Distinct regulatory effect of the p34^{SEI-1} oncoprotein on cancer metastasis in HER2/neu-positive and -negative cells

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Abstract. The p34^{SEI-1} oncoprotein is involved in a transcriptional regulation, cell cycle regulation, apoptosis, development and many other important cellular functions. Our present study suggests that p34^{SEI-1} can promote metastasis by enhancing migration and invasion of cancer cells. Consistently, p34^{SEI-1} expression was found to be increased as the tumor invasiveness progressed in human breast tissues. p34^{SEI-1} may promote cancer metastasis by activating the PI3K/AKT signaling pathway. In this process, p34^{SEI-1} activates two different serine/threonine kinases, AKT or ILK, depending on the expression status of HER2/neu oncogene. In HER2/neu suppressed cancer cells, p34^{SEI-1} promoted metastasis mainly by activating AKT via phosphorylation of the 473 serine residue. In HER2/neu expressing cancer cells, p34^{SEI-1} overexpression downregulates HER2/neu expression, leading to the activation of another crucial serine/threonine kinase ILK due to phosphorylation of the 178 threonine residue instead of AKT. These results suggest that p34^{SEI-1} affects cancer metastasis by regulating two different signaling pathways depending on the HER2/neu expression level, in which AKT and ILK modulation can be stimulated by p34^{SEI-1} overexpression.

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Abbreviations: p34^{SEI-1}, 34-kD protein encoding *SEI-1* (selected with Ink4a-1 as bait) gene; HER2/neu, human epidermal growth factor receptor 2; ILK, integrin-linked kinase; PI3K, phosphoinositide-3 kinase; AKT, serine/threonine-specific protein kinase

Key words: metastasis, 34-kD protein encoding *SEI-1* gene, human epidermal growth factor receptor 2, phosphoinositide-3 kinase/serine/threonine-specific protein kinase, integrin-linked kinase

Introduction

Most of the cancer deaths result from metastasis. In many cases, survival rate of cancer patients with metastatic tumors is much lower than those of patients with localized tumors. Metastasis of breast cancer, the most common form of cancer in woman, is responsible for nearly 90% of deaths from breast cancer. Although breast cancer comprises about 25% of all cancers, the survival rates of patients with breast cancer are relatively high as long as cancer is detected and treated before it metastasizes. Therefore, better understanding of the mechanism that promotes metastasis of breast cancer would be helpful to design effective therapies.

The phosphoinositol-3-kinase (PI3K)/AKT pathway is involved in multiple cellular processes including cell differentiation, proliferation, survival, angiogenesis, invasion and metastasis (1-8). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), yielding phosphatidylinositol 3,4,5-triphosphate (PIP_3), which recruits and activates phosphatidylinositol-dependent kinase 1 (PDK1). Activated PDK1 phosphorylates serine/threonine-specific protein kinase (AKT), also known as protein kinase B (PKB). AKT activation can be induced by phosphatase and tensin homolog (PTEN) abnormality (9-11). PTEN tumor suppressor is a negative regulator of the PI3K/AKT pathway, in which PTEN dephosphorylates PIP_3 to PIP_2 (12). Mutation of deletion of PTEN is frequently found to be mutated or deleted in a broad range of cancers, resulting in the hyper-activation of the PI3K/AKT signaling pathway. In addition, the HER2/neu proto-oncogene (also known as ErbB-2, CD340 or p185) is the most oncogenic signaling activator of PI3K/AKT, in which HER2/neu can phosphorylate PIP_2 and in turn induce AKT activation (13,14). HER2/neu plays important roles in the development and progression of certain aggressive types of breast cancer and is found at a relatively high level in most aggressive tumors. Activated AKT acts as a key regulator for many events related with tumor malignancies including cell survival, proliferation, growth, angiogenesis and metastasis (7,12,14-18). The critical steps to initiate the metastasis are the phosphorylation of AKT on serine 473 residue and regulation of its downstream target proteins. GSK3β is one of important downstream target proteins involved in metastasis. GSK3β inhibition promotes metastasis by affecting β-catenin, a dual

function protein that regulates the coordination of cell-cell adhesion and gene transcription (19-22). Deregulated β-catenin expression is associated with many cancers including malignant breast tumors (21,22). GSK3β can be inhibited in at least two ways in PI3K/AKT signaling pathway: activation of AKT to directly phosphorylate GSK3β on serine 9 residue (19) and directly/indirectly by integrin-liked kinase (ILK) (23). ILK is a multifunctional serine/threonine protein kinase that mediates a variety of cellular responses to integrin stimulation by extracellular matrix proteins. The mechanism of ILK activation is not fully understood but it is reported that p21 activated kinase (PAK) can phosphorylate ILK on threonine 173 for its activation (24). It was demonstrated that purified recombinant ILK phosphorylates recombinant GSK3β *in vitro* (25). It means that ILK can directly phosphorylate GSK3β. Furthermore, inhibition of ILK suppresses tumorigenesis and tumor growth (24,26). Exposure to ILK inhibitor suppresses the snail and β-catenin protein stability and transcriptional activity as well as GSK3β phosphorylation indicating that ILK activity affects the epithelial-mesenchymal transition (EMT) process. Despite these facts, its effect on cancer metastasis still remains unclear and controversial. It has been believed that ILK is a component of the PI3K-AKT pathway by phosphorylating AKT. However, it has been reported that ILK acts as an inhibitor of AKT phosphorylation even though cell proliferation was induced by stably overexpression of ILK (26,27).

With the goal of elucidating the roles of PI3K/AKT signaling pathway in cancer metastasis, we have previously reported the participation of the p34^{SEI-1} oncoprotein in this pathway. p34^{SEI-1} enhances cancer cell survival and promotes tumorigenesis by inducing NEDD4-1-mediated PTEN degradation (28). NEDD4-1 negatively regulates PTEN as a proto-oncogenic E3 ubiquitin ligase for PTEN and in turn activates the PI3K/AKT signaling pathway (29,30). Considering the vital roles of PI3K/AKT signaling pathway in metastasis and the indirect effect of p34^{SEI-1} on this pathway, it was suspected that p34^{SEI-1} may play an important role in the development of cancer metastasis. p34^{SEI-1} has multiple biological functions including transcription regulation, cell cycle regulation, inhibition of apoptosis and tumorigenesis (31-33). Furthermore, p34^{SEI-1} increases chromosomal instability, which is closely related to cancer invasiveness (34,35). We previously showed that the expression level of p34^{SEI-1} is significantly increased in cancer tissue compared to normal tissues, in which p34^{SEI-1} stabilizes X-linked inhibitor of apoptosis protein (XIAP) leading to an anti-apoptotic effect (33). XIAP also promotes progression of metastasis by activating the oncogenic NF-κB transcriptional factor, fibronectin-related gene expression and cell motility kinase such as focal adhesion kinase (FAK) or Src (36,37).

All these data implicate that p34^{SEI-1} may be involved in the progression of metastatic cancers. We therefore investigated whether or not p34^{SEI-1} has metastatic potential and the nature of the mechanism.

Materials and methods

Cell lines, cell culture and materials. Five cancer cell lines were used in this study. MCF7, T47D, HEK293T and MDA-MB-231 cancer cells were cultured in DMEM medium and SKBR3 cells were grown in RPMI medium (Welgene Inc., Daegu, Korea). All media were supplemented with 10% FBS (Gibco-BRL, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (Gibco-BRL). All cultures were grown at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2. LY294004 (cat no. 440202) and Cpd22 (cat no. 407331) were purchased from Calbiochem (La Jolla, CA, USA).

Reverse transcription (RT)-PCR. Total RNA was extracted from MDA-MB-231 cells with the RNeasy mini kit (cat no. 74106; Qiagen, Hilden, Germany) following the manufacturer's instructions. For reverse transcription, 1 μg RNA of each sample was subjected to cDNA synthesis using an oligo (dT) primer and the ImProm-II™ Reverse Transcription System (A3800; Promega, Madison, WI, USA). PCR amplification was performed using 10 ng cDNA, different sets of primers, and AccuPower PCR PreMix system (Bioneer, Daejeon, Korea). The amplification reaction was carried out using a PCR Thermal Cycler Dice (Applied Biosystems, Foster City, CA, USA). Each gene product was amplified using corresponding pairs of primers, in which β-actin gene product was used as an internal control. The oligonucleotide sequences for RT-PCR analysis were: pRT-p34^{SEI-1}-RT forward, 5'-AGGACCTCAGCCACATTGAG-3' and reverse, 5'-GGTGCCCAAAGTTCATTGTC-3'; pRT-HER2/neu-RT forward, 5'-CTGAACTGGTGTATGCAGAT-3' and reverse, 5'-CCACACAGTCACACCATAA-3'; pRT-NEDD4-1 forward, 5'-TGGGACATCACTTTGTGATC-3' and reverse, 5'-TGAGGCTTTTACTGGGGTC-3'; pRT-β-catenin forward, 5'-CATTTCCAATCTACTAATGC-3' and 5'-CTGCATTCTGACTTTCAGTA-3'; pRT-c-MYC forward, 5'-ACCAGCAGCGACTCTGAGGA-3' and reverse, 5'-TGACCCTCTTGGCAGCAGGATAGTCC-3'; pRT-ACTB forward, 5'-AGGTCGGAGTCAACGGATTTG-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3'.

Western blot analysis. Cells were recovered from culture by centrifugation at 3,000 rpm for 1 min and washed twice in an ice-cold phosphate-buffered saline (PBS) buffer. The cells were then lysed in RIPA lysis buffer and the protein was quantified using a protein assay kit (Bio-Rad, Hercules, CA, USA). Approximately 25 μg of total protein per sample was subjected to 12% SDS-PAGE and the resolved proteins were transferred to Immobilon transfer membranes (cat no. IPVH00010, Millipore, Billerica, MA, USA). The filter was blocked in 5% non-fat dry milk/0.1% Tween-20/Tris-buffered saline (TBS) followed by incubation with each corresponding antibody and immune-detection was accomplished using the Power Opti-ECL Western blotting detection reagent (Bionote, Hwaseong, Korea). Antibodies used in this study were purchased as follows: p34^{SEI-1} (ALX-804-645; Enzo Life Sciences, Farmingdale, NY, USA), NEDD4-1 (sc-25508) and PTEN (sc-7974; from Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (cat no. 610181) and N-cadherin (cat no. 610921; from BD Biosciences, Franklin Lakes, NJ, USA), vimentin (sc-7557), fibronectin (sc-8422), HER2/neu (sc-33684; from Santa Cruz Biotechnology), pAKT^{ser473} (cat no. 9271; Cell Signaling, Danvers, MA, USA), pILK^{thr178} (sc-130196; Santa Cruz Biotechnology), pGSK3β^{ser9} (cat no. 2435-1; Epitomics, Burlingame, CA, USA) and γ-tubulin (sc-7396; Santa Cruz Biotechnology).

Overexpression or suppression of p34^{SEI-1} or HER2/neu gene. For overexpression of p34^{SEI-1}, cells were plated at 1x10^6 cells in a 60-mm-diameter culture dish and transfected with 4 μg of either the control empty vector (pEF-BOS-EX) or the C-terminally Flag-tagged human p34^{SEI-1} protein (pEF-p34^{SEI-1}-Flag) for 6 h in serum free medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For knockdown of p34^{SEI-1} expression, MDA-MB231 cells were plated as above and transfected with 4 μg of either empty control vector (pLKO.1) or p34^{SEI-1} directed shRNA expression vector [pLKO.1/p34^{SEI-1}-short hairpin (sh) RNA] for 6 h in serum free medium using Lipofectamine 2000. After replacing the DNA-Lipofectamine complex-containing medium with complete, antibiotic-free growth medium, transfected cells were incubated for 48 h. For knockdown of endogenous HER2/neu, SKBR3 cells were plated 4x10^5 in a 60-mm-diameter culture dish and transiently transfected with 200 pmol of either scramble control RNA (scRNA) or a HER2/neu silencing siRNA (siHER2/neu) for 2 h in serum free medium using Lipofectamine 2000. After replacing the RNA-Lipofectamine complex-containing medium with complete growth medium, transfected cells were incubated for 48 h. pEF-BOS-EX and pEF-p34^{SEI-1}-Flag plasmids were kindly provided by Dr Rikiro Fukunaga (Osaka University, Osaka, Japan) and pLKO.1/p34^{SEI-1}-shRNA plasmid was obtained from OriGene (http://www.origene.com). HER2/neu oligonucleotides were chemically synthesized by ST Pharm Co. Ltd (Seoul, Korea).

Wound healing migration assay. Cell migration was estimated by a wound-healing migration assay and monitored by microscopy. MCF7 and MDA-MB231 cells were transfected with p34^{SEI-1} overexpressing pEF-p34^{SEI-1}-Flag or p34^{SEI-1} suppressing pLKO.1/p34^{SEI-1}-shRNA vector with each corresponding control vector, respectively. Each cell line was fully cultured in a 60-mm-diameter culture dish and a scratch was made on the monolayer using a sterile white tip. The distance of migration by cancer cells was measured after 48 h.

Matrigel invasion assay. In vitro invasion assay was performed using a Transwell membrane apparatus (Corning Life Sciences, Tewksbury, MA, USA) and Matrigel (BD Biosciences, Seoul, Korea). SKBR3, MCF7 and MDA-MB-231 breast cancer cells (2x10^5 cells) were placed in the Matrigel-coated upper chamber of the apparatus. Medium to each cell line was placed in the lower chamber as a source of chemo-attractants. Incubation was carried out for 24 h at 37˚C. The capacity of these cells to invade through the semi-solid Matrigel was estimated by fluorescence.

Immunohistochemistry. IHC data using human tissue samples were kindly provided by Dr Chang-Jin Kim at Soonchunhyang University Hospital (Chonan, Korea) and it was obtained as previously described (28).

Results

Enhanced cancer cell migration and invasion by p34^{SEI-1} overexpression. To investigate whether p34^{SEI-1} oncoprotein is involved in the development of metastasis, the effect of p34^{SEI-1} on migration and invasion was tested by wound healing migration and Matrigel invasion assays. In the wound healing assay, cell mobility was enhanced in MCF7 cells transfected with p34^{SEI-1} overexpressing pEF-p34^{SEI-1}-Flag vector, while it was reduced in MDA-MB-231 cells transfected with p34^{SEI-1} suppressing pLKO.1/p34^{SEI-1}-shRNA vector compared to control cells (Fig. 1A). The Matrigel invasion assay showed that overexpression of p34^{SEI-1} in SKBR3 and MCF7 cells increased the invasiveness compared to control cells, while knockdown of p34^{SEI-1} in MDA-MB 231 cells decreased invasiveness (Fig. 1B). These data strongly suggest that p34^{SEI-1} exerts a positive effect on the cell migration and invasion *in vitro* implying the involvement of p34^{SEI-1} in metastasis. During the EMT process, epithelial markers like E-cadherin are diminished, while expression of mesenchymal markers including N-cadherin, vimentin and fibronectin increase due to activation of matrix metalloproteinases (MMPs) inducing metastasis (38). Accordingly, the expression levels of EMT-related proteins were checked using western blot analysis. The representative epithelial marker E-cadherin was decreased but its antagonist N-cadherin was increased in SKBR3 and MCF7 cells after transfection with pEF-p34^{SEI-1}-Flag vector (Fig. 1C). Furthermore, vimentin another mesenchymal marker, was also increased in SKBR3 and MCF7 cells. The opposite result was obtained in MDA-MB-231 cells transfected with pLKO.1/p34^{SEI-1}-shRNA vector compared to control (Fig. 1C). Taken together, the findings indicate that p34^{SEI-1} promotes metastasis by enhancing migration and invasion of cancer cells.

Change of p34^{SEI-1} expression level in tissue samples with different degrees of tumor invasiveness. To support the conclusion that p34^{SEI-1} has metastatic potential, immunohistochemistry was performed to test whether p34^{SEI-1} expression is clinically related with the metastasis of breast cancer. Indicated tissue samples were stained with p34^{SEI-1} antibody and the degree of positive signals was estimated. Normal lobular and ductal hyperplastic cells showed no strong positive signal (0 of 20 samples) while 8 of 20 (40%) ductal carcinoma *in situ* and 13 of 20 (70%) invasive ductal carcinoma samples displayed strong positive signals (Fig. 2). The invasive carcinoma showed more strong expression than non-invasive ductal carcinoma *in situ*. The representative immunohistochemistry data are shown in Fig. 2, in which p34^{SEI-1} was not or very weakly expressed in normal lobular epithelial or ductal hyperplasia breast cancer tissues, but was more strongly expressed in invasive ductal carcinoma tissue samples than in ductal carcinoma *in situ*. The data indicate that p34^{SEI-1} expression increases as the tumor invasiveness progresses in human breast tissues, strongly relating p34^{SEI-1} with breast cancer metastasis.

Positive effect of p34^{SEI-1} on metastasis via regulation of PI3K/AKT signaling pathway. To elucidate the mechanism of how p34^{SEI-1} promotes metastasis during cancer cell tumorigenesis, western blot analysis was employed to check the expression levels of the main components of the PI3K-AKT pathway, NEDD4-1, PTEN, phosphorylation of AKT on serine 473 residue (pAKT^{ser473}) and phosphorylation of GSK3β on serine 9 residue (pGSK3β^{ser9}) after overexpression or suppression of p34^{SEI-1}. In MCF7, T47D and HEK293T cells transfected with pEF-p34^{SEI-1}-Flag, p34^{SEI-1} induced increased pAKT^{ser473} and pGSK3β^{ser9} protein levels at least partly by inducing NEDD4-1-mediated PTEN degrada-

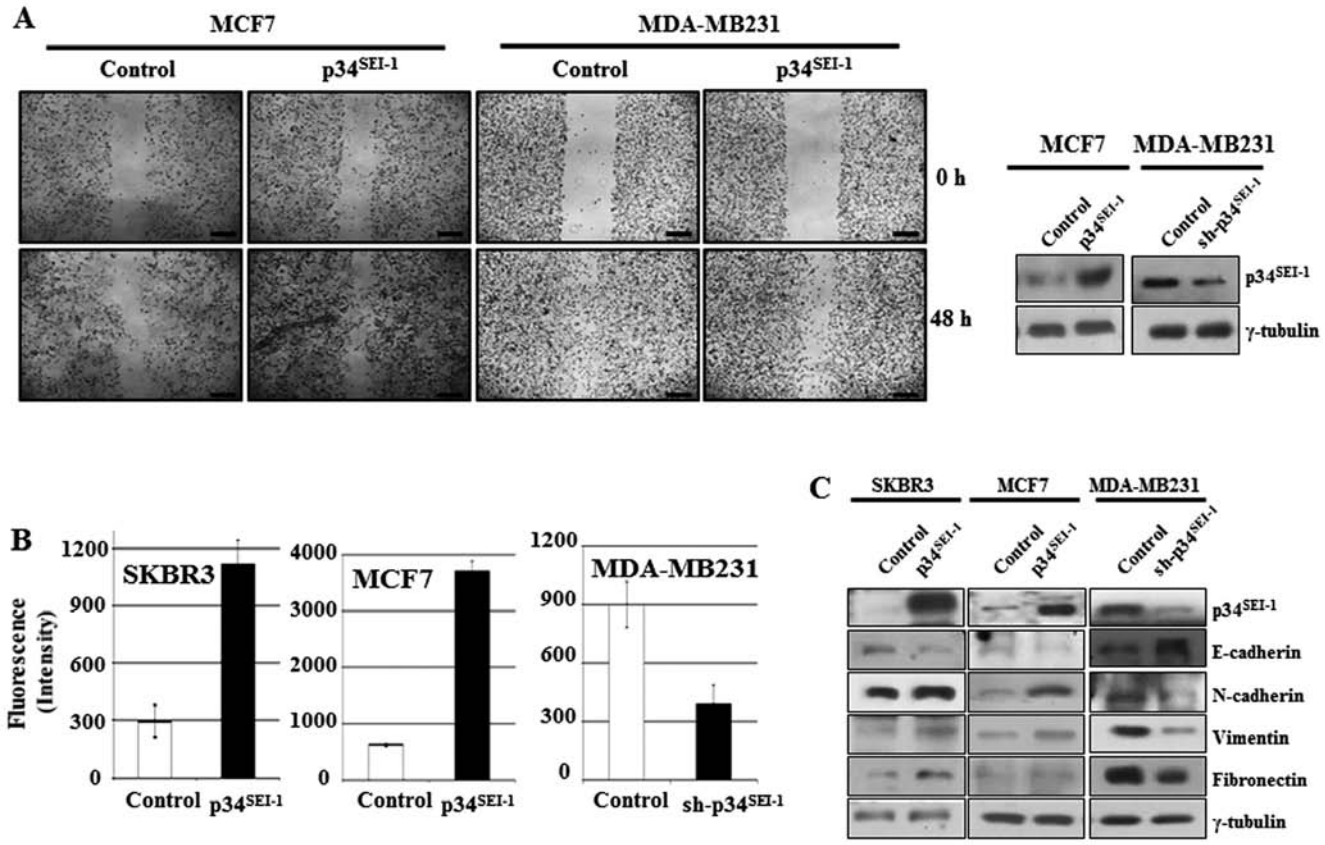

Figure 1. Positive effect of p34^{SEI-1} on cell migration and invasion *in vitro*. (A) Wound healing migration assay. MCF7 or MDA-MB231 cells were transfected with p34^{SEI-1} overexpressing pEF-p34^{SEI-1}-Flag or silencing pLKO.1/p34^{SEI-1}-shRNA vector with each corresponding control vector, respectively. Capacity of cell mobility of both MCF7 and MDA-MB231 was monitored by microscopy and photographed 48 h after monolayer wounding. Representative images are shown. The bar denotes 100 μm. (B) Matrigel invasion assay results. SKBR3 and MCF7 cells were transfected with pEF-BOS-EX control or p34^{SEI-1} overexpressing pEF-p34^{SEI-1}-Flag vector. MDA-MB-231 cells were transfected with pLKO.1 control or p34^{SEI-1} suppressing pLKO.1/p34^{SEI-1}-shRNA vector. Each of the transfected cells was applied to the upper semi-solid surface of the matrigel coated membrane and incubated for 24 h. The capacity of these cells to invade through a semi-solid matrigel was estimated by the resulting fluorescence intensity. (C) Effect of p34^{SEI-1} on the expression of EMT marker proteins. The expression of EMT proteins was examined by western blot analysis after transfection with pEF-p34^{SEI-1}-Flag vector into MCF7 and SKBR3 cells or with pLKO.1/p34^{SEI-1}-shRNA vector into MDA-MB231 cells. γ-tubulin was used as an internal control.

	Total cases	Negative	Weak positive	Strong positive
Normal	20	15	5	0
Ductal hyperplasia	15	8	7	0
Ductal carcinoma in situ	20	7	5	8
Invasive ductal carcinoma	20	3	4	13

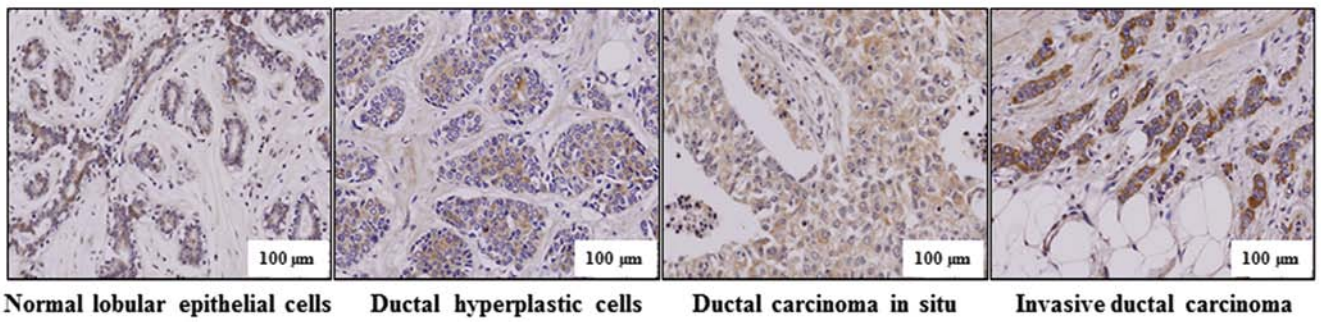

Figure 2. Change in p34^{SEI-1} expression level during development of tumor invasiveness. Immunohistochemistry analysis was performed on breast tissue samples of normal lobular epithelial cells, ductal hyperplastic cells, carcinoma *in situ* and invasive ductal carcinoma. Representative images are shown from each stage in normal lobular cells, hyperplastic cells, carcinoma *in situ* and invasive carcinoma. Weak or strong immune-reactivity against p34SEI-1 is shown in brown color.

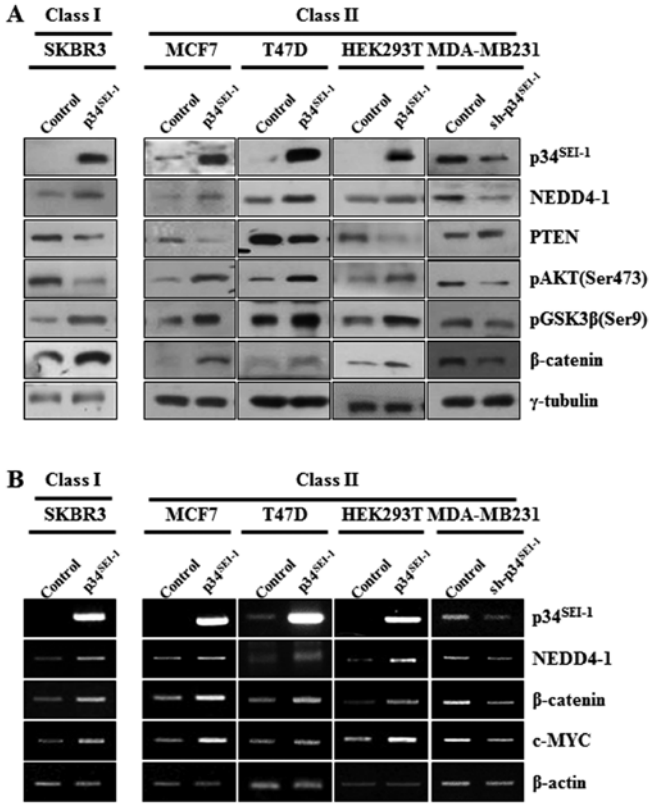

Figure 3. Positive effect of $p34^{SEI-1}$ on metastasis via regulation of the PI3K/AKT signaling pathway. SKBR3, MCF7, T47D and HEK293T cell lines were transfected with either pEF-BOS-EX control or $p34^{SEI-1}$ overexpressing pEF-$p34^{SEI-1}$-Flag vector. MDA-MB231 cells were transfected with either pLKO.1 empty or pLKO.1/$p34^{SEI-1}$-shRNA expression vector. Tested cancer cells were divided into two groups of class I and class II depending on the $pAKT^{ser473}$ protein level responding to $p34^{SEI-1}$ protein. (A) The transfected cells were used for western blot analysis of NEDD4-1, PTEN, $pAKT^{ser473}$, $pGSK3\beta^{ser9}$ and β-catenin. γ-tubulin was used as an internal control. (B) RT-PCR analysis of NEDD4-1, β-catenin and c-MYC in two classes. β-actin was used as an internal control.

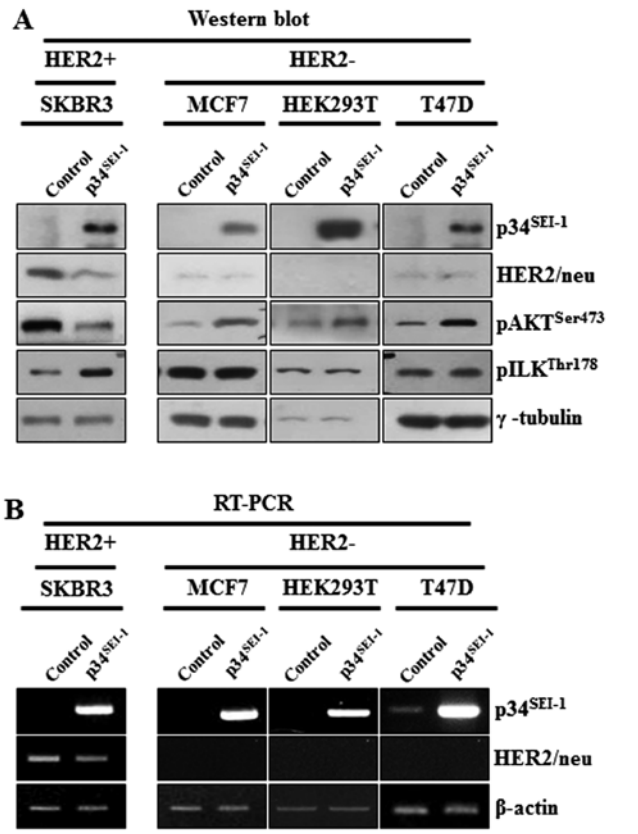

Figure 4. Effect of $p34^{SEI-1}$ overexpression on the activation of AKT and ILK in HER2/neu-positive and -negative cancer cell lines. (A) Western blot analysis of expression level of HER2/neu, $pAKT^{ser473}$ and $pILK^{thr178}$ in HER2/neu-positive breast cancer line (SKBR3) or HER2/neu-negative breast cancer cell lines (MCF7, HEK293T and T47D) after transfection with either pEF-BOS-EX empty control or $p34^{SEI-1}$ overexpressing pEF-$p34^{SEI-1}$-Flag vector, respectively. γ-tubulin was used as an internal control. (B) RT-PCR analysis of HER2/neu gene expression in SKBR3, MCF7, HEK293T and T47D. β-actin was used as an internal control.

tion as we previously reported (20). In MDA-MB231 cells transfected with pLKO.1/$p34^{SEI-1}$-shRNA vector, suppression of $p34^{SEI-1}$ resulted in a decrease of NEDD4-1 and an increase of PTEN compared to the control. Consequently, the protein levels of $pAKT^{ser473}$ and $pGSK3\beta^{ser9}$ decreased (Fig. 3A). However, SKBR3 cells transfected with pEF-$p34^{SEI-1}$-Flag revealed a different expression pattern, in which $pAKT^{ser473}$ was unexpectedly diminished. More interestingly, expression levels of GSK3β phosphorylation and β-catenin, downstream target of GSK3β, were not affected by the AKT inactivation (Fig. 3A). This fact implies the presence of another upstream kinase regulating GSK3β phosphorylation regardless of AKT inactivation. This speculation was supported by RT-PCR results showing that both classes had very similar expression pattern to the NEDD4-1, β-catenin, and its downstream target, c-MYC, at the transcriptional level. Overexpression of $p34^{SEI-1}$ produced an increase of NEDD4-1, β-catenin and c-MYC in SKBR3, MCF7 and HEK293T cells, whereas, in MDA-MB231 cells $p34^{SEI-1}$ was suppressive (Fig. 3B). The data implicated an unknown kinase in this pathway downstream of NEDD4-1 and upstream of GSK3β. Collectively, the data indicate that $p34^{SEI-1}$ may promote cancer metastasis using distinct signaling pathways in two different types of cancer cell lines with different genetic background.

Effect of $p34^{SEI-1}$ overexpression on the activation of AKT and ILK in HER2/neu-positive and -negative cancer cell lines. $p34^{SEI-1}$ seems to promote metastasis by activating the PI3K/AKT signaling pathway. During this process, overexpression of $p34^{SEI-1}$ decreased the phosphorylation of AKT on 473 serine residue in SKBR3 cells but increased phosphorylation in MCF7, T47D and HEK293T cells. Considering that SKBR3 is HER2/neu-positive cell line, but MCF7, T47D and HEK293T are HER2/neu-negative cell lines, it was assumed that HER2/neu might be responsible for the decrease of $pAKT^{ser473}$ protein level in $p34^{SEI-1}$ overexpressing SKBR3 cells, since HER2/neu is a positive regulator of the PI3K/AKT signaling pathway and its expression was significantly decreased by $p34^{SEI-1}$ overexpression (Fig. 4A). This result was consistent with the view that $p34^{SEI-1}$ suppresses HER2/neu expression and downregulated HER2/neu inhibits the phosphorylation of AKT at the 473 serine residue. Unexpectedly, the phosphorylation level of GSK3β on 9 serine residue was increased despite AKT inactivation (Fig. 3A). This finding indicated that GSK3β might be phosphorylated by another factor rather than AKT in SKBR3 cells. To elucidate the mechanism, ILK was at first suspected to be responsible for GSK3β phosphorylation because ILK is known to affect PI3K/AKT signaling pathway

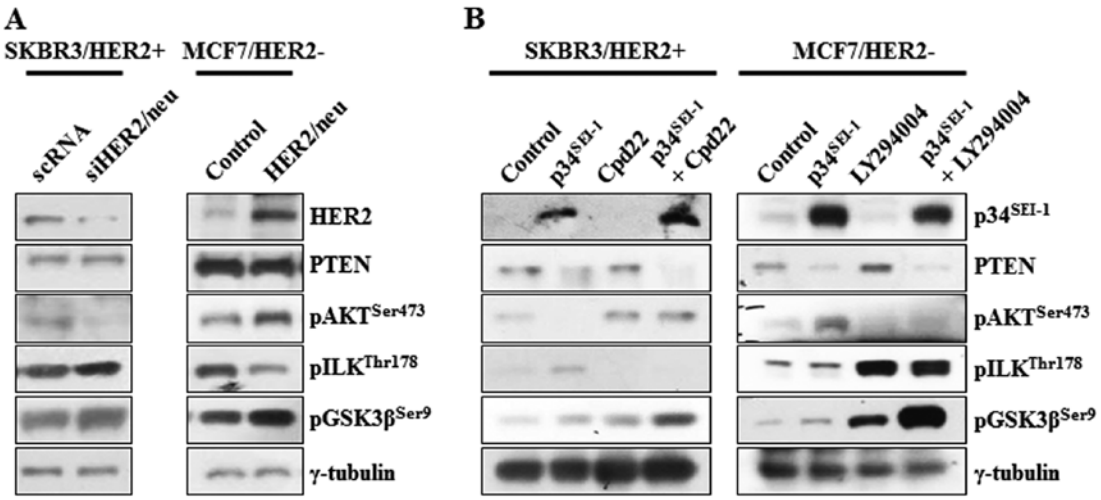

Figure 5. HER2/neu-dependent switching relationship between AKT and ILK signaling pathways. (A) HER2/neu-positive SKBR3 cell line was transfected with either scramble control scRNA or HER2/neu directed siRNA. HER2/neu-negative MCF7 cell line was transfected with either control vector or HER2/neu overexpressing pHER2/neu vectors. Western blot analysis was employed to check the expression levels of PTEN, pAKT^{ser473}, pILK^{thr178} and pGSK3β^{ser9}. (B) SKBR3 or MCF7 cells were treated with 30 μM of Cpd22 or 50 μM of LY294004 for 24 h to inhibit ILK or PI3K/AKT signaling pathway, respectively. Western blot analysis was employed to check the proteins related with PI3K/AKT pathway such as PTEN, AKT, pAKT^{ser473}, pILK^{thr178} and pGSK3β. γ-tubulin was used as an internal control.

by directly phosphorylating the 9 serine residue of GSK3β (23). The phosphorylation levels of ILK on the 178 threonine residue were checked in both HER2/neu-positive and -negative cell lines. pILK^{thr178} protein level was significantly increased in HER2/neu-positive SKBR3 cells while no change was found in HER2/neu-negative MCF7, HEK293 and T47D cells (Fig. 4A). The protein level of pILK^{thr178} was significantly induced when phosphorylation of pAKT^{ser473} was inhibited by decreased HER2/neu. However, it was not changed in HER2/neu-negative cells, in which AKT phosphorylation was induced (Fig. 4A). This observation indicates that p34^{SEI-1} overexpression promotes cancer metastasis by inducing ILK instead of AKT when HER2/neu is diminished or depleted. Further RT-PCR analysis showed that the negative effect of p34^{SEI-1} on HER2/neu expression occurred both at the transcriptional and the translational levels (Fig. 4B).

Taken together, the data demonstrates that p34^{SEI-1} activates the PI3K/AKT signaling pathway using at least two different types of signaling pathways depending on HER2/neu expression status.

HER2/neu-dependent switching relationship between AKT and ILK signaling pathways. Decreased HER2/neu activity by p34^{SEI-1} overexpression might be responsible for the activation of ILK signaling in SKBR3 cells. This idea prompted the assessment of the dependence of the activation of ILK on HER2/neu. The phosphorylation of AKT, ILK and GSK3β was checked at the protein level after SKBR3 and MCF7 cells were transfected with HER2/neu specific siRNA or overexpressing pHER2/neu vector. In HER2/neu silenced SKBR3 cells, pAKT^{ser473} protein level was decreased, probably due to HER2/neu mediated inhibition of PI3K, a direct downstream target of HER2/neu. Surprisingly, both pILK^{thr178} and pGSK3β^{ser9} protein levels were increased after treatment of HER2/neu silencing siRNA, in which GSK3β was thought to be phosphorylated by the ILK rather than AKT (Fig. 5A). On the other hand, the expression

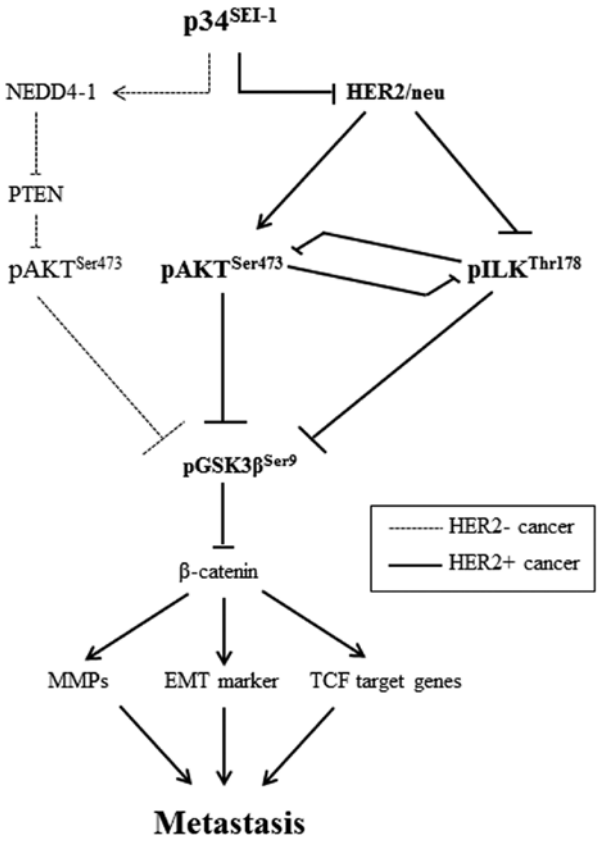

Figure 6. The distinct effect of p34^{SEI-1} on cancer metastasis in HER2/neu-negative and -positive cells. In HER2/neu-negative cells, p34^{SEI-1} onco-protein enhances cancer cell survival and promotes tumorigenesis by inducing NEDD4-1-mediated PTEN degradation, which in turn activates the PI3K/AKT signaling pathway. In HER2/neu-positive cells, p34^{SEI-1} induces downregulation of HER2/neu, which inactivates AKT by dephosphorylating AKT but activates ILK by phosphorylating on 178 threonine residue. In both signaling pathways, abnormally activated AKT or ILK leads to the phosphorylation of GSK3β on 9 serine residue and in turn stabilizes β-catenin, which induces expression of MMPs, EMT markers and TCF target genes, and finally metastasis is promoted.

levels of same proteins were also checked after MCF7 was transfected with HER2/neu overexpressing vector. Overexpression of HER2/neu highly promoted the phosphorylation of AKT on 473 serine residue but reduced that of ILK on the 178 threonine residue. PTEN was not affected by neither HER2/neu inhibition or overexpression (Fig. 5A). In an extended experiment, the switching relationship between AKT and ILK was investigated using Cpd22 of ILK inhibitor and LY29002 of PI3K/AKT inhibitor. In both groups, PTEN was decreased whenever $p34^{SEI-1}$ was overexpressed probably due to $p34^{SEI-1}$ mediated NEDD4-1 activation as we showed before (28). Very importantly, both groups showed an inverse relationship between $pAKT^{ser473}$ and $pILK^{thr178}$ expression levels. When HER2/neu-positive SKBR3 cells were treated with Cpd22, $pAKT^{ser473}$ protein level increased even under $p34^{SEI-1}$ overexpression (Fig. 5B). When HER2/neu-negative MCF7 cells were treated with LY29002, the ratio of ILK phosphorylation was significantly elevated (Fig. 5B). LY29002 treatment after $p34^{SEI-1}$ overexpression produced an even higher level of $pILK^{thr178}$ expression (Fig. 5B). It may be explained by the strong switching relationship between AKT and ILK. In both cases, $p34^{SEI-1}$ overexpression and treatment of Cpd22 and LY29002 tremendously increased the phosphorylation of GSK3β on the 9 serine residue in both HER2/neu-positive and -negative cell lines. This may be the basis for progression of metastasis because either AKT or ILK phosphorylation could promote the metastasis under the circumstance that one of them is inhibited (Fig. 5B).

Taken together, the data demonstrate that $p34^{SEI-1}$ induces the activation of either AKT or ILK signaling in a HER2/neu-dependent manner (Fig. 6).

Discussion

The present study shows that $p34^{SEI-1}$ exerts a positive effect on cancer metastasis by inducing migration and invasion of cancer cells. $p34^{SEI-1}$ appears to promote metastasis by activating the PI3K/AKT signaling pathway, in which two different serine/threonine kinases, AKT and ILK, are alternatively activated depending on HER2/neu expression. In HER2/neu suppressed cells, $p34^{SEI-1}$ overexpression increased $pAKT^{ser473}$ protein level and in turn activated PI3K/AKT signaling pathway at least partly via NEDD4-1 mediated PTEN ubiquitination. However, $pAKT^{ser473}$ protein was unexpectedly diminished despite exuberant $p34^{SEI-1}$ overexpression in HER2/neu expressing SKBR3 cancer cells. In HER2/neu strongly positive SKBR3 cells, $p34^{SEI-1}$ reduced HER2/neu, leading to the inhibition of AKT phosphorylation. Instead, $p34^{SEI-1}$ activated another multifunctional serine/threonine protein kinase ILK to promote metastasis. This result suggests that $p34^{SEI-1}$ affects cancer metastasis in a HER2/neu-dependent manner. Interestingly, our data also showed the inverse relationship in the expression levels of AKT and ILK proteins, implying that AKT and ILK have a switching relationship with each other in HER2/neu-dependent manner. Considering all these results, we suggest that drug resistance or recurrence of metastatic cancer may be caused by the switching relationship between AKT and ILK after the treatment of HER2/neu overexpressing breast cancers with a monoclonal antibody targeting HER2/neu oncogene (39). However, the exact mechanism of HER2/neu inhibition by $p34^{SEI-1}$ overexpression is still not clear and needs further study.

We showed that $p34^{SEI-1}$ can downregulate HER2/neu at the transcriptional and protein levels. We also found that p21 enhancer activator 3 (PEA3) is at least partly responsible for the $p34^{SEI-1}$ mediated HER2/neu downregulation at the transcription level (data not shown). Interestingly, $p34^{SEI-1}$ overexpression increased the expression levels of PEA3 gene at the transcription level (data not shown). PEA3 can bind to the promoter region of HER2/neu directly (40). PEA3 facilitates cancer invasion via regulation of PI3K/AKT-related proteins and MMP13. Inhibition of PEA3 diminishes the non-adherent tumor growth, migration and invasion via downregulation of EMT markers in a variety of tumors including breast cancer (41). Therefore, we assumed that $p34^{SEI-1}$ might negatively affect HER2/neu expression by regulating PEA3 gene expression or by interacting with it. However, more precise experiments such as ChIP assay need to be performed to make the mechanism clear.

In summary, our data demonstrate that $p34^{SEI-1}$ activates the PI3K/AKT signaling pathway by positively regulating at least two different types of AKT or ILK-mediated signaling pathways depending on HER2/neu expression status. Taken together, $p34^{SEI-1}$ would be considered as blocking metastatic breast cancer, and it might be used for the prevention and treatment of metastatic breast cancer.

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