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**Research Paper**

# Assessment of the Role of c-KIT in Salivary Adenoid Cystic Carcinoma Utilizing Immunohistochemical and Molecular Biological Methods

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***Abstract***
***Background:*** *Adenoid cystic carcinoma is one of the most common malignant neoplasms of salivary glands, characterized by aggressive behavior, recurrence, metastasis to distant organs, and relative radio resistance, rendering local control challenging to achieve.*
***Objective:*** *The goal of this work is to shed light on the role of c-KIT in the pathogenesis of salivary adenoid cystic carcinoma at the gene and protein levels.*
***Materials and Methods:*** *c-KIT protein expression and gene levels were determined by real-time immunohistochemistry (IHC) and polymerase chain reaction (PCR) in a total of fifty-two specimens of salivary adenoid cystic carcinoma.*
***Results:*** *The immunoreactivity for c-KIT antibody was observed among all of the studied cases, whereas, thirteen cases tested for c-KIT gene mutations by DNA sequencing showed no detection of genetic mutation despite being positive for immunohistochemistry.*
***Conclusion:*** *Although c-KIT immunopositivity is high in salivary adenoid cystic carcinoma, the absence of gene mutation renders target therapy ineffective.*

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## I. Introduction

Salivary gland carcinomas constitute a large collection of highly heterogeneous tumors that exhibit broad spectrum of histology. These tumors have variable biological behavior ranging from indolent, slow growing to highly aggressive and rapidly fatality **[1].**

Adenoid cystic carcinoma (AdCC) is Libya's second most common malignant salivary gland tumor, following mucoepidermoid carcinoma as reported by Hamad et al., 2021 **[2],** and Elarbi & Khalifa 2018 **[3].** AdCC often develops at a slower rate than other salivary carcinomas. However, AdCC is considered a high-grade tumor, with radical resection followed by postoperative radiotherapy. Moreover, regional and distant recurrences of primary AdCC are rather prevalent after local therapy, and roughly half of patients die as a result of recurrences within 10 to 20 years of treatment. Hematogenous metastasis is common, particularly in the lungs, bones, and liver **[4],[5].**

c-KIT is a tyrosine kinase receptor found on the long arm of chromosome 4. It belongs to the same subclass of genes as platelet-derived growth factor and colony-stimulating growth factor. The c-KIT has been considered a proto-oncogene due to the strict connection existing between alterations in its expression and a number of malignancies (e.g., gastrointestinal stromal tumors, mast cell disease, and acute leukemia). The activation of c- KIT triggers several signal transduction pathways, including JAK/STAT, RAS pathway, PI3 kinase, PLCγ pathway, and SRC pathway, that regulate fundamental biological processes, such as apoptosis, and cell proliferation, differentiation, and migration **[6],[7].**

Overexpression of the c-KIT protein has been thought to be related with the molecular pathogenesis of AdCC and Up to 94% of AdCC patients reported expressing this protein **[8].** As a result of AdCC's poor

response to conventional therapy, molecular targeted therapy is thought to be the best option for treatment, by using small-molecule kinase inhibitors based on c-KIT inhibitor, particularly over 120 small-molecule kinase inhibitors have been approved worldwide for treating various diseases, with nearly 70 Food and Drug Administration (FDA) approvals specifically for cancer treatment such as acute myelogenous leukemia, mast cytosis, mast cell leukemia, and gastrointestinal stromal tumors **[9].**

**Objective:** The study aims to assess the role of c-KIT in the pathogenesis of salivary adenoid cystic carcinoma at the gene and protein levels.

## II. Materials and Methods

**1- Retrieved tissue samples:** The present study was carried out on fifty-two formalin-fixed, paraffin-embedded (FFPE) tissue blocks of salivary AdCC cases, collected from the Pathology Department at the Tripoli University Hospital. The retrieved paraffin blocks were investigated for the aimed IH evaluation and for the DNA sequencing according to the prepared protocol. The selected AdCC group encountered the three histologic types described by Szanto et al., 1984 **[10].** The tumor can be graded into three categories; Grade I – cribriform or tubular pattern, Grade II – <30% solid pattern, and Grade III – more than 30% solid pattern.

**2- Immunohistochemical evaluation:** The antibody for c-KIT, which is a rabbit polyclonal anti-human c-KIT antibody, was in the form of a prediluted antibody, which was ready to be used for the staining procedure obtained from ABclonal Technology, Cat. A0357, U.S.A. The dilution of this product in phosphate buffer solution (PBS) was 1:50–1:200. 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. Mean values of area percentage of positive cells were obtained for the whole specimens in each group.

## III. DNA sequencing:

DNA primers to amplify exons 11 and 17 of c-KIT gene.

| Target sequence | Primers | Primer sequence (5`-3`) | Annealing temp. |
|---|---|---|---|
| c-KIT -exon 11 | F | AGCCCCTGTTTCATACTGAC | 56 ℃ |
| | R | CCAGAGTGCTCTAATGACTG | |
| c-KIT -exon 17 | F | GTTTTCTTTTCTCCTCCAACCTAATA | 56 ℃ |
| | R | TTGAAACTAAAAATCCTTTGCAGGAC | |

The extraction of DNA from formalin-fixed, paraffin-embedded (FFPE) tissues was performed on samples from 13 randomly selected patients who tested positive for c-KIT mutations via immunohistochemistry (IHC). Using a modified polymerase chain reaction (PCR) method, the samples underwent deparaffinization and were then suspended in a DNA extraction buffer, followed by incubation with shaking at 55˚C overnight. The DNA in the supernatant was collected and precipitated using ammonium acetate and 100% ethanol. Subsequent centrifugation at 14,000 rpm for 20 minutes was used to pellet the DNA, which was then dissolved in TE buffer. The concentration and quality of the extracted DNA were assessed using a NanoDrop 1000 spectrophotometer. For amplification, a thermocycler was programmed with specific temperature cycles to generate multiple copies of the target DNA region, using primers designed for that region. The PCR products were evaluated through 2% agarose gel electrophoresis in 1.5% (w/v) agarose gels, utilizing 1× TAE buffer containing 0.5 µg/ml ethidium bromide. The PCR fragments were excised from the gels and transferred into 1.5 ml tubes, to which a binding buffer was added. The gel mixture was incubated at 60°C for 10 minutes, and the PCR products were purified using the Gene JET Gel Extraction Kit as per the manufacturer's instructions. Finally, the PCR products were analyzed with a gel documentation system, confirming that the amplified products were highly specific to the target region, with the c-KIT gene demonstrating the highest sensitivity.

## IV. 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. One Way ANOVA test was used to compare more than two independent groups. The significance of the obtained results was judged at $p < 0.05$ level.

**Results**

The current AdCCs were presented histologically as three patterns which were classified according to Szanto et al. (1984) into fifteen cases of grade I (cribriform or tubular pattern), seventeen cases of grade II (<30% solid pattern), and twenty cases of grade III (more than 30% solid pattern).

**Immunohistochemical findings with c-KIT**

The expression of c-KIT was in the form of diffuse cytoplasmic/membranous and nuclear immunopositivity among the three growth patterns of tumor; the cribriform pattern revealed the immunoreactivity for c-KIT among the vast majority of basaloid cells surrounding the cystic spaces. Meanwhile, only few cells showed negative or nuclear immunoexpression. The connective tissue stromal cells showed a positive reaction as well, (figure 1, A). A tubular pattern; the immunoexpression for c-KIT was restricted to the malignant ductal cells while the adjacent myoepithelial cells revealed either a nuclear or negative reaction. The hyalinised tumor's stromal cells expressed positive reaction, (figure 1, B). A solid pattern; all neoplastic basaloid cells that formed solid masses of tumor, were stained deeply by the c-KIT biomarker, (figure 1, C). Moreover, the surrounding stromal cells, neural tissue and vascular walls displayed positive reaction, (figure 1, D). Statistically, there was a significant difference between mean values of area percentage for c-kit immunoexpression among the histological grades of the studied of AdCCs.

| Histological grades | Mean values of area% (Mean± SD) | P-value |
|---|---|---|
| Grade I (n=15) | 35.1 ±1.59 | <0.001* |
| Grade II (n=17) | 45.7 ±4.73 | |
| Grade III (n=20) | 61.6 ±1.26 | |

*ANOVA test. * statistically significant if p<0.05. n=number. SD= standard deviation*.

**PCR findings with c-KIT gene**

All the thirteen tested samples did not show evidence of c-KIT gene mutation by DNA sequencing despite being positive by IHC. Moreover, the alignment of the nucleotide sequence to the GenBank reference sequence X65998.1 showed that no genetic variations could be detected between the sequences.

## V. Discussion

All of the current AdCC growth patterns revealed strong immunoreaction for c-KIT in the form of diffuse cytoplasmic/membranous and nuclear immunopositivity. This finding was in agreement with previous IHC investigations of El-Nagdy et al., 2013 **[11],** and AbdelMaksoud & AbdAllah 2016 **[12].** Moreover, the immunostaining for c-KIT among the presented cases of AdCC was restricted to the malignant ductal cells, whereas the adjacent myoepithelial cells displayed either a nuclear or negative reaction. This was in accordance with the study of Jain et al., 2016 **[13],** who observed that the c-KIT biomarker was specific for ductal cells, implying a dual origin for AdCC. Furthermore, a statistically significant difference was found between area percentages and histological grades of the studied AdCCs. The finding was accompanied by the concept that the histologically solid pattern of this malignancy exhibits the most aggressive behavior than the others.

In the current work, the surrounding stromal cells of MSGTs also responded positively to c-KIT. This might represent the residence of mast cells in connective tissue as reported by Khatri et al., 2013 **[14],** who revealed that IHC expression of c-KIT was specific for detecting mast cells. Particularly, the c-KIT receptor was reported to regulate mast cells activity. After activation, mast cells could release their secretory granules containing proangiogenic factors into the microenvironment **[15].** It should be noted that the vascular wall in surrounding stroma of the presented cases reacted to the c-KIT antibody. Tacconi et al., 2022 **[16],** demonstrated that c-KIT was normally present in the vascular endothelial cells to promote blood vessel growth. Moreover, the tumor-associated fibroblasts were reported to secrete the stem cell factor, which bind to the c-KIT receptor in endothelial cell membranes and activated its expression **[17].**

On the other hand, the absence of a c-KIT mutation reduces the probability that this cancer may respond to targeted therapy using c-KIT kinase inhibitors. This finding was in disagreement with Vila et al., 2009 **[18],** who claimed that direct PCR sequencing lacks significant sensitivity compared to subcloning of the DNA products because of the heterogeneous DNA from both normal and tumor samples. However, our result was supported by Miller et al., 2022 **[19],** who noticed that molecular targets based on the c-KIT receptor have limited efficacy in clinical trials.

## VI. Conclusion

Although c-KIT immunopositivity is high in salivary adenoid cystic carcinoma, the absence of gene mutation renders target therapy ineffective.

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**Recommendations**
Our study did not include cases of recurrence, lymphovascular invasion, or distant metastasis, so we propose additional research on these parameters.

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**Authors' Contributions**
Everyone in our team contributed to this study. Algharyani A. S., Maghrabi M. M., and Fadel M. B. contributed to the conception and design of the study. Data analysis and acquisition were done by Algharyani A.S., Maghrabi M. M. Laboratory work had been done by Fadel M. B. Manuscript writing and adding Algharyani A.S. and Maghrabi M. M. Statistical analysis of study data was done by Fadel M. B.

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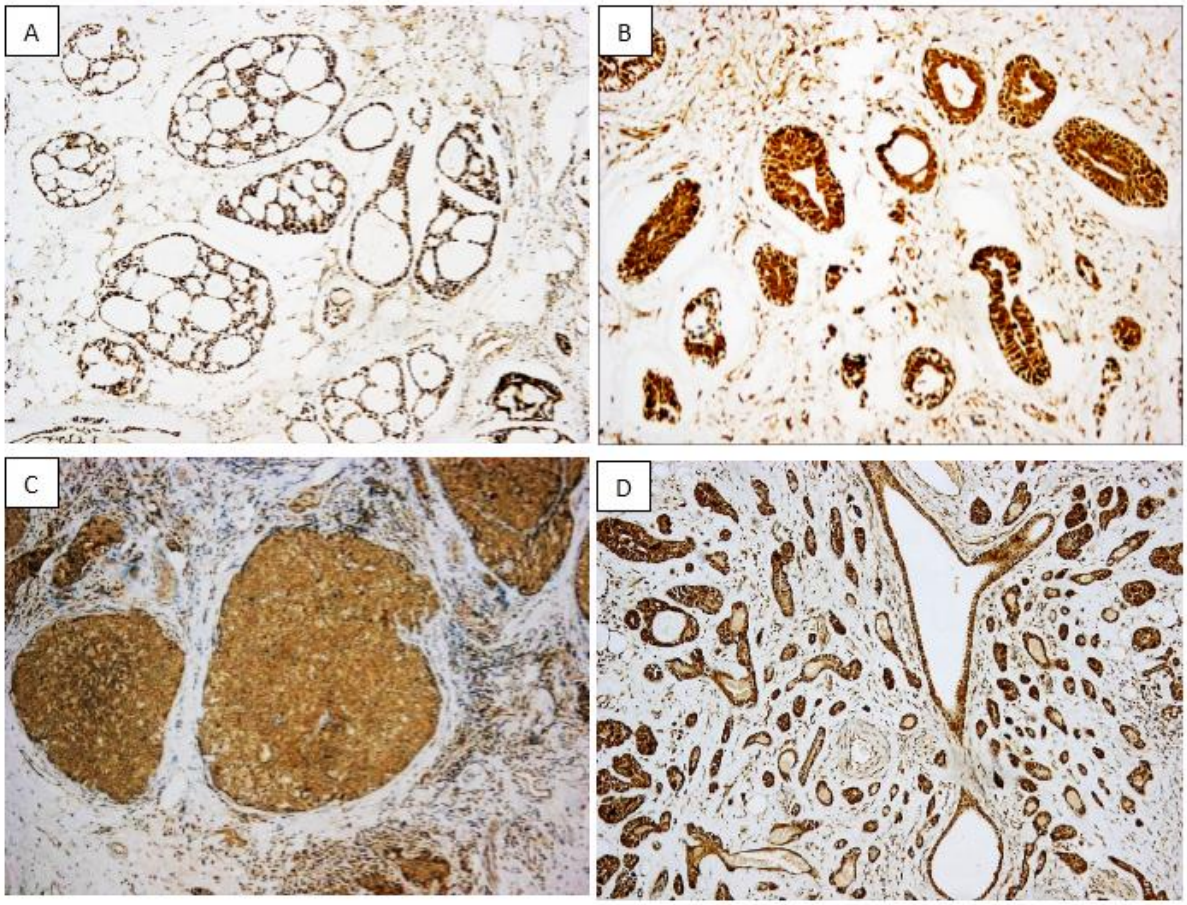


**Figure:** Photomicrograph of AdCC (cribriform pattern) demonstrating immunoreactivity for c-kit among the majority of basaloid cells around cystic spaces, (c-kit antibodyX 100, A). tubular pattern of AdCC showing cytoplasmic/ membranous and nuclear expression among ductal cells, (c-kit antibody X 200, B). solid pattern showing cytoplasmic membranous and nuclear c-kit expression in all basaloid cells. The stromal cells and small vascular wall express positive reaction, (c-kit antibody X 200, C). diffuse c-kit immunopositivity among malignant cells, neural tissue and vascular wall, (c-kit antibody X 100, D).