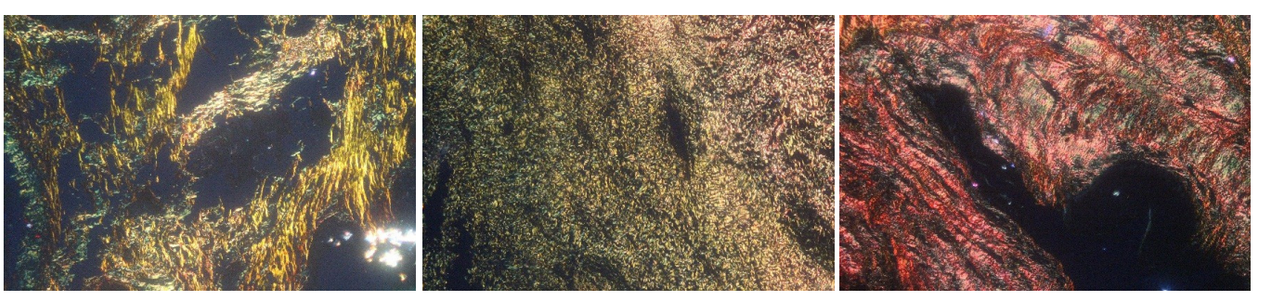

**Fig. 2:** Picrosirius red staining under polarized light microscope showing the yellow-green birefraction of collagen fibers in (a) ameloblastoma, (b) OKC, (c) dentigerous cyst (original magnification x20).

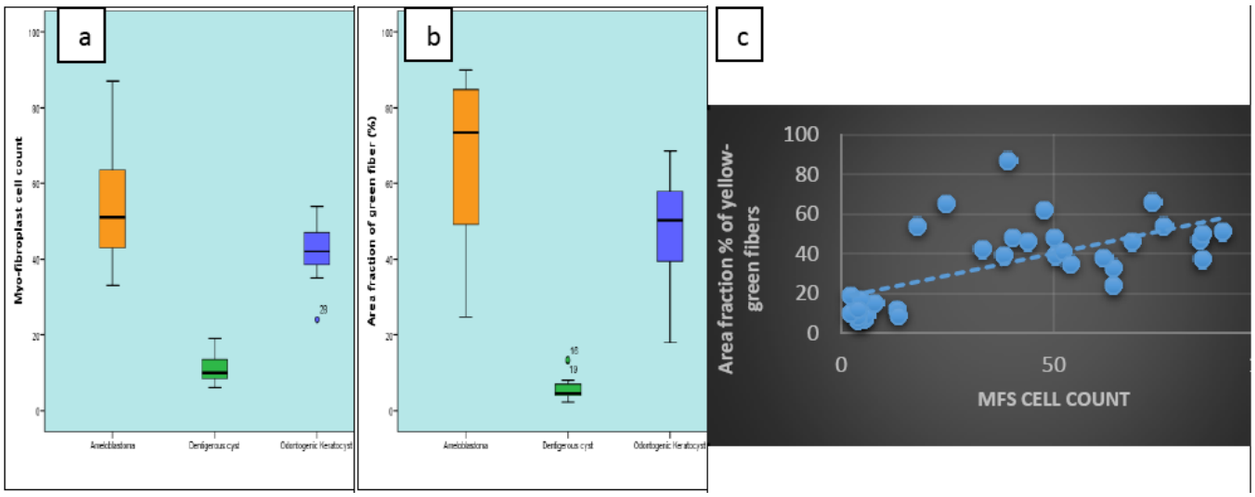


**Fig. 3:** Box plot representing median and range values for (a) MFs cell count, (b) area fraction of yellow-green fibers in the three groups (Circles represent outliers). (c) scatter diagram representing direct correlation between MFs cell count and area fraction % of yellow-green fibers.

**Table 1:** Median, range values and results of Kruskal-Wallis test for comparison between MFs cell counts and area fraction of Yellow-green fibers in the three groups.

| | Ameloblastoma | OKC | Dentigerous cyst | *P-value* | Effect size (Eta Squared) |
|---|---|---|---|---|---|
| MFs count | 51 (33– 87)$^{A}$ | 42 (24– 54)$^{B}$ | 10 (6– 19)$^{C}$ | <0.001* | 2.619 |
| Area fraction of Yellow-green fibers | 73.4 (24.7– 90)$^{A}$ | 50.3(18– 68.5)$^{B}$ | 4.6 (2.2– 13.5)$^{C}$ | | 2.645 |

*: Significant at $P \leq 0.05$, different superscripts are statistically significantly different

## DISCUSSION

OKC is one of the most aggressive odontogenic cysts that show a high rate of recurrence. It is an arguable odontogenic developmental cyst that has experienced several changes in terms of nature and terminology along the years. Whether its nature was cystic or more aggressive, this consequently affected the surgical treatment approach, which varied from simple enucleation of the lesion to resection of the affected area[19,20].

Picrosirius red is a strong, linear anionic dye covering six sulfonate groups. It can associate with cationic collagen fibers, improving their natural birefringence under polarized light. Collagen type I would show an orange-red color, while type III would be yellow-green[21]. However, Lattouf *et al*.[22], deny this explanation and stated that this stain only reflects the configuration of the collagen, in terms of fibers direction in the tissues, with no differentiation between the types. Likewise, Coleman[23] found that this technique is based only on the packing and thickness of the collagen fibers, not on specific collagen type composition within the collagen bundles. On the other hand, the combination of this dyeing technique with morphometric image analysis, keeps it among the supreme methods to study and measure collagen network in different diseases[24]. Several studies demonstrated the importance of this specific staining to detect collagen network abnormalities taking place in connective tissues[25-27], considering it as a valuable tool in assessing the amount of collagen content in normal or pathological tissues[27].

Additionally, collagen degradation could be valuably studied using the sirius polarization method[28] as long as under polarized light the collagen displays birefringence due to its molecular arrangement. Therefore, this birefringence will show a different pattern when comparing pathological and normal tissue.

In this study, the picrosirius red staining showed the highest average percentage for yellow- green fibers in ameloblastoma followed by OKC while dentigerous cyst was the least. Although there was a statistical significant difference between the three groups, yet the average percentage of these fibers in OKC is much closer to that of the odontogenic tumor (ameloblastoma) when compared to that of the odontogenic cyst (dentigerous cyst). These findings coincide with that of Nayak *et al*.[29] who showed that unicystic ameloblastoma showed slightly higher values of yellow-green fibers when compared to that of OKC, with a low statistical significance difference between both lesions. Another study by Singh *et al*.[30] revealed differences in the quality, organization and packing of collagen fibers between the different radiographic patterns of OKC which accounts for alteration in biological behavior of these lesions.

According to Hirshberg *et al*.[31], these yellow-green collagen fibers in OKC possibly represent procollagen, intermediate or pathological collagen. This can correspondingly confirm the aggressive behavior of OKC partially.

The predominance of yellow-green fibers observed in this study in both OKC and ameloblastoma in comparison to dentigerous cyst, may intensify the proposed role of the interplay between the epithelium and connective tissue in the pathogenesis and behavior of odontogenic cysts and tumors. In addition, despite the fact that proliferation of epithelial cells is an indispensable ingredient for cyst formation, connective tissue may be regarded as a functional part of cyst and not just a structural support[32]. In agreement with that, the experimental study of Vedtofte *et al*.[33], who showed that the transplanted epithelium yields new cystic lesion only if supported by its own stroma.

The findings of the immunohistochemical staining of MFs in this study agreed with that of the picrosirius red staining. Ameloblastoma showed the highest MFs cell count followed by that of OKC while dentigerous cyst showed a very low MFs count. There was statistically significant difference between the three groups, however, the averages of ameloblastoma and OKC were close to each other. Our findings were guaranteed with those of Mashhadiabbas *et al*.[15] and Vered *et al*.[34], who observed the lower count of MFs in dentigerous cyst when compared to other odontogenic cysts and tumors.

This further proves that OKC resembles the odontogenic tumor more than that it does with the odontogenic cyst. Keeping in mind the role of MFs in tumor invasive characteristics that observed by Shimasaki *et al*.[35], the higher MFs count in ameloblastoma and OKC detected in our study compared to that of dentigerous cyst may explain the aggressiveness of these two lesions when compared to other benign odontogenic tumors and cysts. The direct relation between MFs density and invasive tumor characteristics had also been suggested by Tuxhorn *et al*.[36], who examined the reactive stroma in human prostate cancer, induction of MFs phenotype and ECM remodeling. Furthermore, Seifi *et al*.[37] noted an increase in α-SMA-positive MFs number in the higher grades of oral squamous cell carcinoma, which supports the role of MFs in invasiveness of tumors.

At the same time, this study showed a statistically significant inverse correlation between MFs cell counts and area fraction of yellow-green fibers, which means that the increase in MFs cell count is associated with decreased quality and packing of the collagen fibers. This finding was in line with the study of Ehrlich and Hunt[38], who concluded the inability of MFs to produce thick collagen fibers. Moreover, in situations like wound healing and pathological conditions, the formed collagen by MFs is not compact as that formed by fibroblasts. Gabbiani[39] stated that the excessive replacement of collagen type I by collagen type III in granulation tissue during wound healing, is associated with appearance of large quantities of MFs. Our finding together with these studies suggested that the presence of MFs in a lesion might be the direct cause of predominance of collagen type III or collagen of a lower quality and less packing, which in turn facilitates the local tissue infiltration and thus reflecting the aggressiveness of this lesion.

## CONCLUSIONS

The immunohistochemical profile of OKC presented by α-SMA positive MFs, as well as histochemical profile as regard collagen packing and quality were much closer to odontogenic tumor (ameloblastoma) than odontogenic cysts (dentigerous cyst) suggesting the neoplastic nature of this debatable lesion. In addition, the MFs seem to have a role in poor quality of collagen and loose packing that is responsible for the aggressiveness of the lesion.

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## CONFLICT OF INTERESTS

There are no conflicts of interest

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**الملخص العربى**

# دور الخلايا الليفية العضلية وجودة الكولاجين في إيضاح الطبيعة المبهمة للكيس الكيراتيني سني المنشأ

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**الخلفية:** إن الكيس الكيراتيني سني المنشأ هو أحد الآفات سنية المنشأ التي عليها جدال من حيث طبيعته. وقد اوصت منظمة الصحة العالمية في عام ٢٠١٦ بالحاجة للمزيد من الدراسات لمعرفة طبيعة هذه الافة. إن تفاعل الانسجة الداعمة للأورام الناشئة من طبقة الخلايا الطلائية يتميز بظهور الخلايا الليفية العضلية التي تظهر تفاعلا إيجابيا ضد الاجسام المضادة المعروفة باسم (الفا اس ام ايه). ومن جهة أخرى فان الياف الكولاجين الموجودة حول الآفات سنية المنشأ لها دور حيوي في حدوث و نمو هذه الآفات.

**اهداف البحث:** مقارنة عدد الخلايا الليفية العضلية التي تظهر تفاعلا إيجابيا ضد الاجسام المناعية المعروفة باسم (الفا اس ام ايه) وكذلك جودة وتكدس الياف الكولاجين في حالة الكيس الكيراتيني سني المنشأ بالنسبة لمثيلتها في الكيس السني والورم المينائي.

**الطرق والمواد المستخدمة:** تم إحصاء عدد الخلايا الليفية العضلية التي تظهر تفاعلا إيجابيا ضد الاجسام المناعية المعروفة باسم (الفا اس ام ايه) ونسبة الالياف التي تظهر باللون الأصفر-الأخضر عند صبغها بالصبغة الخاصة وفحصها بميكروسكوب الضوء المستقطب والتي تعكس بدورها جودة وتكدس الياف الكولاجين ومقارنة النتائج بين احدى عشرة عينة من كل من الكيس الكيراتيني سني المنشأ والكيس السني والورم المينائي. وتم عمل التحليل الاحصائي للنتائج.

**النتائج:** بلغ متوسط عدد الخلايا الليفية العضلية ٥٣,٧- ٤١,٩ -١١,٣ في كل من الورم المينائي و الكيس الكيراتيني سني المنشأ والكيس السني على التوالي مع وجود دلالة إحصائية للفرق في العدد. كما سجل متوسط نسبة الالياف التي تظهر باللون الأصفر-الأخضر ٦٥,٦- ٤٧,٧٢- ٦,١٪ في كل من الورم المينائي و الكيس الكيراتيني سني المنشأ والكيس السني على التوالي مع وجود دلالة إحصائية للفرق في النسب. كما وجدت دلالة إحصائية للعلاقة الطردية ما بين عدد الخلايا الليفية العضلية ونسبة الالياف التي تظهر باللون الأصفر-الأخضر.

**الاستنتاجات:** ان السجل المناعي الهستوكيميائي للأكياس الكيراتينية سنية المنشأ من حيث الخلايا الليفية العضلية التي تظهر تفاعلا إيجابيا ضد الاجسام المضادة المعروفة باسم (الفا اس ام ايه) وكذلك السجل الهستوكيميائي لهذه الآفة من حيث تكدس و جودة الياف الكولاجين هو اقرب للأورام سنية المنشأ متمثلة في الورم المينائي منه الى الأكياس سنية المنشأ متمثلة في الكيس السني مما يرجح الطبيعة السرطانية الحميدة لهذه الآفة التي تثير الجدال.