Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is downregulated in Invasive ductal carcinoma: a potential prognostic marker of breast cancer

Zahra Zahid Piracha (piracha.zahra@gmail.com)
Health Services Academy (HSA)
Umar Saeed
National Institute of Health (NIH)

Research Article

Keywords: brest cancer, prognostic markers, tumor supressors

Posted Date: May 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-557273/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

LRIG1 (leucine-rich repeat and immunoglobulin-like domain containing), belongs to LRIG family of transmembrane proteins containing leucine rich repeats. LRIGs are considered as tumor suppressor as they negatively regulate receptor tyrosine kinases. The role of LRIG1 as an EGFR regulator makes it an important marker to be studied in various epithelial-derived cancers. LRIG1 expression was low in $Erbb2^{+}$ cell lines and more cell motility were observed. AKT/GSK3-β/β-catenin pathway was activated when LRIG1 was downregulated and consequently, Erbb2 was upregulated. So far, no study has reported the expression of LRIG1 in benign forms of tumor such as fibroadenoma. Current study aims to analyze LRIG1 expression in fibroadenoma and Invasive ductal carcinoma (IDC) tissues. In this study, we compared the LRIG1 expression with different clinicopathological parameters of patients having IDC or fibroadenoma. Our results indicated that LRIG1 expression can be significantly correlated with age, Nottingham index and Her2/ neu status of cancer. The expression of LRIG1 in IDC and fibroadenoma were found to be significantly different. The fibroadenoma tissue sections were found to express LRIG1 more intensely as compared to the IDC sections which are in line with the studies reporting reduced copy number of the gene either due to gene deletion or transcriptional inhibition. This further lends supports to the previous studies showing that the down regulation of LRIG1 may lead to a more aggressive and malignant tumor thus acting as a tumor suppressor.

Introduction

Breast cancer is most common cancer of women both in developing and in developed countries. It is estimated that every one in nine women will develop breast cancer at some stage of their life and accounts for 34.6% of cancer in female in Pakistan (1). Different environmental and hereditary risks play role in progression of breast cancer. Fibroadenoma is one of the risk factors that arise from stroma or epithelium of the terminal duct lobular unit and develop into ductal hyperplasia, ductal carcinoma in situ or invasive carcinoma. A woman having fibroadenoma is two times more likely to develop breast cancer in her lifetime than a woman without fibroadenoma (2). Invasive ductal carcinoma (IDC) is most common invasive cancer, according to international statistics, that accounts for 75% of all breast cancer cases (3).

St Gallen guidelines has set different prognostic and predictive factors such as age, tumor grade, Nottingham grading system, hormone receptors and Her2/ neu receptor status to estimate patient survival and degree of aggressiveness of tumor and treatment (4). Various proteins are elevated at abnormal levels or are deregulated in cancers. One such protein is LRIG1. LRIG1 belong to Leucine rich repeats and Immunoglobulin like domains (LRIG) family. It has structural similarities with Drosophilia Kekkon 1 gene; epidermal growth factor inhibitor indicates its important role in signal transduction events. It is expressed in cytoplasmic, nuclear and perinuclear compartments of cells belonging to epithelial, muscle and central nervous system (5-6). LRIG1 regulates cell to cell contact via forming ternary complex between LRIG1, E-cadherin and EGFR (7). LRIG1 ectodomain can be solubilized and is reported to function as non-cell autonomous regulator of EGFR signaling (8). LRIG1 negatively regulates the tyrosine kinases ErbB receptors and MET receptors. ErbB signaling contributes to development of

many epithelial cancers so LRIG1 was first considered as tumor suppressor gene. The LRIG1 locus 3p14.3 is frequently deleted in cancers suggest its role as tumor suppressor (9). It is down regulated in renal cell carcinoma squamous cell carcinoma and glioblastoma due to inhibitory effect on EGFR (10-12). However, this scenario is not always true as LRIG1 expression is up regulated in certain tumors such astrocytoma and in prostate cancer (13). So LRIG1 can be tumor suppressor or tumor promoter depending upon the cellular context (14). Ectopic expression of LRIG1 negatively regulates the oncogenic EGFR mutant EGFRvIII, which is the most common mutant, through inhibiting the activation of PI3K/Akt of EGFR and EGFRvIII signal transduction pathways (15). In breast cancer, the role of LRIG1 is ambiguous where some types of tumor show high expression and some show low expression (16). Different cellular localization, feedback mechanisms, bystander effects and intensity of protein expression may play an important role in defining LRIG1 as tumor suppressor or tumor promoter.

The purpose of the study was to establish a role of LRIG1 in breast cancer malignancy with respect to various clinicopathological features of the breast tumor to clarify the role of LRIG1 in breast cancer. In current study we evaluated LRIG1 expression with different clinicopathological factors of breast cancer. And compared LRIG1 expression in different breast cancer cell lines and found low LRIG1 expression in Erbb2 positive cell lines. Next, we identify the migration pattern of different breast cancer cell lines in absence of LRIG1 and found that breast cancer cells migrate fast when Erbb2 is present and LRIG1 is downregulated. LRIG1 and Erbb2 colocalize together and LRIG1 decreases Erbb2 stability. Also, AKT/GSK3-β/β-catenin pathway is activated when LRIG1 is downregulated and consequently, Erbb2 is upregulated. Furthermore, we compared the LRIG1 expression in fibroadenoma and IDC patients breast tissue samples through immunohistochemistry. The level of expression is significantly different in benign and malignant tumors. Our study indicated that LRIG1 can be correlated with the different clinicopathological features such as age, Nottingham index and Her2/ neu status of breast carcinoma.

Material And Methods

Cell lines and culture

HEK293T, BT20, MCF 7, MDA-MB-231 and MDA-MB-361 cells were maintained in Dulbecco's modified Eagle's medium, as described previously (17-19), AU565 cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS, Gibco BRL) in a humidified atmosphere (37°C/5% CO2). HEK293T cells were passaged every second day, and other cells were passaged every third day, as described previously (17-19).

Determination of migration using wound healing assays

In the wound healing migration assays, AU565 or MCF 7 cells were seeded in 6-well plates at density a of $1x10^6$ cells/well, incubated for 24 h and at 37 °C and scratched using a yellow pipette tip when the cells covered the well. as described previously (17-19). The cells were washed with PBS twice to clear the floating cells and treated with control siRNAs or LRIG1 siRNAs. Images were captured at 0, 24 and 72 h

using a fluorescence microscope at 40x magnification. Subsequent recolonization of the stripped surface was quantitated by measuring the distance between wounded edges.

SDS-PAGE and immunoblotting

Equal quantities (measured in a Bradford Assay) of cell lysate (0.2% NP-40 (IGEPAL, Sigma-Aldrich)-TNE (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. as described previously (17-19). Resolved proteins were then transferred to PVDF membranes and incubated with the appropriate primary antibodies (rabbit polyclonal anti-LRIG1 [1:1,000] (Abcam #ab36707), mouse monoclonal anti-Erbb2 [1:1,000] (Cell Signaling Technology #2242), rabbit polyclonal anti-AKT [1:1,000] (Cell Signaling Technology #9272S), rabbit anti-phospho-AKT (T308) [1:1,000] (Cell Signaling Technology #9275S), rabbit anti-phospho-AKT (S473) [1:1,000] (Cell Signaling Technology #9271S) mouse monoclonal anti-GSK-3β [1:1,000] (Santa Cruz #11B9), rabbit monoclonal anti-β-catenin [1:1,000] (Cell Signaling Technology #D10A8), or mouse anti-GAPDH (1:5,000; Santa Cruz; sc-32233), followed by anti-rabbit secondary antibodies coupled to horseradish peroxidase (1:5,000 dilution) (Thermo Fisher Scientific) or anti-mouse secondary antibodies coupled to horseradish peroxidase (1:5,000 dilution) (Thermo Fisher Scientific). The blots were then visualized by ECL. The relative band intensities were measured using ImageJ. 1.46r.

Erbb2 stability analysis

AU565 cells were treated with 50 µg/ml cycloheximide (Sigma; C1988-1G) 24 h after siRNAs treatment and then harvested at 0, 0.5, 1, or 3 h posttreatment.

Immunofluorescence analysis and confocal microscopy

AU565 (2×10^4) cells grown on coverslips in 24-well plates cells were fixed for 20 min with 4% paraformaldehyde, followed by washing three times in 1×PBS. Then, cells were permeabilized with 0.2% Triton×100 (0.1% saponin [Sigma-Aldrich #S7900-25G]/1% bovine serum albumin [Bovogen Biologicals #BSAS0.1]/0.1% sodium azide [Sigma-Aldrich #S2002-100G] in 1×PBS) for 20 min, followed by washing three times in 1×PBS, as described previously (17-19). Next, cells were incubated overnight at 4°C with anti-LRIG1 (1:300) and anti-Erbb2 (1:300) in permeabilization buffer. Immunofluorescence detection was performed using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM (1:350; Jackson ImmunoResearch #115-095-044) and rhodamine (TRITC)-conjugated goat anti-rabbit IgG (1:350; Jackson ImmunoResearch #115-025-003) in permeabilization buffer at room temperature for 1 h. Cells were mounted with Fluoroshield mounting medium containing DAPI (Abcam #ab104139). Digital images of stained cells were captured under a confocal microscope (LSM710; Zeiss, Germany), as described previously (17-19).

Co-immunoprecipitation

To examine the physical interaction of LRIG1 and Erbb2 in MCF7 and AU565 cells, 1×10^6 cells on 10 cm plates were seeded, as described previously (17-19) For immunoprecipitation, rabbit polyclonal anti-LRIG1 antibody was added to the lysates, as described previously (17-19), followed by immunoblotting with mouse monoclonal anti-Erbb2.

Patients and tissues

Fibroadenoma and Invasive ductal tissues were obtained from 70 different patients (male and female) from Armed Force Institute of Pathology, (AFIP) Rawalpindi, Pakistan. Information on patient demographics (gender, age), tumor features (size of tumor, histological grade, lymph node metastasis, Nottingham index), hormones receptor status (Estrogen and Progesteron) and Her2/ neu status were obtained from hospital records. Study was approved by AFIP and Atta-ur-Rahman School of Applied Biosciences Ethics and Investigation Committee. Biopsies were taken after patient's informed consent. These biopsies were formalin fixed; paraffin embedded for immunohistochemistry.

Immunohistochemistry

In order to determine the expression level of LRIG1 in paraffin embedded and formalin fixed tissue biopsies, immunohistochemistry was done by the protocol mentioned on catalogue of anti-LRIG1 antibody ab36707. The 5µm thick tissue sections were incubated with rabbit polyclonal anti-human/mouse LRIG1 antibody (ab36707; Abcam, UK) at 4°C overnight without performing antigen retrieval step. The HRP labeled goat anti rabbit secondary antibody (ab6721; Abcam, UK) was applied and incubated for 1 hour at room temperature. DAB staining kit (Abcam Cat: ab64238, UK) was used for detecting the expression of LRIG1. Tissue sections were counterstained with hematoxylin, dehydrated and mounted via mounting media. Negative control was PBS without the primary antibody and positive control was normal breast tissue.

Evaluation of Immunohistochemical Staining

Each slide was evaluated by two histo-pathologists who were unaware of clinical outcome and results of the study. Positive staining was defined as brown color staining on the glandular epithelium of normal breast tissue and was measured by counting the positive cells at 400X in three representative high-power fields for each section by using Labomed TCM400 inverted microscope (Labi America Inc., USA) and stained sections were photographed using ProgRes Capture Pro 2.6 (JENOPTIK Laser, Optik, Systeme GmbH, Germany). Immunoreactivity was scored as a). Zero or (-ve) for sections that have less than 10% positive cells. b). One or (+ve) for sections having more than 10% positive cells. Intensity was scored as a). Zero or (-ve) for no staining, b). One or (+ve) for low staining, c). Two or (++) for moderate staining, c). Three or (+++) for high or normal staining (20).

Statistical Analysis

The history of patient such as age, Nottingham grading index, histological findings and Her2/neu status were included in study. Spearman's Rank Order Correlation was used to determine the relationship between expression of LRIG1 and history of patients. One Sample T-test was run in order to evaluate the correlation between the risk factor fibroadenoma and disease IDC. Data are expressed as the mean ± standard deviation. Mean values were compared using Student's *t*-test. *P*- values less than 0.05 were taken as statistically significant in correlation. Statistical analysis was performed using Statistical Package for the Social Sciences, version 16.0 (SPSS Inc, Chicago, IL, USA).

Ethical Declaration:

The experiments performed in current study were in accordance with the relevant guidelines and regulation and approved by institutional review board and ethical review committee of National University of Sciences and Technology.

Results

LRIG1 expression in breast cancer cell lines

We first determined the expression of LRIG1 using a panel of breast cancer cell lines (BT20, AU565, MDA-MB-361, MCF 7, and MDA-MB-231) and compared it with HEK293T cells (Fig. 1A). Our data shows that LRIG1 levels were declined in breast cancer cell lines. Interestingly, LRIG1 level was more significantly reduced in AU565 and MDA-MB-361 cells. It is essential to note that these are cell lines with Erbb2 positive status and there LRIG1 expression is less than Erbb2 negative cell lines MCF7, MDA-MB-231and BT20 (21).

Effects of LRIG1 silencing on breast cancer cell proliferation

An earlier study suggested that there was role of LRIG1 in opposing the breast cancer cell motility and invasion, cellular processes that are fundamental to metastasis (22). In order to evaluate the association between LRIG1 expression and microscopic vascular invasion with relation to Erbb2 in breast cancer cell lines, we carried out siRNA mediated LRIG1 knockdown to elucidate the cellular functions of LRIG1. Independent siRNAs of LRIG1showed efficient LRIG1 knockdown in Erbb-positive AU565 cells (Fig. 1B) and in Erbb-negative cells MCF7 (Fig. 1D) compared with scrambled shRNA (shCont)-transfected cells (Fig. 1B and 1D). The migration of the cells was detected by wound healing assay (Fig. 1C and 1E). In AU565 cells, when LRIG1 is knock downed, the wound is healed 72 hours post-wound as compared to control-siRNAs (Fig. 1C, third panel, lane 2 *vs.* 3). Surprisingly, in MCF7 cells, when LRIG1 is knock downed, the wound is not healed 72 hours post-wound as compared to control-siRNAs clearly demonstrates that knockdown of LRIG1 clearly diminished wound healing capacity (Fig. 1D, third panel, lane 2 *vs.* 3). This data clearly points out that Erbb2 and LRIG1 are showing role in cell motility of breast cancer cell lines.

LRIG1 decreases Erbb2 stability

Since Erbb2 and LRIG1 are together showing role in cell motility of cancer cell lines (Fig. 1), to examine the effect of LRIG1 on stability of Erbb2, cycloheximide (50 µg/ml) was treated for 0, 6, 12 and 24 h to AU565 cells 24h after treatment of cells with respective si-RNAs (Fig. 2A). LRIG1 KD via siRNAs treatment significantly improved Erbb2 stability. Relative Erbb2 levels are shown as graphs at righthand sides (Fig. 2B). The Erbb2 half-life was increased from 3 minutes to 9 minutes by LRIG1 KD. Overall, our results demonstrate that LRIG1 decreases Erbb2 protein stability.

LRIG1 and Erbb2 are co-localized in the cell

It has been reported that endogenous LRIG1 expression is lost when Erbb2 is activated in breast cancer cell lines demonstrating that Erbb2 oncogenic signaling positively contributes to suppression of LRIG1 (23). In order to determine that either LRIG1 or Erbb2 co-localize in the cell, immunoprecipitation was performed (Fig. 3A). In these experiments, lysates from MCF7 cells and AU565 cells were immunoprecipitated with anti-LRIG1 antibody and then immunoblotted with anti-Erbb2 antibody. The results show that LRIG1 interacts with Erbb2 in AU565 cells (Erbb2 positive cell line) (Fig. 3A, lane 3 *vs.* 4). Next in order to further validate this data, confocal microscopy analysis was performed in AU565 cells (Fig. 3B). This data clearly shows that LRIG1 associates with Erbb2 mainly in cytoplasmic compartment of cell.

Decreased expression of LRIG1 in Erbb2 positive cells activate AKT/GSK-3β/β-catenin pathway

The PI3K/AKT pathway is the thought to be the prime focus of several targeted therapeutic agents for a variety of malignancies. In Erbb2-amplified breast cancer, the overactive activation of this signaling pathway is linked with resistance to Erbb2-targeted therapy (24). In order to determine that how LRIG1 is playing role in this pathway, LRIG1 was knocked down by using LRIG1 si-RNAs (Fig. 3C). As shown, the knock down of LRIG1 is successfully done (Fig. 3C, lane 1 and 2 *vs.* 3). Next when LRIG1 is knock downed, Erbb2 protein expression is increased, AKT is activated (i.e., phosphorylation of T308 and S473), GSK-3β activity was inhibited and subsequently, β-catenin levels were increased (Fig. 3C, lane 1 and 2 *vs.* 3). We further examined whether inhibiting PI3K/AKT pathway downregulates LRIG1 expression. To examine the effect of PI3K/AKT signaling cascade inhibition on LRIG1 expression, we exposed AU565 cells (Fig. 3D) to the potent PI3K inhibitor LY294002 (10 μM) (25) from the time of seeding of cells until 72 h. The results of an MTT assay revealed that LY294002 was not cytotoxic (data not shown). Upon treatment of AU565 cells with LY294002, LRIG1 expression was enhanced significantly, Erbb2 protein expression was dramatically decreased, AKT is deactivated (i.e. phosphorylation of T308 and S473), GSK-3β activity was enhanced and subsequently, β-catenin levels were less stabilized (Fig. 3D, lane 1 *vs.* 2). Altogether, this data clarifies that LRIG1 associates with Erbb2 and mediated the PI3K/AKT pathway.

Lower expression level of LRIG1 in invasive ductal carcinoma (IDC) as compared to fibroadenoma

Our results show that LRIG1 is associated with Erbb2 (Fig. 3A and B) and suppress the receptor levels (Fig. 3C). PI3K/AKT pathway is activated and malignancy can be seen in breast tissue. In order to compare the LRIG1 expression in the benign and malignant form of breast tumor, we performed the

immunohistochemical analysis on IDC tissues (35) and fibroadenoma samples obtained from patients (35) (Fig. 4A). Normal breast tissue was used as control (Fig. 4A, top panel). In case of fibroadenoma, LRIG1 was highly expressed (Fig. 4A, second panel). In IDC, the ductal cells show the aggressive growth and invade in stroma and are no longer remaining confined in basal layer. LRIG1 expression was less than normal breast tissue in grade II tumor (Fig. 4A, third panel) and in grade III tumor (Fig. 4A, bottom panel). Figure 4B shows the quantitative analysis of immunohistochemical analysis performed in figure 4A. So, this data from the patient tissues clearly demonstrates that LRIG1 expression is downregulated if the breast cancer goes toward malignant stages.

The association of LRIG1 expression with clinicopathological factors of IDC and fibroadenoma

The expression of LRIG1 in IDC was correlated with different clinicopathological factors of IDC such as Nottingham index (tumor grade), her2/ neu expression and age of patients and for fibroadenoma, age factor was included (Fig. 5). Nottingham grading system is a grading system that measure the histological grade of tumor based on nuclear polymorphism, mitotic index and tubular formation (26). LRIG1 expression was downregulated in grade II tumors (63%) and it was even further decreased (17%) in grade III tumor samples (Fig. 5A). Erbb2 or Her2/neu is a proto-oncogene. Its amplification is associated with rapid progression of breast carcinoma, increased metastatic potential and increased resistance to conventional therapies (27). Our results indicated that expression of Her2/neu is associated with LRIG1 expression (Fig. 5B). Our results indicated a strong correlation of LRIG1 expression with that of age of patients (Fig. 5C). LRIG1 was expressed in patients from age 30 to 39 years and less expression was observed in patients of older age (Fig. 5C).

Our results indicated a strong correlation of LRIG1 expression with that of age of patients of fibroadenoma (Fig. 5D). The patient's age ranges from 15 to 24 had high intensity of LRIG1 staining whereas less LRIG1 expression was scored in older individuals (Fig. 5D). Our data specified that LRIG1 can be correlated with the different clinicopathological features such as age, tumor grade, Nottingham index and Her2/ neu status of breast carcinoma.

Discussion

Nature has distinctive therapeutic options against chronic inflammation, cancer and several other diseases. And there is a strong need to determine such therapeutic potentials. Designing potential therapeutic options after identification of biomarkers could be important for understanding of disease progression (28-35).

In this study, out data shows that LRIG1 is a growth suppressor in breast cancer. We show that LRIG1 expression is decreased in human breast cancer cell lines and that the majority of $Erbb2^{+}$ cells under-express LRIG1 (Fig. 1A). Depletion of endogenous LRIG1 in Erbb2 overexpressing breast cancer cells (AU565) further supplements Erbb2 expression and helps in proliferation of tumor cells (Fig. 1C).

Breast tissues included the tissues of IDC were stained via LRIG1 antibody by Ljuslinder et al in 2009 but their results indicated that LRIG1 staining gave the results that were difficult to interpret because all the tumor cells stained at same intensity without variability (36). The study conducted by us was the first study in which LRIG1 expression in IDC was correlated with the clinicopathological factors of cancer (Fig. 5).

Tumor grading systems or Nottingham grading system combines three morphological features including nuclear grade which indicates change in nuclear size and uniformity, tubule formation which is percentage of cancer composed of tubular structures and mitotic index. Each of these elements is given score on scale of 1-3 and score of these components are added to define grades I to III. Score 3 to 4 defines Grade I and they are well differentiated more like normal cells, score 6 to 7 defined moderately differentiated tumors having bad outcome of Grade II, score 8 and 9 define poorly differentiated tumors of grade III. Grade III patients show poor prognosis than grade I (26,37). In our study, we scored LRIG1 expression in grade I, II and III patients. High LRIG1 expression was scored in Grade I patients as compared to Grade III patients (Fig. 5A). Hence LRIG1 can act as tumor suppressor during early phases of breast cancer.

Her2/neu is found as amplified gene in breast cancer and a product of neu oncogene in rat neuroblastoma (38). Gene is amplified or transcriptional deregulation is seen in 20-30% of breast cancer (39). In breast cancer metastasis, Her2/ neu is over expressed and over expression is also seen in primary tumors (40). This over expression is related to resistance in conventional therapies, tumor behavior, histological grade, tumor cell proliferation index, poor prognosis and enhanced invasiveness (41). Previous studies also established the correlation between LRIG1 expression and her2/neu expression. There are some endogenous mechanisms that limit Her2/neu expression. One of such endogenous post transcriptional regulator is LRIG1. In transgenic mouse model of Her2/neu positive breast cancer, depletion of LRIG1 in Her2/neu positive over expression cancer cells causes the over expression of Her2/neu and proliferation of tumor cells but ectopic expression of LRIG1 results in down regulation of Her2/neu and attenuating the tumor cell proliferation. Activation of Her2/neu causes the down regulation of LRIG1 in feed forward regulatory loop in breast tumor cells and this contributes to Her2/neu over expression, tumor progression and metastasis (23). Current study also indicated that when Her2/neu was expressed, its post transcriptional regulator LRIG1 was also expressed and when no Her2/neu was expressed, LRIG1 expression was very low (Fig. 5B).

PI3K/Akt pathway is downstream signal of EGFR and it plays a central role in the pathogenesis of a variety of tumors (42). Previous studies have also reported that activation of PI3K/Akt signaling pathway through interaction with EGFR leads to metastasis of osteosarcoma (43). Gastric cancer is also proliferated via EGFR-mediated activation of PI3K/Akt pathway (44). Our results were also consistent with previous findings. We also authenticated that LRIG1 suppressed PI3K/Akt signal pathway and attenuated phosphorylation of Akt (Fig. 3).

LRIG1 is an epidermal growth factor inhibitor which indicates its important role in signal transduction events. LRIG1 gene and protein in different tissues was identified and characterized by Nilsson et al (6). In breast tissue, expression of LRIG1 is in glandular epithelium (5-6). The sub cellular localization of LRIG1 proteins is important in defining its role in clinical implications. The nuclear localization holds the significance with the patient survival and good clinical outcome in case of astrocytoma and cytoplasmic localization is considered as just mislocalization of protein (14). In our results, positive controls of normal breast tissue showed the strong LRIG1 expression in nucleus and in cytoplasm. In our study, the IDC tissues expressed LRIG1 in cytoplasm and no nuclear expression was scored. Fibroadenoma, one of risk factor of breast cancer, also shows LRIG1 expression mainly in cytoplasm (Fig. 4).

When we compared expression of LRIG1 in malignant tumor with respect to the risk factor and benign form of tumor within the limitations of small sample size (Fig. 4), the low percentage of expression of LRIG1 in fibroadenoma and high percentage of expression in IDC shows that LRIG1 has role in tumorigenesis. LRIG1 expression between malignant and benign tumor is compared by Tanemura et al and their results indicate that LRIG1 level is up regulated in benign forms of squamous cell carcinoma (SCC) and down regulated in malignant forms of SCC (11). Up regulation of LRIG1 suppress advances in transformation. Hence fibroadenoma patients show high levels of LRIG1 and these tumors remain benign. Similarly, tumors of IDC expressing LRIG1 rarely metastasize to axillary lymph nodes (data not shown). Gur and his colleagues show that LRIG1 works in negative feedback loop with EGFR (45). Hence in the cells where EGFR is abnormally induced and causing proliferation of tumor, LRIG1 may upregulate to limit the proliferation and maintain quiescent integrity of epithelium. High number of receptors expressing LRIG1 in low percentage of fibroadenoma patients shows that LRIG1 act as tumor suppressor and is over expressed in response to EGFR over expression in negative feed-back loop. This means expression is strong and more receptors are positive for the LRIG1. The difference in intensity of staining in squamous cell carcinoma is associated with histological differentiation (11). The molecular mechanisms underlying the expression pattern in malignant and benign tumor have yet to be elucidated. The fibroadenoma tissue sections were found to express LRIG1 more intensely as compared to the IDC sections which are in line with the studies reporting reduced copy number of the gene either due to gene deletion or transcriptional inhibition. Comparatively low or no expression in the metastatic ductal carcinoma sections further lends supports to the previous studies showing that the down regulation of LRIG1 may lead to a more aggressive and malignant tumor thus acting as a tumor suppressor.

References

1. Shaukat U, Ismail M, Mehmood N. Epidemiology, major risk factors and genetic predisposition for breast cancer in the Pakistani population. Asian Pac J Cancer Prev 2013;14(10):5625-9.
2. Kuijper A, Buerger H, Simon R, Schaefer KL, Croonen A, Boecker W, et al. Analysis of the progression of fibroepithelial tumours of the breast by PCR-based clonality assay. J Pathol 2002 Aug;197(5):575-81.

3. Li CI, Anderson BO, Daling JR, Moe RE. Trends in incidence rates of invasive lobular and ductal breast carcinoma. JAMA 2003 Mar 19;289(11):1421-4.
4. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007 Jul;18(7):1133-44.
5. Nilsson J, Vallbo C, Guo D, Golovleva I, Hallberg B, Henriksson R, et al. Cloning, characterization, and expression of human LIG1. Biochem Biophys Res Commun 2001 Jun 29;284(5):1155-61.
6. Nilsson J, Starefeldt A, Henriksson R, Hedman H. LRIG1 protein in human cells and tissues. Cell Tissue Res 2003 Apr;312(1):65-71.
7. Lu L, Teixeira VH, Yuan Z, Graham TA, Endesfelder D, Kolluri K, et al. LRIG1 regulates cadherin-dependent contact inhibition directing epithelial homeostasis and pre-invasive squamous cell carcinoma development. J Pathol 2013 Mar;229(4):608-20.
8. Yi W, Holmlund C, Nilsson J, Inui S, Lei T, Itami S, et al. Paracrine regulation of growth factor signaling by shed leucine-rich repeats and immunoglobulin-like domains 1. Exp Cell Res 2011 Feb 15;317(4):504-12.
9. Hedman H, Nilsson J, Guo D, Henriksson R. Is LRIG1 a tumour suppressor gene at chromosome 3p14.3? Acta Oncol 2002;41(4):352-4.
10. Ye F, Gao Q, Xu T, Zeng L, Ou Y, Mao F, et al. Upregulation of LRIG1 suppresses malignant glioma cell growth by attenuating EGFR activity. J Neurooncol 2009 Sep;94(2):183-94.
11. Tanemura A, Nagasawa T, Inui S, Itami S. LRIG-1 provides a novel prognostic predictor in squamous cell carcinoma of the skin: immunohistochemical analysis for 38 cases. Dermatol Surg 2005 Apr;31(4):423-30.
12. Thomasson M, Hedman H, Guo D, Ljungberg B, Henriksson R. LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT–PCR and immunohistochemical analysis. Br J Cancer 2003 Oct 6;89(7):1285-9.
13. Thomasson M, Wang B, Hammarsten P, Dahlman A, Persson JL, Josefsson A, et al. LRIG1 and the liar paradox in prostate cancer: a study of the expression and clinical significance of LRIG1 in prostate cancer. Int J Cancer 2011 Jun 15;128(12):2843-52.
14. Hedman H, Henriksson R. LRIG inhibitors of growth factor signalling - double-edged swords in human cancer? Eur J Cancer 2007 Mar;43(4):676-82.
15. Stutz MA, Shattuck DL, Laederich MB, Carraway KL, III, Sweeney C. LRIG1 negatively regulates the oncogenic EGF receptor mutant EGFRvIII. Oncogene 2008 Sep 25;27(43):5741-52
16. Ljuslinder I, Malmer B, Golovleva I, Thomasson M, Grankvist K, Hockenstrom T, et al. Increased copy number at 3p14 in breast cancer. Breast Cancer Res 2005;7(5):R719-R727.
17. Piracha ZZ, Kwon H, Saeed U, Kim J, Jung J, Chwae YJ, Park S, Shin HJ, Kim K. 2018. Sirtuin 2 Isoform 1 Enhances Hepatitis B Virus RNA Transcription and DNA Synthesis through the AKT/GSK-3β/β-Catenin Signaling Pathway. J Virol 92:e00955-18.

18. Saeed U, Kim J, Piracha ZZ, Kwon H, Jung J, Chwae YJ, Park S, Shin HJ, Kim K. 2019. Parvulin 14 and Parvulin 17 Bind to HBx and cccDNA and Upregulate Hepatitis B Virus Replication from cccDNA to Virion in an HBx-Dependent Manner. J Virol 93:e01840-18.
19. Piracha ZZ, Saeed U, Kim J, Kwon H, Chwae YJ, Lee HW, Lim JH, Park S, Shin HJ, Kim K. An Alternatively Spliced Sirtuin 2 Isoform 5 Inhibits Hepatitis B Virus Replication from cccDNA by Repressing Epigenetic Modifications Made by Histone Lysine Methyltransferases. J Virol. 2020 Jul 30;94(16):e00926-20.
20. Ghasimi S, Haapasalo H, Eray M, Korhonen K, Brannstrom T, Hedman H, et al. Immunohistochemical analysis of LRIG proteins in meningiomas: correlation between estrogen receptor status and LRIG expression. J Neurooncol 2012 Jul;108(3):435-41.
21. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006 Dec;10(6):515-27.
22. Yokdang N, Hatakeyama J, Wald JH, Simion C, Tellez JD, Chang DZ, Swamynathan MM, Chen M, Murphy WJ, Carraway Iii KL, Sweeney C. LRIG1 opposes epithelial-to-mesenchymal transition and inhibits invasion of basal-like breast cancer cells. Oncogene. 2016 Jun 2;35(22):2932-47.
23. Miller JK, Shattuck DL, Ingalla EQ, Yen L, Borowsky AD, Young LJ, et al. Suppression of the negative regulator LRIG1 contributes to ErbB2 overexpression in breast cancer. Cancer Res 2008 Oct 15;68(20):8286-94.
24. Carmona FJ, Montemurro F, Kannan S, Rossi V, Verma C, Baselga J, Scaltriti M. AKT signaling in ERBB2-amplified breast cancer. Pharmacol Ther. 2016 Feb;158:63-70.
25. Fujiwara M, Izuishi K, Sano T, Hossain MA, Kimura S, Masaki T, Suzuki Y. Modulating effect of the PI3-kinase inhibitor LY294002 on cisplatin in human pancreatic cancer cells. J Exp Clin Cancer Res. 2008 Nov 25;27(1):76.
26. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991 Nov;19(5):403-10.
27. Emi Y, Kitamura K, Shikada Y, Kakeji Y, Takahashi I, Tsutsui S. Metastatic breast cancer with HER2/neu-positive cells tends to have a morbid prognosis. Surgery 2002 Jan;131(1 Suppl):S217-S221.
28. Saeed U, Mazoor S, Jalal N, Zahid Piracha Z. Contemplating the Importance of Toll-like Receptors I and II Regarding Human Viral Pathogenesis. Jundishapur Journal of Microbiology. 2015;8(1):e13348. doi:10.5812/jjm.13348.
29. Saeed U, Waheed Y, Ashraf M. Hepatitis B and hepatitis C viruses: a review of viral genomes, viral induced host immune responses, genotypic distributions and worldwide epidemiology. Asian Pacific J Tropic dis. 2014;4(2):88–96. doi: 10.1016/s2222-1808(14)60322-4. [Cross Ref]

30. Saeed U, Waheed Y, Manzoor S, Ashraf M. Identification of novel silent HIV propagation routes in Pakistan. World J Virol. 2013;2(3):136–8. doi: 10.5501/wjv.v2.i3.136.[PMC free article] [PubMed] [Cross Ref]
31. Piracha ZZ, Saeed U, Khursheed A, Chauhdry WN. Isolation and Partial Characterization of Virulent Phage Specific against Pseudomonas aeruginosa. Global J Med Res. 2014;14(1):1–9.
32. Saeed U, Jalal N. Orange juice: a natural remedy for treatment of Cancer, prevention of chronic inflammation, Metabolic disorders and cardiovascular diseases. JPHBS. 2013;2:183–4.
33. Saeed U, Nawaz N, Waheed Y, Chaudry N, Bhatti HT, Urooj S. n silico identification of BIM-1 (2-methyl-1H-indol-3-yl) as a potential therapeutic agent against elevated protein kinase C beta associated diseases. . African J Biotech. 2012;11(19):4434–41.
34. Saeed U, Waheed Y, Anwar A, Hussain T, Naeem K, Nawaz N. In-Silico Binding of ATP to Protein Kinase C Delta: Insights into the Structure and Active Site. Europ J Sci Res. 2011;52:177–87.
35. Saeed U,, Jalal N,, Ashraf M. Roles of Cyclin Dependent Kinase and Cdk- Activating Kinase in Cell Cycle Regulation: Contemplation of Intracellular Interactions and Functional Characterization. GJMR. 2012;12:47–52.
36. Ljuslinder I, Golovleva I, Henriksson R, Grankvist K, Malmer B, Hedman H. Co-incidental increase in gene copy number of ERBB2 and LRIG1 in breast cancer. Breast Cancer Res 2009;11(3):403.
37. Rampaul RS, Pinder SE, Elston CW, Ellis IO. Prognostic and predictive factors in primary breast cancer and their role in patient management: The Nottingham Breast Team. Eur J Surg Oncol 2001 Apr;27(3):229-38.
38. Stern DF, Heffernan PA, Weinberg RA. p185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol Cell Biol 1986 May;6(5):1729-40.
39. Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer 2009 Jul;9(7):463-75.
40. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007 Oct 4;26(45):6469-87.
41. Ross JS, Fletcher JA, Linette GP, Stec J, Clark E, Ayers M, et al. The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. Oncologist 2003;8(4):307-25.
42. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov, 2006,5(8):671-688
43. Liu JF, Tsao YT, Hou CH. Amphiregulin enhances intercellular adhesion molecule-1 expression and promotes tumor metastasis in human osteosarcoma. Oncotarget, 2015,6(38):40 880-40 895
44. Zhou J, Zhi X, Wang L, et al. Linc00152 promotes proliferation in gastric cancer through the EGFR-dependent pathway. J Exp Clin Cancer Res, 2015,34(1):135
45. Gur G, Rubin C, Katz M, Amit I, Citri A, Nilsson J, et al. LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. EMBO J 2004 Aug 18;23(16):3270-81.

Figures

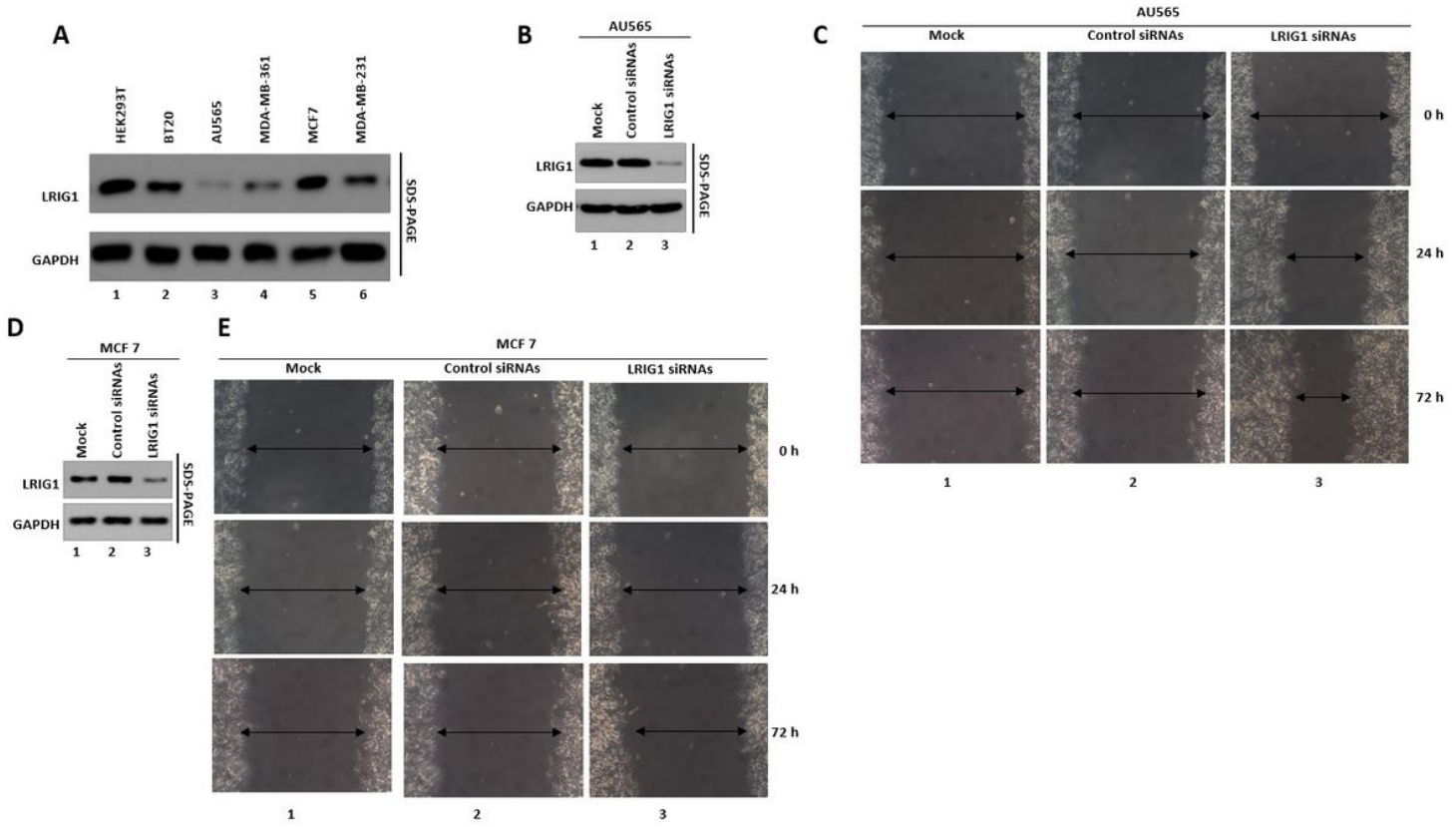


Figure 1

The Erbb2+ breast cancer cells show more proliferation and migration when LRIG1 is knock downed as compared to Erbb2- breast cancer cells. A. Expression of LRIG1 in different breast cancer cell lines. HEK293T cells and BT20 (Erbb1+) (lanes 1 and 2) are used as controls. AU565 and MDA-MB-361 cells (lanes 3 and 4) are Erbb2+ and MCF7 and MDA-MB-231 (lanes 5 and 6) are Erbb2- cells. Cells were freshly cultured in 6 cm dish and lysates were prepared 72 h post seeding. Prepared lysates were subjected to SDS-PAGE and immunoblotting was performed using anti-LRIG1 antibody (1:1000) to detect endogenous LRIG1 protein levels. GAPDH was used as a loading control. B. Knockdown of LRIG1 in AU565 cells. AU565 cells were seeded onto 6 cm dish and siRNAs (40 nM) treatment (control siRNAs lane 2 and LRIG1 siRNAs lane 3) were given to cells one day after seeding. Cell lysates were prepared 48 h post treatment and subjected to SDS-PAGE as described in legend of figure 1A. C. Enhanced ability of wound repair in Erbb2+ cells when LRIG1 is knock downed. AU565 cells were seeded onto 12 cm dish and wound was given 24 h post seeding when cells reached 95% to 100% confluency. The cells were treated with control siRNAs or LRIG1 siRNAs. Images were captured at 0 h (panel 1), 24 h (panel 2) and 72 h (panel 3) using a fluorescence microscope at 40x magnification. Subsequent recolonization of the stripped surface was quantitated by measuring the distance between wounded edges. D. Knockdown of LRIG1 in MCF7 cells. MC7 cells were seeded onto 6 cm dish and siRNAs treatment was given same as described in legend of figure 1B. E. Significant impairment of wound healing ability in in Erbb2- cells

when LRIG1 is knock downed. MCF7 cells were seeded onto 12 cm dish and wound was given same as described in legend of figure 1C. A representative result from three independent experiments is shown.

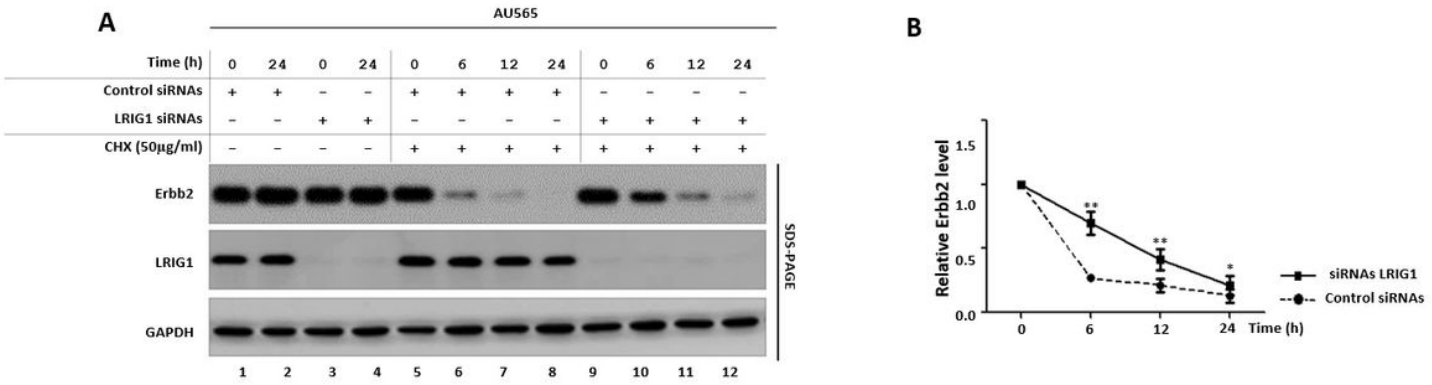


Figure 2

LRIG1 decreases Erbb2 stability. A. Erbb2 stability is decreased in LRIG1-KD cells. Control siRNAs treated (lanes 1, 2, and 5 to 8) and LRIG1 siRNAs treated (lanes 3, 4, and 9 to 12) AU565 cells were treated with nothing (lanes 1 to 4) or 50 μg/ml cycloheximide (lanes 5 to 12). Cells were harvested at the indicated times and analyzed by SDS-PAGE and immunoblotting with anti-Erbb2, anti-LRIG1, and anti-GAPDH antibodies. B. Half-life of Erbb2 effected by LRIG1. Representative results from three independent experiments are shown. Data are presented as mean Erbb2 level ± SD.

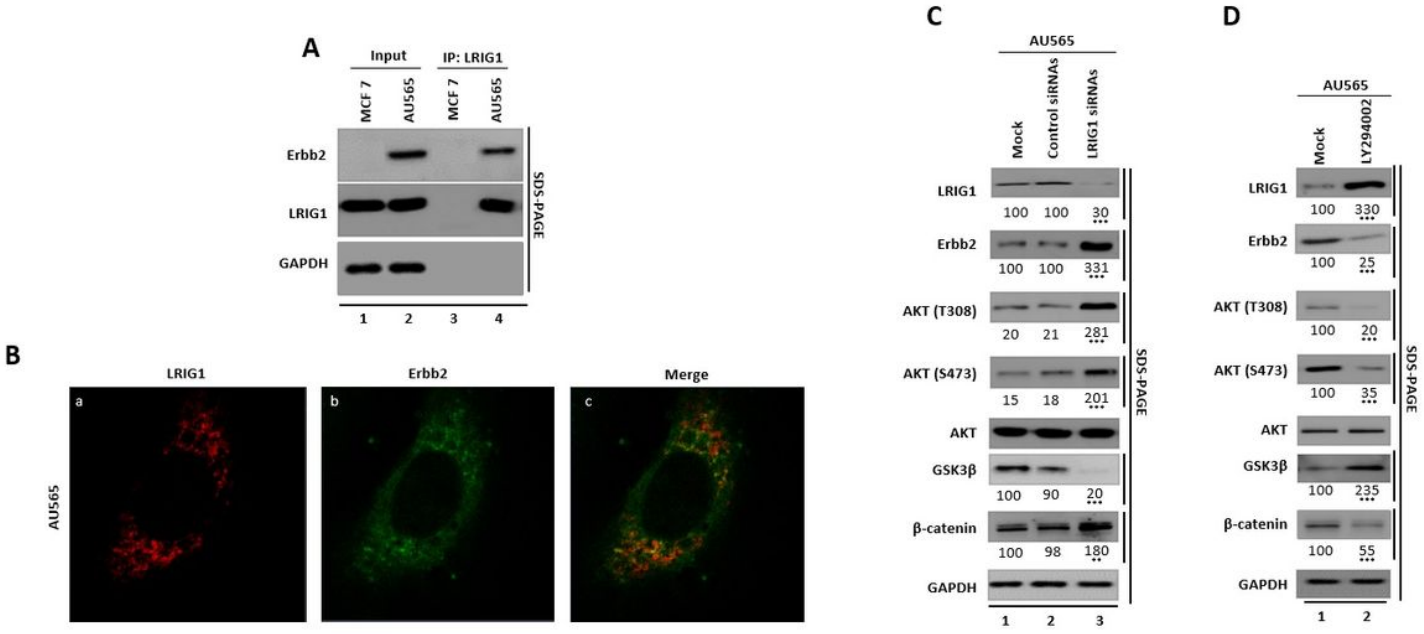


Figure 3

AKT/GSK-3β/β-catenin signaling pathway is activated in LRIG-KD cells. A. LRIG2 and Erbb2 are binding partners. MCF7 (Erbb2-) cells and AU565 cells (Erbb2+) cells were seeded onto 6 cm dish. (Input) At 72 h post seeding, whole-cell lysates were prepared and subjected to SDS-PAGE and immunoblotting (lanes 1

and 2). (IP) Lysates were immunoprecipitated with anti-LRIG1 antibody and immunoblotted with anti-Erbb2 antibody (lanes 3 and 4). B. Colocalizations of LRIG1 and Erbb2 in AU565 cells. Confocal images of of LRIG1using TRITC (a) and Erbb2 using FITC (b) are shown. Merged images are indicated (c). Digital images of stained cells were captured through confocal microscopy (A1R-A1; Nikon, Japan). Representative data is from three independent experiments. C. AKT/GSK-3β/β-catenin signaling pathway is activated when LRIG1 is knockdown in AU565 cells. AU565 cells were mock-treated (lane 1), control siRNAs treated (lane 2) or LRIG1 siRNAs treated (lane 3). Lysates were prepared at 48 h post-treatment. SDS-PAGE and immunoblotting of proteins was performed as described in legend of figure 1A. Levels of total LRIG1, Erbb2, active AKT (pT308 and pS473) relative to those of total AKT, total β-catenin, and total GSK-3β were measured using ImageJ. 1.46r. Data presented as mean value from four independent experiments. Statistical significance was assessed using Student's t-test. *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$, comparative to control. D. AKT/GSK-3β/β-catenin signaling pathway is inhibited by PI3K inhibitor LY294002. AU565 cells were mock-treated (lane 1) or LY294002 treated (lane 2). Lysates were prepared at 72 h post-treatment. SDS-PAGE and immunoblotting of proteins was performed as described in legend of figure 1A. Levels of proteins were measured as described in legend of figure 3C. Data presented as mean value from three independent experiments.

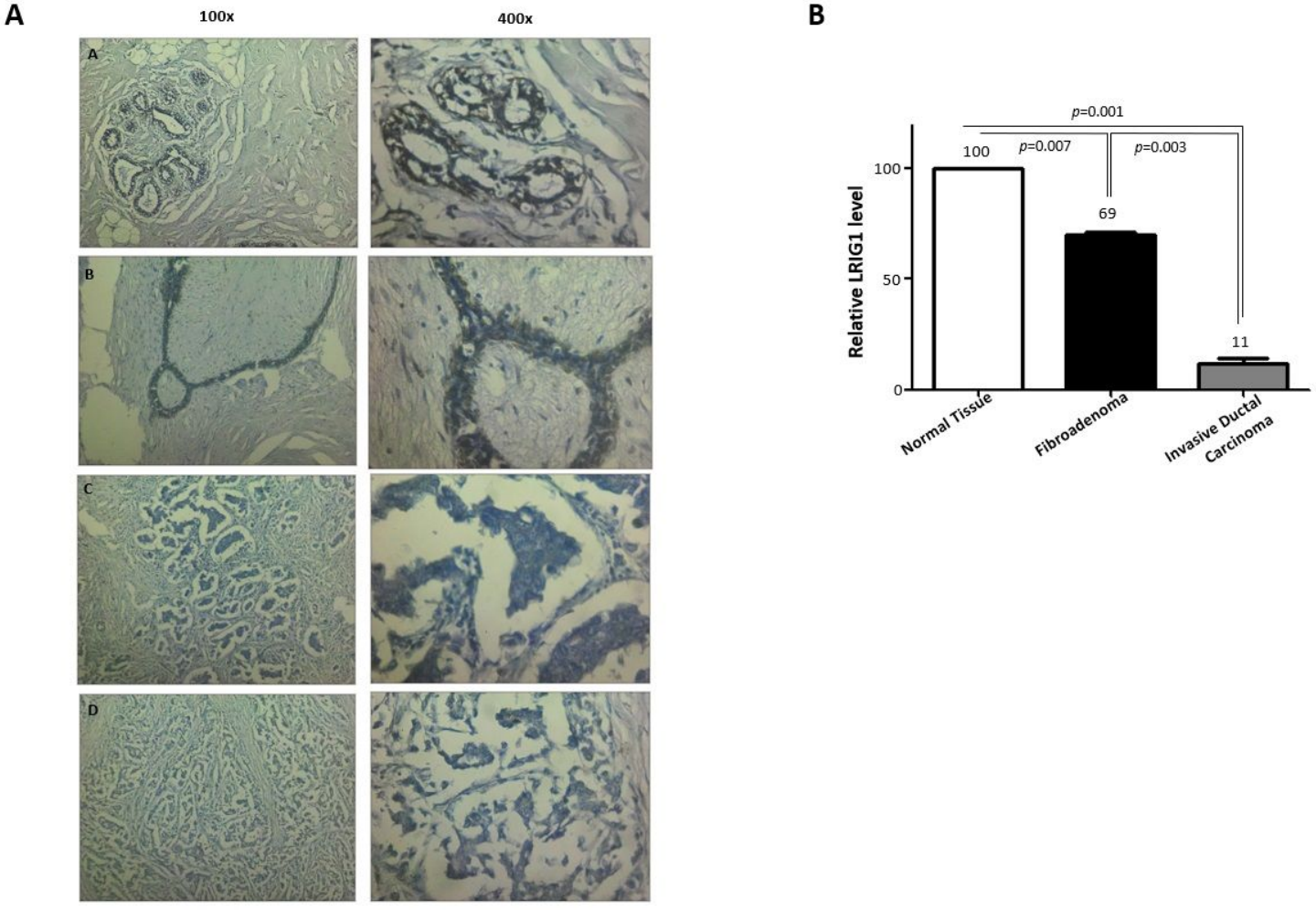


Figure 4

LRIG1 expression is attenuated in IDC tissue as compared to fibroadenoma tissue. A. Immunohistochemical staining of IDC and fibroadenoma tissue sections with LRIG1. a. Normal Breast Tissue. b. Fibroadenoma Breast Tissue. c. Invasive Ductal Carcinoma Breast Tissue (Grade I). d. Invasive Ductal Carcinoma Breast Tissue (Grade II). The brown color is showing the expression of LRIG1 (original magnification 100X and 400X). B. Relative LRIG1 expression level in normal breast tissue and tumor tissue. Relative LRIG1 expression was measured in normal, fibroadenoma and IDC tissue. Statistical significance was assessed using Student's t-test. *p < 0.05, **p < 0.005, and ***p < 0.0005, comparative to control.

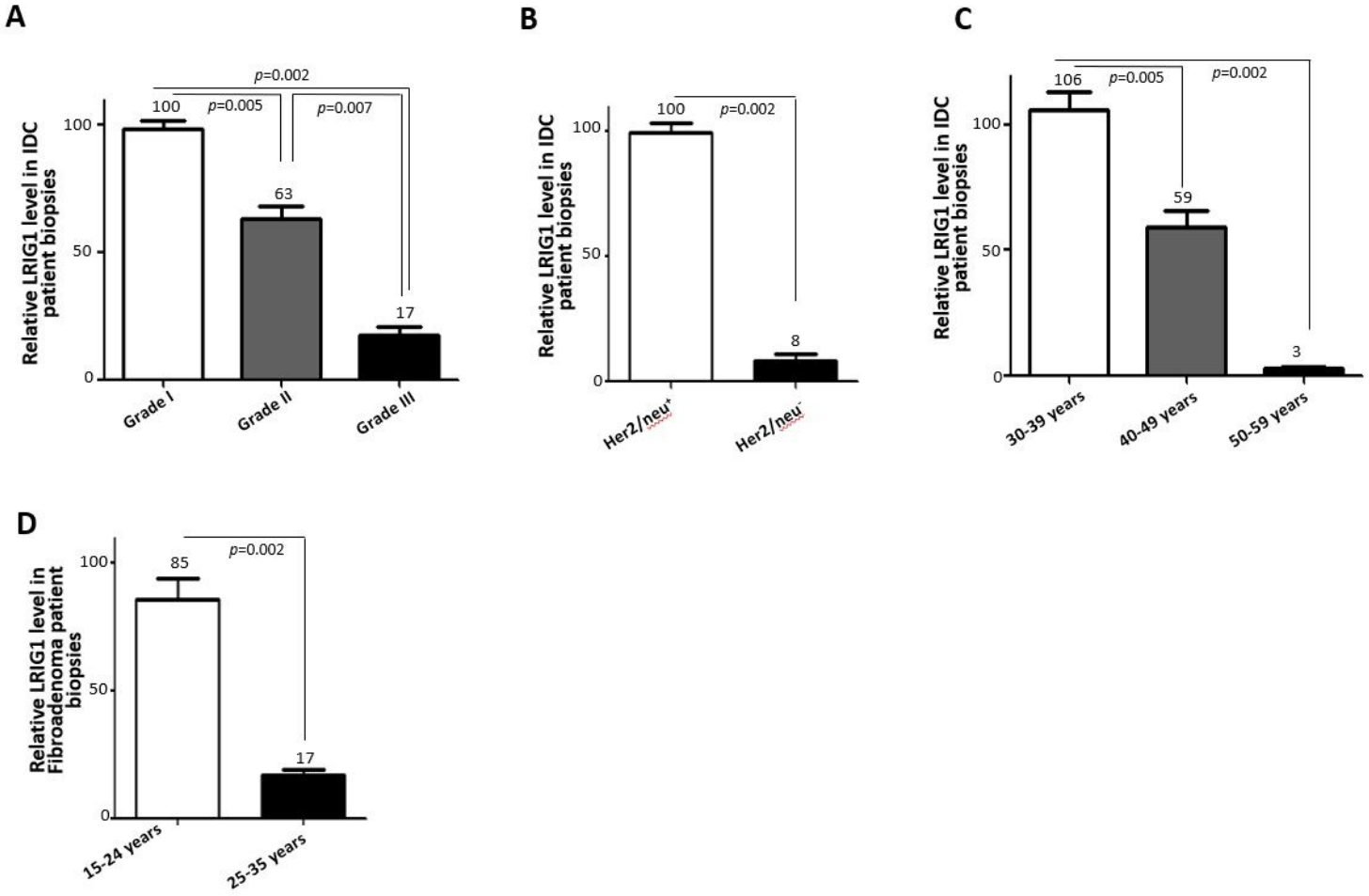


Figure 5

The association of LRIG1 expression with clinicopathological factors of IDC and fibroadenoma. A. Relative level of LRIG1 expression in grade I, II and III of IDC biopsies. B. Relative level of LRIG1 expression in her2/neu+ and her2/neu- samples. C. Relative level of LRIG1 expression in different age groups of IDC patients. D. Relative level of LRIG1 expression in different age groups of fibroadenoma patients. Spearman's Rank Order Correlation was used to determine the relationship between expression of LRIG1 and clinicopathological factors.