Nad(P) Dependent Steroid Dehydrogenase-Like, Involved In Cholesterol Biosynthesis, Regulates Proliferation And Metastasis In Breast Cancer

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Research article

Keywords: Breast cancer, NSDHL, Knock-down, Proliferation, Metastasis, Cholesterol, EGFR

Posted Date: August 4th, 2019

DOI: https://doi.org/10.21203/rs.2.12420/v1

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Version of Record: A version of this preprint was published at BMC Cancer on May 4th, 2021. See the published version at https://doi.org/10.1186/s12885-020-06840-2.
Abstract

Background Cholesterol biosynthesis pathway is a common upregulated pathway in breast cancer. NSDHL (NAD(P) Dependent Steroid Dehydrogenase-Like), a gene which is involved in cholesterol biosynthesis has not been elucidated for its role in breast cancer. Methods After knock-down of NSDHL in human breast cancer cell lines, cell proliferation assay, cell cycle analysis, 3D culture, clonogenic assay, migration and invasion assay, and wound healing assay were performed. We tested erlotinib as a target drug for NSDHL. Xenograft was developed with MDA-MB-231 transfected with NSDHL shRNA in NOD/SCID mice. Public gene expression databases were used for the analysis of prognostic role of NSDHL in breast cancer Results Both NSDHL mRNA and protein levels were higher in a Luminal (MCF7) and triple negative cells (MDA-MB-231, BT-20) than normal breast cancer cell (MCF10A). Silencing of NSDHL by siRNA inhibited cell cycle and proliferation, and also inhibited migration and invasion in MDA-MB-231 and BT-20 cells (p< 0.05). The sensitivity to erlotinib was significantly decreased in siNSDHL treated cells, especially in MDA-MB-231 (p<0.01). Additionally, we found that NSDHL regulated total cholesterol levels in breast cancer cells. In vivo xenograft model, NSDHL knock down in the cancer cell decreased the tumor growth and metastasis. Clinically, higher NSDHL expression in the tumor of 3951 breast cancer patients was associated with significantly lower recurrence-free survival. Conclusions In conclusion, we showed that NSDHL might have a role for the progression of breast cancer. Further studies are needed to clarify that NSDHL can be a therapeutic target in breast cancer.

Background

Breast cancer is common malignancy of women in Korea [1, 2] and the major cause of mortality in females worldwide [3]. Although various progress in early diagnosis, radiation therapy and various combination of surgery improved the prognosis of breast cancer patients [4], the high mortality rates are remained. Furthermore, the identification of novel therapeutic targets is important to discover the potential biomarkers for early diagnosis and prognosis in breast cancer patients.

In previous study, using Whole-exome sequencing in 120 breast tumor and normal paired tissues, they detected 11,684 putative somatic mutations in 7,373 genes. After selected 1,116 genes with 3 or more mutations, 695 genes were chosen using messenger RNA (mRNA) expression in the whole transcriptome sequencing data. Finally, 198 somatic mutations of 50 genes were selected in 64 patients. In this result, NSDHL (NAD(P) Dependent Steroid Dehydrogenase-Like) has 3 mutations and high level of hazard ratios for recurrence using K-M plotter (Hazard Ratio = 1.43, 95% CI = 1.28 - 1.61, P-value < 0.001) [5].

NSDHL (NAD(P) Dependent Steroid Dehydrogenase-Like) is a protein coding gene. Also, localized in the endoplasmic reticulum and involved in cholesterol biosynthesis [6]. Obesity
is considered important risk factor in cancer [7-10] but the cause is not elucidated. Oncogene-transformed cancer cells rapidly grow from elevated cholesterol levels. the cholesterol uptake process is regulated by epidermal growth factor receptor (EGFR) signaling [11-13]. NSDHL, catalyze oxidative decarboxylation of the C4 methyl group from MAS (Meiosis-activating sterols) [14, 15], has critical role to convert squalene to cholesterol [16]. Also, cholesterol is an essential component of the animal cell membranes [17]. NSDHL derived from ER membranes [18], translocated to the plasma membrane from the intracellular compartment by lipid-depleted serum (LDs) and cooperate with lipid rafts to promote metastasis [19-22]. NSDHL function in breast cancer has rarely been studied.

In the present study, in breast cancer cells, NSDHL expression of both mRNA and protein levels in MCF7, MDA-MB-231 and BT-20 cells were higher than non-tumorigenic epithelial cell (MCF10A). Among them, we inhibited NSDHL expression using small interfering RNA (siRNA) and studied proliferation and migration potential in breast cancer cells related with EGFR and cholesterol pathway. Moreover, we found that NSDHL regulated total cholesterol levels. After then, the tumor growth and metastasis were confirmed in NOD scid gamma mice. Clinically, we identified Patients with high NSDHL expression in total of 3951 breast cancer patients showed unfavorable outcomes on recurrence free survival (RFS) rates. Taken together, these results demonstrated that NSDHL is pivotal for the tumorigenesis in breast cancer.

Methods
1. **In vitro**

- **2. Breast cancer cell lines**

Among the mutations confirmed by Whole Exome Sequencing, we evaluated the function of NSDHL. The normal breast cancer cell line (MCF10A; by. Woo Hang Hur), Luminal A (MCF7), Luminal B (ZR-75-1, BT-474), HER2+ (SK-BR-3), TNBC (BT-20, Hs578T, MDA-MB-231) were used in this study. MCF7, Hs578T and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC), BT-20, BT-474, SK-BR-3 and ZR-75-1 cell lines were obtained from Korean Cell Line Bank (KCLB). MCF7, Hs578T and MDA-MB-231 cells were grown in DMEM (Welgene) supplemented with 10% Fetal Bovine Serum (FBS; Welgene) and 1% Antibiotic-Antimycotic (100X) (Gibco, Carlsbad, CA, USA). BT-20, BT-474, SK-BR-3 and ZR-75-1 cells were grown in RPMI 1640 (Welgene) supplemented with 10% Fetal Bovine Serum (FBS; Welgene) and 1% Antibiotic-Antimycotic (100X) (Gibco, Carlsbad, CA, USA). All cells were maintained at 37°C in a humidified atmosphere of 95% air with 5% CO2.

- **Primers, Antibodies and Drugs**

For **Polymerase Chain Reaction** (PCR), we used primers (GAPDH; F: GAGTCCAGGGCGTCTTCA, R: GGGGTGCTAAGCAGTTGGT), **NSDHL**; F: GGTGACGCACAGTGGAAAAC, R: TCGCACGGACTCATTTGACA). For western blot and immunohistochemistry, we used the following antibodies: b-actin (sc-47778), Sterol regulatory element -binding transcription factor 1 (SREBP-1) (sc-365513) from Santa Cruz; NSDHL (ab190353), EGFR (ab52894), Low-Density Lipoprotein Receptor (LDLR) (ab52818) from Abcam; AKT (#9272), Cyclin-dependent kinase 2 (CDK2) (#2546) from Cell Signaling. For cell viability assay with drug treatment, we used Erlotinib HCl (OSl-744) (Selleckchem).

- **cDNA synthesis and quantitative Reverse Transcription- Polymerase Chain Reaction**

Total RNA was extracted from cells using Tri-RNA Reagent (FAVORGEN, Kaohsiung, Taiwan). qRT-PCR reactions were conducted using cDNA kit (Applied Biosystems) and
Power SYBR™ Green PCR Master Mix (Applied Biosystems). Reactions were performed by Real time PCR System (Light Cycler 480 ™, Roche) and the results were analyzed with the comparative Ct to establish relative expression curves.

- **Western Blotting and antibodies**

Cells treated a concentration of 10 nM, 20 nM of siRNA were 48 h transfection. Total cell lysates prepared in lysis buffer (RIPA buffer, Phosphatase Inhibitor, 0.5M EDTA). Proteins concentrations were measured with the Pierce BCA Protein Assay Kit (Thermo scientific). Equal concentration of cell lysates was separated by 10% SDS PAGE. Immobilon - P Transfer Membranes (Merck Millipore L td) were blocked with blocking buffer (5% non-fat dry milk in TBS-T, 5% BSA in TBS-T) for 1 h. Primary antibodies were added to the blocking solution and incubates overnight at 4°C. Blots were washed three times (5 min, 5 min, 10 min) with TBS-T, and incubated with secondary antibodies for 1h at room temperature. After washing with TBS-T three times for 10 min each, immunocomplexes were visualized by chemiluminescence (Bio Molecular Imager, Amersham™ Imager 600, GE Healthcare) with SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific) and estimated molecular weight with Image J (National Institutes of Health).

- **siRNA transfection**

The small interfering RNA (siRNA) targeting NSDHL and control Non-targeting pool were obtained from Dharmacon (Dharmacon Inc., Lafayette, CO). Transient transfection of cells was performed using Lipofectamine 2000 RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Further experiments performed after. The NSDHL siRNA sequence was: GAGGAUAUGCUGUCAAUGU and Non-targeting pool sequence was: UGGUUUACAUUGUCGACUAA.

- **Cell proliferation assay**
A concentration of 20 nM of siRNA, 48 h transfected cells were evaluated by CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) as described in 96 h. Cells were plated at 3,000 cells/100 ml in triplicate in 96-well plate incubated at 37°C. CellTiter-Glo assay buffer was added 100 ml (1:1) and measured by Luminescence (Luminoskan Ascent® Microplate Luminometers, THERMO SCIENTIFIC, SAN DIEGO).

- **Cell cycle analysis**

Treated 1x10⁶ cells were fixed in 700 ml 70% cold ethanol included in 300 ml phosphate buffered saline (PBS) and leave it in overnight, 4°C. Before analyzed, washed the cells twice in cold PBS and added 250 ml of PBS included 5 ml of 10 mg/ml of Rnase A (0.5 mg/ml) at 37°C for 1 h. Keep in 4°C after added 10 ml of 1 mg/ml propidium iodide (10 mg/ml, PI; Sigma, St. Louis, USA). Cell cycle analysis was performed using the ModFit 3.0 (#441622, Verity software House)

- **Three-dimensional Matrigel culture (Sphere formation)**

Cell imaging coverglass, 8 chambers (Eppendorf) were coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 8 mg/ml in 250 ml and incubated at 37°C for 4 h. Extracted cells (1x10⁵ cells/ml) were diluted with 0.5 mg/ml Matrigel and spread on the 8 mg/ml Matrigel (1:1) then incubated at 37°C. Observed chambers for 9 day under a microscope (x40) (Leica DM IL LED, Leica Microsystems). And we measured sphere surface area formation ( )

- **Colony formation assay**

Single cell suspensions of cells were thoroughly suspended and single cells plated at 1.5x10³ cells/ml in full media in 6-well plate. After 9 day, to confirmed the efficiently cell-clusters, we determined microscopically (x40) (Leica DM IL LED, Leica Microsystems) and
measured colony surface area (mm). After then, fixed with 4% formaldehyde for 30 min and stained with 0.1% crystal violet. For measured the concentration of staining cells, washed the stained crystal violet with 400 ml of 10% acetic acid and read in (SpectraMax 190 Microplate Reader, Molecular Devices) (Lm: 570).

- **Drug treatment assay**

The effect of Erlotinib on transfected in MCF7, MDA-MB-231, BT-20 cells were measured using CellTiter-Glo Assay Kit (Promega) and measured by Luminescence (Luminoskan Ascent® Microplate Luminometers, THERMO SCIENTIFIC, SAN DIEGO). Corresponding dilutions of DMSO were added as vehicle. Cells were plated at 3,000 cells/100 ml in triplicate in 96-well plate incubated at 37°C. After 24 h, the complete medium was replaced with various doses (0 mm – 160 mm) of vehicles and incubated at 37°C during 72 h.

- **Transwell migration assay**

Cells transfected with 20 nM of siRNAs for 48 h were seeded in the upper transwell chambers (8.0 mm pores sized) at a density of 5x10^4 cells in 200 ml serum-free medium. Filled with 750 ml full DMEM containing 10% FBS and 1% Antibiotic-Antimycotic. After incubated for 24 h at 37°C, fixed chambers with 400 ml of 4% paraformaldehyde for 30 min and stained with 400 ml of 0.1% crystal violet for 30 min. Washed with distilled water and cleaned upper chamber with a cotton swab. Drying chambers then photographed (x40) (Leica DM IL LED, Leica Microsystems). For measured the concentration of staining cells, washed the stained crystal violet with 400 ml of 10% acetic acid and read in (SpectraMax 190 Microplate Reader, Molecular Devices) (Lm: 570).

- **Invasion assay**

Cells transfected with 20 nM of siRNAs for 48 h were seeded in the upper transwell chambers (8.0 mm pores sized) coated with 100 ml of 1 mg/ml matrigel (BD Biosciences, Franklin Lakes, NJ, USA) before 4 h performed assay. Added a density of 5x10^4 cells in 100
ml serum-free medium (1:1) on the matrigel. Filled with 750 ml full DMEM containing 10% FBS and 1% Antibiotic-Antimycotic. After incubated for 24 h at 37°C, fixed chambers with 400 ml of 4% paraformaldehyde for 30 min and stained with 400 ml of 0.1% crystal violet for 30 min. Washed with distilled water and cleaned upper chamber with a cotton swab. Drying chambers then photographed (x40) (Leica DM IL LED, Leica Microsystems). For measured the concentration of staining cells, washed the stained crystal violet with 400 ml of 10% acetic acid and read in (SpectraMax 190 Microplate Reader, Molecular Devices) (Lm: 570).

**Wound healing assay**

4x10^5 cells/500 ml transfected with siRNAs were seeded in 24-well plate in triplicate and incubated at 37°C for overnight. After cells were full, using 1000 ml pipette tips made a straight scratch and washed in phosphate buffered saline (PBS). Then filled with 500 ml of full media. Observed at intervals of 24 h from 0 h to 24 h (x40) (Leica DM IL LED, Leica Microsystems).

**Total cholesterol assay**

Cells treated a concentration of 20 nM of siRNA were seeded for 48 h transfection. For 1x10^6 cells, extracted with 200 ml of a mixture of chloroform : isopropanol : tritonx-100 (7:11:0.1) and centrifuge (10 min, 15,000 rcf, 25°C). Transfered the liquid to a new e-tube, then air dry at 50°C. Put samples under vacuum (30 min) (SPD1010 & SPD2010, Thermo Fisher SCIENTIFIC). 200 ml of 1X Assay buffer (Total cholesterol Assay Kit (Fluorometric), STA-390) was added in dried lipids and vortexed. After solutions were homogenous and cloudy, measured 50 µl per well in triplicate with cholesterol reaction reagent (diluting the Cholesterol Oxidase 1:50, HRP 1:50, Fluorescence Probe 1:50, and Cholesterol Esterase 1:250 in 1X Assay Diluent. Determine the cholesterol concentration of the samples with the equation obtained from the linear regression analysis of the standard curve.

Total Cholesterol (mm) = Sample corrected absorbance / Slope x Sample dilution,
2. **In vivo**

- **shRNA lentiviral particle transduction**

Plated 4x10^5 MDA-MB-231 cells in 12-well plate 24 h prior to viral infection. Replace with 1 ml of 5 µg/ml Polybrene® (sc-134220) media mixture and 20 µl of control shRNA Lentiviral Particles-A (sc-108080), **NSDHL** shRNA (h) Lentiviral Particles (sc-90849-V). After 24 h, replaced with 1 ml of full media and incubated overnight. Split cells 1:5 and continue incubated for 48 h in full media. Change with 10 µg/ml **Puromycin dihydrochloride** (sc-108071) of full media for 7 day.

- **Cg-Prkdc^{scid} Il2rg^{tm1wjl}/SzJ mice**

NOD scid gamma mice (NSG mice) obtained from The Jackson Laboratory were approved by the Institutional Animal Care and Use Committee (IACUC). NSG mice born between july 31, 2018 and august 9, 2018 were tested on september 21, 2018. 5 shCONTROL mice and 5 shNSDHL mice were injected 100 ml of 1x10^6 cells with cold 1X PBS and 10 mg/ml matrigel (BD Biosciences, Franklin Lakes, NJ, USA) (1:1) into each left breast. We calculated tumor volume using formation. \( V \text{ (Tumor Volume)} = L \text{ (Length)} \times W \text{ (Width)} \times W / 2 \)

After dissection, we were euthanized with Co_2 gas and wrapped in a bean bag and stored frozen.

- **Immunohistochemistry**

Blocks of paraffin-embedded tissue from NSG mouse was cut in serial 4-µm sections and lung tissues were stained for H&E from pathology lab. Tumor tissues were incubated in dry oven (60°C) and deparaffinized in xylene. After 20 min, we rehydrated through ethanol and microwaved in 1X Antigen retrieval solution (S1699, Dako). For blocking tissues, Immuno blocking solution with goat serum (AR-6591-02, Immuno bioscience) was added and incubated in 4°C. On the slides, diluted 1^{st} antibody NSDHL(1:100, ab190353, Abcam) with
Dako antibody diluent with background reducing components (S3022, Dako) at 4°C for overnight. The immunoreactions were detected using DAB kit (K5007, Dako) and stained with hematoxylin and Mayer’s (S330930-2, Dako). After staining, we performed dehydration and determined microscopically (x100) (Light Microscope with imaging system, iSolution Lite, Image & Microscope Technology).

3. Clinical Prognostic Implication of NSDHLExpression level in Survival of Breast Cancer Patients

We have performed Cox proportional hazard ratio model analysis for the effect of NSDHL expression on recurrence free survival (RFS) rates of a total of 3951 breast cancer patients (including cases with luminal A (n=1933), luminal B (n=1149), HER2-positive (n=252), HER2-negative (n=800), Basal (n=618), ER-positive (n=3083), ER-negative (n=873), and TNBC (n=198) subtypes) by using microarray gene expression data and clinical data deposited in Gene expression omnibus (GEO) database and also used in previous investigations [5].

4. Statistics

Each experiment was done in triplicate. Statistical analyses were calculated by using Student’s t Test (*: \( p<0.05 \), **: \( p<0.01 \)) (GraphPad Prism v6.01 software for Windows).

Results

- **Expression of NSDHL in breast cancer cell lines**

In order to identify the function of NSDHL, the NSDHL mRNA and protein levels were detected in 8 breast cancer cell lines (MCF10A, MCF7, ZR-75-1, BT-474, SK-BR-3, BT-20, Hs578T, MDA-MB-231) (Figure 2A, B and C). Based on NSDHL mRNA and protein expressions, we were selected MCF7, MDA-MB-231 and BT-20 cells highly expressed in both mRNA and protein levels than MCF10A cell. By transfection of NSDHL siRNA,
NSDHL was knocked down in these cells \( (p<0.01) \). The mRNA level (Figure 3A and B) and protein expressions (Figure 3C) were downregulated by NSDHL siRNA in these cell lines. NSDHL expression is significantly decreased in breast cancer cell lines at 48 h after transfection with scramble siRNA. These results suggested that suppression of NSDHL lead the inhibition of breast cancer cell lines.

- **Silencing of NSDHL inhibits the proliferation of MCF7, MDA-MB-231 and BT-20 cells**

To determine the effect of NSDHL on cell growth, cell viability by Cell-titer glo assay kit, 3D culture assay, colony formation assay, cell cycle assay were performed after inhibited with siRNA. Compared with the control cells, after 48 h treated 20 nM NSDHL siRNA transfected cell’s (Figure 4B) growth curves showed significantly lower for 96 h of incubation measured by cell viability assay, especially in MDA-MB-231 cells (Figure 4A). Moreover, 3D sphere formation showed that the treated siNSDHL cells slowly differentiated for 9 day than control cells (Figure 4C). Correlated with colony formation, control cell significantly grown than siNSDHL transfected cells (Figure 4D, E). Also, Silencing of NSDHL inhibited the cell cycle than transfected with the siCONTROL (Figure 4F). After 48 h transfection with NSDHL siRNA in MCF7 and MDA-MB-231 cells, the percentage of cells in S and G2/M phases was decreased \( (p<0.01) \), but not in BT-20 cell. In BT-20 cell, G0/G1 phase was decreased \( (p<0.05) \). To support these results, we found that EGFR, CDK2 and LDLR expressions were inhibited in siRNA transfected cells (Figure 8B). These results indicated that NSDHL siRNA regulated the MCF7, MDA-MB-231 and BT-20 in cell cycle, delayed the progression of cell cycle and lead to inhibition of cell proliferation.

- **Effect of erlotinib in silencing of NSDHL in MCF7, MDA-MB-231 and BT-20 cells**

Erlotinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. It inhibits EGF-dependent cell proliferation and blocks cell-cycle progression in the G1 phase. To identify the function of NSDHL and Erlotinib in cell growth, we evaluated IC50 concentration, treated 20 nM after 48 h cells for 72 h incubation. The IC50 value was the range of 0 \( \mu \text{m} \) – 160 \( \mu \text{m} \) and detected by Luminescence using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega). In Table 4 and Figure 5A, B, and C, Erlotinib could
inhibit cell proliferation in a dose-dependent manner. In detail, the IC50 value in MCF (-74.07, + 46.42), MDA-MB-231 (- 83.54, + 27.62) and BT-20 (- 168.2, + 78.28) cells. Furthermore, the highest sensitivity was found in siCONTROL of BT-20 cell (Figure 5C) and the difference between siCONTROL and siNSDHL was largest in MDA-MB-231 cell (Figure 5B). The sensitivity of Erlotinib was significantly lower in siNSDHL treated cells, especially in MDA-MB-231 ($p<0.01$). Also, the extent of proliferation is consistent with the degree of knock-down levels in protein of EGFR and CDK2 (Figure 8B). In these results indicated that Erlotinib effectively reduced transfected NSDHL siRNA in breast cancer cells in relative to EGFR pathway.

- **Downregulation of NSDHL inhibits MDA-MB-231 and BT-20 cells in the migration and Invasion**

Since cancer cell migration play very critical roles in cancer metastasis. Thus, we investigated the effect of NSDHL suppression on transwell, invasion and wound healing assays. After 48 h, treated 20 nM NSDHL siRNA cells demonstrated reduction, especially in MDA-MB-231 cell. The transwell assay revealed that the loss of NSDHL expression could significantly decrease the migration rate by 60% in MDA-MB-231 cell, 30% in BT-20 cells comparison with scrambled siRNA-treated cells (Figure 6A). Results from invasion assay supported these data, reduced by 53% in MDA-MB-231 cells and 10% in BT-20 cells (Figure 6B). Consistently, the wound healing assay also showed that the migration rate was decreased by silencing of NSDHL in TNBC cells (Figure 6C). In detail, compared with the control cell groups, scratch wound reduced differently for 24 h, especially in MDA-MB-231 cells ($p<0.05$). In western blotting assay, we found that binding to specific sterol element, SREBP-1 and LDLR expressions were reduced in siRNA transfected cells (Figure 8B). Therefore, these data indicated that the pivotal role of NSDHL on the migration and invasion in TNBC cells.

- **Effect of NSDHL knock-down total cholesterol level in breast cancer cells**

NSDHL is involved in cholesterol biosynthesis and cholesterol is related with breast cancer. We measured total cholesterol level in MCF7, MDA-MB231 and BT-20 cells. It was
quantified both cholesterol esters and free cholesterol by fluorometric. After 48 hours, *NSDHL* siRNA 20 nM treated cells demonstrated significant reduction in breast cancer cells (*p*<0.01) (Figure 7A), especially in MDA-MB-231 cell. In detail, cholesterol level of BT-20 cell was 90 times higher than MDA-MB-231 and MCF7 cells (*p*<0.01) (Figure 7B). To support these results, we analyzed in western blotting with involved in sterol biosynthesis enzymes. In western blotting assay, we found that binding to specific sterol element, EGFR, SREBP-1 and LDLR expressions were reduced in siRNA transfected cells (Figure 8B). These results were definitely confirmed that *NSDHL* involved in the molecular mechanisms might be associated with cholesterol in breast cancer cells.

- **Predicted Mechanism of *NSDHL* regulation in breast cancer cells**

To determine the biological significance of *NSDHL* in breast cancer cells related with EGFR/biosynthesis pathway of cholesterol, we confirmed by western blotting analysis (Figure 8A, B). In these results, silencing of *NSDHL* decreased whole protein expressions involved in EGFR/biosynthesis pathway related with proliferation and migration.

- **Downregulation of *NSDHL* expression in MDA-MB-231 cells by shRNA Lentiviral Particles Transduction**

We performed short hairpin RNA (shRNA) (h) Lentiviral Particles Transduction in MDA-MB-231 for *in vivo* study. Selected stable clones expression the shRNA via Puromycin dihydrochloride selection were measured mRNA expression levels by RT-qPCR (*p*<0.01) and protein levels in western blot analysis (Figure 9A, B). These results supported that the suppression of *NSDHL* by shRNA could lead to inhibition of MDA-MB-231 cell progression and metastasis in NSG mice.

- **Tumor progression and lung metastasis of NSG mouse was decreased by sh*NSDHL* in *in vivo***
NOD scid gamma mice (NSG mice) born in between July 31, 2018 and August 9, 2018 injected on September 21, 2018. 5 shCONTROL mice and 5 shNSDHL mice were injected into each left breast (Figure 10A). Tumor growth in NSG mouse was significantly inhibited by NSDHL knock-downed cell for 44 day (p<0.01) (Figure 10B). Also, the control and knock-down tumor were different in volume, weight (p<0.01) and immunohistochemistry (Figure 10C, D and E).

3. Clinical implication of NSDHL in breast cancer

As shown in Table 2, high NSDHL expression in a total of 3951 breast cancer patients could be associated with lower recurrence free survival (Hazard Ratio (HR) = 1.419, 95% Confidence Interval (CI) = 1.267–1.59, P-value (log-rank test) = 1.155 x 10-9). luminal A (HR =1.307, 95% CI = 1.103–1.55, P-value = 0.00197) and TNBC (HR = 1.605, 95% CI = 0.9134–2.819, P-value = 0.09691) subtypes, such tendency of the reduction in survival was more obvious upon high NSDHL expression regarding the NSDHL expression in 8 breast cancer cell lines (Figure 2A, B and C).

Discussion

NSDHL (NAD(P)-dependent steroid dehydrogenase-like) play a critical role in meiosis activation [23]. In the skin, MASs activate because of the skin abnormalities caused by cholesterol synthesis in human and animal, including SC4MOL (sterol-C4-methyl oxidase-
like 1) [15, 24] and EGFR may be important in these skin changes in human and animal
through EGFR signaling [11, 15, 25]. In addition, inactivation of SC4MOL and NSDHL
reduced EGFR expression. In many studies, they implicated the function of the cholesterol
pathway in tumor grow and response to treatment [15]. For instance, the sensitivity of
neck and head cancer cells in apoptosis [26, 27] and through EGFR signaling, sterol
composition of the membrane is regulated [15, 28]. Furthermore, NSDHL derived from ER
membranes [18], cooperate with lipid rafts to promote metastasis by lipid-depleted serum
(LDs) [22]. However, the impact of NSDHL on progression and metastasis of breast cancer
remained and underlying mechanism is not clearly demonstrated.

We here use a NSDHL, which was selected as a therapeutic target, function in breast
cancer in in vitro, in vivo and clinal strategy. Our data imply a important functional role for
NSDHL in breast cancer and potential biomarker for diagnosis in breast cancer patients.
From whole-exome sequencing in 120 breast tumor and normal paired tissues, NSDHL has
3 mutations and high level in hazard ratio [5].

To investigate the pathological function of NSDHL in breast cancer, various experiments
were performed in NSDHL silenced breast cancer cell lines. In NSDHL expression in 8
breast cancer cell line, MCF7, MDA-MB-231 and BT-20 cells were higher than MCF10A cell
in both mRNA and protein levels. Then we regulated cells with specific siRNA targeting
NSDHL. These results revealed that NSDHL regulates cell survival, morphogenic
differentiation, colony formation and G0/G1, S, G2/M phases of cell cycle in cell
proliferation and growth. EGFR is upregulated in non-small-cell lung cancer, metastatic
colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer, and breast
cancer. EGFR over-activate downstream signaling pathways, including the RAS-RAF-MEK-
ERK MAPK and AKT-PI3K-mTOR pathways, activate chronic initiation and progression
through G1 cell cycle of the cancer cell proliferation [29]. Also, the PI3K/AKT/mTOR
pathway, implicated in endocrine resistance, is a major intracellular pathway, which leads
to cell proliferation [30, 31]. LDL-cholesterol induced breast cancer cell growth, migration
[32] and LDLR accelerates LDL-cholesterol that increased recurrence and mortality in
breast cancer [33]. We analyzed the regulation of NSDHL and EGFR/AKT/LDLR
expressions in protein level and found through this pathway that affected cell growth.

Erlotinib, a small-molecule epidermal growth factor receptor tyrosine kinase inhibitor has
potent effect in non-small-cell lung cancer proliferation In that results, H322 NSCLC cells
highly expressed EGFR was accompanied by G1/S phase arrest, because of the cell growth
inhibition by erlotinib [34]. And regardless of EGFR expression, the ability of erlotinib to
inhibit CDK2 activity is important role for cellular sensitivity to erlotinib and the p27 expression in the cytoplasm also involved in erlotinib resistance [35]. Additionally, depletion of SC4MOL and NSDHL, sensitizes tumor cells to EGFR inhibitor [15]. Therefore, we tested the sensitivity of Erlotinib, a small-molecule EGFR tyrosine kinase inhibitor, related with NSDHL in breast cancer cells. These results also indicated that erlotinib is effectively reduced breast cancer cell growth with NSDHL relation to CDK2 protein expression.

In addition, NSDHL has impact on metastasis in TNBC cell lines. Specifically, in NSDHL induced cells, migration and invasion ability were increased consistent with the degree of wound closer. In previous study, SREBP-1 involved in cholesterol pathway promotes migration and invasion in breast cancer [36]. NSDHL translocated to the plasma membrane from the intracellular compartment and promote metastasis relation to LDLR expression relation to LDL-cholesterol [22, 32]. We found that regulated NSDHL reduced SREBP-1 and LDLR expression of protein level in breast cancer cells.

We were concerned about the amount of cholesterol in breast cancer, total cholesterol levels were measured in the cells, resulting in a high cholesterol level in order of BT-20, MDA-MB-231 and MCF7 cells. After then, we are going to consider the relationship between EGFR, cholesterol and breast cancer in relation to the amount of cholesterol expressed by EGFR.

To reveal the mechanism of NSDHL in cholesterol pathway, we analyzed various enzymes, involved in cholesterol pathway. For example, EGFR, AKT, SREBP-1 and LDLR [37]. As well as we tested CDK2. In previous study, the LDLR expression is stimulated by activated SREBP-1 and in cholesterol homeostasis regulation [38] and SREBP-SCAP complex to move to the Golgi is promoted by activated EGFR/mTOR signaling [37]. In these results, we found out NSDHL regulated by EGFR/AKT pathway. And we predicted that NSDHL affected to cell cycle and proliferation from CDK2 and translocated to LDLR related with cholesterol. But, in migration and invasion, NSDHL was affected by SREBP-1 relation to LDLR.

After then, we confirmed the progression and metastasis ability of NSDHL in NSG mice. The downregulation of NSDHL induced a decrease in tumor growth and NSDHL expression through immunohistochemistry. Also, metastasis in Hematoxylin and eosin staining (H&E staining) of lung was inhibited compared with control cell.

Finally, the Cox Proportional Hazard Ratio Model Analysis in 3951 breast cancer patients revealed that NSDHL is a significantly meaningful gene in breast cancer clinically.
Therefore, these results indicated that \textit{NSDHL} is a pivotal molecule for prognosis in breast cancer.

**Conclusion**

In conclusion, \textit{NSDHL} is critical for de novo progression and metastasis in breast cancer cells and NSG mice. Therefore, we indicated that \textit{NSDHL} is novel therapeutic targets [22, 39] for breast cancer. Further studies are needed to clearly identify the association of EGFR and cholesterol mechanisms with \textit{NSDHL}.

**Abbreviations**

\textit{NSDHL}; NAD(P) DEPENDENT STEROID DEHYDROGENASE-LIKE  
ER; estrogen receptor  
TNBC; triple negative breast cancer  
HER-2; human epidermal growth factor 2,  
CI; confidential interval,  
HR; hazard ratio  
EGFR; epidermal growth factor receptor  
ATCC; American Type Culture Collection  
KCLB; Korean Cell Line Bank  
FBS; Fetal Bovine Serum  
PBS; phosphate buffered saline  
PCR; \textit{Polymerase Chain Reaction}  
qRT-PCR; Quantitative Real-Time Polymerase Chain Reaction  
mRNA; messenger RNA  
siRNA; small interfering RNA  
CDK2; Cyclin-dependent kinase 2  
shRNA; short hairpin RNA  
SREBP-1; Sterol regulatory element -binding transcription factor 1  
LDLR; Low-Density Lipoprotein Receptor  
\textbf{NSG mice}; NOD scid gamma mice  
H&E stain; Hematoxylin and eosin stain
Declarations

*Ethics approval and consent to participate

No ethics approval and consent were needed for this study.

*Consent for publication

Not applicable

*Availability of data and material

The datasets supporting the background of this article are included within additional files.

Additional file 1. Table2

Additional file 1. Table3

*Competing interests

All authors declare that they have no competing interests.

*Funding

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*Authors' contributions

SHY is an employee of this study. SHY designed and performed the experiments. BSH and EJK taught of an experiment. RNK performed the clinically statistical analysis. SHY wrote the manuscript. And SYJ,
HBL, HGM, DYN gave comments about experiments and design. All authors read and approved this manuscript.

*Acknowledgements*

Thank you for funded by breast cancer lab (pf. WSH) of Seoul national university and Woo Hang Hur gave me a MCF10A. All professors and researchers kindly coached the experiments and design.

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**Tables**

**Table 1.** 198 somatic mutations of 50 genes in 64 patients through whole exome sequencing of 120 breast cancers [5]
| Gene   | Description                              | Position     | R_ base | A_ base | No. of mutation |
|--------|------------------------------------------|--------------|---------|---------|----------------|
| NSDHL  | NAD(P) dependent steroid dehydrogenase-like | X:15201879   | G       | C       | 1              |
|        |                                          | X:152027402  | G       | A       | 1              |
|        |                                          | X:152037444  | C       | A       | 1              |

Table 2. Hazard ratios for recurrence of NSDHL in 3951 breast cancer patients using Cox proportional hazard ration model

Additional excel file

Table 3. Univariate and multivariable analysis by NSDHL

Additional excel file

Table 4. IC$_{50}$ of erlotinib in silencing of siCONTROL and siNSDHL in breast cancer cells

| Cell lines (siNSDHL) | Drug name | IC$_{50}$(mM) |
|----------------------|-----------|---------------|
| MCF7 -               | Erlotinib | 74.07         |
| MCF7 +               | Erlotinib | 46.42         |
| MDA-MB-231 -         | Erlotinib | 83.54         |
| MDA-MB-231 +         | Erlotinib | 27.62         |
| BT-20 -              | Erlotinib | 168.2         |
| BT-20 +              | Erlotinib | 78.28         |
Figures

Figure 1

Scheme of this study
Figure 2

NSDHL expression in 8 breast cancer cell lines in (a) RT-qPCR (b) PCR and (c) western blot analysis.
Figure 3

Downregulation of NSDHL expression in MCF7, MDA-MB-231 and BT-20 cells by siRNA transfection. (a), (b) Relative expression levels of NSDHL mRNA level of
Figure 4

Knock-down of NSDHL inhibited breast cancer cells proliferation. (a) Cell viability of NSDHL in cells treated with siRNA 20 nM, 48 h post transfection) for 96 h. These data showed cell growth inhibition by silencing of NSDHL treatment. Treated siNSDHL in MDA-MB-231 cell were more effectively reduced than MCF7 and BT-20 cells. (b) 2D culture (x40) and (c) 3D culture assay (x40), coated with matrigel indicated that reduction of proliferation in siNSDHL transfection cells for 9 day. Correlated with colony formation, (d) colony area and (e) concentration of stained control cells significantly more than siNSDHL transfected cells. (f) NSDHL function in cell cycle, in these data showed that cells treated siNSDHL, increased in G0/G1 phase of MCF7, MDA-MB-231 cells but S and G2/M phases in BT-20 cell. These data demonstrated that cell proliferation was significantly inhibited by siNSDHL treatment in breast cancer cells. Data were expressed as mean ± standard deviation. Each experiment was done in triplicate. * p<0.005; ** p<0.001 (Multiple t-test).

Figure 5

Figure 5. EGFR inhibitor reduces cell sensitivity in silencing of NSDHL in breast cancer cells. Erlotinib could inhibit cell proliferation in a dose-dependent manner. Control and siRNA transfection cells were treated with erlotinib in concentrations ranging to 0 to 160 μM at 72 h and determined by Cell Titer-Glo® assay. As shown in (a), (b), (c), the sensitivity of erlotinib on knock-down cells were decreased. In detail,
the IC50 value in MCF (-74.07, +46.42), MDA-MB-231 (-83.54, +27.62) and BT-20 (-168.2, +78.28) cells. Given that erlotinib is reduced cell viability. These results indicated that EGFR pathway is affected by MCF7, MDA-MB-231 and BT-20 cells in relation to NSDHL. Data were expressed as mean ± standard deviation. Each experiment was done in triplicate. * p<0.005; ** p<0.001 (Multiple t-test).

Figure 6

Figure 6. NSDHL regulated migration and invasion of MDA-MB-231 and BT-20 cells. (a) transwell assay and (b) invasion assay demonstrated that NSDHL knock-down inhibited cell migration (x40) in TNBC si treated cells as compared with Control cells. These assays were performed for 24 h (48 h post transfection) to assess cell migration. The migrated cells were stained with crystal violet and imaged by microscopy. The concentration was measured with 10% acetic acid and read by Spectramax 190 (Lm: 570). We compared siNSDHL cells by standardizing siCONTROL to 100%. (c) Consistently, the migration was confirmed in the wound healing assay (x40). The wound was closed slowly in NSDHL knock-down of MDA-MB-231 and BT-20 cells. The length of the wound was measured and compared based on control cells. These results showed that NSDHL small interfering RNA significantly reduce migration and invasion in TNBC cells. Data were expressed as mean ± standard deviation. Each experiment was done in triplicate. * p<0.005; ** p<0.001 (Multiple t-test).
Figure 7

Figure 7. NSDHL inhibited total cholesterol level in breast cancer cells. Decreased total cholesterol level in NSDHL knock-down MCF7 and MDA-MB-231 and BT-20 cells. It was quantified both cholesterol esters and free cholesterol by fluorometric. (a) Total cholesterol level regulated by siNSDHL was significantly reduced. (b) In order to confirm the association with cholesterol, we analyzed the amount of cholesterol between MCF7, MDA-MB-231 and BT-20 cells were compared. In detail, total cholesterol level of BT-20 cell was significantly higher than MDA-MB-231 and MCF7 cells. Data were expressed as mean ± standard deviation. Each experiment was done in triplicate. * p<0.005; ** p<0.001 (Multiple t-test).
Figure 8

Figure 8) Predicted mechanism of NSDHL regulation in breast cancer cells. To determine the biological significance of NSDHL in breast cancer cells related with EGFR/ biosynthesis pathway of cholesterol (a), we confirmed EGFR, AKT, CDK2, SREBP-1 and LDLR expressions by western blotting analysis (b).
Figure 9

Figure 9. shRNA (h) Lentiviral Particles Transduction in MDA-MB-231 for in vivo study. Selected stable colonies were measured mRNA expression levels by (a) RT-qPCR and protein levels in (b) western blot analysis. These results supported that the suppression of NSDHL by shRNA could lead to inhibition of MDA-MB-231 cell proliferation. Each experiment was done in triplicate. * p<0.005; ** p<0.001 (Multiple t-test).
Figure 10

Figure 10. Tumor growth of NSG mouse was inhibited by shNSDHL in in vivo. (a), (b) Tumor progression was significantly inhibited by NSDHL knock-downed cell for 44 day and the control and knock-down tumor were different in (c) volume, (d) weight and (e) immunohistochemistry. * p<0.005; ** p<0.001 (Multiple t-test).
Figure 11

Figure 11. (a) Lung metastasis of NSG mouse was verified with (b) H&E staining.
Figure 12

Figure 12. Recurrence free survival data by NSDHL expressions in 3951 breast cancer patients

Supplementary Files

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