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THE UBIQUITINATION OF KCTD12 IN S PHASE PROMOTES THE FORMATION OF BREAST CANCER

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Abstract: ***In order to study the role of KCTD12 in breast cancer, the tissue microarray and bioinformatics methods were used. At the same time, in vitro we used MDA-MB-231 a cell line of breast cancer to research the influence of KCTD12 on breast cancer cells, the ability of proliferation and colony formation of 231 which with over expression KCTD12 were detected. At last we used the method of synchronization preliminary to discusse the mechanism of KCTD12 regulated the cell cycle and promoted the formation of breast cancer. We found that KCTD12 was highly expressed in breast cancer tissues and that patients with high KCTD12 expression had low survival rate. At the same time, in vitro experiments also confirmed that KCTD12 promoted cell proliferation and enhanced the ability of cell colony formation. Meanwhile, by means of synchronization, we found that KCTD12 was ubiquitination in S phase, which may be the reason for its regulation of cell cycle.***

Keywords : KCTD12, breast cancer, ubiquitination, S phase, tumorigenesis

I. INTRODUCTION

The incidence of breast cancer ranks the first among female malignant tumors, It is a major disease that seriously endangers women's health. The study of the pathogenesis of breast cancer can provide a theoretical basis for the treatment of clinical breast cancer.

Potassium channel tetramerization donmaincontaining (KCTD). Currently, there are 25 members in this family, The gene family has been reported involve in neurodevelopmental and neuropsychiatric disorders[1]. They are similar in structure, with a BTB/POZ domain at the N terminal and a unique domain at the C terminal [2-4]. The function of the BTB/POZ domain has been reported to be not only a voltage-gated potassium channel [5], but also involved in transcriptional inhibition [6-8], skeletal regulation [9-11] etc. It also interacts with ubiquitinated E3 ligases to form complexes [10, 11].

KCTD12 is a member of the KCTD family. KCTD12 has two tightly bound structural domains. Like all family members, it has a BTB/POZ domain rich in spiral and pleat folds at the n-terminal, while at the c-terminal it is a domain rich in pleat folds [12].

It has been reported that KCTD12 promotes the formation of cervical cancer and lung cancer by regulating the transition of G2/M phase[13]. Another report showed that KCTD12 could inhibit esophageal squamous cell carcinoma through the WNT signaling pathway[14]. Thus, the role of KCTD12 in tumor remains controversial.

In this study, the expression of KCTD12 in breast cancer tissues was detected by tissue microarray, and the relationship between the expression of KCTD12 and the survival rate was analyzed by drugsurv database. At the same time, in vitro overexpression method was used to detect the effect of KCTD12 on the ability of proliferation and colony formation to breast cancer cell line MDA-MB-231, and to explore the mechanism of KCTD12 effect on tumor. This study expected to provide a new theoretical basis for the treatment of breast cancer.

II. METERIALS AND METHODS

A. cell culture

Breast cancer cell line MDA-MB-231 was purchased from ATCC and cultured in DMEM medium containing 10%FBS at 37℃ and 5% CO2 in incubator.

B. Immunohistochemistry

Tissue microarray of human Breast cancer was presented by Shanghai outdo biotech. The tissue microarray contains 15 pairs breast cancer tissues and adjacent normal tissues. The

tissue microarry was put in an 60℃ oven for 30 minutes, dewaxed in xylene, rehydrated in a graded series of ethanol solutions, incubated with 3% hydrogen peroxide for 5 minutes, rinsed by distilled water, antigen repair 15 minutes, cool to room temperature, then sealed with serum for 30 minutes, KCTD12 antibody(Abcam, Massachusetts, US) incubated overnight at 4℃ , washed with PBS three times, then incubated with the appropriate peroxidase-conjugated secondary antibody(SantaCruz, CA, USA) for 30 minutes, Immunostaining was visualized using 3,3'-diaminobenzidine(DaKo, ON, Canada), which served as a chromogen, and the section was counterstained with hematoxylin, dehydrated in a graded series of ethanol solutions, transparent in xylene, sealed the piece with Permount TM Mounting Medium. KCTD12 immunostaining was evaluated based on scores representing the percentage of positively stained tumor cells and the staining intensity grade. The percentage of positive tumor cells were scored according to the following scale: 0, <10% 1, 10% - 30% 2, 30% - 50% 3, 50% - 80% and 4, 80%-100%. The staining intensity was divided into the following four categories: 0, no staining; 1, weak staining; 2, moderate staining; 3, Strong staining.

C. Plasmids and transfection

V-KCTD12 was generated by PCR amplification of sequences obtained from breast cancer cDNA library and cloned into a pCMV-N-flag vector. The following primers were used to generate the KCTD12, forward 5'-CCGGAATTCCACCTCTCTGTCATGGCTCT-3' and reverse 5'-CTGCAGAGAACTCAGCACCAAG-3'.Transfection was performed using Lipofectamine^{TM}2000 reagent(Thermo Fisher, MA, USA)

D. WST-1 assay cell proliferation

Cell proliferation was measured by a WST-1 Cell Proliferation and Cytotoxicity Assay Kit(Beyotime Biotechnology, Shanghai, China). respectively collected the 231 cells which transfected V-NC or V-KCTD12, then made the cell suspension, every hole seeded 5000 cells in 96 - well plates, each group with five holes, and detected at 1, 2, 3, 4, 5, and 6 days. At the time point, WST-1 was added and incubated for 2h at 37℃, and the absorbance of 450nm was detected by an enzyme marker

E. Flow cytometric cell cycle analysis

Flow cytometric was used to detection the cell cycle. Collected V-NC 231 cells and V-KCTD12 231 cells, the cells were fixed with 70% alcohol for 2h at -20℃, stained with propidium iodide(PI)staining buffer(PBS with 33 g/mL PI, 0.13 mg/mL RNaseA, 10 mmol/mL EDTA, 0.5% tritonx-100) for 10 min at room temperature.Cell cycle analysis was performed on a BD Accuri C6 Analyzed(BD, CA, USA).

F. Colony formation

Collected V-NC 231 cells and V-KCTD12 231 cells, Seeded 500 cells each hole in six orifice plate, cultured for 10 days. Then the cells were washed twice with PBS, and fixed with anhydrous methanol for 10 min, stained with crystal violet of 0.5% for 10 min. Washed with PBS twice to remove non-specific staining and background, a scanner was used to scan the results.

G. Cell synchronization

Cell cycle synchronization was performed by thymidine double block method. 231 were inoculated into five number respectively in cell culture bottles (1), (2), (3), (4), (5). At the same time, Thymidine(Sigma, San Francisco, CA, USA) with a final concentration of 2.5 mM was added and cultured for 18 hours for the first time block. then washed the cells with PBS for 3 times, thoroughly remove Thymidine, added 37℃ preheated medium for the first release, and cultured for 10 hours. Thymidine with a final concentration of 2.5 mM was added again and blocked for the second time, and cultured for 16 hours. Then collected bottles of G1/S phase cells. The other 4 bottles were washed with PBS for 3 times, Thymidine was thoroughly removed, and the medium preheated at 37oC was added for the second release and continued to be cultured. The second release was followed 4 hours later then collected the S phase cells. And 2 bottles were added with the final concentration of 100 ng/mL Nocodazole(Sigma, San Francisco,CA, USA) and continued to be cultured. collected bottles of G2 phase cells 8 hours after the second release. collected bottles of M phase cells 10 hours after the second release. the G1 phase cells of the bottle were collected 15 hours after the second release.

H. Western blotting

The cell were lysed with SDS lysis buffer(beyotime, shanghai, China) containing 1mM PMSF(Sigma, San Francisco, CA, USA) and 1mM protease inhibitors(Sigma, San Francisco, CA, USA), The protein samples were heated at 100 °C last 5 min, then the SDS-PAGE gel were employed for the proteins separation, the following antibodies were used: anti- KCTD12(Abcam, Massachusetts, US); anti-actin (SantaCruz, CA, USA), the result were detected by ECL reagent(bio-rad, CA, USA) .

I. Statistical Analysis

The results were analyzed using GraphPad PRISM software. And the date from different experiments using T test, the comparison between the multiple sets of data using multiple factors gap analysis, $P < 0.05$ showed significant difference, $P < 0.01$ showed very significant difference.

III.RESULTS

A. KCTD12 is highly expressed in breast cancer tissues

The expression of KCTD12 in breast cancer tissues and adjacent tissues was detected by breast cancer tissue microarray. hematoxylin and eosin staining(HE) and immunohistochemical staining were used in this study. Results as shown in figure 1, KCTD12 was highly expressed in breast cancer tissues.

At the same time, The effect of KCTD12 expression on the survival rate of breast cancer patients was analyzed using drugsurv database, result as shown in figure 2, and we found that the survival rate of patients with low expression of KCTD12 was high, while the survival rate of patients with high expression of KCTD12 was low. So KCTD12 is an oncogene in breast cancer.

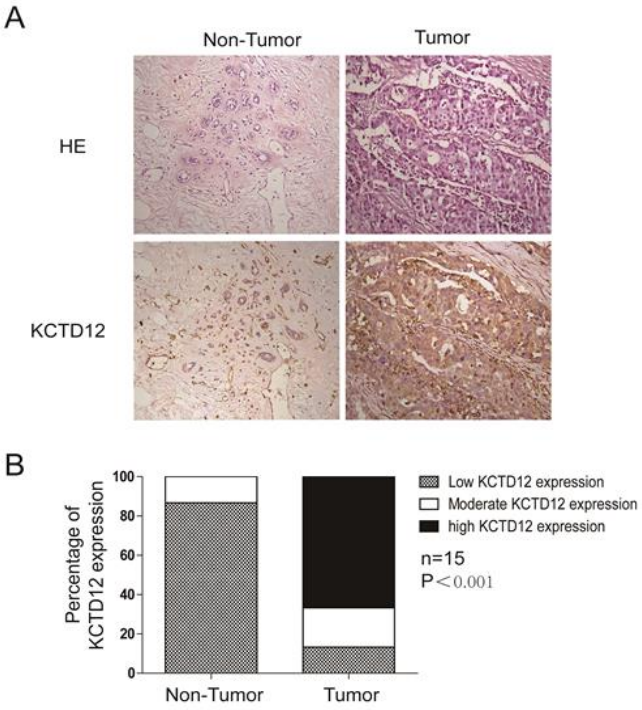

Fig. 1 Clinical significance of KCTD12 in breast cancer. (A) Representative results of HE staining and immunohistochemical staining for KCTD12 expression in breast cancer tissue compared with non-tumor tissues. (B) statistical analysis showed that KCTD12 expression was significantly increased in breast cancer tissues compared with non-tumor tissues.

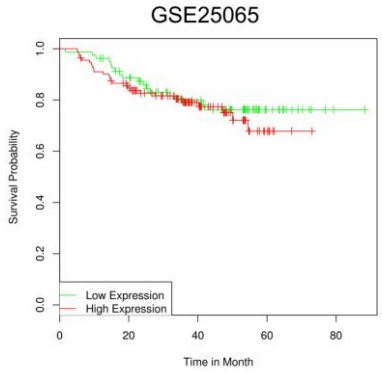

Fig. 2 Relationship between the expression of KCTD12 and the survival rate of the breast cancer patient. Data from DRUGSURV DATABASE, the underlying database is GEO.

B. KCTD12 promotes breast cancer cell proliferation and improved the ability of colony formation

We verified the role of KCTD12 in clinical breast cancer, and then we studied the specific function of KCTD12 in tumor formation. We constructed an overexpression plasmid of KCTD12 and transfected it into 231 cells. It was found that KCTD12 promoted cell proliferation and enhanced cell tumorigenosis, but had little effect on the cycle. The results show as figure 3. which suggests that KCTD12 promotes breast cancer formation by promoting cell proliferation and enhancing cell tumorigenesis.

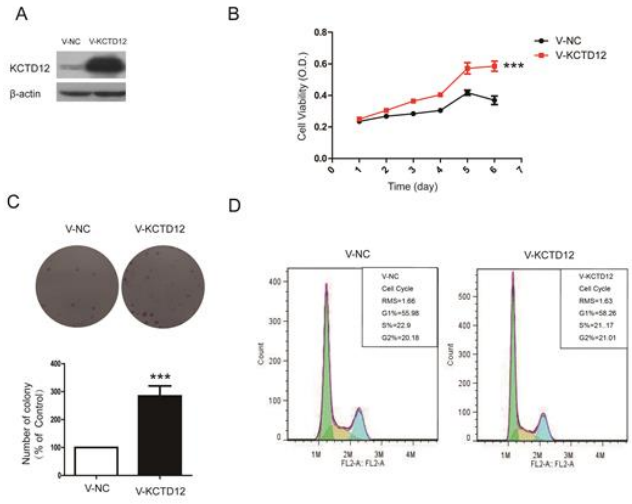

Fig. 3 KCTD12 promotes cell proliferation in breast cancer cells. (A) 231 cells were transfected with V-KCTD12 plasmid or V-NC control plasmid, KCTD12 and actin expression leves detemined by western bolting. (B) Cell proliferation was examined by WST-1. (C) KCTD12 enhanced the ability of cell colony formation, Bars, s.d; *P<0.05,**P<0.01,***P<0.001 compared with control cells. (D) KCTD12 had little affecte on cell cycle. Cell cycle were detected by flow cytometry.

C. KCTD12 promotes cell proliferation by degradation during the S phase

We have found the role and function of KCTD12 in breast cancer, and we would discuss its mechanism. By using the method of synchronization, we found that the expression of KCTD12 changed periodically with the change of cell cycle, which was relatively high in the G1/S phase, and decreased from the S phase to the least G2 (figure 4A). KCTD12 has a BTB/POZ domain, which has been reported to interact with ubiquitin ligase E3 to form a complex, suggesting that KCTD12 may be ubiquitinated and degraded in the S phase. At the same time of synchronization, we used the proteasome inhibitor MG132 and foud that KCTD12 did not decrease in the S phase after added MG132 (FIG. 4B), which suggested that KCTD12 was ubiquitinized in the S phase. Next, we continued to explore the function of its ubiquitination. We examined the cell cycle and found that after added MG132, not only did KCTD2 not decrease, but the cell cycle was also blocked (FIG. 4C). which suggested that the ubiquitination of KCTD12 at the S phase may promote the progression of the cell cycle, thereby promoting cell proliferation and tumor formation.

IV. DISCUSSION

One of the important ways of cell cycle regulation is through periodic expression and degradation of cyclins. Most of the degradation is regulated by ubiquitination. For example, CyclinA is synthesized from G1 to S and degraded from G2 to M. This degradation is regulated by ubiquitination, which in turn regulates the cell cycle.

Several KCTD family proteins have been reported to mediate ubiquitination and degradation of specific target proteins, and to regulate some signal path way. Such as Cullin-3 binging to KCTD10 may regulate action organization and other cell functions by degrading Rho GTPases RhoA or RhoB[10, 11].

In this study, the ubiquitination of KCTD12 at the S stage leads to its degradation, which also suggests that it may degradated some specific target proteins and participate in the regulation of cell cycle, thereby promoting cell proliferation and breast cancer progression.

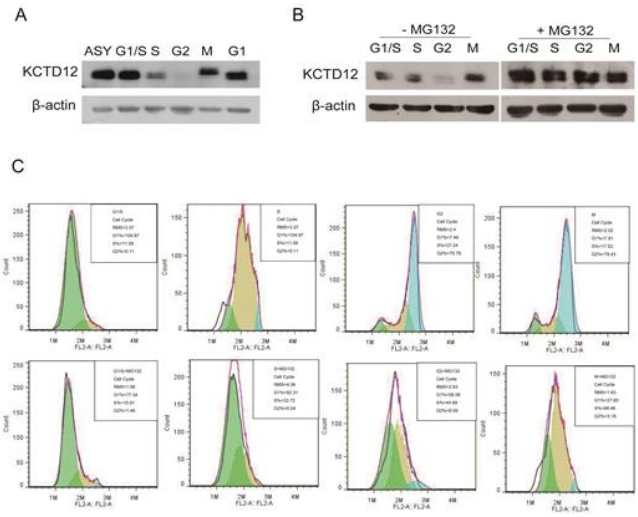

Fig. 4 KCTD12 was ubiquitinated in the S phase. (A) The expression of KCTD12 in each stage were detected by western blotting. (B)The expression of KCTD12 in each stage were detected by western blotting. (C) The effect of cell cycle after added MG132 were tested by flow cytometry.

In conclusion, KCTD12 was high expression in breast cancer, and the breast cancer patients with high expression KCTD12 had a low survival rate. In vitro, KCTD12 promoted the proliferation of breast cancer cell 231 and enhances its ability of colony formation. This suggested that KCTD12 play an important role in the pathogenesis of human breast cancer. At the same time, we also found that KCTD12 was ubiquitinated in the S phase, which suggested that KCTD12 may promote the progression of breast cancer through the S phase ubiquitination.

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