Unraveling the Role of KIAA1199, a Novel Endoplasmic Reticulum Protein, in Cancer Cell Migration

Nikki A. Evensen, Cem Kuscu, Hoang-Lan Nguyen, Kevin Zarrabi, Antoine Dufour, Pournima Kadam, You-jun Hu, Ashleigh Pulko-Scott, Wadie F. Bahou, Stanley Zucker, Jian Cao

Manuscript received September 5, 2012; revised July 9, 2013; accepted July 18, 2013.

Correspondence to: Jian Cao, MD, Stony Brook University, 100 Nicolls Rd, Life Sciences Bldg, Rm 004, Stony Brook, NY 11794 (e-mail: jian.cao@stonybrookmedicine.edu).

Background

Cell migration is a critical determinant of cancer metastasis, and a better understanding of the genes involved will lead to the identification of novel targets aimed at preventing cancer dissemination. KIAA1199 has been shown to be upregulated in human cancers, yet its role in cancer progression was hitherto unknown.

Methods

Clinical relevance was assessed by examining KIAA1199 expression in human cancer specimens. In vitro and in vivo studies were employed to determine the function of KIAA1199 in cancer progression. Cellular localization of KIAA1199 was microscopically determined. SNAP-tag pull-down assays were used to identify binding partner(s) of KIAA1199. Calcium levels were evaluated using spectrofluorometric and fluorescence resonance energy transfer analyses. Signaling pathways were dissected by Western blotting. Student t test was used to assess differences. All statistical tests were two-sided.

Results

KIAA1199 was upregulated in invasive breast cancer specimens and inversely associated with patient survival rate. Silencing of KIAA1199 in MDA-MB-435 cancer cells resulted in a mesenchymal-to-epithelial transition that reduced cell migratory ability in vitro (75% reduction; P < .001) and decreased metastasis in vivo (80% reduction; P < .001). Gain-of-function assays further demonstrated the role of KIAA1199 in cell migration. KIAA1199-enhanced cell migration required endoplasmic reticulum (ER) localization, where it forms a stable complex with the chaperone binding immunoglobulin protein (BiP). A novel ER-retention motif within KIAA1199 that is required for its ER localization, BiP interaction, and enhanced cell migration was identified. Mechanistically, KIAA1199 was found to mediate ER calcium leakage, and the resultant increase in cytosolic calcium ultimately led to protein kinase C alpha activation and cell migration.

Conclusions

KIAA1199 serves as a novel cell migration–promoting gene and plays a critical role in maintaining cancer mesenchymal status.


Cell migration is a complicated and incompletely understood process required for cancer invasion. Cell migration is often a consequence of epithelial-to-mesenchymal transition (EMT) of cancer cells, which leads to a more aggressive phenotype. Reversal of EMT (mesenchymal-to-epithelial-transition) results in decreased cell migration. Identification of specific genes involved in cancer cell migration is critically important in preventing cancer dissemination.

To identify novel genes involved in cancer cell invasion, we used a polymerase chain reaction–based suppression subtractive hybridization method, which has been demonstrated to be effective in isolating, normalizing, and enriching differentially expressed genes >1000-fold in a single round of hybridization. Because concanavalin A enhances cell surface proteolytic activity and cell migratory ability, differential gene expression in concanavalin A–treated HT-1080 human fibrosarcoma cells was examined. This approach resulted in the identification of a marked upregulation of a previously obscure gene, KIAA1199, which is reported in the Human Unidentified Gene-Encoded Large Proteins database.

The current literature and sequence homology provide few clues as to the function of KIAA1199. Based on a publication that describes genetic mutations of KIAA1199 in families with nonsyndromic hearing loss, this gene appears to be essential for auditory function, although the function was not investigated. Clinical relevance of KIAA1199 in cancers has been highlighted by reports of increased KIAA1199 mRNA expression in human gastric and colorectal cancers; an association was shown between KIAA1199 expression level and disease stage/5-year survival rates. However, the function of KIAA1199 in cancer remains unknown.

In this study, we discovered that KIAA1199 is a novel endoplasmic reticulum (ER) resident protein that plays a critical role in...
cancer cell migration and invasion. Moreover, KIAA1199 enhances cell migration through its interaction with ER glucose-regulated protein 78/binding immunoglobulin protein (GRP-78/BiP), leading to ER calcium release. Increased cytosolic calcium results in the activation of protein kinase C alpha (PKCα), ultimately leading to enhanced cell migration.

Methods

Materials

Oligo primers were synthesized by Operon. RNAi-Ready pSIREN Retro-Q vector for specific gene silencing and pQCXIP retro viral vector for generation of stable cells were purchased from Clontech (Mountain View, CA). D1ER expression plasmid was kindly provided by Dr Roger Tsien (University of California–San Diego) (9). Mouse anti–Myo monoclonal antibody was purchased from Roche (Indianapolis, IN). The pcDNA3.1-myc expression vector, rabbit anti–PKCγ pT674 polyclonal antibody, and Organelle Lights reagents were purchased from Invitrogen (Grand Island, NY). Rabbit anti-KIAA1199 polyclonal antibody was produced by PrimmBiotech (Cambridge, MA) using the C-terminus of the KIAA1199 protein between Gly1108-Thr1340 as an antigen. Mouse anti–protein disulfide isomerase monoclonal antibody was purchased from AssayDesign (Farmingdale, NY). Rabbit anti-BiP monoclonal, -α/β-tubulin polyclonal, -β-actin monoclonal, and -Twist-1 polyclonal were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-XBP-1 polyclonal, -pan-PKCγ polyclonal, and mouse anti-cytokeratin 8/18 monoclonal were purchased from Santa Cruz Biotechnology (Dallas, TX). Mouse anti–PKCα monoclonal and rabbit anti–PKCB monoclonal were purchased from Enzo Life Sciences (Farmingdale, NY). Mouse anti-N-cadherin monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA). Mouse anti-vimentin monoclonal antibody, concanavalin A, and phalloidin were purchased from Sigma (St. Louis, MO). SNAP-capture beads and rabbit anti-SNAP polyclonal antibody were purchased from New England Biolabs (Ipswich, MA). PKCγK380R cDNA (Addgene plasmid 21239) and PKCB cDNA (Addgene plasmid 16378) (10) were purchased from Addgene (Cambridge, MA). All antibodies were used at 1:1000 dilution unless otherwise specified in each individual experiment. The human breast cancer tissue microarray samples (BR804, BC08013a, and BR10010a) were purchased from US Biomax Inc (Rockville, MD).

SNAP Pull-Down Assay and Proteomic Analysis

COS-1 cells transiently transfected with a control plasmid containing a SNAP tag or KIAA1199–SNAP plasmids were lysed, followed by incubation with SNAP beads (New England Biolabs, Ipswich, MA). For proteomic analysis, bound proteins were released by trypsin digestion. Resulting peptides were analyzed on a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA), and the resulting mass spectra were analyzed by Inspect search. For the BiP pull-down assay, bound proteins were eluted using sodium dodecyl sulfate (SDS)-sample buffer under reducing conditions, followed by Western blotting using anti-BiP antibody.

Fluorescent Recovery After Photobleaching (FRAP)

Living COS-1 cells expressing KIAA1199–green fluorescent protein (GFP) were treated with cycloheximide for 3 hours before FRAP to prevent nascent protein expression. KIAA1199–GFP protein was irreversibly photobleached by a laser flash within a restricted area (7 × 5 μm²). Fluorescence recovery, as determined by the exchange of bleached protein by nonbleached protein, was measured using an attenuated laser beam.

ER Calcium Measurement

ER cytosolic calcium (Ca²⁺) was measured using the ER-cameleon D1ER (9). COS-1 and MCF-7 cells stably expressing empty vector or KIAA1199 cDNA were transfected with D1ER cDNA, and live imaging was conducted using a Zeiss LSM 510 Meta NLO two-photon laser scanning confocal microscope system with 5% carbon dioxide and 37°C. D1ER was excited at 458 nm, and emission filters of 522–704 (yellow fluorescent protein [YFP], fluorescence resonance energy transfer channel) and 458–490 (cyan fluorescent protein [CFP]) were used. Baseline images were taken for 20 to 30 cells per sample, excluding highly fluorescent or dim cells. Cells were treated with 3 mM EGTA + 2 μM ionomycin, and images were taken to determine R_yfp and R_yair, respectively (11). A region of interest was formed for each image, and the YFP/CFP, proportional to the level of ER calcium, was calculated.

Statistical Analyses

Student unpaired two-sided t test was used to assess differences, with P values less than or equal to .05 considered statistically significant. Pooled data were presented as either mean ± standard deviation for all real-time reverse-transcription polymerase chain reaction analyses or mean ± standard error of the mean for other analyses of three independent experiments. GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA) was used for the determination of standard deviation and standard error of the mean as well as t test analyses. A χ² test was used to analyze difference in intensity staining grade among breast cancer tissue samples in different stages. Patient survival curve was estimated based on the Kaplan–Meier method and compared using a log-rank test. StatXact 9 (Cytel Inc., Cambridge, MA) software was used for these analyses. All experiments were repeated at least three times. All statistical tests were two-sided. Additional methods are available in the Supplementary Methods (available online).

Results

Upregulation of KIAA1199 in Aggressive Human Cancers

To determine the clinical relevance of KIAA1199 expression in cancer progression, human breast cancer cells and benign epithelial cells in formalin-fixed, paraffin-embedded specimens isolated using laser capture microdissection (12) were examined. KIAA1199 mRNA was statistically significantly upregulated in both ductal carcinoma in situ (fivefold change; P = .03) and invasive breast cancer cells (12-fold change; P = .01) compared with benign epithelial cells (Figure 1A). Using immunohistochemical (IHC) analyses with an anti–KIAA1199 polyclonal antibody (Supplementary Figure 1, available online), high levels of KIAA1199 were detected in the cytoplasm of breast carcinoma cells, whereas minimal
Figure 1. Clinical relevance of KIAA1199 in human breast cancer. A) KIAA1199 mRNA expression analysis using real-time reverse-transcription polymerase chain reaction of RNA isolated from formalin-fixed, paraffin-embedded tissue sections of human ductal carcinoma in situ (DCIS), invasive breast cancer, and benign breast epithelium control by a laser capture microdissection approach. Each bar represents the mean ± standard deviation. Student’s t test was used to assess differences. All statistical tests were two-sided. B and C) Tissue distribution of KIAA1199 in breast cancer tissues. Samples from benign breast, DCIS, and invasive ductal and lobular breast cancers tissues (B) and from human breast cancer tissue microarrays containing invasive breast cancers and benign breast tissues (US Biomax Inc) (C) were examined by immunohistochemical analyses (IHC) with anti-KIAA1199 antibody. Representative images of tissue types (B) and staining intensities of invasive breast cancer tissue array samples (C) are shown. Bar = 50 µm. Overall results summarized in KIAA1199 IHC charts where + indicates intensity level of KIAA1199 staining and the number equals the number of cases with indicated intensity level for each tissue type. A χ² test was performed to assess differences in intensity level of KIAA1199 staining. All statistical tests were two-sided. D) Association of patient survival probability (Kaplan–Meier analysis) with KIAA1199 expression (DNA microarray data mining) in three cohorts of patients with breast cancer. Log-rank (Mantel Cox) was used to determine statistical significance. All statistical tests were two-sided.
to no expression of KIAA1199 was found in adjacent normal epithelial cells, stromal cells, or benign breast tissues (Figure 1B). Furthermore, IHC analyses using human breast cancer tissue microarrays (US Biomax), which contained a total of 135 samples, revealed a statistically significant difference in KIAA1199 staining in invasive cancer tissue as compared with benign breast tissue samples ($P < .0001$) (Figure 1C). However, there was no statistically significant difference between grades of invasive tissue.

To elucidate the clinical significance of KIAA1199 on the survival probability of breast cancer patients, three publicly available DNA microarray datasets (13–15) were analyzed. When these samples were grouped based on KIAA1199 mRNA expression dichotomized at the mean, high KIAA1199 expression was inversely associated with patient survival in all datasets (Stockholm cohort: $P = .03$; Rotterdam cohort: $P = .02$; Uppsala cohort: $P = .02$) (Figure 1D). Together, these results suggest an important role of KIAA1199 in cancer progression.

**KIAA1199 as Novel Cell Migration Promoting Gene**

To unravel the function of KIAA1199 in cancer, we first surveyed KIAA1199 expression in cell lines derived from different cancers. Notably, KIAA1199 is highly expressed in invasive cell lines compared with corresponding minimally invasive lines in all lines tested, which included prostate, breast, and colon cancer lines (Supplementary Figure 2, available online). A targeted gene-silencing technique was then employed to silence KIAA1199 in metastatic human MDA-MB-435 breast cancer cells (16) because they were found to express high levels of endogenous KIAA1199 (Supplementary Figure 2, available online). Surprisingly, the KIAA1199-silenced cells (85% reduction in KIAA1199 mRNA; $P < .001$) (Figure 2A and Figure 4C) gradually changed their morphology from a typical mesenchymal-like shape to a polarized epithelial-like shape (Figure 2B), which was accompanied by a reorganization of F-actin from stress fibers to a cortical ring-like staining pattern. Similar morphological changes were observed in MDA-MB-231 cells expressing KIAA1199 small hairpin RNA (shRNA) (Supplementary Figure 3A, available online). Further analysis revealed a state of mesenchymal-to-epithelial-transition as indicated by a decrease in mesenchymal markers (vimentin, N-cadherin, and Twist-1) and an increase in epithelial markers (cytokeratin-8/18) (Figure 2C).

Because mesenchymal-to-epithelial-transition phenotypic changes often result in decreased cell migration, we hypothesized that KIAA1199 may be associated with cancer cell migration. Indeed, silencing of endogenous KIAA1199 resulted in a statistically significant decrease (75% reduction; $P < .001$) in migratory ability as examined by a Transwell chamber migration assay (Figure 2D). The migratory ability was rescued by overexpression of KIAA1199 cDNA in the silenced cells (Figure 2E). Similar results were observed in invasive MDA-MB-231 cells examined by a phagokinetic cell migration assay (Supplementary Figure 3B, available online). Additionally, overexpression of KIAA1199 cDNA in minimally invasive MCF-7 cells (Supplementary Figure 3C, available online) statistically significantly induced cell migration (2.75 fold increase; $P < .001$), comparable with that of cells transfected with the highly migratory matrix metalloproteinase 14 (MMP-14) (threefold increase; $P < 0.001$), a membrane anchored-matrix metalloproteinase (Figure 2F). This increased migratory ability of MCF-7 cells is associated with phenotypic changes associated with an EMT, as demonstrated by increased mesenchymal markers (Twist-1) and decreased epithelial markers (E-cadherin and cytokeratin-8/18) in cells stably expressing KIAA1199-GFP (Figure 2G).

Because cell migration is a critical determinant of invasion, we examined the function of KIAA1199 in cancer invasion using a three-dimensional invasion assay (17). Silencing of KIAA1199 in MDA-MB-435 cancer cells abrogated invasion into surrounding matrices (Figure 2H) without notably affecting cell proliferation (Figure 3B). Collectively, these results further support the role of KIAA1199 as a key molecule involved in maintaining an aggressive mesenchymal phenotype of cancer cells.

**Attenuation of Cancer Metastasis by Silencing KIAA1199**

Enhanced cell invasion has been shown to promote metastasis (1,18). To probe the relevance of our in vitro findings in an in vivo setting, we employed an orthotopic mouse model previously used to study the link between cell migration/invasion and metastasis (19). MDA-MB-435 cancer cells expressing KIAA1199 shRNA or luciferase control shRNA were selected and maintained as pools of resistant cells to avoid artifacts due to the use of single clones. This cell line was chosen because MDA-MB-435 cells express a high level of endogenous KIAA1199 and possess a high metastatic potential that could be used for in vivo evaluation. The cells were orthotopically injected into the mammary fat pads of immunodeficient mice, and tumorigenicity was monitored. Tumor growth of MDA-MB-435 cells stably expressing KIAA1199 shRNAs grew slowly compared with uninjected MDA-MB-435 cells and luciferase shRNA control groups over 4 weeks of observation (Figure 3A). The reduced tumor volume in the KIAA1199 shRNA group was not due to an effect on cell proliferation because silencing of KIAA1199 did not alter cell proliferation as examined by CellTiter-Glo (Promega, Madison, WI) (Figure 3B). At necropsy, histological analyses revealed tumor micrometastases in the lungs of six of eight (75%) mice from control groups compared with one of eight (12.5%) mice bearing cells expressing KIAA1199 shRNA. Among metastasized tumors in mice bearing KIAA1199-silenced cells, the number and size of metastases were statistically significantly reduced (80% reduction in number; $P < .001$) (Figure 3C and D). IHC analyses confirmed knockdown of KIAA1199 expression within the tumor tissue, strengthening the conclusion that endogenous expression of KIAA1199 leads to a more invasive and metastatic phenotype (Figure 3E). To further validate the role of KIAA1199-mediated cell migration in tumor metastasis, an experimental metastasis mouse model was employed by injecting tumor cells ($2 \times 10^7$ per mouse) into the lateral tail vein of 5-week-old immunodeficient mice. Six nonconsecutive tissue sections were microscopically examined for tumor cell extravasation to and growth in the lungs after 8 weeks. Although mice injected with parental MDA-MB-435 or MDA-MB-435 cells expressing luciferase shRNA displayed macrometastases in the sections, silencing of KIAA1199 in the cells statistically significantly diminished experimental metastasis in the lungs ($P < .0001$) (Figure 3F). This observation suggests that impairment of KIAA1199-mediated tumor cell migration efficiently reduces tumor extravasation, hence preventing tumor metastasis.

**Subcellular Localization of KIAA1199**

To unravel the mechanism underlying KIAA1199-mediated cell migration, we first determined the subcellular localization of KIAA1199 by employing immunostaining and
KIAA199 is a novel cell migration–promoting gene. A) Western blotting analysis of luciferase- (Luc-) and KIAA199-silenced MDA-MB-435 cells confirms knockdown of KIAA199 endogenous expression. COS-1 cells transfected with vector and KIAA199 cDNAs were used as controls. Tubulin was used as a loading control (top panel). Total RNA was analyzed by real-time reverse-transcription polymerase chain reaction using KIAA199-specific primers. The expression of KIAA199 was normalized using housekeeping gene HPRT-1 (bottom panel). Labels for the bottom panel correspond to that in the top panel. Each bar represents the mean ± standard deviation. *** P < .001. All statistical tests were two-sided. B) Morphological examination of MDA-MB-435 cancer cells expressing Luc small hairpin RNA (shRNA) control and KIAA199 shRNA (phase contrast images). The distribution of filamentous actin in KIAA199-silenced and control MDA-MB-435 cells was examined by TRITC-phalloidin staining (F-actin). Scale bars = 20 µm. Representative images of three individual experiments. C) Western blotting analysis of total cell lysate from MDA-MB-435 cells (control) or MDA-MB-435 cells expressing Luc or KIAA199 shRNAs using antibodies against epithelial-to-mesenchymal transition (EMT) markers. β-Actin was used as a loading control. Representative images of three individual experiments. D) Transwell chamber migration assay performed using MDA-MB-435 cells expressing shRNAs as indicated. Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t-test was used to assess differences. **P < .01. All statistical tests were two-sided. E) Western blotting analysis of Luc- and KIAA199-silenced MDA-MB-435 cells transiently transfected with KIAA199 cDNA (top panel). β-Actin was used as a loading control. Representative images of three individual experiments. Cell migratory ability was assessed using Transwell chamber migration assay (bottom panel). Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t test was used to assess differences. All statistical tests were two-sided. F) Transwell chamber migration assay performed using MCF-7 cells transfected with cDNAs as indicated (top panel). Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t test was used to assess differences for both experiments. ***P < .001. All statistical tests were two-sided. The cell migratory ability was also assessed using a dot-based cell migration assay by monitoring cell movement away from the initial dot (bottom panel). Representative images of three individual experiments. G) Western blotting analysis of cell lysates from MCF-7 cells stably expressing either green fluorescent protein (GFP) (control) or KIAA199-GFP cDNAs using antibodies against EMT markers and KIAA199. β-Actin was used as a loading control. Representative images of three individual experiments. H) Three-dimensional invasion assay performed in MDA-MB-435 cells expressing shRNAs as indicated. Invaded cells were microscopically determined by monitoring cell movement away from the initial three-dimensional cell-matrix dot. Images represent day 8 under phase contrast microscopy. Scale bar = 50 µm. Representative images of three individual experiments performed with duplicates.
Figure 3. Silencing of KIAA1199 in MDA-MB-435 cells results in attenuated tumor growth and inhibits lung metastasis. A) MDA-MB-435 cells stably infected with control luciferase (Luc) small hairpin RNA (shRNA) or KIAA1199 shRNAs were injected into the mammary fat pads of 4–5-week-old female nude mice. Parental cells (No infection) were used as a control. Tumor size was measured every 4 days. n = 8 mice per group. B) Cell proliferation assay was performed using MDA-MB-435 cells stably expressing Luc shRNA or KIAA1199 shRNA-2 using CellTiter-Glo over 5 days. Parental cells (No infection) were used as a control. Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. C) The number of lung micrometastases from each mouse was microscopically determined at the end of the experiments. Student t test was used to assess differences. ***P < .001. All statistical tests were two-sided. D) Representative hematoxylin and eosin-stained images of the lungs with (Luc shRNA) or without (KIAA1199 shRNA-1, and -2) metastasis are shown. Arrow lines indicate metastasized tumors in the lungs. Insets represent enlarged areas of the lung tissues. Scale bar = 100 μm. E) Representative immunohistochemical images of tumor tissue from mice injected with either MDA-MB-435 cells expressing Luc shRNA or KIAA1199 shRNA-1 or -2 stained with anti-KIAA1199 antibody. Minimal to no staining was observed in tissue expressing KIAA1199 shRNAs. Scale bar = 100 μm. F) Incidence of pulmonary metastasis in mice 8 weeks after receiving tumor cells by tail vein injection. A χ² test with exact P value was performed to assess differences. All statistical tests were two-sided. Representative hematoxylin and eosin-stained lung tissue sections are presented. n = 7 mice per group. Scale bar = 50 μm.
fluorescent tagging approaches. KIAA1199 fused with a GFP at the C-terminus exhibits a polygonal network of interconnected tube-like structures with lengths ranging from 1 to 2.5 μm when transfected into COS-1 cells and colocalizes with calreticulin–orange fluorescent protein (OFP), an ER marker fused with OFP (Invitrogen) (Figure 4A). This characteristic ER staining pattern, along with an absence of nuclear staining, is also observed in transfected COS-1 and MCF-7 examined by immunostaining (Supplementary Figure 4, available online). To rule out the possibility that KIAA1199 ER localization is due to temporal ER retention of the overexpressed proteins, MMP-14-GFP chimeras (with signal peptide; plasma membrane localization) and GFP (lacks signal peptide; diffuse throughout cells) were used as controls. The KIAA1199 distribution pattern was distinguishable from GFP and MMP-14 (Figure 4A). Immunostaining of endogenous KIAA1199 in MDA-MB-435 cells revealed 78.8% colocalization with protein disulfide isomerase, another well-characterized ER marker (Figure 4B). No staining was observed in KIAA1199-silenced MDA-MB-435 cells, demonstrating specificity of the anti-KIAA1199 antibody (Figure 4C).

To investigate the dynamics of KIAA1199 in the ER, FRAP was performed in living KIAA1199-GFP expressing COS-1 cells pretreated with cycloheximide to inhibit protein synthesis (20). After photobleaching, almost complete recovery of fluorescence occurred within 7 ± 0.2 minutes compared with an unbleached area (Figure 4D). This recovery represents the diffusional exchange of unbleached KIAA1199-GFP within the ER for bleached KIAA1199-GFP, as recovery was abolished when fixed cells were photobleached (data not shown). These data indicate that KIAA1199 is an ER resident protein but not a structural component of the ER because it rapidly traffics within the ER compartment.

**Molecular Mechanism of KIAA1199 ER Retention**

Because KIAA1199 lacks an ER retention signal (KDEL), we investigated the requirement for a specific region of KIAA1199 for ER retention. A series of KIAA1199 deletion mutants with a C-terminal Myc tag, which allows for detection of mutants regardless of loss of epitope, was generated (Figure 5A). ER retention was assessed based on the release of recombinant protein to the extracellular medium. As shown in Figure 5B, deletion from amino acid 296 (KIAA1199ΔC), resulted in secretion of the mutant. However, mutant KIAA1199ΔD, which is deleted from amino acid 591, is retained within the cell, indicating that the motif spanning Ala295 to Thr591 (termed the B domain) is required for KIAA1199 ER retention. Deletion from the C-terminal half of the B-domain (KIAA1199ΔC–Myc) resulted in partial secretion of the protein.

The requirement of the B-domain in ER retention of KIAA1199 was further evaluated by fusing the B-domain to the C-terminus of an unrelated secretory protein, soluble MMP-14 (SolMMP14), an MMP-14 mutant that lacks the transmembrane and cytoplasmic domains (SolMMP14-B) (21). Secretion of SolMMP14 was disrupted by addition of the B-domain, as shown in COS-1 cells transfected with SolMMP14-B cDNA (Figure 5C). Furthermore, SolMMP14-B chimera exhibits a meshwork staining pattern that is distinguishable from wild-type and SolMMP14 cellular distributions (Figure 5D). However, retention of SolMMP14 in the ER was unable to induce cell migration comparable with wild-type MMP-14 (Figure 5E). Taken together, our data suggest that although the B domain is necessary for KIAA1199 ER retention, it alone is insufficient for increased cell migration.

To investigate the mechanism of KIAA1199 ER retention, a SNAP-tag pull-down assay followed by proteomic analysis was performed. The ER chaperone protein, GRP78/BiP, was specifically pulled down by KIAA1199 (Supplementary Table 1, available online). The authenticity of this interaction was verified by coimmunoprecipitation, which showed that wild-type, but not KIAA1199ΔB, coprecipitated with BiP in transfected COS-1 cells (Figure 5F). Further analyses suggest that this interaction is not due to BiP’s role in the induction of the unfolded protein response due to ER stress (Supplementary Figure 5, A and B, available online), as a transient chaperone for nascent protein folding (Supplementary Figure 5C, available online) or in degrading misfolded proteins (Supplementary Figure 5D, available online).

Because BiP has been shown to enhance cell migration and metastasis (22,23), the role of the KIAA1199–BiP interaction in cell migration was examined by transiently silencing BiP using an shRNA approach (Supplementary Figure 6, available online). KIAA1199-mediated cell migration was abrogated in BiP-silenced COS-1 cells expressing KIAA1199 cDNA (42% reduction as compared with luciferase shRNA control cells expressing KIAA1199, P = .03) (Figure 5G). No effect on pcDNA vector control cell migration was observed upon knockdown of BiP, indicating that the effect observed was specific to KIAA1199-enhanced migration. In support of this, COS-1 cells overexpressing KIAA1199ΔB, which failed to interact with BiP, resulted in no enhancement of cell migration (Figure 5H). Taken together, these data indicate that KIAA1199 forms a stable complex with BiP, leading to ER retention and cell migration.

**Signaling Cascade of KIAA1199-Induced Cell Migration**

Based on the role of BiP in calcium homeostasis and the regulation of cell migration by calcium signaling (24–26), we analyzed Ca²⁺ levels in KIAA1199-expressing cells. Using flow cytometry to analyze cells loaded with the Ca²⁺-sensitive dye calcium green-1AM, we observed an increase in the percentage of cells exhibiting an elevated level of cytosolic Ca²⁺ in COS-1 cells transfected with KIAA1199 cDNA (13.8%) compared with control cells expressing vector (2.39%), MMP-14 (2.78%), and SolMMP14-KDEL (3.64%), an engineered ER-resident protein (a chimera of SolMMP14 and the KDEL motif) (Figure 6A; Supplementary Figure 7, A–C, available online). Increased cytosolic Ca²⁺ was further confirmed using Fura-2AM and spectrophotometry in both COS-1 and MCF-7 cells stably expressing KIAA1199 (Figure 6B).

Because KIAA1199 is localized in the ER and interacts with BiP, we hypothesized that the increased cytosolic Ca²⁺ is due to Ca²⁺ leakage from ER stores. To test this hypothesis, we performed fluorescence resonance energy transfer using the ER-targeted Ca²⁺ sensor, D1ER, a cameltoe peptide that consists of fusions of CFP and YFP separated by Ca²⁺-responsive elements (9). A statistically significantly diminished level of ER Ca²⁺, as evidenced by a lower YFP/CFP ratio, was found in both COS-1 (80% reduction; P < .001) and MCF-7 cells (75% reduction; P < .001) stably expressing KIAA1199 as compared with pQXIP vector control cells (Figure 6C; Supplementary Figure 7D, available online).
Figure 4. KIAA1199 is a novel endoplasmic reticulum resident (ER) protein. A) Microscopic determination of KIAA1199 cellular localization using Calreticulin–orange fluorescent protein (OFP)–expressing COS-1 cells transfected with green fluorescent protein (GFP), MMP-14–GFP, and KIAA1199–GFP chimeric cDNAs, followed by confocal microscopic examination. Dashed arrow lines represent orientation from nucleus to plasma membrane. Insets represent enlarged areas showing polygonal meshwork. Scale bar = 10 µm. Representative images from three individual experiments. B) Confocal microscopic examination of immunofluorescent staining of endogenous KIAA1199 in MDA-MB-435 cancer cells using antibodies against KIAA1199 and protein disulfide isomerase (PDI) (top panel). Using Zeiss LSM 3.2 software, 78.8% colocalization was found between KIAA1199 and PDI staining (bottom panel). Images represent part of the cell to display ER polygonal meshwork. Scale bar = 20 µm. Representative images from three individual experiments. C) Microscopic examination of immunofluorescent staining of endogenous KIAA1199 in MDA-MB-435 cells expressing luciferase (Luc) small hairpin RNA (shRNA) or KIAA1199 shRNA-2 using antibodies against KIAA1199. No staining was observed in KIAA1199 silenced cells. Scale bar = 20 µm. Representative images from three individual experiments. D) Dynamics of KIAA1199 in the ER. Fluorescence intensities from fluorescent recovery after photobleaching analyses in cycloheximide-treated COS-1 cells expressing KIAA1199-GFP were plotted against time for KIAA1199-GFP in the ER (top panel). Representative cell images before bleaching, immediately after bleaching, and after 600 seconds are shown (bottom panel). Box 1 shows the bleached area. Box 2 shows the unbleached area. Representative images from three individual experiments.
Figure 5. Endoplasmic reticulum (ER) retention of KIAA1199 by binding immunoglobulin protein (BiP) interaction is required for migration. A) A schematic diagram of deletion mutants of KIAA1199. All mutations contain KIAA1199 signal peptide (green box) and a Myc tag (pink box) that was fused to the C-terminus of each mutant. B) Western blotting analysis of trichloroacetic Acid (TCA)-precipitated conditioned media (CM) and cell lysates from COS-1 cells transfected with the series of KIAA1199 deletion mutants using anti-Myc antibody. Tubulin was used as a loading control. Representative images from three individual experiments. C) Western blotting analysis of TCA-precipitated CM and cell lysate in COS-1 cells transfected with cDNA as indicated using anti-MMP-14 antibody. β-Actin was used as a loading control. Representative images from three individual experiments. D) Immunostaining of COS-1 cells transfected with cDNA as indicated using anti-MMP-14 antibody. Insets represent an enlarged area for detailed structures. Scale bars: top left panel = 5 μm; other panels = 10 μm. Representative images from three individual experiments. E) Transwell chamber migration assay of COS-1 cells transfected with cDNAs as indicated. Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t test was used to assess differences. All statistical tests were two-sided. F) SNAP pull-down assay using COS-1 cells transfected with cDNA as indicated, followed by Western blotting analysis (top panel). Input (SNAP antibody; middle panel) and loading controls (tubulin antibody; bottom panel) were examined by Western blotting. Representative images from three individual experiments. G and H) Transwell chamber migration assay using COS-1 cells transiently expressing BiP or Luciferase (Luc) small hairpin RNA (shRNA) along with vector pcDNA3 and KIAA1199 cDNAs (G) or COS-1 cells transfected with cDNAs as indicated (H). Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t test was used to assess differences. ***P < .001. All statistical tests were two-sided.
Figure 6. KIAA1199 expression in the endoplasmic reticulum (ER) results in ER Ca\(^{2+}\) release and increased cytosolic Ca\(^{2+}\) leading to enhanced cell migration. A) Flow cytometry of cytosolic Ca\(^{2+}\) in transfected COS-1 cells loaded with calcium green1-AM and propidium iodide. Vector, plasma membrane anchored MMP-14 and ER-retained soluble MMP-14 were used as controls. Representative images from three individual experiments. B) Spectrofluorometry of cytosolic Ca\(^{2+}\) in FURA-2AM-loaded COS-1 and MCF-7 cells stably expressing KIAA1199 or vector control cDNA. The ratios of the fluorescence intensities (emission at 340/380 nm; Ca\(^{2+}\) bound/unbound) were plotted against time for cytosolic Ca\(^{2+}\). RFU = relative fluorescence units. Representative images from three individual experiments performed with duplicates. C) Fluorescence resonance energy transfer analysis for determination of ER Ca\(^{2+}\) level in vector and KIAA1199 stably expressing COS-1 and MCF-7 cells transfected with D1ER cDNA. Ratio of yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) was determined by measuring individual cells. Vector pQCXIP: COS-1 (n = 23) and MCF-7 (n = 29); KIAA1199: COS-1 (n = 32) and MCF-7 (n = 16). R\(_{\text{min}}\) was determined by treating cells with 3 mM EGTA + 2 mM ionomycin. Student t test was used to assess differences. ***P < .001. All statistical tests were two-sided. Representative images from three individual experiments each with a minimum of 15 cells per cell condition. D and E) Scratch wound healing assay for assessing the effect of 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) on KIAA1199-mediated COS-1 and MCF-7 stable cell migration. Representative images of COS-1 cells taken at 0 hours and 18 hours under ×4 magnification from one of three individual experiments are shown (D, top panel). Quantification of fold change in wounded area was analyzed using NIS Elements BR 3.2 software (Nikon, Melville, NY) (D, bottom panel, and E). Scale bar = 100 μm. Each bar represents the mean ± standard error of the mean of three independent experiments performed with triplicates. Student t test was used to assess differences. All statistical tests were two-sided.
To link KIAA1199-induced cytosolic Ca\(^{2+}\) increase with enhanced cell migration, the intracellular Ca\(^{2+}\) chelator 1,2-Bis (2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) was used in a cell migration assay. Treatment of COS-1 stable cells with BAPTA-AM statistically significantly reduced KIAA1199-mediated cell migration but had little effect on control cell migration (Figure 6D). Similar results were obtained using MCF-7 stable cells (Figure 6E). These results suggest that the KIAA1199-induced ER Ca\(^{2+}\) leakage, leading to elevated cytosolic Ca\(^{2+}\), is required for the enhanced cell migration.

To elucidate the signaling cascade of KIAA1199-induced cell migration, an antibody array was performed (Kinex Microarray, Kinexus Bioinformatics Corp, Vancouver, British Columbia, Canada). The array revealed a statistically significant increase in the Thr\(^{674}\) phosphorylated form of the protein kinase C-gamma isoform (PKC\(\gamma\)) in MCF-7 cells stably expressing KIAA1199 cDNA as compared with vector control (data not shown) using an antibody that cross-reacts with the phosphorylated PKC\(\alpha\), \(\beta\), and \(\gamma\) isoforms (Invitrogen). Validation of the array was demonstrated by increased phosphorylation of PKC in whole cell lysates of KIAA1199-expressing MCF-7 stable cells using the cross-reactive phospho-PKC\(\gamma\) antibody (Figure 7A). Immunostaining revealed a redistribution of PKC from the cytosolic compartment in control cells to the plasma membrane in KIAA1199-expressing cells (Figure 7B).

Because this translocation of PKC to the plasma membrane is required for activation and function of PKCs (27), crude membrane fractions were analyzed using pan-specific antibodies to determine which cross-reacting PKC isoform(s) is specifically translocated in KIAA1199-expressing cells. PKC\(\alpha\) showed increased membrane localization in KIAA1199-expressing MCF-7 stable cells as compared with control cells (Figure 7C, top panel). Neither the \(\beta\) nor \(\gamma\) isoforms were detected (Supplementary Figure 8, available online); these results agree with reports showing no \(\beta\) or \(\gamma\) expression in MCF-7 cells (28).

Furthermore, decreased membrane localization of PKC\(\alpha\) was observed upon silencing of KIAA1199 in MDA-MB 231 cells (Figure 7C, bottom panel), which is consistent with the decreased migratory capacity of these cells (Supplementary Figure 3B, available online). Increased PKC\(\alpha\) membrane localization was also recapitulated in KIAA1199-expressing COS-1 cells (Figure 7D). Furthermore, incubation of KIAA1199-expressing cells with BAPTA-AM substantially diminished membrane-associated PKC\(\alpha\) in COS-1 stable cells (Figure 7D), as well as in MCF-7 stable cells (data not shown). These data demonstrate a functional link between increased cytosolic Ca\(^{2+}\) and PKC\(\alpha\) activation.

To examine whether PKC\(\alpha\) is required for KIAA1199-mediated migration, PKC\(\alpha\) was silenced in COS-1 cells using an shRNA approach. Decreased PKC\(\alpha\) mRNA and protein expression were verified in cells infected with PKC\(\alpha\) shRNA (Figure 7E, a and b). Silencing of PKC\(\alpha\) diminished KIAA1199-enhanced cell migration (Figure 7E, c), confirming the cascade of KIAA1199-BiP–ER calcium leakage–PKC\(\alpha\) in KIAA1199-mediated cell migration.

Discussion

Before this work, the only studies on KIAA1199 were in regard to its upregulation in various forms of cancer. However, the pathological consequences of this upregulation were unknown. The key findings reported here are the unveiling of the pathological role of KIAA1199 in cancer cell migration and the elucidation of the signaling pathway downstream of KIAA1199. We show that elevated levels of KIAA1199 are exhibited in both invasive human cancer cell lines and breast cancer tissues. Furthermore, we demonstrate that KIAA1199 induces cancer cell migration by a BiP–Ca\(^{2+}\)–PKC\(\alpha\) cascade. In vivo analysis links the role of KIAA1199 in cell migration to cancer dissemination, as cell migration is one of the critical determinants of metastasis. Hence, this study establishes KIAA1199 as both a potential biomarker for cancer diagnosis and a target for cancer therapy aimed at preventing metastasis.

Our studies link KIAA1199 expression to cell migration and invasion based on the reversal of EMT upon silencing of endogenous KIAA1199 in invasive cancer cells. Subsequent studies showed that overexpression of KIAA1199 causes an EMT, leading to an increased migratory capacity of nonaggressive cancer cells. Multiple cell lines in loss- or gain-of-function studies were employed to validate these observations. Interestingly, knockdown of KIAA1199 inhibited both primary tumor growth and metastasis in a spontaneous metastasis mouse model without affecting cell proliferation in vitro. It is generally accepted that metastasis correlates closely with tumor burden (29), which is supported by the finding that inhibition of primary tumor growth often delays spontaneous metastasis (30). Based on our study, we speculate that a collaborative effort between reduced cell migration and subsequent decreased primary tumor growth contributes to reduced lung metastasis in mice bearing KIAA1199-silenced tumor cells. To precisely investigate the association of KIAA1199-mediated cell migration with metastasis, an in vivo experimental metastasis model was employed that demonstrated diminished lung metastasis in mice intravenously injected with KIAA1199-silenced cancer cells exhibiting impaired cell migration. This observation is consistent with the concept that both increased proteolytic activity and cell migratory ability are required for cancer invasion and blocking either biological role can reduce cancer invasion and metastasis.

Our study demonstrates that ER localization of KIAA1199 is a requisite for enhanced cell migration because loss of ER retention results in abrogation of cell migration. KIAA1199 has previously been reported to be localized primarily in the cytoplasm of cells (6–8), although nuclear localization has been reported in colon adenocarcinomas (31). Nevertheless, detailed cellular distribution has not been previously reported. Here we have demonstrated for the first time that both endogenous and exogenous KIAA1199 are localized in the ER, which requires a special motif (the B-domain) rather than the conventional ER retention signals. Additional data suggest that cooperation of this motif with other KIAA1199 domains is required for cell migration.

Further characterization reveals that KIAA1199 interacts with BiP by the B-domain to remain in the ER. We provide evidence to suggest a functional interaction between KIAA1199 and BiP: 1) deletion of the B-domain results in loss of interaction with BiP, KIAA1199 ER-retention, and inhibition of cell migration; and 2) silencing of BiP results in reduced KIAA1199-mediated cell migration. Collectively, our data indicate that the KIAA1199–BiP interaction is a specific cellular event required for ER retention and subsequent KIAA1199-mediated cell migration. Although the exact role of this interaction in KIAA1199-mediated cancer cell migration will require further study, BiP has been shown to negatively
Figure 7. KIAA1199-mediated cell migration involves a signaling cascade of KIAA1199– binding immunoglobulin protein (BiP)–endoplasmic reticulum (ER) calcium release– protein kinase C alpha (PKC\(\alpha\)). A) Western blotting analysis of MCF-7 cells stably expressing vector control or KIAA1199 cDNAs using nonselective phospho-PKC\(\gamma\) (p-PKC) antibody. Representative images from three individual experiments. GFP = green fluorescent protein. B) Immunofluorescence staining of MCF-7 cells stably expressing vector control or KIAA1199 using anti-phospho-PKC\(\gamma\) antibody. The signal strength of fluorescence was scanned along the white arrow lines. Graphs were plotted using Metamorph software. Scale bar = 10 μm. Representative images from three individual experiments. C) Western blotting analysis of whole cell (WC) and fractionated cell lysates (cytosolic [Cyto]; membrane [Mem]) of MCF-7 stable cells (top panel) and fractionated cell lysates from MDA-MB 231 wild-type (WT), luciferase (Luc) small hairpin RNA (shRNA), and KIAA1199 shRNA-1 and -2 cells (bottom panel) using anti-pan PKC\(\alpha\) antibody. Tubulin was used as a loading control. Representative images from three individual experiments. D) Western blotting analysis of fractionated cell lysates of COS-1 stable cells treated with DMSO or 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) using anti-pan PKC\(\alpha\) antibody. Tubulin was used as a loading control. Representative images from three individual experiments. E) Real-Time reverse-transcription polymerase chain reaction (a) and Western blotting (b) analyses of PKC\(\alpha\) in COS-1 cells expressing shRNAs against Luc or PKC\(\alpha\). Representative of three independent experiments each with two replicates. Effect of silencing PKC\(\alpha\) in COS-1 cells transfected with KIAA1199 or vector control on cell migration was examined by a Transwell chamber migration assay (c). Each bar represents the mean ± standard deviation of three independent experiments performed with triplicates. Student t test was used to assess differences for both experiments. ***P < .001. All statistical tests were two-sided.
correlate with patient survival, and silencing of BiP has been shown to abrogate gastric and head and neck cancer cell invasion (23, 32).

The mechanism underlying KIAA1199-mediated cell migration relies on ER Ca$^{2+}$ leakage followed by PKC$\alpha$ activation. Because of calcium’s involvement in numerous cellular processes, including development, apoptosis, transcriptional regulation, and motility (33), it is strictly maintained within the cell, with the ER serving as the major storage site (34). A rise in intracellular Ca$^{2+}$ has been shown to lead to activation of various signaling cascades, including mitogen-activated protein kinase (MAPK), PKC, and integrin signaling, all of which play roles in cell movement (35). Our study demonstrates a requirement for KIAA1199-induced cytosolic Ca$^{2+}$ accumulation in cell migration. Although the exact mechanism of KIAA1199-induced ER Ca$^{2+}$ release needs further elucidation, recent studies have reported proteins capable of causing changes in steady-state Ca$^{2+}$ levels, including Bcl-2 and Presenilin (36, 37). Of particular interest is another KIAA protein, known as Fam38A or KIAA0233, that is also localized to the ER. Fam38A has been shown to mediate cell adhesion by a pathway that requires increased release of calcium, resulting in downstream signaling that ultimately leads to integrin activation (38). Furthermore, elevated cytosolic Ca$^{2+}$ has been shown to result in increased migration by PKC activation (35). These findings are in agreement with our data demonstrating involvement of PKC$\alpha$ downstream of Ca$^{2+}$ signaling in KIAA1199-mediated cell migration. Additionally, PKC$\alpha$ has been shown to be involved in cancer cell motility and invasion, and it has been correlated with aggressiveness (39).

Although our study links KIAA1199 to enhanced cancer cell migration, it seems likely that there is not a unique route through which tumor cells gain the capacity to migrate. Pinpointing the requirement for KIAA1199 in the complicated signaling network involved in cancer cell migration will determine whether KIAA1199 is a key druggable target in prevention of cancer metastasis. One of the limitations of this study is the lack of a deep understanding of the minimal binding motif of KIAA1199 required for the BiP interaction. Although we identified a region within KIAA1199 that is required for this interaction and retention within the ER, further characterization of this minimal motif can potentially provide a novel treatment strategy aimed at blocking KIAA1199-mediated cell migration. Another limitation of this study is the cell line used in the animal models. Although the role of KIAA1199 has been validated in different cell lines using various in vitro models, only MDA-MB-435 cells have been examined in spontaneous and experimental metastasis mouse models. Because the origin of MDA-MB-435 cells is still debatable, additional in vivo studies using cell lines from other cancer types may strengthen our observations and ensure that the role of KIAA1199 described here is applicable to other cancer cell types. The lack of a knockout mouse model, which would allow for further testing of the physiological role of KIAA1199, is an additional limitation of this study. Nonetheless, this study widens our understanding of cancer cell migration and provides a novel target for preventing cancer invasion and metastasis. Overall, our observations have reinforced the connections between KIAA1199,
BiP, ER Ca\(^{2+}\) leakage, and PKC\(\alpha\) activation in KIAA1199-induced cell migration (Figure 8). In addition, recently published data on KIAA1199 promoter regulation now provides insights into the mechanism of upregulation of KIAA1199 expression in human cancer by direct binding of nuclear factor kappa-light-chain-enhancer of activated B cells to the KIAA1199 promoter (40). However, our understanding of other potential pathways involved in KIAA1199-mediated cell migration is far from complete. Further work will be required to understand the complete mechanistic basis of the effects we have observed. Given the long half-life of KIAA1199 and constant association with BiP, KIAA1199 may also function as part of an undiscovered BiP chaperoning complex, which we are currently investigating. Nonetheless, this study widens our understanding of cancer cell migration and provides a novel target for preventing metastasis. Our data demonstrate that alterations in KIAA1199 expression contribute to the loss of epithelial cell architecture and aggressiveness, making KIAA1199 a strong candidate for a bona fide human invasion-promoting gene.

References


**Funding**

This study was supported in part by the National Institutes of Health (NIH)/National Cancer Institute (grants R01CA113553 and R01CA166936 to JC) and the Carol Baldwin Breast Cancer Foundation (to JC); the NIH/NIH Heart, Lung, and Blood Institute (grant R01HL091939 to WFB); and a Merit Review grant from the Department of Veterans Affairs (to SZ).

**Notes**

N.A. Evensen and C. Kuscu contributed equally to this work.

This article is dedicated to the memory of Dr Yong-jun Hu who lost his battle with colon cancer recently. We thank Dr Stephen J. Weiss (University of Michigan) for critical reading of this manuscript. We thank Dr Jie Yang, Dept of Preventive Medicine, Stony Brook University, for assistance with statistical analysis.

**Affiliations of authors:** Department of Medicine/Cancer Prevention (NAE, CK, H-LN, KZ, AD, AP-G, JC), Department of Pathology (YH, JC), Department of Medicine/Hematology & Oncology (WFB, SZ), Stony Brook University, Stony Brook, NY; Department of Research, Veterans Affair Medical Center, Northport, NY (H-LN, PK, SZ); Centre for Blood Research and Departments of Biochemistry and Molecular Biology and Oral Biological and Medical Sciences, University of British Columbia, Vancouver, BC, Canada (AD).