Regulation of cell proliferation and migration by keratin19-induced nuclear import of early growth response-1 in breast cancer cells

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Abstract

Purpose: Keratin19 (KRT19) is the smallest known type I intermediate filament and is used as a marker for RT-PCR-mediated detection of disseminated tumors. In this study, we investigated the functional analysis of KRT19 in human breast cancer.

Experimental Design: Using a shRNA system, we silenced KRT19 in breast cancer cells. KRT19 silencing was verified by western blot analysis and immunocytochemistry. We further examined the effect of KRT19 silencing on breast cancer cells by cell proliferation, migration, invasion, colony formation assay, cell cycle analysis, immunocytochemistry, immunohistochemistry and mouse xenograft assay.

Results: Silencing of KRT19 resulted in increased cell proliferation, migration, invasion, and survival. These effects were mediated by up-regulation of Akt signaling as a result of reduced PTEN mRNA expression. Silencing of KRT19 decreased nuclear import of early growth response-1 (Egr1), a transcriptional factor for PTEN transcription, through reduced association between Egr1 and importin-7 (Imp7). We also confirmed that silencing of KRT19 increased tumor formation in a xenograft model.

Conclusions: KRT19 is a potential tumor suppressor that negatively regulates Akt signaling through modulation of Egr1 nuclear localization.

Translational Relevance

Keratin19 (KRT19) is used as a marker for RT-PCR-mediated detection of tumor cells and its positivity is considered as a prognostic indicator. On the contrary, we found that silencing of KRT19 increased cell proliferation, migration, invasion, and survival in human breast cancer cells. We also confirmed that silencing of KRT19 increased tumor formation in a xenograft model. These effects were mediated by up-regulation of Akt signaling as a result of reduced PTEN mRNA expression. Silencing of KRT19 decreased nuclear import of early growth response-1 (Egr1), a transcriptional factor for PTEN transcription, through reduced association between Egr1 and importin-7 (Imp7). To our knowledge, this is the first report on KRT19-mediated tumor suppression and this result may indicate that the potential of KRT19 as a prognostic marker should be carefully re-evaluated.

Introduction

Keratins (KRTs) are a subgroup of intermediate filament proteins that are present in epithelial
KRT19 regulates cell proliferation and migration

tissues (1). There are two types of KRTs: low molecular weight acidic type I KRTs and high molecular weight basic or neutral type II KRTs (2). One of the most biologically interesting KRTs is KRT19, at 40 kDa the smallest known intermediate filament. KRT19 is used as a marker for RT-PCR-mediated detection of tumor cells disseminated in lymph nodes, peripheral blood, and bone marrow of breast cancer patients, and its positivity is considered a prognostic indicator (3-5).

The early growth response-1 (Egr1) transcription factor regulates the expression of several genes including phosphatase and tensin homologue, deleted on chromosome 10 (PTEN) (6, 7). Egr1 directly binds to the consensus Egr1-binding motif in the PTEN promoter and activates PTEN gene transcription. PTEN is a tumor suppressor in human cancers that functions as a phosphatase to dephosphorylate phosphatidylinositol-trisphosphate (PIP3), the product of phosphoinositide 3-kinase (PI3K), resulting in the formation of PIP2 (8). Increased levels of PIP3 result in recruitment of Akt to the membrane to promote tumorigenesis (9). As a downstream effector of PI3K, Akt regulates cellular survival and metabolism via phosphorylation of many downstream molecules (9).

The S/T-P-S/T nuclear translocation signal (NTS) domain in extracellular signal-regulated kinase-2 (ERK2) was identified and characterized as a novel nuclear localization signal (NLS) domain (10). Importin-7 (Imp7) binds the phosphorylated NTS domain of target molecules and mediates nuclear localization of the cargo as the complex is released from nuclear pore proteins (10). The NLS of Egr1 was previously identified as a zinc finger DNA binding domain (11). Recently, it was reported that Egr1 also contains a S-P-S NTS domain in its C-terminal region (12). The NTS of Egr1 is required for the nuclear localization of Egr1, which is dependent on association between Egr1 and Imp7. In this study, we showed that KRT19 regulated cell proliferation, migration, invasion, and survival through KRT19-mediated association between Egr1 and Imp7 and subsequent nuclear import of Egr1 in human breast cancer cells.

Materials and Methods

Cell lines, antibodies, reagents, and plasmids

T47D, MCF7 vec, MCF7 HER2, BT474, MDA-MB-231 and SKBR3 human breast cancer cells and 293T human kidney cells were grown and routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were incubated at 37°C in 5% CO2 and 95% humidified air. KRT19 silenced cells were generated by transfection with KRT19 shRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). The following antibodies were used: p-HER2, p-PI3K, PI3K, p-Akt, Akt, p-GSK 3β, GSK 3β, p-IKK α/β, IKK α, IKK β, p-IκB α, IκB α, p-NFκB, NFκB, p-c-Raf, c-Raf, p-MEK1/2, MEK1/2, p-ERK1/2, ERK1/2, p-Elk, Elk, actin, HA, caspase-3, Egr1 and c-Jun antibodies (Cell Signaling Technology); PTEN, Egr1, Imp7, β-Tubulin and normal rabbit or mouse immunoglobulin G antibodies (Santa Cruz Biotechnology); KRT19 antibody (Chemicon); HER2
KRT19 regulates cell proliferation and migration

(ERBB2/neu) antibody (Lab Vision); PARP antibody (BD Pharmingen); anti-mouse IgG-Cy3 (Zymed); and anti-Mouse IgG-PE antibody (Abcam). The following reagents were used: Alexa Fluor 488 phalloidin (Invitrogen); doxorubicin (Calbiochem); cycloheximde and MG132 (Sigma-Aldrich); Herceptin (Roche). KRT19 and HER2 plasmids were purchased from Addgene. HA-FOXO3a (13), NFXB-Luc (14), and FOXO-Luc (15) plasmids were previously described. PTEN-Luc and Egr1 plasmid was kindly provided by Prof. Young Han Lee (Konkuk University, Seoul, Korea). PCR products amplified from -500 bp to +273 bp of the promoter region of the PTEN gene were cloned into the pGL3-Basic vector using KpnI and BglII restriction enzymes. Using the full length Egr1 (WT) construct as a backbone, Egr1 NLS domain deletion mutant (ΔNLS) and NTS (482APA484) mutant were made as described (Supplementary Materials and Methods).

**Western blot analyses**

Cells were lysed in lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na3VO4 and protease inhibitor cocktail (Roche). Samples were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Whatman). After blocking with 5% skim milk in TBS-T, membranes were incubated with the appropriate primary antibodies overnight, followed by 2 h incubation with HRP-conjugated secondary antibodies. Protein bands were visualized with the WEST ZOL plus System (iNtRON, Seongnam, Korea).

**Immunoprecipitation**

Cell lysates (1 mg) were precleared by addition of 30 μL Protein A or G Sepharose (Invitrogen) for 2 h at 4°C. After centrifugation at 1000g, the supernatant was incubated with either primary antibodies or normal rabbit/mouse IgG overnight at 4°C. Protein A or G Sepharose (30 μL) was added and incubated for 3 h at 4°C. After centrifugation, the pellet was washed three times with cell lysis buffer. Immunoprecipitated proteins were resolved by SDS–PAGE and analyzed by western blotting.

**Immunocytochemistry**

Cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 15 min. Fixed samples were blocked with 3% skim milk in PBS for 1 h, followed by incubation with primary antibody diluted in 1% skim milk in PBS for 1 h. After washing with PBS, the samples were treated with anti-mouse IgG-Cy3. For DNA staining, samples were incubated with Hoechst 33342 (1 μg/mL) for an additional 10 min. Immunofluorescence was monitored with an Olympus upright fluorescence microscope (BX50F).

**Immunohistochemistry of tumor tissue sections**

Tumor tissues were fixed in Bouin’s solution (Sigma). Tissues were dehydrated, washed in 70%
ethanol, embedded in paraffin, and prepared as 5-μm thick sections. Paraffin sections were mounted on poly-L-lysine coated slides and incubated in a dry oven at 60°C for 1 h followed by deparaffinization in xylene. The slides were rehydrated by sequential incubation in 100%, 90%, 80%, and 70% ethanol, with two 5-min incubations in each solution. The slides were then incubated with primary antibodies in PBS with 5% FBS in a humidified chamber overnight followed by incubation for 30 min with a biotin-conjugated secondary antibody (Vector Laboratories). The avidin-biotin complex (ABC) reaction was generated according to the manufacturer’s protocol. Sections were counterstained with Mayer’s hematoxylin.

**Dual luciferase assays**

Cells grown in 12-well plates were transfected with 0.2 μg of reporter constructs and 0.002 μg of pCMV-Rl as an internal control using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, dual luciferase assays were performed according to the manufacturer’s protocol (Promega).

**Cycloheximide decay assay**

Cycloheximide (100 μg/mL, Sigma) was added to block further protein synthesis. After treatment, cells were harvested at each of the indicated time points and subjected to western blot analyses.

**Cell fractionation**

Cells were washed with ice-cold PBS and harvested in cytoplasmic extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) with agitation for 10 min at 4°C. After addition of NP-40 (final 0.5%), the samples were further agitated for 10 min at 4°C and subjected to centrifugation at 13,000 rpm in a microcentrifuge (Eppendorf) for 5 min. The supernatant was collected as the cytosolic fraction. The nuclear pellets were washed two times with cold PBS and resuspended in a nuclear extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). The nuclear extracts were agitated for 10 min at 4°C, centrifuged at 13,000 rpm at 4°C, and the supernatants were collected as the nuclear fraction.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Expression of transcripts was assessed using the following primers: PTEN, Fwd 5’-AAA GCT GGA AAG GGA CGA AC-3’ and Rev 5’-CAG GTA ACG GCT GAG GGA AC-3’; Egr1, Fwd 5’-TCA GGC GGA CAC GGG CGA GC-3’ and Rev 5’-TGC GCA GCT CAG GGG TGG GC-3’. Quantification of actin expression was performed as an internal control using an RT-PCR primer and control primer set (Invitrogen).
Proliferation assays

Cells were seeded at 1 × 10^5 cells/well in 12-well dishes, trypsinized, and counted with a hemocytometer in triplicate every 24 h for 5 days.

Cell cycle analyses by flow cytometry

Cells were harvested with 0.25% trypsin and washed once with PBS. After centrifugation, cells were fixed in 100% ice-cold methanol overnight at -20°C. Fixed cells were incubated with 50 μg/mL propidium iodide in PBS and 1 mg/mL RNAase in PBS for 30 min. Cell cycle analyses were performed on a BD FACS (Becton & Dickinson Biosciences).

Soft agar and matrigel colony formation assays

As a base agar, 0.7% low melting point agar gel (Bio-Rad) containing 20% FBS in 2×DMEM was added to 12-well dishes. Cells (1 × 10^4/well) were plated in 0.4% low melting point agar gel with 20% FBS in 2×DMEM and allowed to grow for 4, 8, and 12 days. Matrigel colony forming assays were performed according to the manufacturer’s protocol (BD Bioscience).

Cell migration and invasion assays

Cell migration assays were conducted using 8-μm pore size transwell chambers (Corning). The lower chamber was filled with medium containing 10% FBS. Cells were suspended in DMEM with 1% FBS and plated into the upper chamber. After 6, 12, and 24 h, the number of cells on the bottom surface of the polycarbonate membranes was counted visually using crystal violet dye and a light microscope. Cell invasion assays were performed using the same procedure except that the upper chamber was coated with matrigel.

Xenografts

SKBR3 Cont sh and KRT19 sh cells were passaged in 8-week-old BALB/C nude mouse (Orient Bio, Seongnam, Korea) by subcutaneous injection of 1 × 10^6 or 5 × 10^6 cells into the lower flanks of mice. The mice were monitored for tumor formation and growth. Tumor weight was determined after sacrificing all the mice together at 39 days post tumor formation. The mice were cared for and treated in compliance with institutional guidelines (HY-IACUC-10-048).

Statistical analyses

All of the results were confirmed in at least three independent experiments. Data are presented as mean ± SD. Comparison of results from treated versus control cells was performed using t-tests. A value of \( p < 0.05 \) was considered statistically significant.
KRT19 regulates cell proliferation and migration

Results

Silencing of KRT19 expression increases proliferation of breast cancer cells

We established KRT19 silenced cells (KRT19 shRNA cells) and control cells (Cont shRNA cells) using BT474, SKBR3, T47D, MCF7 vec and MCF7 HER2 human breast cancer cell lines and a shRNA transfection system. Western blot and immunocytochemical analyses confirmed the down-regulation of KRT19 in KRT19 shRNA cells (Fig. 1A and quantified in Supplementary Fig. S6A; Supplementary Fig. S1A and S1B). We examined the cell morphology and rearrangement of the actin filament cytoskeleton following silencing of KRT19 using F-actin staining and found that KRT19 silencing did not significantly affect cell morphology and actin rearrangement (Supplementary Fig. S1B). First, we compared the cell proliferation rate of KRT19 shRNA cells with that of Cont shRNA cells. As shown in Figure 1B and Supplementary Fig. S1C, the growth rates of five KRT19 shRNA cell lines were up-regulated compared with those of Cont shRNA cell lines, suggesting that KRT19 silencing induced cellular event(s) associated with increased cell proliferation. To confirm the effect of KRT19 on cell proliferation, MTT assays were performed in KRT19 negative/low 293T and MDA-MB-231 cells using KRT19 overexpressing system (Supplementary Fig. S1E). The proliferation rates of KRT19 overexpressing cells were down-regulated compared with that of control cells (Supplementary Fig. S1F). When the cell cycle profiles of KRT19 shRNA and Cont shRNA cells were compared, we found that the S phase fractions were significantly increased in KRT19 shRNA cells with a concomitant reduction in G1 phase (Fig. 1C and Supplementary Fig. S1D). We also investigated the effect of KRT19 silencing on three-dimensional colony formation. The results of both soft agar and matrigel colony formation assays suggest that KRT19 silencing resulted in a significantly increased rate of colony formation (Fig. 1D and Supplementary Fig. S2A).

KRT19 silencing up-regulates cell migration, invasion, and survival

Next, we examined the effect of KRT19 silencing on cell migration and invasion. As shown in Figure 2A, 2B, Supplementary Fig. S2B and S2C, the migration and invasion rates of KRT19 shRNA cells were up-regulated compared with those of Cont shRNA cells. To determine the effects of KRT19 silencing on cell survival and apoptosis, the cells were exposed to stress conditions including serum starvation, treatment with the DNA damaging agent doxorubicin (500 nM), or treatment with Herceptin (20 µg/mL), a therapeutic antibody against human epidermal growth factor receptor-2 (HER2) for 48 h. Herceptin was chosen for this study because both BT474 and SKBR3 cell lines express robust levels of HER2. The cell death induced by these treatments was attenuated in KRT19 shRNA cells compared with that of Cont shRNA cells (Fig. 2C, upper panels). We also demonstrated efficient down-regulation of the apoptotic markers of proteolytic cleavage of caspase3 and poly ADP ribose polymerase (PARP) after treatment with doxorubicin or Herceptin in KRT19 shRNA cells compared with Cont shRNA cells (Fig. 2C, lower panels). Conversely, we could observe sensitizing
KRT19 regulates cell proliferation and migration

effect of KRT19 overexpression on cytotoxicity of doxorubicin (Supplementary Fig. S1G). These results indicate that KRT19 silencing might confer resistance to apoptosis. Together, the results of Figure 1 and 2 suggest that KRT19 might act as a tumor suppressor in human breast cancer through the regulation of cancer cell proliferation and survival.

**KRT19 silencing induces Akt activity through down-regulation of PTEN mRNA expression**

To investigate the mechanisms responsible for KRT19 silencing-mediated up-regulation of cell proliferation, migration, invasion, and survival, we examined the status of intracellular signaling molecules. HER2 activates a number of intracellular signal cascades including the PI3K/Akt pathway (16). We found that silencing of KRT19 induced down-regulation of HER2 (Fig. 3A), possibly via blockade of proteosomal-mediated degradation of HER2 (Ju et al., unpublished data). Interestingly, despite the down-regulation of HER2 by KRT19 silencing, we found that phosphorylation of PI3K, Akt, and Akt downstream factors including GSK3β and IκB kinase (IKK)/IκB/NFκB was up-regulated in all five KRT19 shRNA cell lines (Fig. 3A and quantified in Supplementary Fig. S6B; Supplementary Fig. S3A). Accordingly, NFκB-dependent transcriptional activities were significantly increased in KRT19 shRNA cells (Fig. 3B, left panel). To further assess the effect of KRT19 on activity of Akt and its downstream signaling, we re-introduced pSuper shRNA resistant KRT19 mutant in BT474 pSuper KRT19 shRNA clone (Supplementary Fig. S3E). We confirmed that KRT19 shRNA exclusively regulates its level and Akt signaling. We also examined the nuclear localization and transcriptional activity of FOXO, an transcription factor downstream of Akt1 that is negatively regulated by Akt activity (17). Basal levels of FOXO-dependent transcriptional activities were effectively decreased by KRT19 silencing (Fig. 3B, right panel). Localization of ectopic FOXO3a in nucleus was also reduced in KRT19 shRNA cells, as revealed by cell fractionation assays (Supplementary Fig. S3B). The results in Figure 3A and 3B collectively suggest that silencing of KRT19 efficiently increased PI3K/Akt-downstream signaling.

To investigate the mechanism by which KRT19 silencing up-regulated the Akt pathway despite down-regulation of HER2 levels, we considered the possibility that KRT19 silencing regulates PTEN, a negative modulator of the PI3K/Akt pathway. We found that mRNA and protein levels of PTEN were significantly down-regulated in KRT19 shRNA cells compared with those of Cont shRNA cells (Fig. 3C and quantified in Supplementary Fig. S6C; Supplementary Fig. S3C and S3E). Transcriptional activity of a reporter construct containing the promoter region of PTEN (-500/+273 bp) was also decreased by KRT19 silencing (Fig. 3D). Accordingly, co-transfection of KRT19 increased PTEN promoter activity in 293T cells (Supplementary Fig. S3D). To determine whether KRT19 directly affects the stability of PTEN protein, we measured PTEN protein level after treatment with MG132, a 26S proteasome inhibitor. MG132 treatment resulted in a slight increase in of PTEN protein level (Supplementary Fig. S4A). PTEN protein level is known to be regulated by NEDD4-1
E3 ligase via ubiquitination-mediated proteosomal degradation (18). Although the decreased basal expression level of PTEN in KRT19 shRNA cells was restored by treatment with MG132, the levels were still lower than the PTEN level in Cont shRNA cells after MG132 treatment (Supplementary Fig. S4A, lane 2 vs. 4 and lane 6 vs. 8). These results suggest that silencing of KRT19 did not down-regulate PTEN protein level through a decrease in protein stability of PTEN. In cycloheximide decay assays, the decay slopes of PTEN were similar between KRT19 and Cont shRNA cells (Supplementary Fig. S4B). These findings confirmed that transcriptional control of PTEN mRNA is the major molecular mechanism responsible for KRT19 silencing-induced down-regulation of PTEN.

KRT19 regulates nuclear localization of Egr1 transcriptional factor

We next focused on transcription factors that might be responsible for the regulation of PTEN transcription. Egr1 is a well-known transcription factor that up-regulates PTEN transcription (6). We investigated the effect of Egr1 knock-out on PTEN expression in vivo using an Egr1-deficient mouse model. As depicted in Figure 4A, western blot analyses revealed that PTEN protein level was down-regulated in the mammary glands of Egr1-deficient mice compared with their wild type littermates. Phosphorylated Akt was up-regulated in Egr1-deficient mice as a result of reduced PTEN expression. Based on these in vivo observations, we investigated whether the KRT19 silencing-induced down-regulation of PTEN is mediated by Egr1. Interestingly, we observed that both mRNA and protein expression levels of Egr1 were decreased by KRT19 silencing in HER2-positive MCF7 HER2, BT474, and SKBR3 cells, but not in HER2-negative/low T47D and MCF-7 vec cells (Fig. 4B, 4C, Supplementary Fig. S5A and S5B). To explain the differential effect of KRT19 silencing on Egr1 expression between HER2-positive and -negative/low cells, we examined the possible modulation of extracellular signal-regulated kinase (ERK) signaling by KRT19 silencing because Egr1 transcription is regulated by the ERK/E twenty-six (ETS)-like transcription factor (Elk) pathway (19, 20). Our results indicated that activities of ERK signaling after KRT19 silencing were vastly different between HER2-positive and -negative/low cells (Supplementary Fig. S5C). In HER2-positive MCF7 HER2, BT474, and SKBR3 cells, the activities of c-Raf/mitogen-activated protein kinase kinase (MEK), ERK, and Elk were down-regulated by KRT19 silencing, as determined by their phosphorylation status, while activities of c-Raf/MEK/ERK/Elk in HER2-negative/low T47D, and MCF7 vec cells were not significantly changed after KRT19 silencing. Needless to say, activation of the ERK pathway in HER2 negative/low cells was achieved by a HER2-independent mechanism, while that of HER2-positive cells was HER2-dependent (21, 22). Down-regulation of HER2 by KRT19 silencing (Fig. 3A) decreased activity of the c-Raf/MEK/ERK/Elk cascade, culminating in inhibition of Egr1 expression. These results suggest that down-regulation of Egr1 expression by KRT19 silencing was a HER2-dependent phenomenon present only in HER2-positive cells.

We also evaluated the possible modulation of Egr1 nuclear translocation by KRT19 silencing.
because Egr1 acts as a transcription factor within the nucleus. KRT19 silencing decreased nuclear Egr1 levels, as determined by cell fractionation assay and immunocytochemistry, regardless of HER2 expression status (Fig. 4C, 4D and Supplementary Fig. S5B). We confirmed KRT19-mediated nuclear translocation of Egr1 using transient transfection experiments with 293T cells (Supplementary Fig. S5D). As a consequence of Egr1 nuclear translocation by KRT19, PTEN expression increased and Akt activity decreased. These results indicate that up-regulated cell proliferation, migration, invasion, and survival caused by KRT19 silencing (Fig. 1 and 2) were most likely attributable to modulation of Egr1 nuclear translocation and subsequent changes in PTEN expression.

**KRT19 mediates association between Egr1 and Imp7**

The phosphorylated S/T-P-S/T NTS domain regulates nuclear localization of molecules through interaction with Imp7 (10). Egr1 contains a NTS domain that binds Imp7 for nuclear localization (12). Based on these findings, we were interested in whether KRT19 silencing affected the association between Egr1 and Imp7. Co-immunoprecipitation studies (Fig. 5A) revealed that KRT19, Egr1, and Imp7 formed a ternary complex in all five cell lines. Control immunoprecipitates with normal IgG did not contain KRT19, Egr1, or Imp7. In KRT19 shRNA cells, the Egr1-Imp7 association was significantly impaired compared with control cells (Fig. 5B). Therefore KRT19 might facilitate association between Egr1 and Imp7 thereby inducing nuclear targeting of Egr1.

In addition to the NTS domain, classical NLS domain in the zinc finger domain regulates nuclear localization of Egr1 (11). If KRT19-mediated increased association between Egr1 and Imp7 only occur through potentiation of Imp7 binding to NTS domain of Egr1, the classical NLS-mediated nuclear localization of Egr1 may still happen in KRT19-silenced cells. So we further hypothesized that Egr1 nuclear localization by classical NLS domain was also achieved by a KRT19-dependent mechanism. Because NLS domain of Egr1 plays a dominant role in Egr1 nuclear localization as compared to NTS domain (12), it was indeed less likely to conclude that KRT19 silencing-induced perturbation in nuclear targeting of Egr1 is mainly dependent on decreased binding of Imp7 to NTS domain of Egr1. To precisely determine the interaction motifs in Egr1-KRT19-Imp7 ternary structure, we sub-cloned full length Egr1 (WT), NLS domain deletion mutant (ΔNLS), NTS alanine substitution mutant (APA) and NLS/NTS double mutant (ΔNLS/APA). Egr1 constructs were transiently co-transfected with KRT19 wild type construct into 293T cells and the cell lysates were divided into nuclear and cytosolic fractions. Our results showed that KRT19 could effectively translocate Egr1-WT, ΔNLS and APA from cytosol to nucleus, but not double mutant (Fig. 5C). In addition, Egr1-WT and Egr1-APA could effectively bind KRT19 while Egr1-ΔNLS and double mutant exhibited reduced affinity toward KRT19 (Fig. 5D, 1st and 2nd panel, lane 3, 7 vs 5, 9). These data indicated that the binding of Egr1 to KRT19 is mainly mediated by the interaction between NLS domain of Egr1 and KRT19. When the interaction between Imp7 and Egr1 was examined, both Egr1-WT and Egr1-ΔNLS
KRT19 regulates cell proliferation and migration could effectively bind Imp7 and this association between Imp7 and Egr1 was enhanced by KRT19 co-expression (Fig. 5D, 3rd panel, lane 2 vs 3 and 4 vs 5). However, both Egr1-APA and double mutant could not bind Imp7 (lane 6-9). Interestingly, we found that the association between KRT19 and Imp7 was Egr1-independent phenomena, since KRT19 could bind Imp7 even in the presence of Egr1 double mutant in which the interaction site between Egr1 (NLS) and KRT19 and the site between Egr1 (NTS) and Imp7 were deleted (Fig. 5D, 4th panel, lane 9). Taken together, the data in Fig. 5 collectively suggest that KRT19 is a necessary requirement for the association between Imp7 and NTS domain of Egr1.

KRT19 regulates in vivo tumorigenesis via modulation of Egr1 subcellular localization in mouse xenograft model

We next examined the effect of KRT19 silencing on in vivo tumor formation using a xenograft mouse model (Fig. 6A). When 1×10^6 SKBR3 KRT19 shRNA and Cont shRNA cells were inoculated subcutaneously into both sides of the lower flank of BALB/C nude mice, none of the mice examined developed tumors (Fig. 6A and B). When 5×10^6 SKBR3 KRT19 shRNA cells were inoculated, all mice developed xenograft tumors. In contrast, tumors developed at only two out of 10 injection sites inoculated with 5×10^6 SKBR3 Cont shRNA cells. In addition, the weights of SKBR3 KRT19 tumors were approximately 6-fold higher than those of control tumors (Fig. 6C). Immunohistochemistry experiments revealed that SKBR3 KRT19 tumors exhibited up-regulation of phosphorylated Akt and down-regulation of PTEN compared with control cell tumors (Fig. 6D). The KRT19 level was still efficiently down-regulated in SKBR3 KRT19 shRNA tumors. These results are in accordance with those obtained using in vitro cultured cells in Figure 3. We also confirmed that Egr1 nuclear localization was significantly impaired in SKBR3 KRT19 shRNA tumors (Fig. 6E) compared with control tumors whereas the subcellular distribution of Imp7 appeared to be similar in the two types of tumor.

Discussion

This study uncovers a new mechanism by which KRT19-mediated association between Egr1 and Imp7 down-regulates breast cancer cell proliferation, migration, invasion, and survival through up-regulated transcription of PTEN and subsequent inhibition of Akt by promoting nuclear localization of Egr1 both in vitro and in vivo. Egr1 is a crucial transcription factor that is induced by stress, injury, mitogens, and differentiation signals (7, 23, 24). Egr-1 up-regulates the expression of multiple genes, including PTEN and several tumor suppressor genes. Transcription of Egr1 was previously shown to be induced by MAPK pathways, especially by ERK signaling through phosphorylation and activation of Elk1 using MEK inhibitors or the natural anti-cancer compound curcumin, in various types of tumors (25-28). In addition to transcriptional regulation of Egr1 by ERK, we show here that KRT19
KRT19 regulates cell proliferation and migration

plays a role in regulating the nuclear localization of Egr1 by modulating its association with Imp7.

KRTs are dynamically regulated intermediate filaments that are fully integrated within the cellular framework and interact with a range of cellular proteins (1). Many of these proteins, including various kinases, receptors, adaptors, and other types of effector, function in signaling networks that regulate the cell cycle, apoptosis, and the cellular response to stress (1). It has been shown that KRT8 and KRT18 bind tumor necrosis factor receptor-2 (TNFR2), reflecting their ability to specifically interact with other signaling proteins (29). This interaction affects the TNF-dependent activation of downstream effectors such as c-Jun N-terminal kinase (JNK) and NFκB. KRT17 up-regulates protein synthesis and cell growth via the Akt/mTOR pathway by binding to the adaptor protein 14-3-3σ (30).

One of the most interesting keratins is KRT19, the smallest known intermediate filament protein (31). KRT19 has a highly conserved α-helical central domain that is essential for filament formation, but lacks the C-terminal non-helical tail domain present in other acidic KRTs (32). A previous study demonstrated that absence of KRT19 induced skeletal myopathy in a KRT19 knock-out mouse model (33). However, compared with other KRT isoforms, KRT19-mediated intracellular signaling has not been widely studied.

It was previously reported that KRT19 expression is coupled with HER2 expression in breast cancer (34). Our in silico analysis also revealed that KRT19 expression is positively correlated with HER2 (Supplementary Fig. S7). In our experiments, when KRT19 was knocked-down, HER2 expression was dramatically down-regulated. Tumorigenicity of HER2-amplified breast cancer cells such as SKBR3 and BT474 is presumably dependent on HER2 (35, 36). Since a HER2-targeted therapy would be used for a HER2 overexpressing tumor, this may suggest that any intracellular genetic event(s) which completely silences KRT19 levels could lead to resistance to HER2-targeted therapies. However, it is also possible that a modest decrease in HER2 level by attenuation of KRT19 would lead to a decrease in tumorigenic potential of HER2 by inhibition of HER2-downstream signaling.

It was reported that Egr1 nuclear localization and transcriptional activity are regulated by α-tubulin cytoskeleton protein in benign prostate cells but not in malignant prostate cells (37). The authors showed that Egr1 associates with α-tubulin in benign prostate cells but not in prostate cancer cells using immunofluorescence confocal analyses and co-immunoprecipitation experiments. Furthermore, α-tubulin-mediated Egr1 localization in benign prostate cells is unique among the Egr family since other related proteins including Egr2, Egr3, Sp1, or WT1 do not exhibit such behavior. Interestingly, they also showed that KRT co-localized with Egr1 in the perinuclear region of DU-145 prostate cancer cells using pan-KRT antibody and immunofluorescence confocal analyses. We speculate that microtubules and KRTs may compete with each other for binding with Egr1 or may together constitute molecular cross-talk to regulate Egr1 nuclear targeting. The phosphorylated S/T>P>S/T NTS domain is a newly identified nuclear translocation signal that induces nuclear localization of ERK2,
KRT19 regulates cell proliferation and migration

SMAD3, and MEK1 by binding to Imp7 (10). Recently, Chen et al. reported that Imp7 regulates Egr1 nuclear localization through binding to the S-P-S NTS domain in the C-terminal region of Egr1(12). They also showed that Imp7-dependent nuclear localization of Egr1 is not solely dependent on the previously identified classical NLS.

Based on these previous studies, we investigated the possibility that KRT19 might mediate nuclear localization of Egr1 by facilitating Egr1-Imp7 complex formation in breast cancer cells. KRT19 was indeed required for proper Egr1 nuclear localization via formation of a KRT19-Egr1-Imp7 ternary complex. Upon nuclear localization of Egr1, PTEN was expressed through transcriptional activity of Egr1, resulting in down-regulation of PI3K/Akt activity in breast cancer cells. The PI3K/Akt pathway is centrally involved in cell proliferation, migration, invasion, and survival (9, 38).

As summarized in Figure 6F, KRT19 silencing up-regulated the activity of Akt, and subsequently increased cell proliferation, migration, invasion, and survival. It was recently reported that activation of Akt could be subject to negative feedback regulation by PTEN (7). Akt-mediated phosphorylation of Egr1 induces SUMOylation of Egr1 by p14ARF, which is required for Egr1-mediated transcription of its target genes including PTEN (7). We also identified KRT19 as a necessary component of the negative feedback loop that down-regulates PI3K/Akt pathway. Although KRT19-silenced cells exhibit high Akt activity, the absence of KRT19 fails to establish negative feedback on PI3K/Akt pathway due to impaired nuclear import of Egr1. The lack of negative feedback regulation of Akt activity may account for the dramatic rise in Akt activity as well as the tumorigenic potential of KRT19 silenced cells in vivo.

Disclosure of Potential Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Conception and design: Ji-hyun Ju, Incheol Shin
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Analysis and interpretation of data: Ji-hyun Ju, In-Sun Chu, Incheol Shin
Writing, review, and/or revision of the manuscript: Ji-hyun Ju, Incheol Shin
Administrative, technical, or material support: Ji-hyun Ju, Soon Young Shin, Myung Chan Gye, Incheol Shin
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KRT19 regulates cell proliferation and migration

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Figure Legends

Figure 1. Inhibition of KRT19 up-regulates cell proliferation. A, BT474, and SKBR3 cells were transfected with KRT19 shRNA (KRT19 sh) or control shRNA (Cont sh). Cell lysates from each cell lines were subjected to western blot analyses using KRT19 antibody to detect endogenous KRT19 protein expression. Actin was used as a loading control. B, Cont sh and KRT19 sh cells were seeded at a density of 1 × 10^4 cells per well in 12-well dishes and counted with a hemocytometer over 5 days (*p < 0.05, **p < 0.005). C, for cell cycle profiles, the cells were fixed in methanol and incubated in PBS containing 50 µg/mL propidium iodide and 1 mg/mL RNase. Propidium iodide-labeled nuclei were analyzed by flow cytometry (*p < 0.05). D, cells were plated on soft agar or matrigel. The
KRT19 regulates cell proliferation and migration

The number of colonies (> 20 µm diameter) was counted at days 4, 8, and 12 (*p < 0.05, **p < 0.005).

**Figure 2.** KRT19 inhibition up-regulates cell migration, invasion, and survival. BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells were plated on transwell plates for A, migration assay or B, invasion assay. The number of cells on the bottom of the membrane was counted at 6, 12, and 24 hours (*p < 0.05, **p < 0.005). C, at 24 h hours after cell seeding, BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh and SKBR3 Cont sh cells were treated with serum-free medium containing doxorubicin (500 nM) or Herceptin (20 µg/mL). After a further 24 hours, the cells were counted with a hemocytometer. Cell lysates were analyzed by western blotting using antibodies against p-Akt, caspase3, and PARP, and anti-actin antibody as a loading control.

**Figure 3.** KRT19 inhibition induces Akt activity through down-regulation of PTEN expression. A, total cell lysates were prepared from BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells and subjected to western blot analyses with the indicated antibodies. B and D, BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells were transfected with NFκB, FOXO, or PTEN-Luc reporter constructs, harvested after 48 h, and analyzed by dual luciferase assays (*p < 0.05, **p < 0.005, ***p < 0.0005). C, mRNA from each cell line was analyzed by RT-PCR using primers specific for PTEN and actin (as a loading control). Total cell lysates were prepared from each cell line and subjected to western blot analyses with PTEN and actin antibodies.

**Figure 4.** KRT19 regulates nuclear localization of Egr1 transcriptional factor. A, mammary gland tissue lysates from Egr1 knock-out mice and wild type littermates (10-weeks-old, n = 4 for each) were analyzed by western blotting with antibodies against Egr1, PTEN, p-Akt, Akt, and actin. Mammary gland tissues were extracted in lysis buffer, homogenized with a homogenizer, incubated for 30 min at 4°C, and centrifuged for 15 min at 4°C. B, mRNA from each cell line was analyzed by RT-PCR using primers specific for Egr1 and actin (as loading control). C, cells were fractionated into cytosolic and nuclear fractions and subjected to western blot analyses with antibodies specific for Egr1, c-Jun (as nucleus marker), and actin (as cytosol marker). D, subcellular localization of Egr1 was monitored by immunocytochemistry. For DNA staining, cells were incubated with Hoechst 33342 (1 µg/mL). Scale bar, 20 µm.

**Figure 5.** KRT19 mediates association between Egr1 and Imp7. A and B, cell lysates were immunoprecipitated with KRT19, Egr1, Imp7 or normal IgG antibodies and Protein A or G sepharose. The immunoprecipitates were analyzed by western blotting with KRT19, Egr1, and Imp7 antibodies. C, Full length Egr1 (WT), NLS domain deletion mutant (ΔNLS), NTS alanine substitution mutant (A-P-A) and double mutant (ΔNLS/A-P-A) were sub-cloned into pcDNA3-Flag vector between the
KRT19 regulates cell proliferation and migration

BamH I and Xho I restriction sites. Egr1 constructs were co-transfected with KRT19 into 293T cells, and the lysates were divided into nuclear and cytosolic fractions. Each fractions were subjected to western blot analyses with the indicated Flag, KRT19, c-Jun (as nucleus marker), and β-Tubulin (as cytosol marker) antibodies. Actin antibody was used to verify equal loading. D, the lysates were immunoprecipitated with Flag, KRT19, Imp7 antibodies or normal IgG and Protein A or G sepharose. The immunoprecipitates were analyzed by western blotting with Flag, KRT19 or Imp7 antibodies.

**Figure 6.** KRT19 regulates tumor growth in mouse xenograft models by modulation of Egr1 subcellular localization. A, SKBR3 KRT19 sh and SKBR3 Cont sh cells were passaged in 8-week-old BALB/C nude mice by subcutaneous injection of $1 \times 10^6$ or $5 \times 10^6$ cells into both sides of the lower flank of mice. Upper panels show tumor growth on mice and lower panels show photographs of each tumor after biopsy at 39 days. B, Tumor volumes were measured every 3 days. Tumor volume was calculated by the formula: tumor volume (mm$^3$) = length (mm) × width (mm) × height (mm) × 0.5. C, bar graph representing final tumor weight after biopsy. D and E, tumor tissues from mouse xenograft model were analyzed by immunohistochemistry with the indicated antibodies. Panels in E are zoomed-in images of the boxed region in D. Quantification of immunohistochemical staining performed in triplicate is represented in E (*p < 0.05, **p < 0.005, ***p < 0.0005). At least 1,000 cells were scored for each image. Scale bar, 200 μm. F, schematic model showing the role of KRT19 in mediating the association between Egr1 and Imp7 and subsequent regulation of Akt signaling.
Figure 1

A

B

C

D

Fig. 1
Fig. 2
Figure 3

(A) Western blot analysis of p-HER2, HER2, p-PI3K, PI3K, p-Akt, Akt, p-GSK3β, GSK3β, p-IKKα/β, IKKα, IKKβ, p-IκBα, IκBα, p-NFκB, NFκB, and PTEN in BT474 and SKBR3 cells with and without KRT19 shRNA.

(B) Bar graphs showing fold change in NFκB-dependent transcriptional activity and FOXO-dependent transcriptional activity in BT474 and SKBR3 cells with and without KRT19 shRNA.

(C) Western blot analysis of p-IκBα, p-IκBβ, p-NFκB, and PTEN in BT474 and SKBR3 cells with and without KRT19 shRNA.

(D) Bar graphs showing fold change in FoxO-dependent transcriptional activity in BT474 and SKBR3 cells with and without KRT19 shRNA.

* p < 0.05, ** p < 0.01, *** p < 0.001
Fig. 4
A

WB: Imp7
WB: KRT19
WB: Egr1

WB: KRT19
WB: Imp7
WB: Egr1

WB: KRT19
WB: Imp7
WB: Egr1

WB: KRT19
WB: Imp7

WB: Egr1

WB: Imp7

WB: Imp7

WB: Imp7

B

IP: KRT19
IP: Flag
IP: KRT19

IP: KRT19
IP: Flag
IP: KRT19

IP: KRT19
IP: Flag
IP: KRT19

IP: KRT19
IP: Flag
IP: KRT19

C

NTS: SPS
NLS: zinc finger domain

1 314 425 533

D

Flag-Egr1-WT
Flag-Egr1-∆NLS
Flag-Egr1-APA
Flag-Egr1-∆NLS/APA
KRT19-WT

Fig. 5
Regulation of cell proliferation and migration by keratin19-induced nuclear import of early growth response-1 in breast cancer cells

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