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# Evaluation of KCTD12 and Cripto-1 as novel upregulated proteins participating in breast cancer development and drug resistance

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**Abstract**

**Background**: Potassium channel tetramerization domain containing 12 (KCTD12) showed contradictory roles in many cancers, however, its role in breast cancer is unknown. Cripto-1 participates activates the Notch signaling pathway which was inhibited by KCTD12 in esophageal squamous cell carcinoma (ESCC). Uncoordinated 51-like kinase 2 (ULK2) with established roles in cancer was negatively regulated by KCTD12. Multidrug resistance protein 1 (MDR1) promotes resistance by an active transport efflux pump. KCTD12 downregulates the expression of drug-resistant proteins as ABCC4 and ABCG2 in ESCC patients.
**Purpose**: We investigated the markers role in the early breast cancer.
**Methods**: Sixty-five patient's serum samples were compared with 10 controls and 15 benign ones.
**Results**: Higher serum levels of KCTD12, Cripto-1, MDR1, and ULK2 in all malignant cases suggesting novel roles with better sensitivity and specificity along with CA15.3, where the resistant group was the highest suggesting value for de-novo breast cancer diagnosis.
**Conclusion**: Positive correlations for KCTD12 with Cripto-1, MDR1, and ULK2 suggesting a new role of KCTD12. The cumulated ROC curves with CA15.3 revealed better sensitivity and specificity highlighting the add on of using more than one marker.
**Keywords**: Breast Cancer; Cripto-1; MDR1; Notch signaling; Potassium channel tetramerization domain containing 12; Uncoordinated 51-like kinase 2

## 1. Introduction

Breast cancers (BC) is the most common cancer in women (24.2%, i.e., about one in 4 of all new cancer cases diagnosed in women worldwide are breast cancer), also is the leading cause of cancer deaths in women (15.0%). In Egypt, the estimated numbers of female BC were not so far, about (28.4%) incidence and (20%) mortality rates for females diagnosed with cancer [1, 2].

Rapid detection can provide early diagnosis and higher survival chances with an improved understanding of the molecular pathways and genetic alterations in different subtypes leading to more targeted and personalized approaches to treatment [3]. Although mammography screening and other tumor markers are available, there is an ongoing interest in improving early detection and prognosis.

Carcinoembryonic antigen (CEA) and CA 15-3 are very often detected in BC despite the controversial roles observed; where they were reported as complementary markers in the detection of reoccurrence but limited by their low sensitivities and specificities especially in early stages [4, 5].

As well, it has been shown that the deregulation of

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cellular signaling pathways is extensively involved in BC progression and drug resistance [6].

Clinical studies showed the presence and expression of the Notch proteins and their ligands in BC were higher than normal tissue [7]. Notch inhibitors along with antiestrogens could be more powerful in ER$\alpha$(+) breast cancers [8].

Potassium Channel Tetramerization Domain Containing 12 (KCTD12), also known as pfetin, is an auxiliary subunit of GABA-B receptors that alter the G-protein signaling of the receptors. A contradictory role of KCTD12 was observed in cancer. For instance, KCTD12 expression was significantly upregulated in cervical and lung cancers [9].

On the other hand, it has been shown that downregulation of KCTD12 is detected in colorectal cancer (CRC), and a low level of KCTD12 is associated with a poor prognosis of patients with CRC [10].

On the same manner, the expression of KCTD12 is reversely associated with gastrointestinal stromal tumors (GIST) in which the five years recurrence-free survival rate of patients without KCTD12 expression was only 16.7% compared with 95.6% in those with KCTD12 expression high KCTD12 expression indicates a favorable prognosis and could act as an independent prognostic factor for GIST [11, 12]. Moreover, KCTD12 acts as a tumor suppressor in esophageal squamous cell carcinoma (ESCC) through inhibiting various pathways including Notch signaling, so it may be used as an efficient therapeutic marker in ESCC [13].

Cripto-1, a transforming growth factor-beta (TGF-β) family member and the main positive regulator of Wnt and Notch pathways is critically important in early embryogenesis, stem cell maintenance, and malignant progression through the generation or expansion of tumor-initiating cells bearing stem-like characteristics and via its role during the epithelial-mesenchymal transition that enhances cell migration in many diseases besides cancer [13-16].

Cripto-1 was inhibited by KCTD12 in ESCC [13]. Moreover, Cripto-1 is suggested to be a novel therapeutic target for triple-negative breast cancer where it acts as a potential driver of mammary tumorigenesis and its knockout inhibited proliferation in vitro and tumor volume in vivo [17].

Uncoordinated 51-like kinase 2 (ULK2) is currently known to regulate autophagy. The silencing of ULK2 significantly reduced the expression of epithelial marker protein such as E-cadherin, whereas expression of mesenchymal marker proteins such as vimentin and fibronectin was significantly enhanced by silencing of ULK2 [18, 19].

A study illustrated that KCTD12 prevented ULK2 autophosphorylation that is required for its activity and thereby negatively regulates the ULK2 function [20].

Tumor reoccurrence still one of the major obstacles for cancer treatment due to drug resistance. Multidrug resistance protein 1 (MDR1, also known as P- glycoprotein or P-gp) belonging to the ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) transporters, can deliberate resistance to chemotherapy by active transport efflux pump. Drug concentration inside tumor cells can be reduced showing poor prognosis in patients with high levels of MDR1 exhibiting lower sensitivity [21-23].

Likewise, KCTD12 plays a role in drug resistance via the downregulation of the expression of ABCC4 and ABCG2 in ESCC patients via these transporters [13].

Thus, the current study was designed to investigate the expression of KCTD12, Cripto-1, ULK2, and MDR1 in female patients with breast cancer compared with healthy control and benign subjects, as well as to clear up their role, correlation, and significance for early detection of breast cancer compared with the already used markers as CEA and CA 15.3.

## 2. **Experimental**

### *2.1. Subjects and Methods*

The study was conducted on 90 subjects who were distributed into four main groups: Group I was 10 control apparently healthy female volunteers, Group II was 15 cases with benign breast tumor, Group III was 15 resistant breast cancer cases based on clinical and therapy response data and Group IV which was sub-divided in agreement with the patient hormonal receptors profile as estrogen receptor (ER) and progesterone receptor (PR) expression and human epidermal growth factor receptor 2 (HER2) obtained from (NCI) for de-novo 50 cases of breast cancer who have not undergone any surgery or received any chemotherapy or radiotherapy into Group IV a (n=6): (ER-PR- Her2+) Group IV b (n=11): (ER+PR+Her2+) Group IV c (n=8): (ER-PR-HER2-) Group IV d (n=25): (ER+PR+Her2-). Subjects were also categorized according to metastasis occurrence into non-metastatic and metastatic groups. A detailed clinical history was taken with complete general and systemic examinations including tumor classification and type. Breast Imaging Reporting and Data System (BIRADS) established by the American College of Radiology was used to categorize the patients where 0- incomplete, “1” negative, “2” benign findings, “3” probably benign, “4” suspicious abnormality, “5” highly suspicious of malignancy and “6” known biopsy with proven malignancy. The characteristics of patients are shown in Table 1.

### *2.2. Ethical approval*

The study was approved by the Ethical Committee of Research, Faculty of Pharmacy, Ain Shams University (188) and by the Ethical Committee of National Cancer Institute, Cairo University.

Additionally, the study was carried out in accordance with the regulations and recommendations of the Declaration of Helsinki. Informed consent was obtained from every patient.

*2.3. Exclusion criteria*

Subjects with any other type of cancer, blood disorders, liver cirrhosis, and uterine diseases were excluded.

*2.4. Blood samples*

Five mls of blood was collected into gel separator tubes for serum preparation. Sera samples were divided into 150µl aliquots then stored at -80 ºC until biochemical assessment.

*2.5. Biochemical assays*

Immunohistochemistry was used to detect receptors types at the (NCI). CEA and CA 15.3 were assayed using human ELISA Kit (Immunospec Corporation, USA); while KCTD12, Cripto-1, ULK2, and MDR1 proteins were measured using human ELISA Kit (Bioassay Technology Laboratory, China) as stated by the sandwich-ELISA technique. The micro-ELISA plate was pre-coated with antibodies specific to human each marker. Standards and samples were pipetted into corresponding wells to bind to the antibodies. Biotinylated detection antibodies targeting marker and streptavidin-HRP conjugate were then pipetted successively into each well. After incubation, washing for unbound components was done. When the substrate solution had been added, only the wells having human each marker, biotinylated antibody, and avidin–HRP complex were colored blue. Then, an acidic stop solution was added. Optical density (OD) was measured with spectrophotometry at wavelength of 450 nm ± 2 nm.

Table 1 Demographic data of patients included in the study

| | ***Control*** | ***Benign*** | ***Resistant*** | ***ER- PR- Her2+*** | ***ER+ PR+ Her2+*** | ***ER- PR- Her2-*** | ***ER+ PR+ Her2-*** |
|---|---|---|---|---|---|---|---|
| ***Number (N)*** | 10 | 15 | 15 | 6 | 11 | 8 | 25 |
| ***Mean age ± SD (years)*** | 42.1 ± 11.57 | 48.19 ± 6.86 | 48.20 ±6.37 | 57.50 ±6.69 | 46.64 ± 9.63 | 52.89 ± 11.40 | 46.85 ± 10.30 |
| ***Type of cancer*** | | | | | | | |
| ***Ductal Carcinoma*** | - | - | 11 | 5 | 10 | 7 | 19 |
| ***Lobular Carcinoma*** | - | - | 3 | - | - | - | 1 |
| ***Other types*** | - | - | 1 | 1 | 1 | 1 | 5 |
| ***Stage*** | | | | | | | |
| ***I and II*** | - | - | 12 | 5 | 11 | 6 | 23 |
| ***III and IV*** | - | - | - | 1 | - | 2 | 2 |
| ***Unidentified*** | - | - | 3 | - | - | - | - |
| ***TNM stage*** | | | | | | | |
| ***T1*** | | | 2 | - | 1 | - | - |
| ***T2*** | | | 5 | 1 | 4 | 2 | 7 |
| ***T3*** | | | 5 | 2 | 1 | 2 | 13 |
| ***T4*** | | | 3 | 3 | 4 | 4 | 5 |
| ***N0*** | | | 2 | - | 1 | 1 | 1 |
| ***N1*** | | | - | 3 | 10 | 4 | 19 |
| ***N2*** | | | 5 | 1 | - | 3 | 4 |
| ***N3*** | | | 8 | 3 | - | - | 1 |
| ***M0*** | | | 10 | 6 | 8 | 6 | 18 |
| ***M1*** | | | 5 | - | 3 | 2 | 7 |
| | **Control** | **Benign** | **Resistant** | **ER- PR- Her2+** | **ER+ PR+ Her2+** | **ER- PR- Her2-** | **ER+ PR+ Her2-** |
| ***BIRADS*** | | | | | | | |
| ***2*** | - | 9 | - | - | - | - | - |
| ***3*** | - | 6 | - | - | - | - | - |
| ***4*** | - | - | 2 | 2 | 6 | 5 | 10 |
| ***5*** | - | - | 11 | 4 | 4 | 2 | 15 |
| ***6*** | - | - | 2 | - | 1 | 1 | - |

*2.6. Statistical Analysis*

Statistical analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA) and IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA). Data are presented as medians and interquartile range (25th -75th percentile). Comparisons between independent groups for non-parametric data were carried out using Kruskal–Wallis test followed by Dunn's test for multiple comparisons using a statistical hypothesis, while the Mann-Whitney test was used to compare between metastatic and non-metastatic groups. The receiver-operating characteristic (ROC) curves illustrating the ability to differentiate between control and malignant cases were plotted for markers with the determination of area under the curve (AUC) and cut-off values except for MDR1 was built up between control and resistant group. Cumulated ROC analysis was done to illustrate the add on of using two markers together. Finally, the Spearman correlation test was carried out to investigate the correlation between variables for nonparametric data. The confidence interval was set to 95%, while the probability of error (P-value) < 0.05 was considered significant.

## 3. Results

The benign group did not show any significant differences for all markers compared with the control group. On the other hand, both resistant and ER-PR-HER2- groups showed a very high significant difference vs control group ($p < 0.001$), while other groups revealed a variety of significant differences as shown in Table 2.

Comparative data of biomarkers between groups are shown in Figure 1.

We compared biomarkers concentration between metastatic and non-metastatic groups as shown in Table 3.

Table 2 Serum marker level for metastatic and non-metastatic groups

| Biomarker | Control | Benign | Resistant | ER-PR-Her2+ | ER+PR+Her2+ | ER-PR-HER2- | ER+PR+Her2- |
|---|---|---|---|---|---|---|---|
| **CEA (ng/L)** | 0.63 | 0.87 | 1.99*** | 1.37** | 1.09** | 1.23** | 0.99** |
| **CA 15.3 (ng/L)** | 41.53 | 90.48 | 495.3*** | 185.3** | 115.5* | 169.1*** | 140.3** |
| **KCTD12 (ng/L)** | 65.71 | 79.16 | 493*** | 439.5* | 462.3*** | 565.6*** | 353.1*** |
| **Cripto-1 (ng/L)** | 47.38 | 79.71 | 277.1*** | 214.3* | 260.4*** | 279.6*** | 255.2*** |
| **MDR1 (ng/ml)** | 1.2 | 1.42 | 5.57*** | 5.475*** | 4.01** | 5.25*** | 4.16*** |
| **ULK2 (ng/L)** | 118.5 | 103.3 | 557.9*** | 360.5* | 350.8* | 435.9*** | 389.1** |

*The serum concentrations of biomarkers are presented as median using the Kruskal Wallis test, * P<0.1 ** P<0.001 *** P<0.001.*

*Abbreviations: CEA carcinoembryonic antigen, KCTD12 potassium channel tetramerization domain 12, MDR1 multidrug resistance protein 1, ULK2 Uncoordinated 51-like kinase 2*

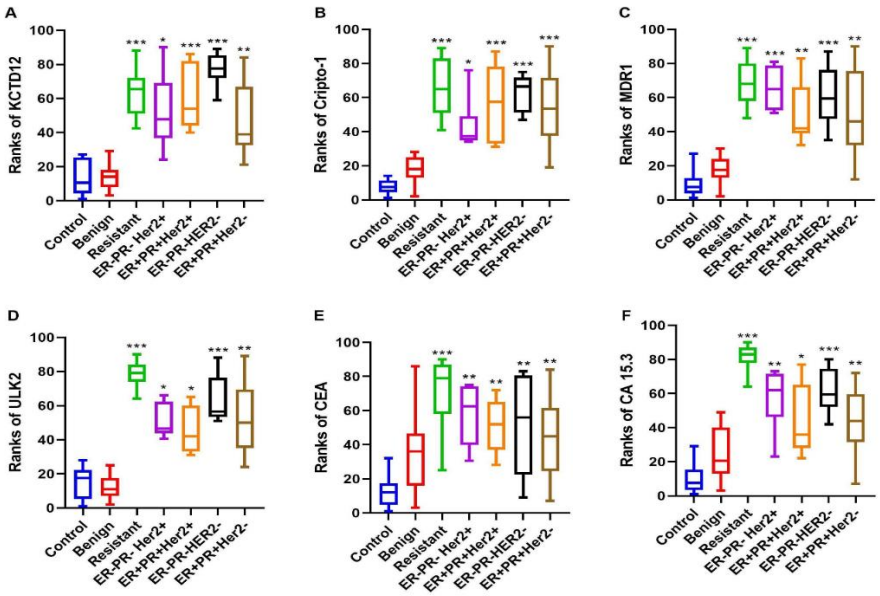


Figure 1 Comparative data for biomarkers ranks in serum using the Kruskal-Wallis test for each serum marker level (A) KCTD12 (B) Cripto-1 (C) MDR1 (D) ULK2 (E) CEA (F) CA 15.3 followed by Dunn's post hoc test presented as median (interquartile ranges) where * P <0.05, ** P < 0.01, *** P < 0.001 compared with Control group.

*Abbreviations: KCTD12 potassium channel tetramerization domain 12, MDR1 multidrug resistance protein 1, ULK2 Uncoordinated 51-like kinase 2*

ROC curves were built up for each marker to compare the diagnostic values to forecast malignancy Figure 3. The AUC indicated decent predictive potential where data showed that these markers can be detected at a higher level in malignant cases serum differentiating patients with breast cancer from healthy control subjects. Cut off values for each marker were determined with both the highest sensitivity and specificity as shown in Table 4.

The combined ROC curve between CA 15.3 with one of the other markers showed higher efficacy with higher AUC and 95% CI.

Finally, Spearman's correlation revealed a strong positive correlation between KCTD12 and the other markers at p-value <0.001 where the strongest correlation appeared with ULK2 as shown in Figure 4.

## 4. Discussion

Notch signaling overexpression has oncogenic activity and may predispose for breast cancer through cell proliferation, differentiation, and apoptosis [24]. A strong argument was suggested for KCTD12 role in tumors either to be elevated as in lung, cervical cancers, ESCC [9, 13]; or knocked down in other tumors so could be used as an encouraging marker for diagnosis, prognosis and survival of patients as in GISTs and CRC [10-12, 25]. Its role in breast cancer patients has not been elucidated yet. In our study, we found KCTD12 level marked up in malignant cases compared to control and benign ones, where it is most elevated in ER-PR-HER2- and resistant groups followed by other malignant groups. Besides that, KCTD12 showed a very high significant difference between metastatic and non-metastatic groups laying out its capability to differentiate between both groups. Positive correlations of KCTD12 with both CEA and CA 15.3 match our findings. These results together suggest the ability of KCTD12 to be used as a useful biomarker for diagnosis of breast cancer.

Table 3 Serum marker level for metastatic and non-metastatic groups

| Biomarker | Non-metastatic (N=48) | Metastatic (N=17) |
|---|---|---|
| **KCTD12 (ng/L)** | 439.5(221.8-492.1) | 538.3(502.8-588.6) *** |
| **Cripto-1 (ng/L)** | 242.8(206.8-271.7) | 420.6(356.0-483.9) *** |
| **MDR1 (ng/ml)** | 4.205(1.803-5.398) | 6.620(5.955-7.360) *** |
| **ULK2 (ng/L)** | 357.5(152.0-504.7) | 567.7(522.1-612.0) *** |

*The serum concentrations of biomarkers are presented as median (interquartile ranges) using the Mann Whitney U test, *** P<0.001.*

*The results declared very high significant differences (P <0.001) between them as shown in Figure 2*

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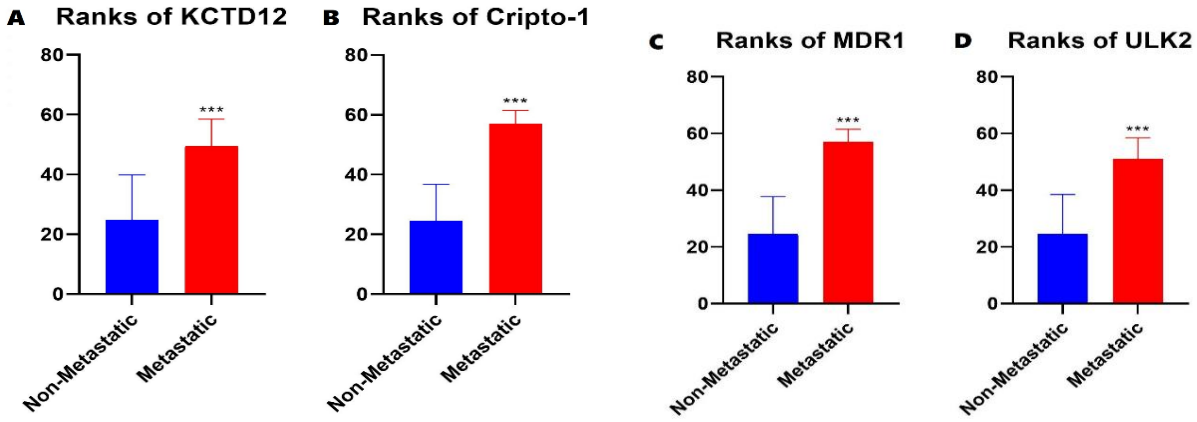


Figure 2 Comparison between metastatic and non-metastatic groups using Mann Whitney U test presented as median with interquartile range for serum markers levels (A) KCTD12 (B) Cripto-1 (C) MDR1 (D) ULK2.

Table 4 ROC curves for serum biomarker for malignant cases vs control group

| Biomarker | Cut off | AUC | 95% CI | P-value |
|---|---|---|---|---|
| **KCTD12** | > 129.8 | 0.875 | 0.786 – 0.963 | <0.001 |
| **Cripto-1** | > 68.14 | 0.964 | 0.923 – 1.0 | <0.001 |
| **MDR1*** | > 2.985 | 1.00 | 1.0 | <0.001 |
| **ULK2** | > 138.1 | 0.770 | 0.650 – 0.891 | <0.01 |
| **KCTD12 + CA 15.3** | - | 0.993 | 0.98 – 1.0 | <0.001 |
| **Cripto-1 + CA 15.3** | - | 0.993 | 0.98 – 1.0 | <0.001 |
| **ULK2 + CA 15.3** | - | 0.959 | 0.911 – 1.0 | <0.001 |

** ROC curve for MDR1 was designed between control and resistant groups*

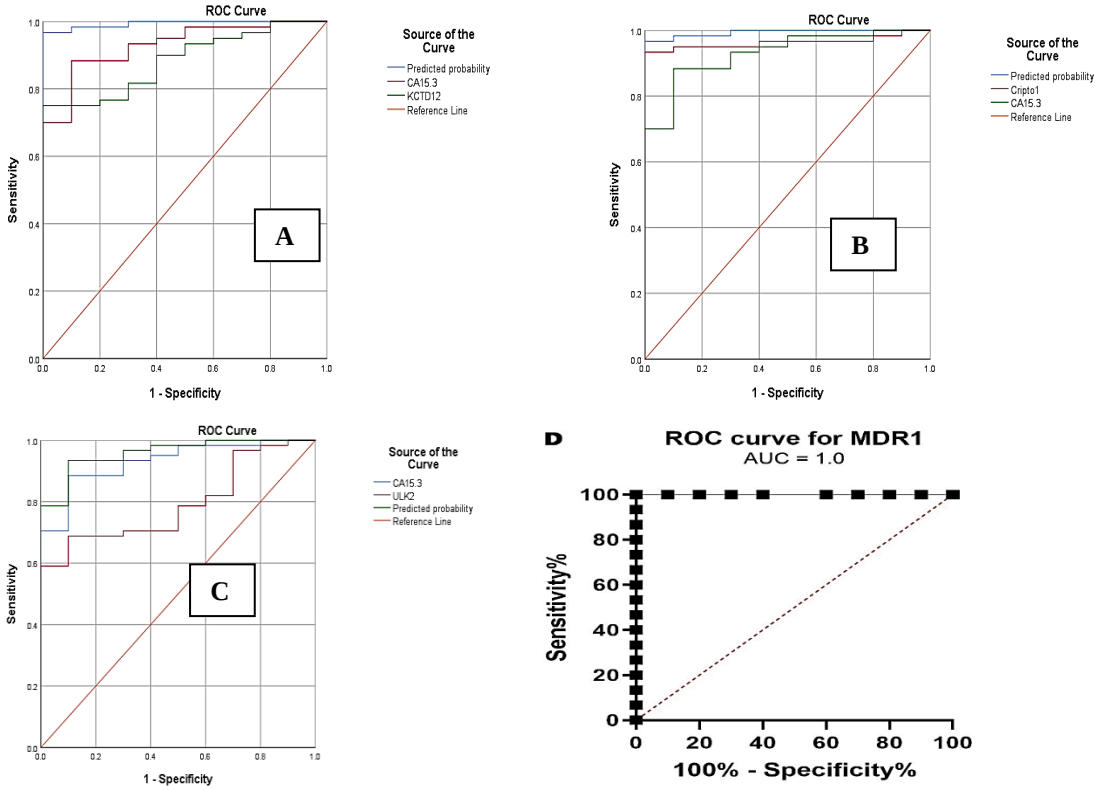


Figure 3 Cumulated Receiver operator characteristic (ROC) analysis of the control group and patients with breast cancer for serum marker CA 15.3 level with (A) KCTD12 (B) Cripto-1 (C) ULK2 serum levels. (D) ROC curve for MDR1 between the control and the resistant groups. The area under the curve (AUC) with 95 % CI were computed for each ROC curve.

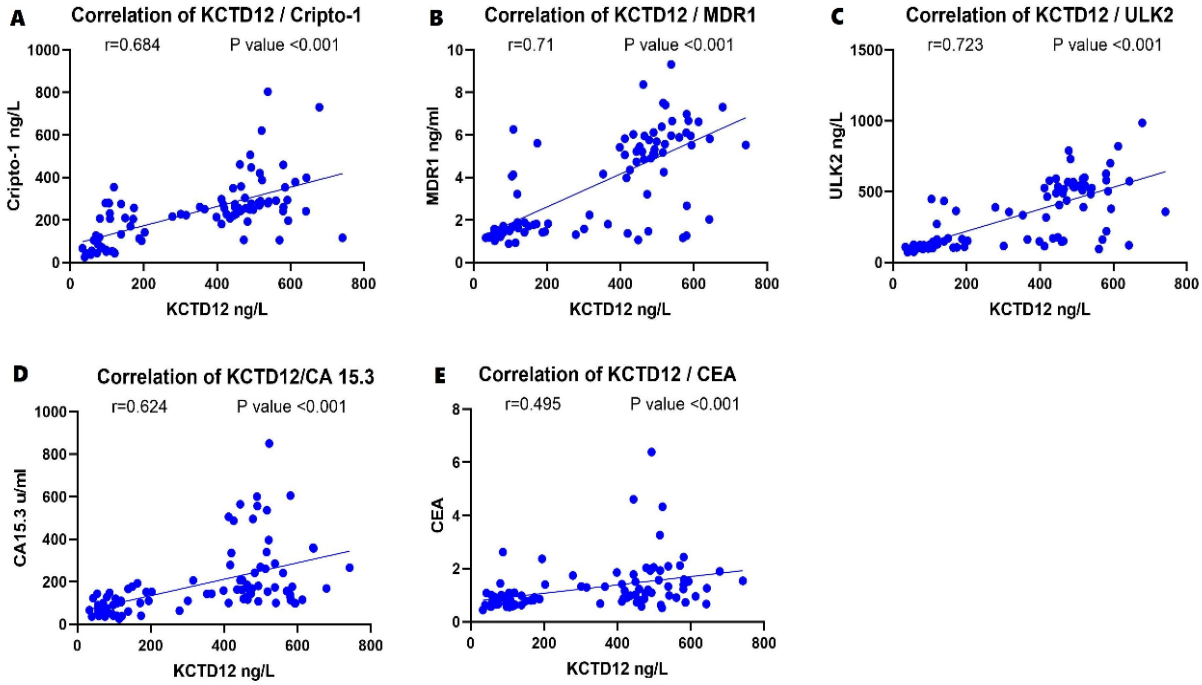


Figure 4 Correlation between serum KCTD12 level with (A) Cripto-1 (B)MDR1 (C) ULK2 (D) CA 15.3 (E) CEA levels using nonparametric Spearman correlation test (r) showing a strong positive correlation between them at P <0.001.

To reveal the relation between KCTD12 and the Notch signaling pathway in breast cancer, Cripto-1 - one of the main regulators of the Notch pathway - was measured. Previous studies showed the downregulation of Cripto-1 by KCTD12 in (ESSC) via chromatin remodeling inhibition exhibiting the suppressing role of KCTD12 over the Notch signaling pathway [13]. Moreover, Cripto-1 levels were much higher in breast cancer patients than healthy controls [26]. Our study is not far from these results where Cripto-1 is higher in malignant groups than control and benign ones. Both resistant and ER-PR-HER2- groups showed the highest levels. Unlike the inhibitory action of KCTD12 over Cripto-1 observed in (ESSC), our data lays out a strong positive correlation between both markers confirmed by Spearman's correlation negating the inhibitory role of KCTD12 over Cripto-1 in breast cancer rather than (ESSC).

On the other side, ULK2 is known to regulate autophagy. Earlier studies suggested that ULK2 suppressed tumor growth and increased cisplatin sensitivity in a certain type of lung cancer [27]. On the contrary, our subjects showed exceeding levels in the malignant cases than the non-malignant ones. As well, a previous study showed a negative correlation between KCTD12 and ULK2 that conceal ULK2 autophosphorylation required for its function or shifting the subcellular localization of Ulk2 to avert initial endosomal association [20, 28]. Our study outlines a strong positive correlation between both markers confirmed by Spearman's correlation denying the suppressing role of KCTD12 over ULK2 in breast cancer.

MDR1 was linked to failure in achieving a therapeutic response. We observed the highest levels in resistant groups emphasizing this fact and is more confirmed by the ROC curve between control and resistant subjects. Also, a new positive correlation between KCTD12 and MDR1 is suggested confirmed by Spearman's correlation unlike the negative correlation between KCTD12 and drug-resistant proteins in ESCC.

Cumulated ROC curves suggested add on in differentiation between malignant and control subjects when combining each marker with CA 15.3 resulting in increasing AUC and both sensitivities and specificities.

## 5. Conclusions

To our knowledge, we firstly suggest the role of KCTD12 in breast cancer other than different types of cancer by illustrating its action on the Notch signaling pathway. Both Cripto-1 and ULK2 are significantly raised in breast cancer subjects than controls with suggested positive correlations between these markers and KCTD12. KCTD12 is much more elevated in resistant subjects and confirmed by a positive correlation with MDR1 which leads to therapeutic resistance. The present results suggest that KCTD12 is elevated and may be used as a useful and reliable biomarker in breast cancer patients. The use of these markers along with already validated tumor marker as CA 15.3 provide better sensitivity for detection advocating more affirmation research with this combining.

## 6. Funding

We did not receive any funding support for the work.

## 7. Conflicts of interest

All authors do not claim any conflict of interest.

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