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CHL1 is involved in human breast tumorigenesis and progression

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ABSTRACT

Neural cell adhesion molecules (CAM) play important roles in the development and regeneration of the nervous system. The L1 family of CAMs is comprised of L1, Close Homolog of L1 (CHL1, L1CAM2), NrCAM, and Neurofascin, which are structurally related trans-membrane proteins in vertebrates. Although the L1CAM has been demonstrated play important role in carcinogenesis and progression, the function of CHL1 in human breast cancer is limited. Here, we found that CHL1 is down-regulated in human breast cancer and related to lower grade. Furthermore, overexpression of CHL1 suppresses proliferation and invasion in MDA-MB-231 cells and knockdown of CHL1 expression results in increased proliferation and invasion in MCF7 cells *in vitro*. Finally, CHL1 deficiency promotes tumor formation *in vivo*. Our results may provide a strategy for blocking breast carcinogenesis and progression.

1. Introduction

Breast cancer is one of the most common human malignancies, accounting for 22% of all cancers in women worldwide [1]. Although the incidence remains high, the increase of the overall survival has been attributed to advances in early detection and therapeutic modalities [2]. Breast cancer represents a complex and heterogeneous disease that comprises distinct pathologies, histological features, and clinical outcome. Although recent advancements in early detection, prevention, and treatment, breast cancer mortality rate, improvements of prevention and therapy remain a major public health concern. Molecularly, breast cancer is a heterogeneous disease with respect to molecular alteration, cellular composition, and clinical outcome [3]. Our complete understanding of mechanisms responsible for breast cancer development and progression will help us to select means in effective controls of breast cancer.

Neural cell adhesion molecules (CAM) of the immunoglobulin superfamily play important roles in the development and regeneration of the nervous system [4]. The L1 family of CAMs is structurally related trans-membrane proteins in vertebrates, including L1, Close Homolog of L1 (CHL1, L1CAM2), NrCAM, and Neurofascin [5]. L1CAM has been demonstrated to be overexpressed in a variety of human cancers and to be associated with poor prognosis, including breast cancer [6], colon cancer [7,8], ovarian and endometrial tumors [9], gallbladder carcinoma [10], pancreatic ductal adenocarcinoma [11], and non-small cell lung cancer [12]. However, very few studies focus on the expression and function of CHL1 in human breast cancer.

The CHL1 gene is a member of the L1 gene family of neural CAM. It is a neural recognition molecule that may be involved in signal transduction pathways. CHL1 is expressed in normal tissues besides the brain and is expressed in a variety of human cancer cell lines and primary tumor tissues [13,14]. The CHL1 gene is involved in general cognitive activities and some neurological diseases [15–17]. Recently, CHL1 was silenced to facilitate in situ tumor growth as a putative tumor suppressor [18]. Thus these reports suggest that CHL1 is involved in carcinogenesis, not only in neuronal activities.

In our current study, we found that CHL1 is down-regulation in breast cancer tissues. Furthermore, overexpression of CHL1 suppresses proliferation and invasion, whereas knockdown of CHL1 expression results in increased proliferation and invasion in breast cancer cells *in vitro*. Finally, CHL1 deficiency promotes tumor formation *in vivo*. Thus, these results indicate that CHL1 is involved in human breast cancer.

2. Materials and methods

2.1. Cell culture and specimens

MDA-MB-435, MDA-MB-231, T47D, MCF7, and MCF10A cells were obtained from the American Type Culture Collection (Manas-

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sas, VA, USA). MDA-MB-435, T47D, and MCF7 were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA), and MDA-MB-231 was cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen). MCF10A was cultured in DMEM/F12 supplemented with 5% horse serum. The cells were incubated at 37 °C in a humidified chamber with 5% CO_2.

Paired samples of human breast cancer tissue and the corresponding adjacent normal breast tissue were obtained from the Provincial Hospital Affiliated to Shandong University. No patients received chemotherapy or radiotherapy prior to the operation. This study was approved by the Institutional Review Board of the Provincial Hospital Affiliated to Shandong University and written consent was obtained from all participants.

2.2. Plasmid, siRNA, and transfection

To construct the CHL1 expression vector, the entire coding sequence of the CHL1 was amplified by PCR using the Pfu DNA Polymerase (Thermo Scientific, Rockford, IL, USA). The PCR product was cloned into the pcDNA3.1 (Invitrogen). The construct was confirmed by sequencing. siRNAs targeting CHL1 were purchased from RiboBio (Shanghai, China). For transient transfection, 2×10^5 cells were plated into 6-well plates and kept in antibiotic-free medium for 24 h before transfection. The cells were then transfected with the siRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For generation of stable depleted CHL1 expression, cells were transfected with CHL1 shRNA. Two days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St. Louis, MO, USA) for about 2 weeks.

2.3. Western blot

Cells and specimens were lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.55 NP-40) with 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). 40 μg proteins were mixed with SDS sample buffer and boiled for 10 min before loading into a 10% SDS–PAGE gel. After electrophoresis, the proteins were blotted onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with anti-CHL1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. Next day, the membranes were incubated with horseradish peroxidase conjugated-secondary antibody, and the bands were visualized using ECL detection reagents (Millipore). Actin served as the loading control.

2.4. Real-time quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions. 4 μg of total RNA was converted to first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions. The targeted gene relative quantification was given by the CT values, and the CT value of Actin was subtracted to obtain ΔCT. The relative mRNA expression level of targeted genes was determined as $2^{-\Delta CT}$. The experiment was performed in triplicate.

2.5. MTT, colony formation, and transwell assays

Both MTT and colony formation assays were used to evaluate the ability of cell proliferation. For MTT assay, MDA-MB-231 and MCF7 cells, following transfection, 5×10^3 cells were seeded in 96-well plates per well. Then 24, 48, 72 h after transfection, the cells were incubated with 10 μl MTT (0.5 mg/ml; Sigma–Aldrich) at 37 °C for 4 h. The medium was then removed, and precipitated Formosan was dissolved in 100 μl DMSO. The absorbance at 570 nm was detected using a micro-plate auto-reader (Bio-Rad, Richmond, CA, USA).

For colony formation assay, the MDA-MB-231 and MCF7 cells 500 cells were counted and seeded in 6-well plates per well after transfection. The plates were incubated at 37 °C and 5% CO_2 in a humidified incubator. Fresh culture medium was replaced every 3 days. After 2 weeks of culture, the cells were stained with crystal violet, and the numbers of colonies containing more than 50 cells were counted.

Transwell assay was used to evaluate the ability of cell invasion. MDA-MB-231 or MCF7 cells in 0.2 ml DMEM without FBS were placed on the top chamber of each insert (BD, Biosciences, San Jose, CA, USA) with 40 μl of 1 mg/ml Matrigel. The lower chamber was filled with 600 μl of DMEM medium with 10% FBS to act as the nutritional attractant. 24 or 36 h later, the migrant cells that had attached to the lower surface were fixed with 20% methanol and stained for 20 min with crystal violet. The membranes were then carved and embedded under cover slips with the cells on the top. Cells in three different fields of view were counted and expressed as the average number of cells per field of view. All assays were performed in triplicate.

2.6. Xenograft assay

Twenty-four female SCID mice (Institute of laboratory animal sciences, CAMS, Beijing, China) 4–6 weeks old were randomly divided into four groups, preparing for the xenografting studies. 1×10^7 viable cells were washed and harvested in 0.1 ml phosphate-buffered saline and subcutaneously injected into fat pad of the left lower quadrant. All experiments involving mice were carried out in strict accordance with institutional and local government guidelines on handling of laboratory animals. Tumor growth was recorded once a week with a caliper-like instrument. Tumor volume was calculated according to the formula volume = $(\text{width}^2 \times \text{length})/2$. Five weeks after inoculation, mice were killed, and the final volumes of tumor tissues were determined. The tumor tissues were visualized by hematoxylin and eosin (H/E) staining.

2.7. Statistical analysis

The hypothesis test for significance between two groups with complete randomized design or paired design utilized the Student's *t*-test, The statistical significance was set to $P < 0.05$.

3. Results

3.1. CHL1 is down-regulated in human breast cancer

To determine whether CHL1 has a role in human breast cancer carcinogenesis, we examined CHL1 mRNA expression levels in 30 cases of primary breast cancer tissues and the adjacent normal breast tissues. We observed that CHL1 mRNA levels were down-regulated in breast cancer tissues compared with normal breast tissues by RT-qPCR (Fig. 1A). Furthermore, CHL1 protein levels were also down-regulated in breast cancer tissues by western blot (Fig. 1B). Importantly, we found that CHL1 expression was significantly lower in high grade tumors than in low grade tumors and the normal breast (Fig. 1C and D). Lower CHL1 expression levels were also evident in breast cancer cell lines (MDA-MB-435, MDA-MB-231, T47D, and MCF7) than the normal breast cell line

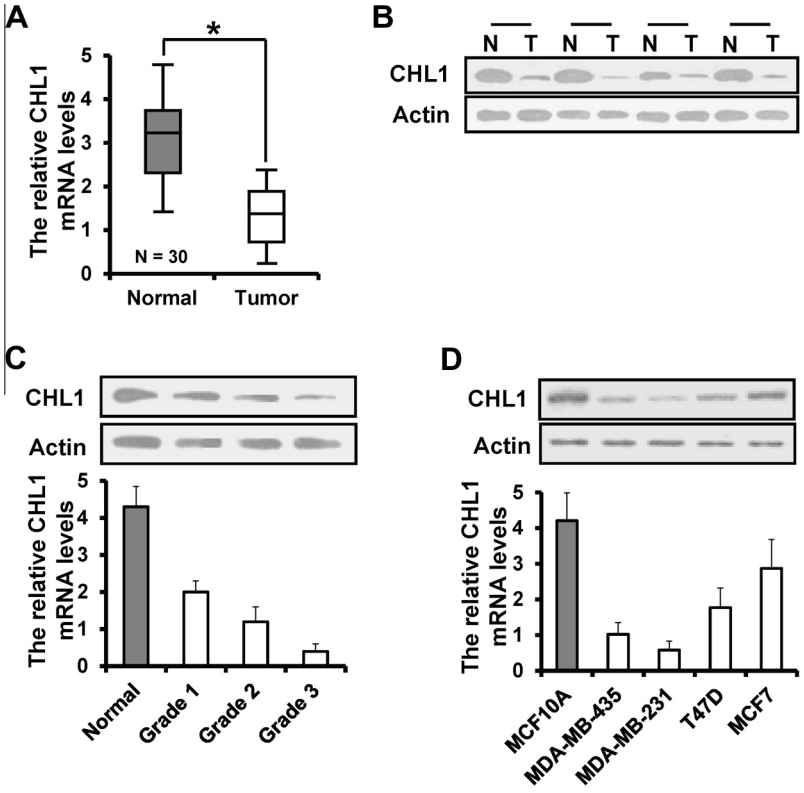

Fig. 1. The CHL1 expression in human breast cancer. (A) The CHL1 mRNA expression in primary breast cancer and the adjacent normal breast determined by RT-qPCR ($n = 30$). (B) The CHL1 protein expression in primary breast cancer (T) and adjacent normal breast (N) determined by western blot ($n = 4$). (C) The CHL1 expression in primary breast cancer with different grade determined by RT-qPCR and western blot. (D) The CHL1 expression in normal breast cell line and breast cancer cell lines determined by RT-qPCR and western blot. Actin as the loading control.

(MCF10A) (Fig. 1E and F). Together, these results indicate that CHL1 is down-regulated in human breast cancer.

3.2. Overexpression of CHL1 suppresses the proliferation and invasion of human breast cancer cells in vitro

To determine whether CHL1 affected cell proliferation and invasion, MDA-MB-231 cells were transfected with pcDNA3.1-CHL1. The CHL1 expression levels were significantly increased in CHL1-transfected MDA-MB-231 cells by RT-qPCR and western blot (Fig. 2A). Next, we performed the MTT assay to evaluate proliferation. The result indicated that pcDNA3.1-CHL1 significantly decreased MDA-MB-231 proliferation at 48 and 72 h after transfection, compared with the parental and control cells (Fig. 2B). Furthermore, we performed transwell assay to demonstrate the effects of CHL1 on the invasive potential of MDA-MB-231 cells. The ectopic expression of CHL1 resulted in an approximately 50% decrease in MDA-MB-231 cell invasion (Fig. 2D). These data indicate that CHL1 suppresses the proliferation and invasion of breast cancer cells *in vitro*.

3.3. Depletion of CHL1 stimulates breast cancer cell proliferation and invasion in vitro

To evaluate the effect of depletion of CHL1 on breast cancer proliferation and invasion, we knock down the CHL1 expression in MCF7 cells using siRNAs targeting CHL1. Cell proliferation analyses showed that CHL1 deficiency in MCF7 significantly promoted cell growth. siRNA-1 and siRNA-2 cells grew much faster than the parental or control cells in MTT assay (Fig. 3B). Soft agar colony formation assay also revealed that siRNA-1 and siRNA-2 cells formed more colonies than parental or control cells (Fig. 3C). Furthermore, we performed transwell assay to evaluate the effects of CHL1 on the invasive potential of MCF7 cells. As shown in Fig. 3D, MCF7 cells transfected with CHL1 siRNAs display higher transwell migratory rates than the parental or control cells. Together, these results indicate that depletion of CHL1 stimulates breast cancer cell proliferation and invasion *in vitro*.

3.4. CHL1 deficiency promotes tumor formation in vivo

To further determine the effect of altered CHL1 expression on breast cancer cell growth, we established two stable CHL1 siRNA-transfected MCF7 cell lines. RT-qPCR and western blot analyses showed decreased levels of CHL1 expression in the CHL1 siRNA-transfected MCF7 cell lines (Fig. 4A). We then injected these cells into SCID mice (1×10^7 per mouse) to evaluate the effect of CHL1 knock down on breast tumor growth. Tumor formation and volume of the tumor in each mouse were examined, measured and recorded for five weeks and tumor growth curves were determined. As shown in Fig. 4B, tumor formation and tumor growth of CHL1 siRNA-transfected cells were significantly increased in nude mice when compared with those of control or parental cells. Five weeks after inoculation, mice were killed and tumor xenografts were obtained. Consistently, tumor xenografts from control or parental cells were much smaller than those from CHL1 siRNA-transfected cells (Fig. 4C). These results indicate that CHL1 deficiency promotes the tumorigenicity of human breast cancer cells.

4. Discussion

Neural cell adhesion molecules (CAM) molecules play important roles in specifying cell–cell interaction and mediating cell–cell and cell–matrix interactions during development, regeneration and plasticity of nervous system [18,19]. Alterations in CAM

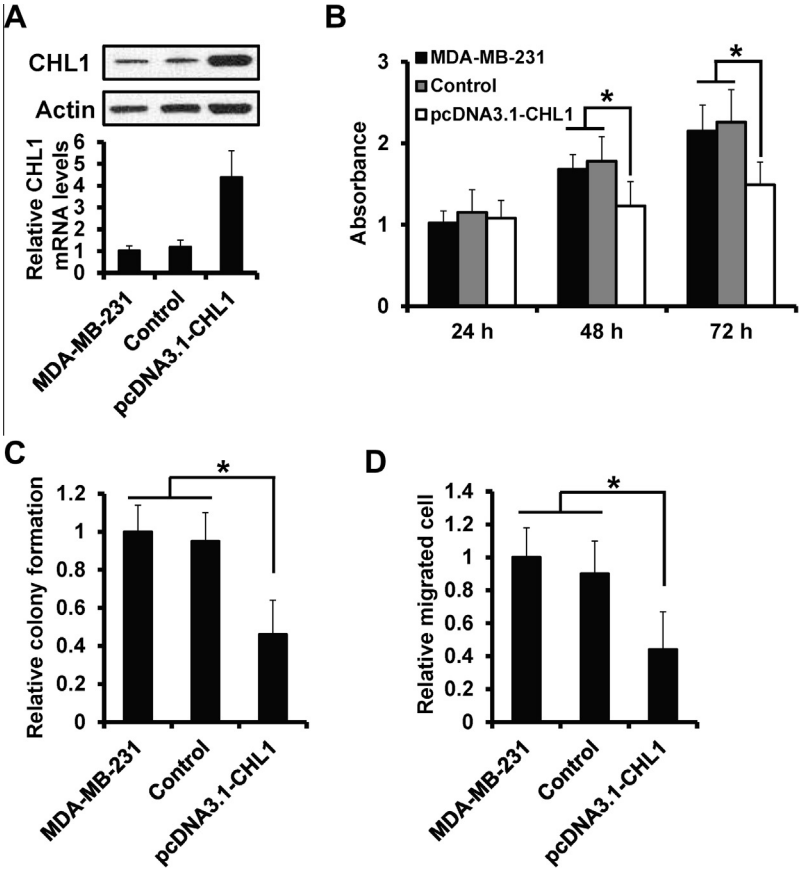

Fig. 2. Overexpression of CHL1 inhibits breast cancer cell proliferation and invasion *in vitro*. (A) MDA-MB-231 cells were transfected with pcDNA3.1-CHL1 or the negative control, and CHL1 expression was detected by RT-qPCR and western blot. (B) Cells as in (A) were analyzed by MTT assay. (C) Cells as in (A) were analyzed by soft agar colony formation assay. (D) Cells as in (A) were analyzed by Transwell assay. $^{*}P < 0.05$.

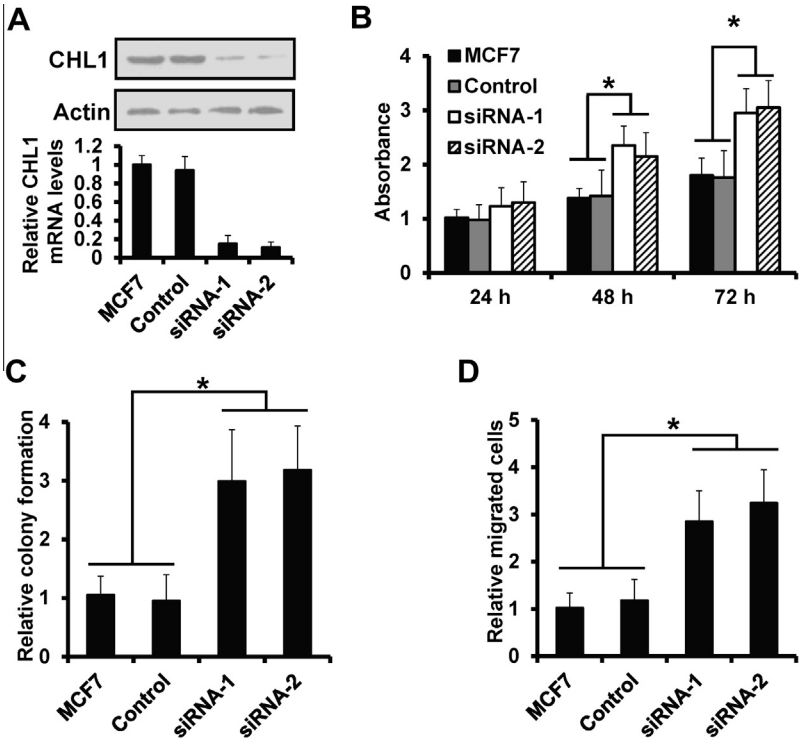

Fig. 3. CHL1 deficiency promotes breast cancer proliferation and invasion*in vitro*. (A) MCF7 cells were transfected with siRNAs targeting CHL1 or the negative control, and CHL1 expression was detected by RT-qPCR and western blot. (B) Cells as in (A) were analyzed by MTT assay. (C) Cells as in (A) were analyzed by soft agar colony formation assay. (D) Cells as in (A) were analyzed by Transwell assay. $^{*}P < 0.05$.

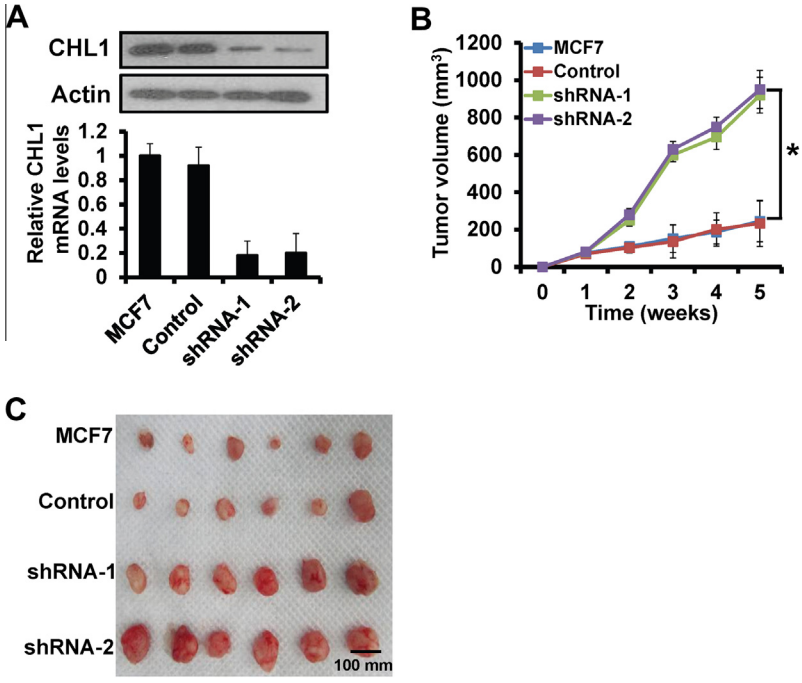

Fig. 4. CHL1 deficiency promotes breast cancer proliferation and invasion*in vivo*. (A) MCF7 cells stably expressing CHL1 siRNA (shRNA-1 and shRNA-2) or (Control) were lysed and CHL1 expression was detected by RT-qPCR and western blot. (B and C) 1×10^7 cells as in (A) were inoculated into the mammary fat pad of SCID mice and tumor growth was recorded with a caliper-like instrument. Tumor volumes were calculated according to the formula volume = (width^2 × length)/2 (B). After 7 weeks, all mice were killed and final tumor tissues were photographed (C).

expression and functions have been reported in the development of different tumor types, including melanoma [21], prostate [22], ovary [23,24], and colon cancer [25]. CHL1, a member of the L1 family of neural recognition molecules, is a subgroup of the immunoglobulin superfamily characterized by six immunoglobulin-like domains [20]. CHL1 is located at 3p26 and is involved in some neurological diseases [26]. However, the function of CHL1 on tumor progression was less studied, and the mechanism of CHL1 in tumor progression is a key question in biology. Here, we demonstrated that CHL1 deficiency promotes breast cancer cell proliferation and invasion both *in vitro* and *in vivo*. Consistent with our work, recent study has been indicated that knockdown of CHL1 expression results in increased colony formation activity, migration and invasion in human cervical cancer cells [5].

The recognition molecules L1 have been reported to regulate neural precursor cell proliferation and neuronal subtype-specific differentiation [27]. CHL1 deficiency on the proliferation of neural progenitor cells (NPCs) is accompanied by means of enhanced activation of ERK1/2 mitogen-activated protein kinase (MAPK) and the inhibitor of ERK1/2MAPK eliminates the effect of CHL1 deficiency on the proliferation of NPCs. Consistent with this view, we found that CHL1 shows anti-proliferative activity and anti-malignant transformation in MCF7 and MDA-MB-231 cells.

MAPK pathway plays a key role in regulating cell proliferation and differentiation, which also is involved in tumorigenesis and progression. It was reported that CHL1 negatively regulates the proliferation and neuronal differentiation of neural progenitor cells through activation of the MAPK pathway [28]. In addition, PAK family kinases was known to be closely related with tumor migration and invasion [29,30] and CHL1 cooperates with PAK1–3 to regulate morphological differentiation of embryonic cortical neurons [31]. Thus, CHL1 deficiency may lead to dysregulation of the MAPK and PAK pathways, and then affects the downstream molecules, finally contributes cell growth, migration and invasion in human breast cancer cells. The mechanism by which CHL1 deficiency enhanced activation of MAPK and PAK is an issue that will be investigated in future studies.

In conclusion, we have demonstrated that CHL1 expression is significantly down-regulation in breast cancer tissues and down-regulation of CHL1 is associated with high grade. Functional analyses indicated that overexpression of CHL1 suppresses tumorigenicity of breast cancer cells whereas knockdown of CHL1 promotes the tumorigenicity both *in vitro* and *in vivo*. Whether the CHL1 is a biomarker to evaluate patient prognosis is an issue that need to be investigated in future.

Acknowledgments

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