Caveolin-1 facilitated KCNA5 expression, promoting breast cancer viability

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Abstract. Potassium voltage-gated channel subfamily A member 5 (KCNA5) is a voltage-gated potassium channel protein encoded by the *KCNA5* gene. A large number of studies have shown that KCNA5 is associated with the survival of malignant tumors, including breast cancer, but the detailed mechanism remains inconclusive. Our previous study found that KCNA5 is co-expressed with a scaffolding protein, caveolin-1 in MCF-10A-neoT non-tumorigenic epithelial cell. In the present study, KCNA5 and caveolin-1 were expressed in breast cancer tissues and cell lines. Exposing MCF-10A-neoT to 2 mM of methyl-β-cyclodextrin, an agent to disrupt caveolae and lipid rafts led to a downregulation of caveolin-1 that reduced the expression of KCNA5. Furthermore, following caveolin-1 knockdown, the expression of KCNA5 was decreased in MDA-MB-231 human breast cancer and MCF-10A-neoT non-tumorigenic epithelial cell lines. In subsequent experiments, the MTT assay showed that increased caveolin-1 and KCNA5 expression promoted the survival of MCF-7 human breast cancer cells, but cell survival was not affected following KCNA5 overexpression alone. Using small interfering RNA technology, KCNA5-silenced MCF-10A-neoT cells were established and a decreased level of phosphorylated-AKT serine/threonine kinase (AKT) was observed in the cells compared with the parental cells. Overall, these results suggested that caveolin-1 facilitated KCNA5 expression and may be associated with AKT activation.

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Abbreviations: K_v, voltage-gated K^+ channel; MCF-10A-neoT, non-tumorigenic epithelial cell line; MCF7 and MDA-MB-231, human breast cancer cell lines; MAPK, ras/mitogen-activated protein kinase; AKT, serine/threonine protein kinase B; Cav-1, caveolin-1; PCNA, proliferation cell nuclear antigen; MβCD, methyl-β-cyclodextrin

Key words: caveolin-1, KCNA5, human breast cancer cells, PI3K/AKT pathway

Introduction

Voltage-gated potassium (K_v) channels play a key role in several physiological processes, such as the membrane potential maintainance, Ca^{2+} signaling, and the cell volume regulation. In addition, they are also involved in cell survival and migration (1). Most studies have focused on the function of K_v channels in viability of tumor cells, particularly tumor cells of epithelial origin, such as human mammary epithelial cell (2,3). Accumulated evidence indicated that the expression of K_v channels is related to tumor development (4). However, the mechanism of K_v channels in these cells is still unknown. Thus, many drugs and toxins that specifically block K_v channels have been tested for their effect on cell proliferation. Among the K_v channels subunits, potassium voltage-gated channel subfamily A member 5 (KCNA5) specifically has been shown to be involved in the viability and apoptosis of oligodendrocytes, hippocampus microglia, macrophages and human mammary epithelial cells (5-7). It has reported that most tumor cells had increased expression of KCNA5 (8). Furthermore, the transition of quiescent cells into the G_1 phase is accompanied by the over-expression of Kv1.3 and KCNA5 proteins in rat oligodendrocyte precursor cells. Blocking KCNA5 sufficiently slowed the viability of astrocytes rather than oligodendrocytes, indicating that this channel may play different roles in different cells.

AKT phosphorylation is transduced and amplified through downstream kinase cascades, inducing cell survival, growth, differentiation as well as metabolic changes (2). In

general, K_v channels are modulated by mitogenic signals, such as growth factor-mediated signaling (8,9). In HEK293 cells, IGF-1 induces the expression of several K_v channels in response to mitogenic signals (10). The mitogenic stimulation of G_0 phase activates K^+ channels, drives the cells into G_1 phase, and then initiates proliferation (3,11). K_v channels play a major role in advancing the cell cycle when activated by mitogenic factors (12,13). Interestingly, K_v channels are upstream modulators of growth factor-mediated MAPK and PI3K/Akt pathways (14,15). And many current studies suggest that K^+ channels involved in the initial mitogenic signaling events occur at the membrane level. Moreover, the activation of K^+ channels may lead to receptor clustering, thereby facilitating transmembrane signaling (2).

Lipid rafts support numerous cellular events in membrane trafficking and signal transduction mediated by multiple membrane proteins (16-18). Caveolae are a type of lipid raft containing specific scaffolding proteins, like caveolin (19). Various lipid rafts share similar lipid proteins (20-22). The caveolin family has three members, including caveolin-1 (Cav-1), Cav-2 and Cav-3, of which Cav-3 is restricted to muscle cells (23,24). Many signaling molecules are directly related to Cav-1 (25,26). Some proteins have been well-characterized, including Kv channels that interact with caveolin, which are to concentrate the cargo proteins in the caveolae (27,28). It has been reported that the expression and localization of Kv channels are important to play their function fully in cells (29). Numerous Kv channels localize to the lipid raft domains and/or caveolae in the plasma membrane. KCNA5 has been found in raft microdomains and their functions are influenced by lipid-protein interactions. Martens *et al* showed that KCNA5 can localize to caveolae microdomains, and KCNA5 was associated with caveolae (27). However, we are unknown for the mechanisms controlling their interactions and the physiological functions of this localization. Recent research has found a role of Cav-1 in transporting proteins to the cell membrane (30). And according to some recent studies, Cav-1 regulates proteins that co-localize with it, such as estrogen receptor (ER), KCNA5, and desmoglein 2 (Dsg2) (31-33). However, the role of Cav-1 in mediating the membrane localization of KCNA5 channel has not been elucidated.

Our previous study demonstrated that K_v channels were required for the viability of the normal MCF-10A-neoT cells (7). In this study, we described that KCNA5 and Cav-1 co-localize in the cytoplasm of MCF-7 human breast cancer cells. The study also found that the knockdown KCNA5 inhibited the PI3K/AKT signaling pathway in MCF-10A-neoT cells, and cells upregulated with Cav-1 and KCNA5 promoted survival in MCF-7 cells through PI3K/AKT signaling. In addition, it was showed that the downregulation of Cav-1 decreased the expression of KCNA5, indicating that Cav-1 was involved in the KCNA5-promoted survival of human mammary cells.

Materials and methods

Plasmids and antibodies. The KCNA5 plasmid was from Dr Jie Zheng (University of California, Davis). The Cav-1 plasmid and siRNA plasmid specific for Cav-1 (target sequence Oligo 1, 5'-ACCTCATTAAGAGCTTCCTGATTGAGTCAAGAGCTCAATCAGGAAGCTCTTAATTT-3', Oligo 2, 5'-CAAAAAATTAAGAGCTTCCTGATTGAGCTCTTGACTCAATCAGGAAGCTCTTAATG-3') were obtained from the Cancer Center at Creighton University.

Anti-KCNA5 (rabbit polyclonal, 1:500; EMD Millipore, Billerica, MA, USA), anti-Cav-1 (mouse monoclonal, 1:1,000, Santa cruz biotechnology), anti-p-MAPK (mouse monoclonal, 1:1,000), anti-MAPK (rabbit polyclonal, 1:1,000), anti-p-AKT (rabbit monoclonal, 1:1,000) (all from Cell Signaling Technology, Danvers, MA, USA), anti-AKT (goat polyclonal, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), 1:500), anti-PCNA (mouse monoclonal, 1:500) and anti-β-actin (mouse monoclonal, 1:1,000) (all from Wuhan Boster Biological Technology, Ltd., Wuhan, China). HRP-conjugated goat anti-rabbit, anti-mouse or anti-goat specific secondary antibody (1:6,000; Zhongshan Golden Bridge Biotechnology, Beijing, China).

Patients. A total of 23 breast cancer tissues were obtained from patients in the First Affiliated Hospital of Dalian Medical University. All the patients were females aged 29-83 with infiltrative non-specific breast cancer. The selected tissue samples express both ERα and ER-α36 under immunofluorescence observation, and without any radiation, chemotherapy, or endocrinotherapy treatment before surgical resection. We got the patients' relatives written informed consent for the procedures, which were also approved by the Ethics Committee on the Use of Human Subjects (the First Affiliated Hospital of Dalian Medical University).

Cell culture and transfection. The MCF-10A-neoT, MCF-7 and MDA-MB-231 cells were purchased from ATCC (Rockville, MD, USA). Stable clones (designated as MCF-10A-neoT^{CE}) were established as described in our previous study (34,35). The MCF-10A-neoT and MCF-10A-neoT^{CE} cells were cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5% horse serum (HyClone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 μg/ml) (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), hydrocortisone ($1.4x10^{-6}$ M; HyClone), insulin (10 μg/ml), cholera toxin (100 ng/ml) and EGF (20 ng/ml) (both from Sigma-Aldrich, Merck KGaA). MCF7, MDA-MB-231 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were maintained in a humidified atmosphere at 37˚C in 5% CO_2. Lipofectamine 2000^{TM} (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection according to the manufacturer's instructions. After 24-48 h of transfection and subsequent culture in 1 μM wortmannin or 50 μM Ly294002 (Sigma-Aldrich, Tokyo, Japan), cells were harvested for western blot analysis or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Methyl-β-cyclodextrin (MβCD) was used to disrupt caveolae. MCF-10A-neoT cells pretreated with MβCD (2 mM) for 90 min were used for immunofluorescent microscopy analysis.

Western blotting. Cells were harvested and then lysed in a cold lysis buffer (20 mmol L^{-1} Tris-HCl, pH 7.5, 70 mmol L^{-1} NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100 and

1% PMSF) to extract protein (35). The concentration of total protein was determined by the Bradford method. The protein samples were then subjected to 10% SDS-PAGE. After electrophoresis, protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane, and then blocked in PBS-T (pH 7.4) containing 5% dried skim milk. Then the PVDF membrane was probed with the specified primary antibody, followed by the appropriate secondary antibody, and finally visualized using the ECL™ which is a Western blotting chemiluminescent reagent kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Immunoblot data were quantified using ImageJ software (NIH, Bethesda, MD, USA). The region of interest was marked and measured in every lane, and the background was subtracted to give the final band intensity.

RNA interference. The small interfering RNAs (siRNA) against the KCNA5 mRNA sequence (GenBank accession number NM_002234) were predesigned and synthesized in Takara (Dalian, China), meanwhile, an unrelated siRNA serving as a negative control was randomly designed. The sequences of the KCNA5 siRNA vector plasmid (target sequence Oligo 1, 5'-GATCCACCAGGGAACCCATTTCTCTCTGTGAAGCCACAGATGGGAGAGAAATGGGTTCCCTGGTTTTTTTAT-3', target sequence Oligo 2, 5'-CGATAAAAAAACCAGGGAACCCATTTCTCTCCCATCTGTGGCTTCACAGAGAGAAATGGGTTCCCTGGTG-3'), and the negative control siRNA plasmid (target sequence Oligo 1, 5'-GATCCAGATCCTCACGATACCGTCTCTGTGAAGCCACAGATGGGAGACGGTATCGTGAGGATCTTTTTTTAT-3', target sequence Oligo 2, 5'-CGATAAAAAAAGATCCTCACGATACCGTCTCCCATCTGTGGCTTCACAGAGACGGTATCGTGAGGATCTG-3').

Knocking down the basal expression levels of KCNA5 or Cav-1 in MCF-10A-neoT or MDA-MB-231 cells were performed using the respective siRNAs, as well as scrambled control siRNA. The cells were transfected with Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 4 μg of Cav-1-siRNA according to the manufacturer's recommendation. After 48 h of transfection, culture medium with G418 (Amresco, Solon, OH, USA) was added to the cells. The cells were harvested when the monoclone formed and were processed for further analysis.

Survival assay. For the MTT assay, 20 μl MTT reagent was added to each well and incubated for 4 h. The supernatant was discarded and replaced with DMSO to dissolve the formazan product, which was measured by a spectrophotometric plate reader at 490 nm.

Hematoxylin and eosin staining. Human breast cancer specimens were fixed for 1 week in 4% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were stained with hematoxylin and eosin, then deparaffinized with xylene. All sections were studied by using a AxioVision zeiss (Olympus, Tokyo, Japan) microscope.

Immunofluorescent staining. Cells were grown on glass coverslips coated with poly-L-lysine, and cultured for 24 h. The cells were then fixed in 4% ice cold paraformaldehyde for 15-20 min. Cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 min. After blocking with 5% bovine serum albumin (BSA) for 1 h, the cells were then incubated with appropriate antibodies at 4˚C overnight, followed by further incubation with fluorescein-conjugated affinipure goat anti-mouse antibodies (1:300) and Rhodamine-conjugated affinipure goat anti-rabbit antibodies (1:300) (both from Zhongshan Golden Bridge Biotechnology). Nucleus was stained using DAPI (0.2 μg/ml; Sigma-Aldrich, Merck KGaA). Fluorescence was imaged with a Bio-Rad MRC 600 confocal imaging system. Images were taken with a Leica TCS SP2 multiphoton confocal microscope.

Statistical analysis. All data were expressed as the mean ± SE Unpaired Student's t-test was used to test for statistical significance between the control and test groups. Comparisons of multiple groups were analyzed using a one- or two-way ANOVA followed by post hoc Tukey's test. P-value <0.05 was considered significance.

Results

Cav-1 and KCNA5 are co-expression in human breast cancer cell lines. Cav-1 and KCNA5 have been shown to be related to epithelial cell growth and survival, and are abnormally expressed in malignant tumors including breast cancer. The study demonstrated that K_v channels activate MAPK and AKT pathways, and Cav-1 and KCNA5 were co-localized in the membrane of MCF-10A-neoT non-tumorigenic epithelial cell line (36). The mechanism by which KCNA5 activates AKT signaling has not been fully determined, but it has been speculated to be activated through Cav-1. To examine the expression of KCNA5 and Cav-1 in breast cancer tissues and cells, hematoxylin and eosin staining was used to determine the region of cancer (Fig. 1A). Next, the study tried to determine whether there was co-expression between Cav-1 and KCNA5 in breast cancer tissues and cells. Cav-1 and KCNA5 both expressed and co-expressed in breast cancer tissues (Fig. 1A) and cells (Fig. 1B). After enlarging MCF-7 fluorescence images, it could be found KCNA5 and Cav-1 are co-expression in cytoplasm and cell membrane, but Cav-1 mainly expressed in cytoplasm of MCF-7 (Fig. 1B). Here, we examined KCNA5 and Cav-1 expression in MCF-10A-neoT, MCF-7 and MDA-MB-231 cells. Western blot analysis showed that both KCNA5 and Cav-1 expression are lower in MCF-7 breast cancer cell compared with MCF-10A-neoT non-tumorigenic epithelial cell and KCNA5 and Cav-1 expressions are significantly higher in MDA-MB-231 breast cancer cell than MCF-7 (Fig. 1C).

KCNA5 and Cav-1 are coupled in caveolae of human breast cancer cells. Our lab already has generated stable MCF-10A-neoT^{CE}, which is a Cav-1 knockdown cell line (34). Compared with control cells, immunofluorescence (Fig. 2) and immunoblot (Fig. 3A) both showed reduced expression of KCNA5 in MCF-10A-neoT^{CE} cells. Quantification of the immunoblots demonstrated that the siRNA reduced Cav-1 by ~80% and KCNA5 by ~50% in MCF-10A-neoT^{CE}. To further confirm the association of Cav-1 with KCNA5,

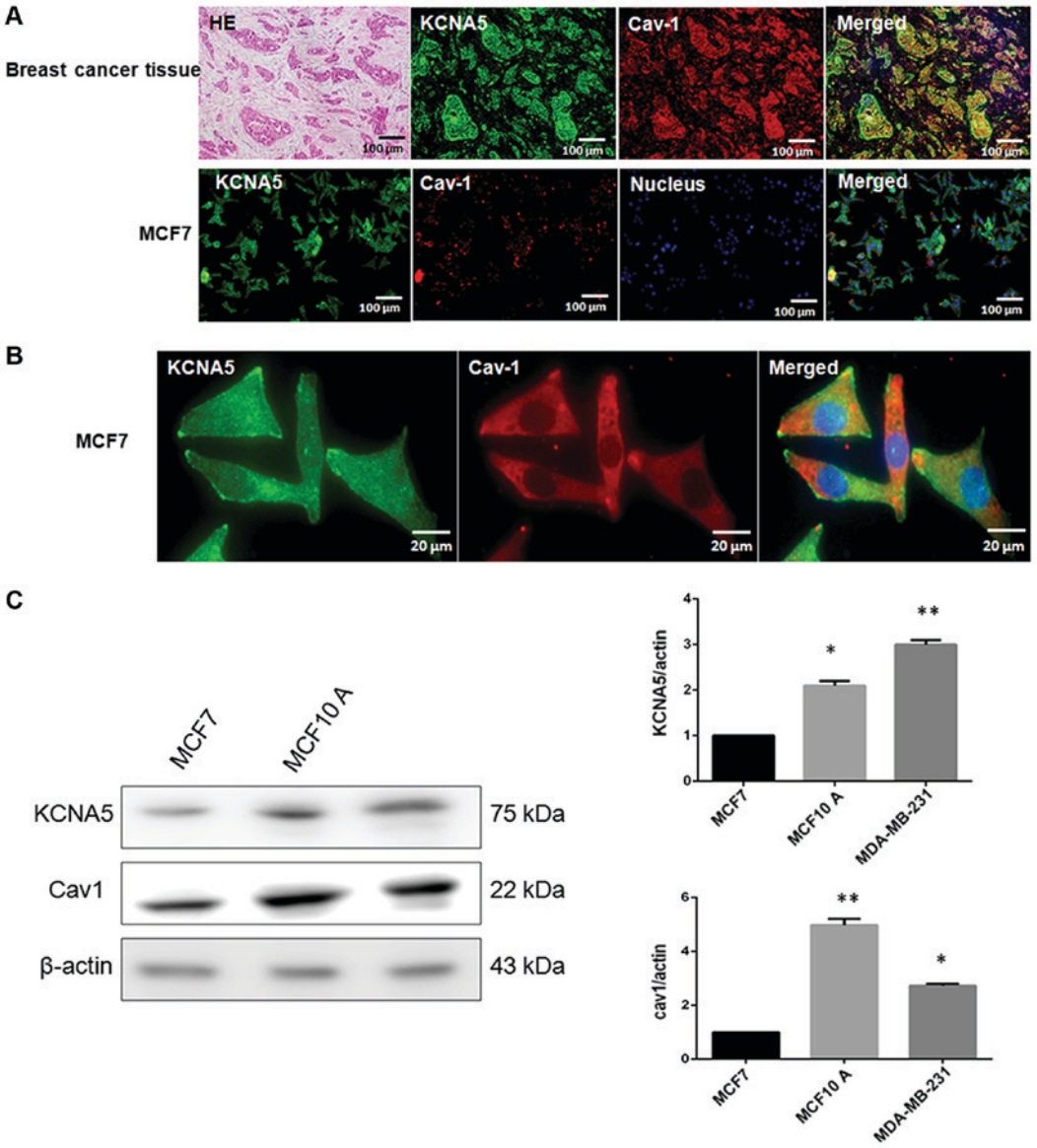

Figure 1. The co-expression of KCNA5 and Cav-1 in human breast cancer cells. Hematoxylin and eosin staining for human breast cancer tissue. (A) Immunofluorescence staining with KCNA5 and Cav-1 antibody in breast cancer tissue (KCNA5 for green, Cav-1 for red, Hoechst 33324 to label nuclear DNA). Bar, 100 μm. (B) MCF-7 human breast cancer cells (KCNA5 for green, Cav-1 for red, Hoechst 33324 to label nuclear DNA), bar, 100 μm or bar, 20 μm. Representative images were obtained by a Leica TCS SP 2 multiphoton confocal microscope. (C) Western blot analyses of Cav-1 and KCNA5 expression in MCF-7, MDA-MB-231 and MCF-10A-neoT cells. The experiment was repeated four times; SE. *P<0.05 and **P<0.01 for MCF-10A-neoT and MDA-MB-231 cells vs. MCF-7 cells. KCNA5, potassium voltage-gated channel subfamily A member 5; Cav-1, caveolin-1.

MCF-10A-neoT was treated with 2 mM MβCD to disrupt caveolae and lipid rafts, and it showed that the expressions of Cav-1 and KCNA5 both significantly decreased in the cell membrane of MCF-10A-neoT (Fig. 2). Collectively, our data demonstrated that the knockdown of Cav-1 reduced KCNA5 levels in MCF-10A-neoT. Similar results were also obtained in MDA-MB-231 (Fig. 3B), which indicated that Cav-1 increased KCNA5 expression in human breast cancer cells. However, the expression of Cav-1 in MCF-10A-neoT-siKCNA5 and MCF-10A-neoT-vector were not changed (Fig. 3C). We over-expressed KCNA5 in MCF7 cell that Cav-1 is low expression, however, we found that overexpression of KCNA5 does not affect Cav-1 expression in MCF7 cells (Fig. 3D).

KCNA5 promotes human breast cancer cells survival through Cav-1. The PI3K/AKT and MAPK signaling pathways are the two major pathways involved in the regulation of cell viability (37). To test the function of Cav-1 and KCNA5 in MCF-7 cells, we made a Cav-1 and KCNA5 vectors co-transfection. Intriguingly, cells with increased Cav-1 and KCNA5 promoted cell survival, and the AKT signaling pathway inhibitor, Wortmanin significantly inhibited cell survival in MCF-7 (Fig. 4A-C). What's more, cells with both upregulated Cav-1 and KCNA5 had increased AKT activation (Fig. 4D). Therefore, we believe KCNA5 involves in cells survival, only when Cav-1 is overexpression. And PI3K/AKT signaling pathway involves in KCNA5-dependent cell survival.

To determine whether KCNA5 survival through Cav-1 in human breast cancer cell lines, we used MCF-10A-neoT as a cell model, which has an overexpression Cav-1 showing in Fig. 1C. Besides, by knocking down KCNA5 in MCF-10A-neoT, we detected whether or not the reduced expression of KCNA5

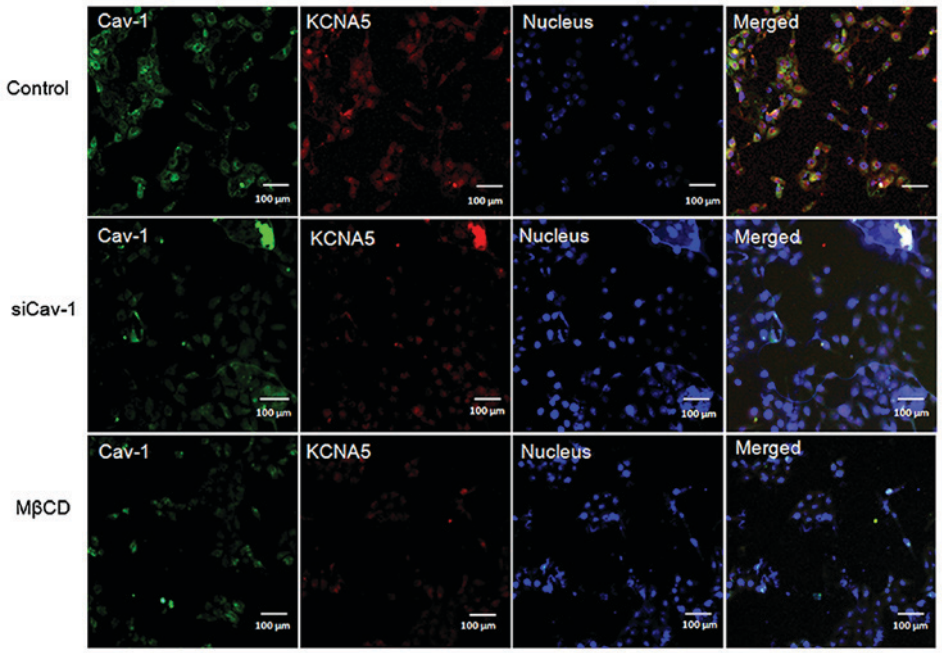

Figure 2. KCNA5 and Cav-1 are coupled together in caveolae. Immunofluorescence staining with KCNA5 and Cav-1 antibody in MCF-10A-neoT. Control: Co-expression of KCNA5 (red) and Cav-1 (green) in MCF-10A-neoT cells; Cav (-/-): Co-expression of KCNA5 (red) and Cav-1 (green) in Cav-1 expression knocked-down cells, MCF-10A-neoT^{CE}; MβCD: 2 mM methyl-β-cyclodextrin destroy the caveolae structure, and examined co-expression of KCNA5 (red) and Cav-1 (green) in MCF-10A-neoT, Bar, 100 μm. KCNA5, potassium voltage-gated channel subfamily A member 5; Cav-1, caveolin-1.

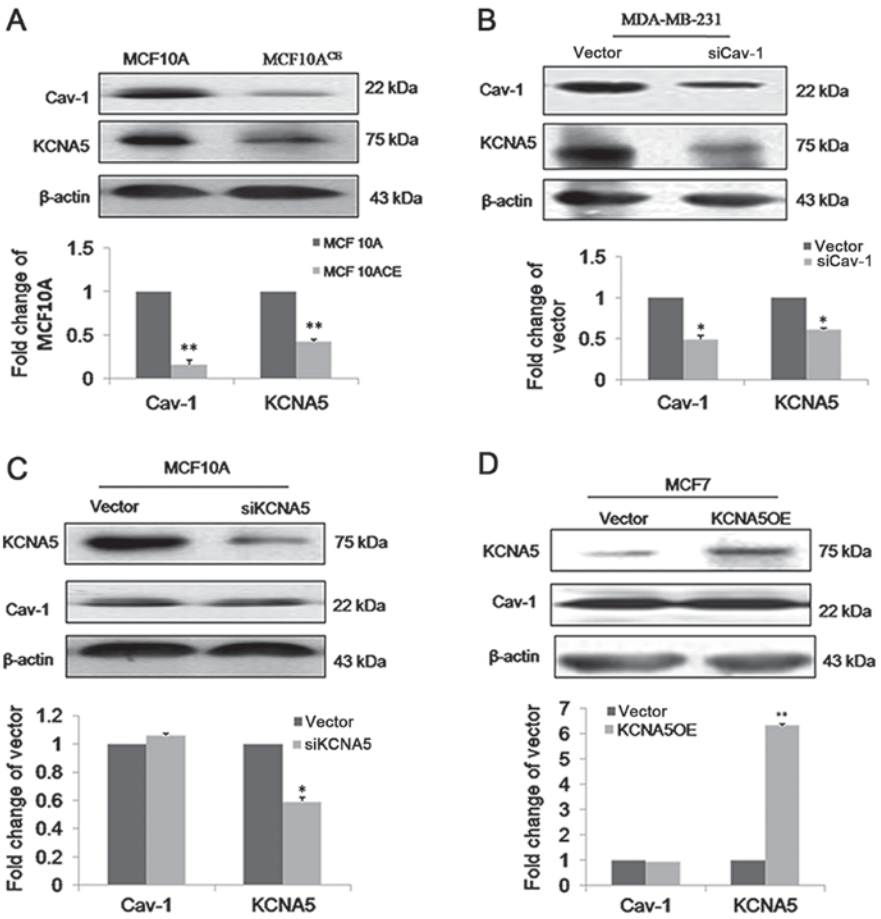

Figure 3. Effect of Cav-1 on the expression of KCNA5. (A) Western blotting analyzed the expression of KCNA5 in MCF-10A-neoT and Cav-1 expression knocked-down cells, MCF-10A-neoT^{CE}. (B) Cav-1 high expression MDA-MB-231 cells were transfected with the Cav-1 siRNA plasmid, western blot analyses of KCNA5 expression. (C) Western blot analyses of Cav-1 expression in MCF-10A-neoT cells and KCNA5 expression knocked-down cells MCF-10A-neoT-siKCNA5. (D) KCNA5 low expression MCF-7 cells were transfected with KCNA5 expression vector for 48 h, western blot analyses of Cav-1 expression. The experiment was repeated three times. *P<0.05 and **P<0.01 vs. vector. KCNA5, potassium voltage-gated channel subfamily A member 5; Cav-1, caveolin-1.

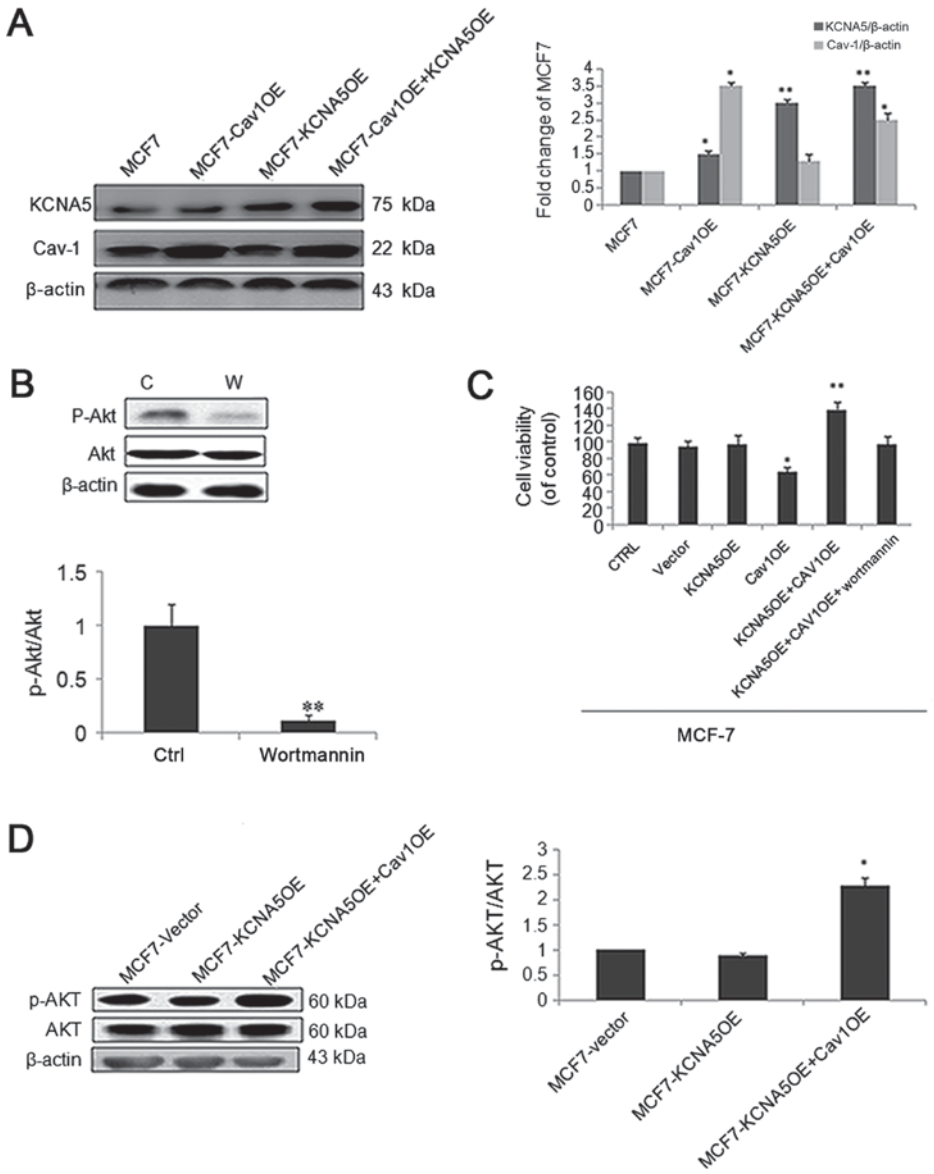

Figure 4. KCNA5 promoted human breast cancer cells survival is dependent on Cav-1. MCF-7 cells were transfected with KCNA5 or Cav-1 vector alone, or co-transfected with both Cav-1 and KCNA5 vectors for 48 h. (A) Western blot analyses of KCNA5 and Cav-1 expression. (B) Western blot analysis of Akt phosphorylation after wortmannin treatment. (C) Cells upregulated Cav-1 and KCNA5 were treated with wortmannin (1 μM) for 24 h. The survival of MCF-7 cells was examined by the MTT assay. (D) Western blot analysis of AKT phosphorylation in different MCF-7 variants; control cells MCF-7-vector (transfected with the empty expression vector); MCF-7-KCNA5 OE (transfected with the KCNA5 expression vector); MCF-7-KCNA5 OE+Cav-1 OE (co-transfected with the KCNA5 and Cav-1 expression vector. The columns represent the means of five experiment; SE. *P<0.05 and **P<0.01 for cells transfected with Cav-1 or KCNA5 expression vectors vs. empty expression vector. KCNA5, potassium voltage-gated channel subfamily A member 5; Cav-1, caveolin-1.

inhibited cell survival through the PI3K/AKT and/or MAPK signaling pathways in MCF-10A-neoT. As shown in Fig. 5A, there was no obvious change of p-MAPK in the two cell lines, but the level of p-AKT was decreased.

To study whether KCNA5 directly promote the cells survival through PI3K/AKT pathway, we observed the effects of wortmannin, LY 294002, inhibitors of AKT and PI3K on expression of KCNA5 and phospho-AKT protein in both MCF-10A-neoT and MCF-10A-neoT-siKCNA5 cell lines. As shown in Fig. 5B, the level of KCNA5 was not changed after treatment with two inhibitors in the MCF-10A-neoT. MCF-10A-neoT treated with both inhibitors, AKT activation was inhibited compared with control (Fig. 5C). In addition, compared with control, the level of p-AKT was significantly inhibited by wortmannin, however, LY29002 had no effect on p-AKT in the MCF-10A-neoT-siKCNA5 cells (Fig. 5D). Finally, we summarized the possible mechanism of how KCNA5 enhances breast cancer cells viability in Fig. 6.

Discussion

The study demonstrated that KCNA5 and Cav-1 co-expression in breast cancer and normal mammary tissue, and in MCF-10A-neoT and MCF-7 cells. And it was expounded that KCNA5 and Cav-1 upregulation in human breast cancer cells promoted cell survival and activated AKT. Furthermore, KCNA5 knockdown with siRNA resulted in a decreased phosphorylation of AKT, but not inhibit MAPK signaling in MCF-10A-neoT. Additionally, Cav-1 knockdown led to decreased KCNA5 expression in MCF-10A-neoT and

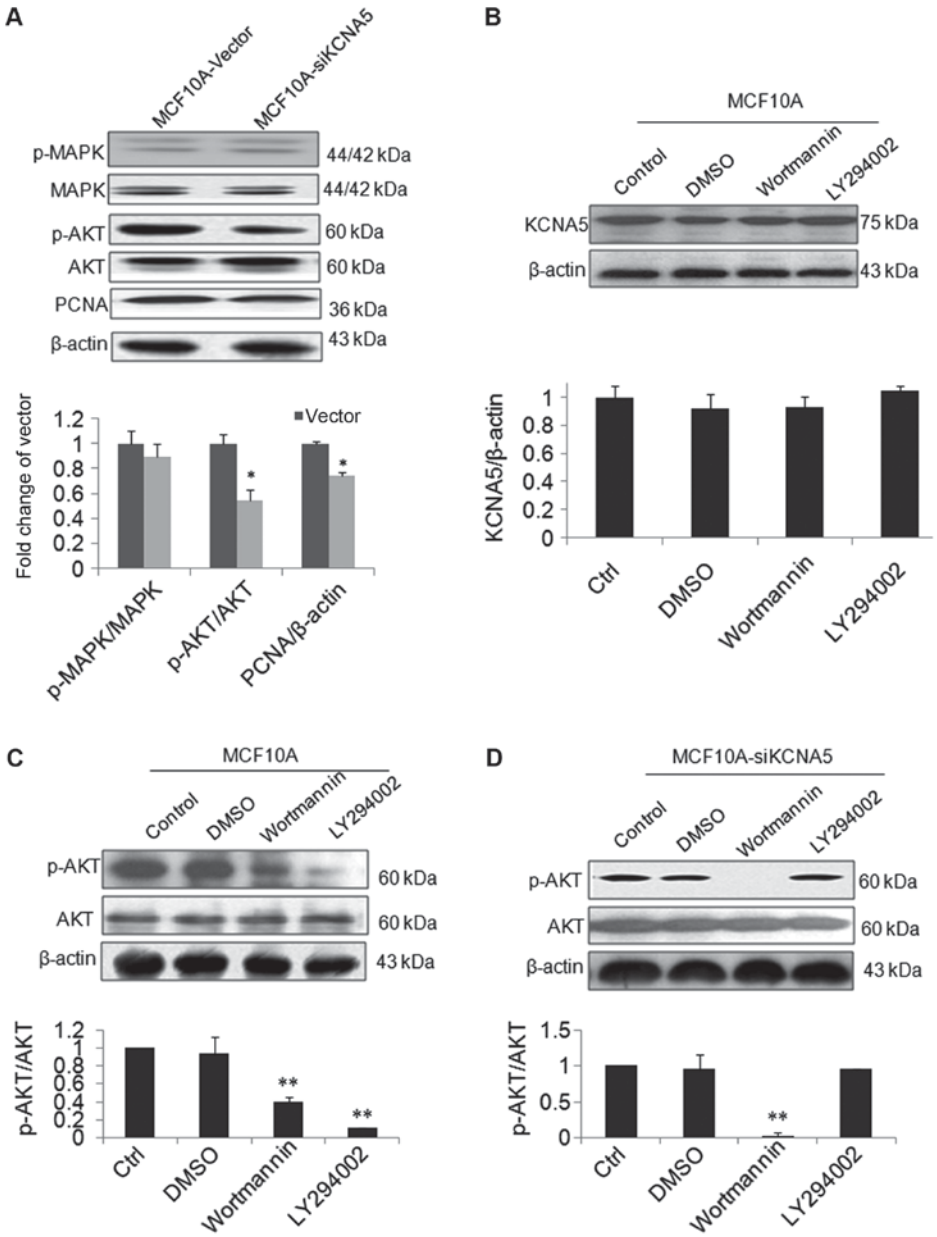

Figure 5. The PI3K/AKT signaling pathway is involved in KCNA5-dependent cell survival. Cells were cultured in serum-free medium for 4 h, and then treated with serum for 10 min, proteins were harvested and the expression of p-AKT and p-MAPK analyzed by western blot analysis. Statistical analyses of p-AKT and p-MAPK levels. (A) Western blot analyses of AKT, MAPK phosphorylation and PCNA expression in MCF-10A-neoT-vector (transfected with the empty expression vector) and MCF-10A-neoT-siKCNA5 (transfected with KCNA5 siRNA plamid). (B) Western blot analyses of KCNA5 expression after MCF-10A-neoT cells were treated with wortmannin (1 μM) and LY294002 (50 μM) for 24 h. (C and D) Cells were cultured in serum-free medium for 4 h, and then treated with wortmannin (1 μM) and LY294002 (50 μM) for 10 min. Western blot analyses of AKT activation in MCF-10A-neoT and MCF-10A-neoT-siKCNA5. The experiment was repeated three times. *P<0.05 and **P<0.01 vs. vector or Ctrl. KCNA5, potassium voltage-gated channel subfamily A member 5.

MDA-MB-231 cells. Overall, these data suggested that the expression and function of KCNA5 are related to Cav-1, and that KCNA5 facilitates the activation of AKT signaling with lipid rafts.

Kv channels are often observed in the MCF-7 human breast cancer cells. Abdul *et al*, found that the potassium channel activator, minoxidil promoted the survival of MCF-7 cells (38). Moreover, the use of specific and non-specific K^+ channels blocker on MCF-7 cells resulted in apoptosis, which implicated the involvement of ATP sensitive channels, SK channels, and Kv channels were related to cells survival in the MCF-7 cells (39). KCNA5 was involved in the survival of many mammalian cells. Wonderlin and Strobl found that KCNA5 was involved in survival of human breast cancer cells (40). From our previous study, Kv channels increased the survival of MCF-10A-neoT non-tumorigenic epithelial cell line (7), but the relevant mechanism is still unclear. In our present study, MCF-10A-neoT normal cells expressed high levels of KCNA5 and Cav-1 while MCF-7 human breast cancer cells expressed low levels of KCNA5 and Cav-1. We established a KCNA5 knockdown cell line with MCF-10A-neoT cells. Interestingly, we observed that KCNA5 knockdown inhibited AKT phosphorylation. And wortmannin and LY294002 PI3-kinase inhibitors have differential effects on p-AKT in MCF-10A-neoT-siKCNA5 cells. According to some studies, LY294002 potently block K_v currents and promote

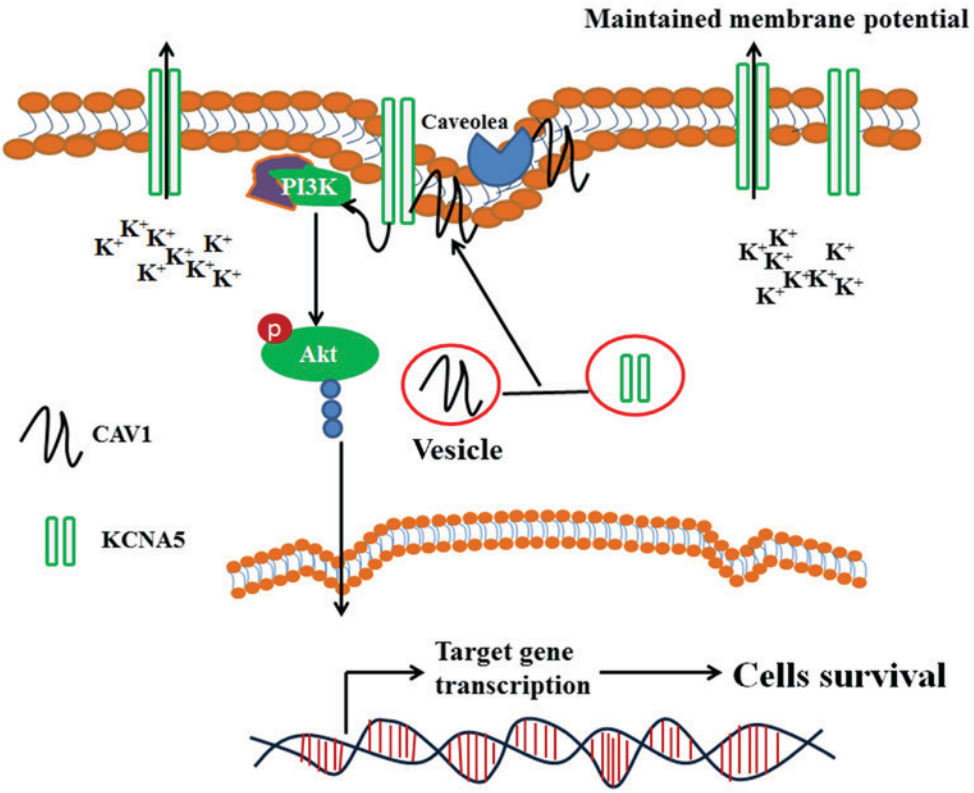

Figure 6. The proposed machanism underlying the observed cell survival effect of KCNA5 and Cav-1. Cav-1 expressed in membrane and cytoplasm and facilitated KCNA5 coupling caveoae. KCNA5 expressed in caveolae or non-caveolae. KCNA5 located in non-caveolae potentially. KCNA5 located in caveolae with Cav-1 together, which activated AKT signaling and promoted cell survival. KCNA5, potassium voltage-gated channel subfamily A member 5; Cav-1, caveolin-1.

increase in $[Ca^{2+}]i$ operates independently of PI3K (35,41). Our result showed that LY294002 failed to activate AKT in MCF-10A-neoT-siKCNA5 cells, which is related to cell membrane depolarization and Ca^{2+} decrease. In MCF-7 cells, KCNA5 had no distinct effect on cell survival, but the overexpression of Cav-1 and KCNA5 promoted cell survival. It has been reported that the suppression of Cav-1 increased AKT phosphorylation facilitated the survival of MCF-10A-neoT^{CE} cells, which downregulated Cav-1 expression, indicating that Cav-1 may be involved in KCNA5-mediated AKT phosphorylation (42). Therefore, KCNA5 is related to survival of human breast cancer cells via Cav-1 that has not been reported so far.

Cav-1, a signature protein in the integrated membrane component of caveolae, plays a scaffolding role in molecular signaling and endocytic trafficking (41). Various membrane proteins are endocytosed through a caveolin-dependent pathway, such as fibronectin, estrogen receptors, and ion channels proteins (43,44). Previous evidence suggested that lipid rafts regulate channels function in different ways (45,46). The interplay of ion channels and proteins, which regulates channel function, can impact the channel behavior. One important finding of our study is that Cav-1 associates with KCNA5 in order to promote cell survival, which is consistent with previous studies that Cav-1 regulates the trafficking of KCNA5 to lipid raft microdomains in cardiac cells stably transfected with human vectors of KCNA5 and caveolin (47). In our study, we found Cav-1 is mainly expressed in the cytoplasm, but there is low level of Cav-1 expressed in the cell membrane in MCF-7 cells. And immunofluorescence suggested that a mass of Cav-1 and KCNA5 cannot co-express in membrane. Therefore, a large amount of KCNA5 cannot be located in caveolae. At the same time, it was showed that Cav-1 and KCNA5 were co-localized in cell membrane of MCF-10A-neoT cells, and the immunoprecipitation was also used to determine the interaction between KCNA5 and Cav-1 in MCF-10A-neoT cells (34). The MCF7 cells with KCNA5 overexpression had no effect on cell survival; but cells with both KCNA5 and Cav-1 overexpression had increased cell survival. Our study speculated that KCNA5 regulated cell survival when located in the caveolae. Moreover, cholesterol-depleting experiment showed the decreased expression of KCNA5 and Cav-1 in the plasma membrane, which indicated that channel function, is related to the cholesterol in the membrane microenvironment. Our results were consistent with ones obtained from previous studies in different cell lines (48,49). Some studies have focused on the multiple function of Cav-1 in different cells and tissues, different stages of development, different physiological and pathological processes. Since 2000, our lab has been forces on the multiple function of Cav-1 in different cells and tissues, different stages of development, different physiological and pathological processes (50). To date interestingly, as functional marker, it plays a role either as a tumor suppressor or an oncogene depending on the tumor type and context of tumor progression. Disruption of Caveolae integrity or downregulation of Cav-1 appears to be a common theme in oncogenic transformation in breast cancer cells.

Disruption of Caveolae integrity or downregulation of Cav-1 appears to be a common theme in oncogenic transformation of breast cancer cells. In addition, it has been shown that

Cav-1 can inhibit the activity of several caveolae-associated signaling molecules, including Src, H-Ras and G-proteins. On the other hand, evidence in support of an oncogenic role for Cav-1 was provided by studies showing that Cav-1 promotes cell survival in metastatic prostate cancer cells. Indeed, Cav-1 expression is upregulated in numerous cancer cell lines and tumor specimens. Our lab also reported that knockdown Cav-1 increased cell proliferation, and colony formation in mammary epithelial cells, which expression of KCNA5 is more than that in MDA-MB-231 than MCF-7 cells (34,51,52). In this study, it has been shown that expression of Cav-1 was more in MDA-MB-231 metastatic breast cancer cell than MCF-7. In this study, the important interaction between KCNA5 and Cav-1 in mammary cells was showed, and it was proved that Cav-1 is required for expression of KCNA5 channel for cell survival. In addition, our data showed a strong co-expression of Cav-1 and KCNA5 in MCF-10A-neoT non-tumorigenic epithelial cell line. And it is reported that the interaction also exists in both heart and vascular smooth muscle (28,53). Our results strongly suggest that the KCNA5 channel increased survival of human breast cancer cells through the PI3K/AKT signaling pathway rather than the MAPK pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CQ, JS, and YL were involved in conceptualization of the study, data acquisition, formal analysis, and writing of the original draft. XW, CH, LW and QC were involved in methodology planning and data analysis. TG, YZ, HL and YW analyzed data. WZ and JL designed the project, and acquired funding and resources.

Ethics approval and consent to participate

Patients' relatives provided written informed consent for the procedures. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University for the use of human subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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