NR4A1 regulates tamoxifen resistance through suppressing ERK signaling pathway in estrogen receptor-positive breast cancer

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Directed by Professor Myoung Hee Kim

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Breast cancer is one of the most prevalent carcinomas in worldwide. The estrogen receptor (ER)-positive breast cancer accounts for about 70% and is treated with endocrine therapy. Tamoxifen, commonly used treatment drug, is effective in this subtype. Nonetheless, approximately one-third of patients treated with tamoxifen gain acquired tamoxifen resistance, resulting in therapeutic challenges. Moreover, molecular mechanism targeting tamoxifen resistance still remains unclear. NR4A1 is known to play key roles in processes associated with carcinogenesis, apoptosis, DNA repair, proliferation, and inflammation. In recent studies, NR4A1 has been reported to act as an oncogene or a tumor suppressor in various cancer models including breast cancer. However, the role of NR4A1 in tamoxifen-resistant ER-positive breast cancer has not yet been defined. In this

study, we demonstrate the clinical significance and functional role as well as molecular mechanistic effects of NR4A1 in tamoxifen-resistant ER-positive breast cancer. NR4A1 gene expression was downregulated in tamoxifen-resistant MCF7 (TamR) and T47D (T47D-TamR) compared to that in MCF7 and T47D cells. Kaplan-Meier plots were used to identify high expression of NR4A1 correlated with increased survival rates in patients with ER-positive breast cancer following tamoxifen treatment. Gain and loss of function experiments showed that NR4A1 restores sensitivity to tamoxifen by regulating cell proliferation, migration, invasion, and apoptotic abilities. In addition, NR4A1 localized to the cytoplasm enhanced the expression of apoptotic factors. Mechanistically, NR4A1 enhanced responsiveness to tamoxifen through suppressing ERK signaling in ER-positive breast cancer by *in silico* and *in vitro* analyses, suggesting that the NR4A1/ERK signaling axis modulates tamoxifen resistance. Taken together, our study reveals that NR4A1 could be a potential therapeutic strategy to overcome tamoxifen resistance in ER-positive breast cancer.

Key words : NR4A1, ERK signaling, tamoxifen resistance, breast cancer

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I. INTRODUCTION

Breast cancer is one of the most common cancers in women worldwide[1]. Breast cancer can be classified into four molecular subtypes based on gene expression profiling[2]. Among these subtypes, estrogen receptor (ER)-positive breast cancer accounts for about 70% of all breast cancers and are generally treated with endocrine therapies, such as tamoxifen and aromatase inhibitor[3]. Tamoxifen, which is the most widely used endocrine therapy, has significant therapeutic effects; but, approximately one-third of ER-positive breast cancer patients treated with tamoxifen for 5 years acquire resistance to the tamoxifen, which reduces its therapeutic effect and shortens the survival of patients[4-6]. However, the molecular targets for predicting tamoxifen resistance are still insufficient. Therefore, discovering molecular mechanisms and genes that are associated with tamoxifen resistance is required to find novel therapeutic

strategies to overcome tamoxifen resistance for ER-positive breast cancer patients.

The NR4A subfamily, which consists of NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor1), belongs to the orphan nuclear receptor whose ligands are unknown and acts as transcription factors[7]. NR4A subfamily, an early response genes that is affected by various signals in a variety of cells and tissues, is known to be involved in cellular functions such as proliferation, migration, apoptosis, DNA repair, inflammation, metabolism, and angiogenesis[8,9]. These functions of NR4A depend on cellular localization and post-translational modification as well as gene expression[10]. Among NR4A subfamily members, the function of NR4A1 is the most known, and NR4A1 acts as an oncogene or a tumor suppressor in diverse cancer models, such as colon, liver, lung cancer, melanoma, and acute myeloid leukemia[11-15]. Similarly, the role of NR4A1 in breast cancer has also been controversial. In recent studies, ectopic expression of NR4A1 is known to promote invasion and metastasis by activating TGF-β/SMAD signaling in breast cancer[16]. Moreover, NR4A1 translocated from nucleus to cytoplasm has been found to induce apoptosis in all-trans retinoic acid -induced breast cancer cells[17]. NR4A1 also inhibits growth and metastasis in triple-negative breast cancer[18]. However, the functional role and associated molecular mechanism of NR4A1 in tamoxifen-resistant breast cancer have not been elucidated.

The mitogen-activated protein kinase (MAPK) cascade, including

extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase, and p38, is a signaling pathway that regulates various cellular functions[19] and is activated in most cancer models[20]. In particular, many studies have suggested that the ERK signaling pathway has important roles in breast cancers[21]. Activation of ERK signaling in breast cancer is not only associated with augmented tumor growth and metastasis[22,23] but also with tamoxifen resistance[24]. Activated ERK signaling reduces the response and effectiveness to tamoxifen treatment and affects poor prognosis in ER-positive breast cancer patients[25,26]. Therefore, the inhibition of ERK signaling associated with tamoxifen resistance improves the survival rate in ER-positive breast cancer patients by increasing the response to tamoxifen therapy.

In this study, we explored the role and regulatory mechanism of NR4A1 in tamoxifen-resistant ER-positive breast cancer cells and patient sample data obtained from a public database. We found that NR4A1 increases responsiveness to tamoxifen, resulting in reduced cell growth, invasion, and migration, and cytoplasmic NR4A1 is involved in apoptosis. Mechanistically, NR4A1 re-sensitizes the response to tamoxifen through suppressing the ERK signaling pathway. Our results demonstrate that NR4A1 reduces tamoxifen resistance by regulating ERK signaling in ER-positive breast cancer, suggesting that it could be a novel diagnostic marker and/or therapeutic target to overcome tamoxifen resistance.

II. MATERIALS AND METHODS

1. Cell culture and treatment

MCF7, T47D, tamoxifen-resistant MCF7 (TamR), and T47D (T47D-TamR) breast cancer cells were used in this study. TamR cells were established as an in vitro model for tamoxifen resistance by treated with 1 μM 4-hydroxytamoxifen (Sigma, MO, USA) in MCF7 cells for at least 6 months[27]. T47D and T47D-TamR cells were provided by Dr. Mi-Ock Lee (Seoul National University). MCF7 and TamR cells were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; WelGENE Inc., Daegu, Korea) and 1% penicillin-streptomycin (WelGENE Inc., Daegu, Korea). T47D and T47D-TamR cells were cultured in RPMI 1640 (WelGENE Inc., Daegu, Korea) with the same supplementation. Both TamR cells were cultured in a medium containing 1 μM 4-hydroxytamoxifen (Sigma, MO, USA). Cells were grown at 37 ℃, 5% CO2 incubator. For some experiments, 50 μM ERK1/2 inhibitor U0126 (Calbiochem, CA, USA) was treated to NR4A1-knockdown cells for 2 hours prior to analysis.

2. Plasmid and siRNA transfection

For the overexpression study, the full-length coding sequence of the NR4A1 gene was cloned into a pcDNA3-HA-tagged vector, and 7.5×10^5 cells were transfected with pcDNA3-NR4A1 plasmid for 24 hours using the Attractene (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To generate stable cell lines, pcDNA3-NR4A1 plasmid-transfected cells were treated with 500 μg/ml G418 for 2 weeks. For the knockdown study, 3.5×10^5 cells were transfected with 50 nM of NR4A1-siRNA (Genolution, Seoul, Korea) or control siRNA (Genolution, Seoul, Korea) for 48 hours using the G-fectin (Genolution, Seoul, Korea) following the manufacturer's protocol. siRNAs were designed and purchased from Genolution.

3. Total RNA isolation and quantitative real-time PCR

Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's procedures. cDNA was synthesized from 1 μg of total RNA by reverse transcription using ImProm-II Reverse Transcriptase (Promega, WI, USA). PCR was performed by Taq DNA polymerase (Bioneer, Seongnam, Korea) with the following conditions: initial denaturation for 5 min at 95 ℃, followed by 28-36 cycles of at 95 ℃ for 40 sec, at 56 ℃ for 20 sec (depending on target genes), and at 72 ℃ for 30 sec. For quantitative real-time PCR, Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) and

StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA) were used. All PCR reactions were performed at least in triplicate, and gene expression was normalized relative to that of human β-Actin and GAPDH, which were used as internal controls.

4. Cell proliferation assay

Cell proliferation assay was performed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan) as following the manufacturer's protocol. Briefly, 7.5×10^3 cells per well were seeded on 96-well plates and cultured in media with or without 8 μM tamoxifen. The cells were stained with 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) for 3 hours at 37 ℃, 5% CO2 incubator on designated days. The absorbance was measured by ELISA reader (Softmax Pro., Molecular Devices, CA, USA) at 450 nm. Tamoxifen treatment was refreshed every 24 hours to maintain a constant concentration.

5. Invasion and migration assays

Invasion assay was performed using the Matrigel™ (BD, Franklin Lakes,

NJ, USA) as previously described[28], while migration assay was carried out using the same way in the absence of Matrigel™. Approximately, 5×10^4 cells were seeded in each chamber and incubated at 37 ℃ for 48 hours. Invasive and migrating cells were stained with fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and observed via fluorescence microscopy. The acquired images were analyzed using the ImageJ software.

6. Apoptosis assay by annexin V-FITC/PI staining

Apoptotic cells were determined by the EzWay™ Annexin V-FITC apoptosis detection kit (Komabiotech, Seoul, Korea). Approximately 5×10^5 cells were seeded and incubated for 24 hours with 15 μM tamoxifen treatment. Subsequently, the cells were harvested, washed with cold PBS, and stained with Annexin V-FITC and propidium iodide (PI). Stained cells were analyzed using BD FACS LSRII (BD Biosciences, San Jose, CA, USA), and data were acquired using BD FACSDiva™ software.

7. Nuclear and cytoplasmic protein fractionation

For nuclear and cytoplasmic protein fractionation, cells were lysed in

cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1mM DTT, protease inhibitor) for 5 min on ice. NP-40 was then added to give a final concentration of 0.6%. The cytoplasmic protein of the total cell lysate was obtained by centrifugation for 30 sec at 4 ℃. The nuclear cell pellet was washed with cold PBS and lysed in cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor) for 15 min on ice. Nuclear proteins were harvested by centrifugation for 15 min at 4 ℃, and protein extracts were analyzed using western blot analysis.

8. Immunocytochemistry (ICC)

For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS for 15 min and rinsed with cold PBS. Cell permeabilization was performed with 0.25% Triton X-100 in PBS for 10 min. The cells were then blocked with 5% bovine serum albumin for 1 hour. After blocking, cells were incubated with primary antibody against NR4A1 (Abcam, ab13851, Cambridge, UK) at 4 ℃ overnight. The cells were subsequently washed with PBS and incubated with goat anti-rabbit IgG Alexa Fluor 594 (Abcam, ab150080, Cambridge, UK) for 1 hour in the dark. DAPI was added to the cells and incubated for 20 min. Fluorescence images were acquired by Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

9. Western blot analysis

All breast cancer cell lines were treated under the proper conditions and lysed in NP-40 lysis buffer, after which their protein concentrations were estimated using the Pierce BCA Protein Assay Kit (Thermo Scientific, MA, USA). Each lysate was separated by 8 - 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to Immobilon-P PVDF transfer membranes (Merck Millipore, MA, USA). Immunoreactive bands were detected by suitable primary antibodies and the appropriate HRP–conjugated secondary antibodies. Bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, MA, USA). Anti-NR4A1 (ab13851, Abcam, Cambridge, UK), anti-Caspase-7 (#12827, Cell Signaling Technology, MA, USA), anti-Caspase-9 (#9508, Cell Signaling), anti-PARP (#9542, Cell Signaling), anti-Src (#2110, Cell Signaling), anti-phospho-Src (#6943, Cell Signaling), anti-MEK1/2 (#4694, Cell Signaling), anti-phospho-MEK1/2 (#9154, Cell Signaling), anti-ERK1/2 (#9102, Cell Signaling), anti-phospho-ERK1/2 (#9101, Cell Signaling), anti-HDAC1 (ab7028, Abcam) and anti-β-Actin (ab6276, Abcam) were used to detect each proteins.

10. *In silico* analysis

A. Gene expression omnibus (GEO) dataset

NR4A1 mRNA levels in luminal A type breast cancer cohorts treated with tamoxifen monotherapy were assessed in the GEO dataset (GSE1378) obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/).

B. Kaplan-Meier survival analysis

The Kaplan-Meier plotter (http://kmplot.com) was used to assess the effect of genes on the survival rate in human breast cancer samples. To investigate the prognostic value of NR4A family genes, patient samples were classified into low- and high-expression groups using the median as the cutoff value.

C. cBioportal

The web-available database cBioportal (http://www.cbioportal.org) was used to evaluate the molecular profile changes of NR4A1 in breast cancer tissues. The patient sample data were obtained from the Breast Invasive Carcinoma (TCGA, Nature 2012).

D. Gene Ontology (GO) functional enrichment analysis

Gene Ontology (GO) functional enrichment analysis was analyzed for the differentially expressed genes (DEGs) according to NR4A1 mRNA levels using the DAVID database (version DAVID 6.8; http://david.ncifcrf.gov/) to annotate biological processes. Both upregulated and downregulated DEGs were used, and p-value < 0.01 was considered to indicate a statistically significant difference.

E. Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed to evaluate potential gene sets and pathways enriched in breast cancer tissue samples with high and low expression of NR4A1 obtained from the cBioportal database. The GSEA software was installed from the Broad Institute online website (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). The enriched gene sets with a p-value < 0.05 and FDR q-value < 0.05 were regarded statistically significant.

11. Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM)

of at least three different experiments. Statistical differences were determined using the paired Student's t-test. A p-value < 0.05 was considered statistically significant.

Table 1. Primers used for quantitative real-time PCR

Gene	Primer Sequences (5' - 3')
Human NR4A1	Forward : CCAAGTACATCTGCCTGGCTA Reverse : GACAACTTCCTTCACCATGCC
Human NR4A2	Forward : CCCAGTGGAGGGTAAACTCAT Reverse : TGTCTCTCTGTGACCATAGCC
Human NR4A3	Forward : CTTTGCAACGCTGACGGTG Reverse : CGACTCATGGGAGAGCACAG
Human SERPINE1	Forward : GGAGAAACCCAGCAGCAGATT Reverse : CTGTGGTGCTGATCTCATCCTT
Human STAT1	Forward : GCACGCTGCCAATGATGTTT Reverse : ACATCTGGATTGGGTCTTCCTG
Human ELK1	Forward : CTTCACGGGATGGTGGTGAA Reverse : CCGGCTGAGCTTGTCGTAAT
Human JUNB	Forward : CCCTACCGGAGTCTCAAAGC Reverse : TGCTGTTGGGGACAATCAGG
Human DUSP1	Forward : TCCCAACCATTTTGAGGGTCA Reverse : AAACACCCTTCCTCCAGCATT
Human FOS	Forward : TTACTACCACTCACCCGCAG Reverse : GCAGTGACCGTGGGAATGAA
Human GAPDH	Forward : TATAAATTGAGCCCGCAGCC Reverse : CCCAATACGACCAAATCCGTTG
Human β-ACTIN	Forward : CATGTTTGAGACCTTCAACACCCC Reverse : GCCATCTCCTGCTCGAAGTCTAG

III. RESULTS

1. NR4A1 is associated with tamoxifen sensitivity in ER-positive breast cancer

To investigate NR4A1 gene expression in tamoxifen-resistant (TamR) breast cancer cells, we examined the mRNA and protein expression levels of NR4A1 in MCF7 and TamR cells by qRT-PCR and western blot assay. The results showed that the mRNA and protein levels of NR4A1 were significantly downregulated in TamR cells compared to those in MCF7 and T47D cells **(Fig. 1A-B and Fig. 2)**. We also compared the expression of NR4A1 in clinical breast cancer patient samples using a publicly available dataset (GSE1378, METABRIC). The expression levels of NR4A1 decreased in recurred (tamoxifen-resistant) luminal A type breast cancer patients treated with tamoxifen therapy (GSE1378) and hormone therapy (METABRIC) compared with those in non-recurred (tamoxifen-sensitive) luminal A type breast cancer patients **(Fig. 1C and Fig. 1D)**.

Further, we examined whether the elevated NR4A1 expression was associated with an increased survival rate in human breast cancer using NR4A1 gene expression. To evaluate the clinical relevance between NR4A1 expression and survival rate, we analyzed the survival of ER-positive breast cancer patients using Kaplan-Meier survival analysis. The high expression of NR4A1 correlated with good overall survival (OS) and relapse-free survival (RFS) in ER-positive

breast cancer patients with following tamoxifen therapy, although there was no significant relevance in untreated patients **(Fig. 1E and F)**. Other NR4A family genes NR4A2 and NR4A3 showed no significant difference between the mRNA levels of MCF7 and TamR cells, although high NR4A3 level represented favorable OS and RFS in patients treated with tamoxifen therapy, but not NR4A2 level **(Fig. 3A-F)**. Taken together, these results show that NR4A1 is associated with tamoxifen sensitivity in human ER-positive breast cancer.

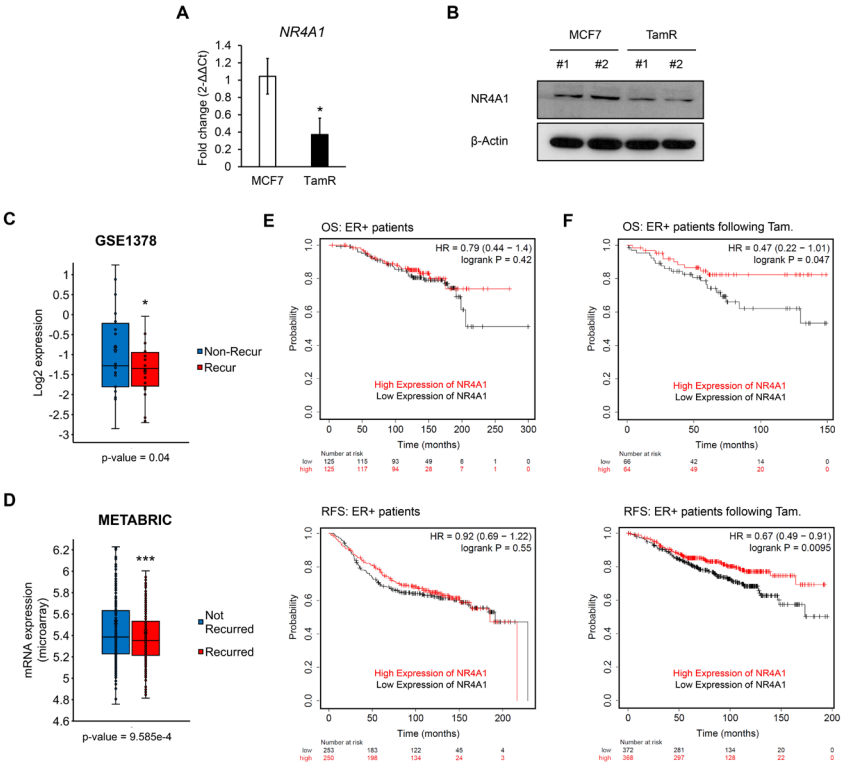

Figure 1. NR4A1 is associated with tamoxifen sensitivity in ER-positive breast cancer. mRNA **(A)** and protein **(B)** expression levels of NR4A1 in MCF7 and TamR breast cancer cells. **(C)** The mRNA expression levels of NR4A1 in luminal A type breast cancer patients and recurred patients treated with tamoxifen monotherapy for 5 years (GSE1378) (n = 52, p = 0.04) are shown by log2 expression values. **(D)** The mRNA expression levels of NR4A1 in ER-positive breast cancer patients and recurred patients treated with hormone therapy (METABRIC) (n = 1,079, p = 9.585e-4) are shown by log2 expression values. Data was derived from cBioportal. **(E)** Kaplan-Meier analysis curves of overall

survival (OS) (upper panel; n = 250, $p = 0.42$) and recurrence-free survival (RFS) (lower panel; n = 503, $p = 0.55$) rates for ER-positive breast cancer patients receiving no treatment. **(F)** Kaplan-Meier analysis curves of OS (upper panel; n = 130, $p = 0.047$) and RFS (lower panel; n = 740, $p = 0.0095$) rates for ER-positive breast cancer patients with following tamoxifen therapy. The NR4A1 (211143_x_at) probe was used and analyzed for survival analysis. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

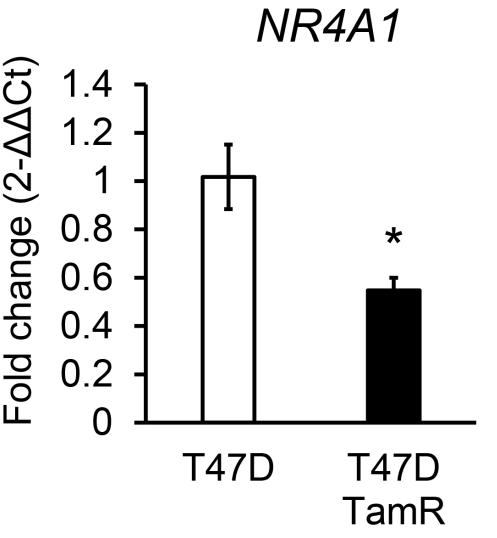

Figure 2. NR4A1 is downregulated in T47D-TamR cells. RT-qPCR analysis of NR4A1 in T47D and T47D-TamR cells. * $p < 0.05$.

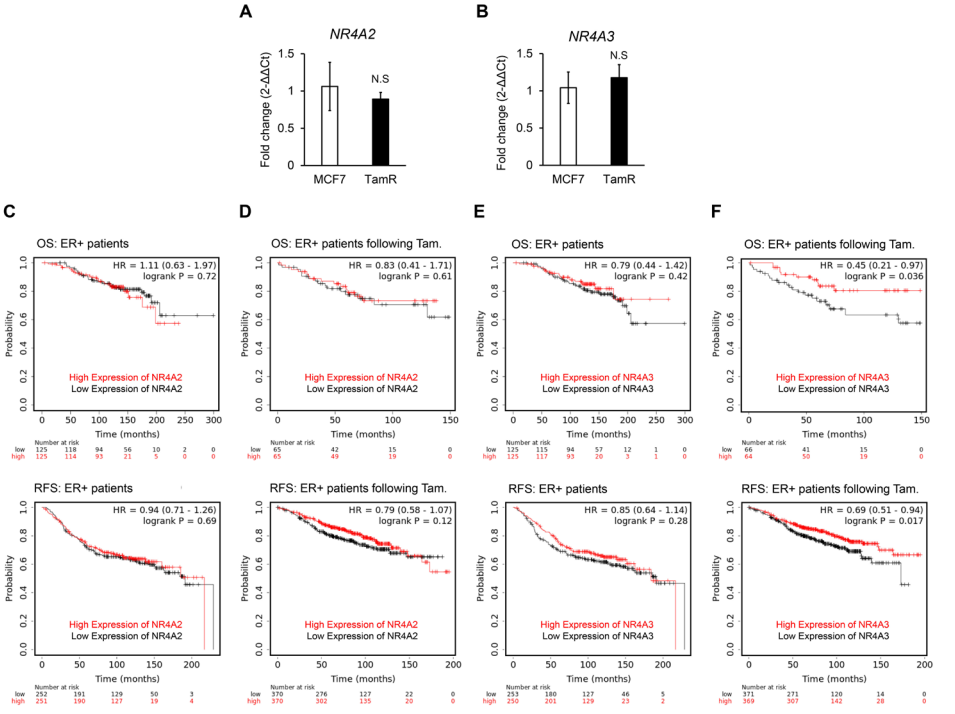

Figure 3. NR4A2 and NR4A3, other NR4A family genes, are not associated with tamoxifen sensitivity in ER-positive breast cancer. (A) and **(B)** mRNA expression levels of NR4A2 and NR4A3, respectively, in MCF7 and TamR breast cancer cells. **(C)** Kaplan-Meier analysis curves of overall survival (OS) (upper panel; n = 250, p = 0.72) and recurrence-free survival (RFS) (lower panel; n = 503, p = 0.69) rates for ER-positive breast cancer patients receiving no treatment. **(D)** OS (upper panel; n = 130, p = 0.61) and RFS (lower panel; n = 503, p = 0.12) curve (lower panel) for patients with following tamoxifen therapy. The NR4A2 (204622_x_at) probe was used and analyzed for survival analysis **(E)** Kaplan-Meier analysis curves of OS (upper panel; n = 250, p = 0.42) and RFS (lower

panel; n = 503, p = 0.28) rates for ER-positive breast cancer patients receiving no treatment. **(F)** OS (upper panel; n = 130, p = 0.036) and RFS (lower panel; n = 503, p = 0.017) curves for patients with following tamoxifen therapy. The NR4A3 (209959_at) probe was used and analyzed for survival analysis. N.S = not significant.

2. NR4A1 suppresses proliferation, migration, and invasion ability, while increasing tamoxifen-induced apoptosis in TamR cells

To confirm tamoxifen resistance in our cell line, we compared cell proliferation between MCF7 and TamR cells by CCK-8 assay. TamR cells were resistant to tamoxifen treatment **(Fig. 4A)**. To investigate the functional role of NR4A1 in TamR cells, we confirmed the ectopic overexpression of NR4A1 in TamR cells and knockdown of NR4A1 in MCF7 cells by RT-PCR **(Fig. 5A)**. There was no difference in cell proliferation when NR4A1 was knocked down in MCF7 cells in the absence of tamoxifen (No Tam.). Interestingly, knockdown of NR4A1 increased the resistance to tamoxifen in MCF7 cells treated with tamoxifen for 4 days **(Fig. 5B)**. Consistent with the results of NR4A1 knockdown, overexpression of NR4A1 significantly restored sensitivity to tamoxifen in TamR cells **(Fig. 5C)**. These results suggest that NR4A1 expression regulates sensitivity to tamoxifen in ER-positive breast cancer cells.

Because tamoxifen-resistant breast cancer cells have migratory and invasive properties[29], we confirmed that TamR cells exhibit higher migratory and invasive abilities than those of MCF7 cells through transwell migration and invasion assays **(Fig. 4B)**. We then evaluated the effect of NR4A1 expression on migration and invasion abilities in TamR cells. As shown in **Fig. 5D**, overexpression of NR4A1 significantly reduced the migration and invasion abilities of TamR cells.

Since it is known that NR4A1 has a function to induces cell apoptosis in many types of tumors[30], we investigated whether the expression of NR4A1 re-sensitizes TamR cells to tamoxifen treatment by increasing apoptotic ability. Flow cytometry analysis was performed using annexin V/PI staining to compare the apoptotic levels between MCF7 and TamR cells under the absence and presence of tamoxifen. High-dose tamoxifen (15 μM) treatment induced apoptosis in MCF7 cells, while there were insignificant changes in TamR cells **(Fig. 4C and Fig. 4D)**. In addition, overexpression of NR4A1 in TamR cells (TamR-NR4A1) significantly increased the percentage of apoptotic cell death compared to that in control cells (TamR-vec) when tamoxifen was treated **(Fig. 5E and Fig. 5F)**. These results demonstrated that overexpression of NR4A1 enhanced tamoxifen-induced apoptotic ability of tamoxifen-resistant ER-positive breast cancer cells. Thus, NR4A1 inhibits cell proliferation, migration, and invasion ability, while enhancing tamoxifen-induced apoptosis in TamR cells.

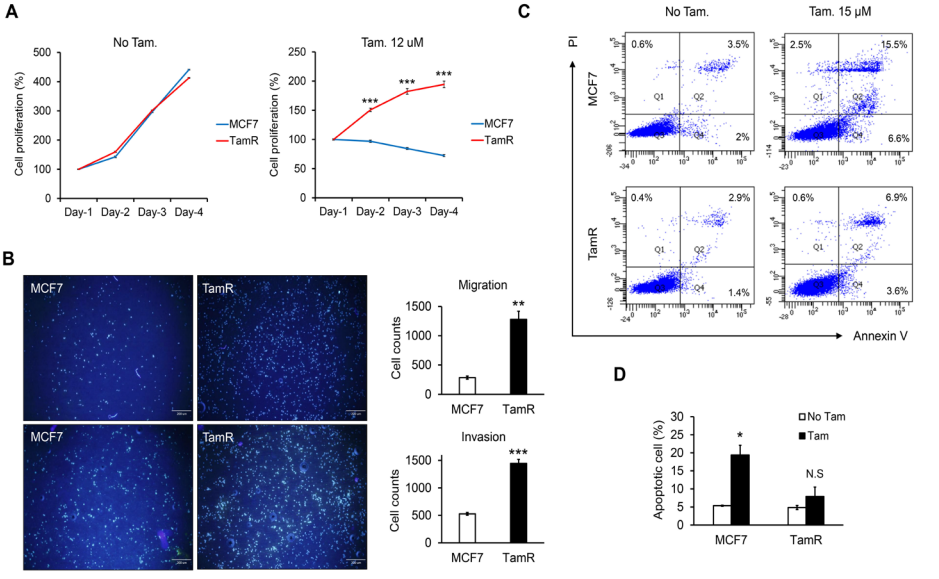

Figure 4. TamR cells are more resistant to tamoxifen compared to MCF7 cells. (A) Cell proliferation curves of MCF7 and TamR cells untreated (No Tam.) or treated with 12 μM tamoxifen (Tam.) for 4 days. **(B)** Matrigel invasion and migration assays in MCF7 and TamR cells. Migrated and invaded cells were stained with DAPI. **(C)** FACS analysis from apoptotic cell death in MCF7 and TamR cells untreated or treated with 15 μM tamoxifen. The apoptotic cells were stained by Annexin V/PI. **(D)** Percentage of apoptotic cells from **(C)** are represented by bar graph. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

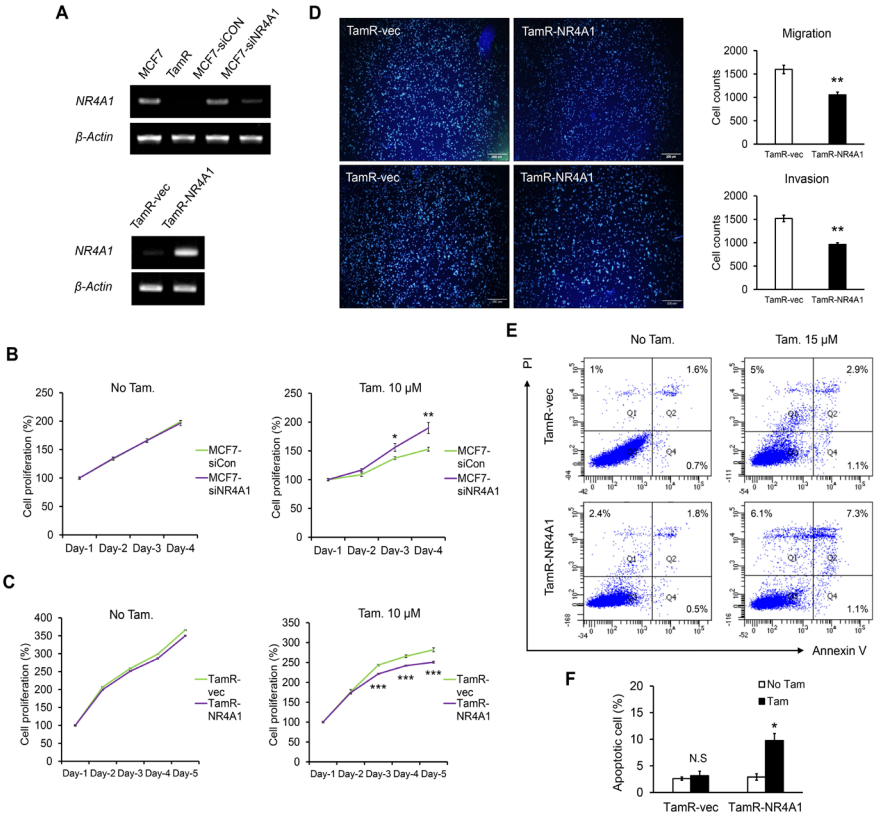

Figure 5. NR4A1 suppresses proliferation, migration, and invasion, while increasing tamoxifen-induced apoptosis in TamR cells. (A) mRNA expression levels of NR4A1 in MCF7, TamR, MCF7 cells transfected with siControl (siCon) or NR4A1 siRNA (siNR4A1), and TamR cells transfected with pcDNA3 (empty vector) or pcDNA3-NR4A1. **(B)** Cell proliferation curves of siCon / siNR4A1 MCF7 cells untreated (No Tam.) or treated with 10 μM tamoxifen (Tam.) for 4 days. **(C)** Cell proliferation curves of TamR-vec and TamR-NR4A1 cells untreated (No Tam.) or treated with 10 μM tamoxifen (Tam.) for 5 days. **(D)** Matrigel invasion and migration assays in empty vector transfected TamR and

stable NR4A1-overexpressing TamR cells. Migrated and invaded cells stained with DAPI were calculated by imageJ. **(E)** FACS analysis from apoptotic cell death in TamR-vec and TamR-NR4A1 cells untreated or treated with 15 μM tamoxifen. The apoptotic cells were stained by Annexin V/PI. **(F)** Percentage of apoptotic cells from **(E)** are represented by bar graph. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. NR4A1 localized in the cytoplasm affects apoptosis

Since NR4A1 has different functions depending on subcellular localization in various types of cancer models[31], we compared the distribution and subcellular localization of NR4A1 between MCF7 and TamR cells by nuclear and cytoplasmic fractionation. The results showed that NR4A1 is primarily localized to the cytoplasm in both cells and upregulated in MCF7 than TamR cells **(Fig. 6A)**. Immunocytochemistry analysis also revealed that localization of NR4A1 predominantly localizes in the cytoplasm in MCF7 cells **(Fig. 6B)**. Although NR4A1 also existed slightly in the nuclear fraction, we intended to focus on the function of cytoplasm, as NR4A1 was more abundant in the cytoplasm. It has been reported that NR4A1, which translocases from the nucleus to the cytoplasm, releases cytochrome c and induces apoptosis[32]. In that sense, we hypothesized that high expression of cytoplasmic NR4A1 in MCF7 cells might cause apoptosis and explored the expression of pro-apoptotic genes by western blot analysis. The results showed that the protein levels of cleaved caspase-7, -9, and PARP, and uncleaved PARP were higher in MCF7 cells compared to that in TamR cells. When NR4A1 was knocked down in MCF7 cells, the levels of cleaved caspase-7, cleaved PARP, and uncleaved PARP decreased, while the level of cleaved caspase-9 did not change. Conversely, when NR4A1 was overexpressed in TamR, the cleaved forms of caspase-7, -9, and PARP increased, although the uncleaved forms did not change **(Fig. 6C)**. Additionally,

we performed immunocytochemistry to confirm the subcellular localization of NR4A1 when NR4A1 was overexpressed in TamR and found that NR4A1 was mainly distributed in the cytoplasm **(Fig. 7)**. These results confirmed that NR4A1 localized in the cytoplasm plays an essential role in inducing the expression of apoptosis-related factors.

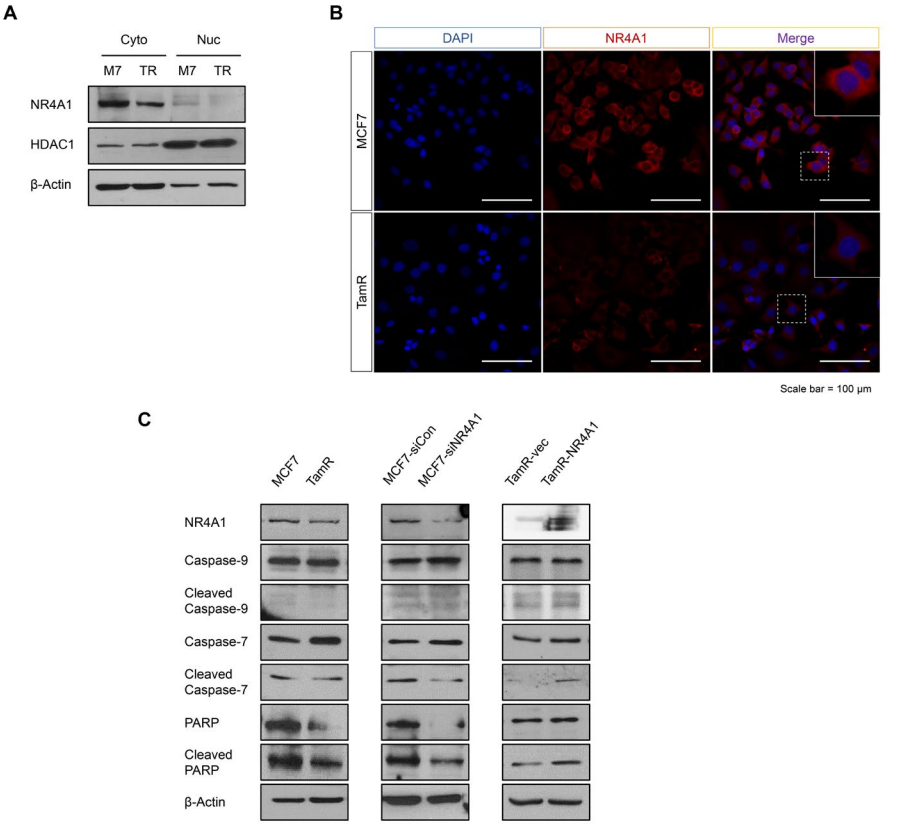

Figure 6. NR4A1 localized in the cytoplasm influences apoptosis. (A) Western blot analysis for NR4A1 protein of cytoplasmic and nuclear fractions from MCF7 and TamR cells. **(B)** Representative images of immunofluorescence for NR4A1 localization in MCF7 and TamR cells. Scale bar = 100 μm. **(C)** Western blot analysis for apoptosis-related proteins in MCF7, TamR, MCF7 cells transfected with scramble (negative) control (siCon), NR4A1 siRNA (siNR4A1), and TamR cells transfected with pcDNA3 and pcDNA3-NR4A1.

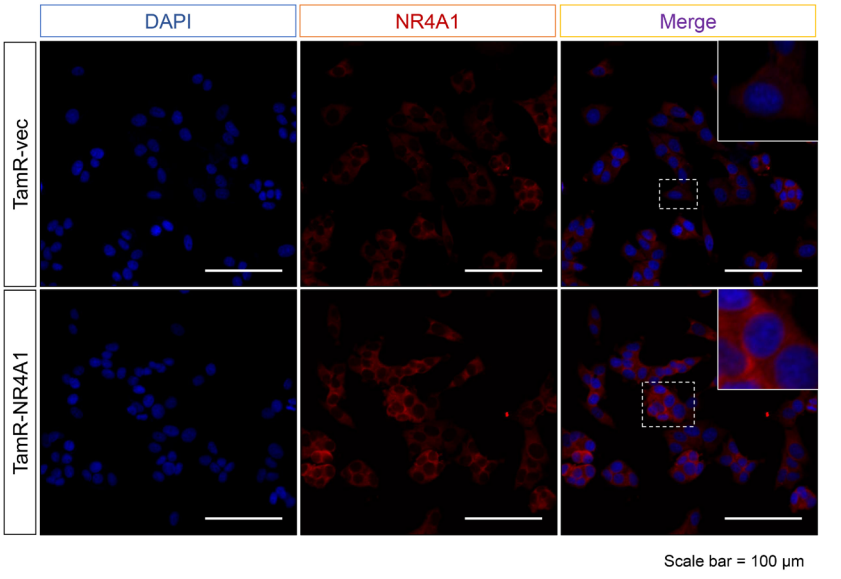

Figure 7. NR4A1 localizes in the cytoplasm following NR4A1 overexpression in TamR cells. Representative images of immunofluorescence for NR4A1 localization in TamR-vec and TamR-NR4A1 cells. Scale bar = 100 μm.

4. NR4A1 alters tamoxifen sensitivity in ER-positive breast cancer cells through suppressing ERK signaling pathway

To investigate the molecular mechanism by which NR4A1 regulates tamoxifen sensitivity, we performed gene expression profiling using the breast cancer patient dataset obtained from the cBioportal. We ultimately identified 199 DEGs according to NR4A1 mRNA levels **(Fig. 8A)**. To determine the biological function of the target genes, DEGs were applied to gene ontology functional enrichment analysis using the DAVID database. The result showed that these NR4A1 target genes were significantly enriched in the negative regulation of ERK1 and ERK2 cascade **(Fig. 8B)**. Moreover, GSEA conducted between the NR4A1 high and low groups revealed that high mRNA levels of NR4A1 are correlated with the negative regulation of MAPK cascade **(Fig. 8C and Fig. 8D)**. Furthermore, previous studies have reported that cytoplasmic NR4A1 is associated with ERK signaling and a good prognosis[33]. This evidence raises the possibility that NR4A1 genes may contribute to the negative regulation of the ERK signaling pathway in breast cancer. Therefore, we checked the expression levels of proteins related to the ERK signaling pathway. Firstly, the results demonstrated that the protein levels of p-SRC, p-MEK1/2, and p-ERK1/2 were higher in TamR cells compared to that in MCF7 cells. In addition, we confirmed that NR4A1 could downregulate the levels of p-SRC, p-MEK1/2, and p-ERK1/2 by western blot analysis **(Fig. 9A)**. Because NR4A1 changes the level of

phosphorylated ERK protein, which actually functions, we assumed that NR4A1 would also change the expression of target genes that are directly regulated by ERK. Therefore, the transcription levels of several ERK target genes were confirmed by quantitative real-time PCR. The results showed that ERK target genes such as STAT1 and DUSP1 were also regulated according to the expression of NR4A1 **(Fig. 9B)**.

It is also known that the ERK signaling pathway plays an important role in the tamoxifen-resistant phenotype[24]. To evaluate whether the induction of ERK signaling is necessary for silenced-NR4A1 mediated tamoxifen resistance, we treated U0126, an ERK1/2 inhibitor, in MCF7-siNR4A1 cells and performed CCK-8 assay. The results showed that U0126 could reverse the ability of cell proliferation which was enhanced by NR4A1 knockdown **(Fig. 10B)**. Additionally, we measured the expression level of p-ERK1/2 after treatment with U0126. As shown in **Fig. 10A**, the inhibitor treatment efficiently inhibited the phosphorylation of ERK1/2, which was increased by knockdown of NR4A1 without affecting the total ERK1/2 level. Taken together, our data suggest that NR4A1 regulates tamoxifen sensitivity by inhibiting the ERK signaling pathway in ER-positive breast cancer.

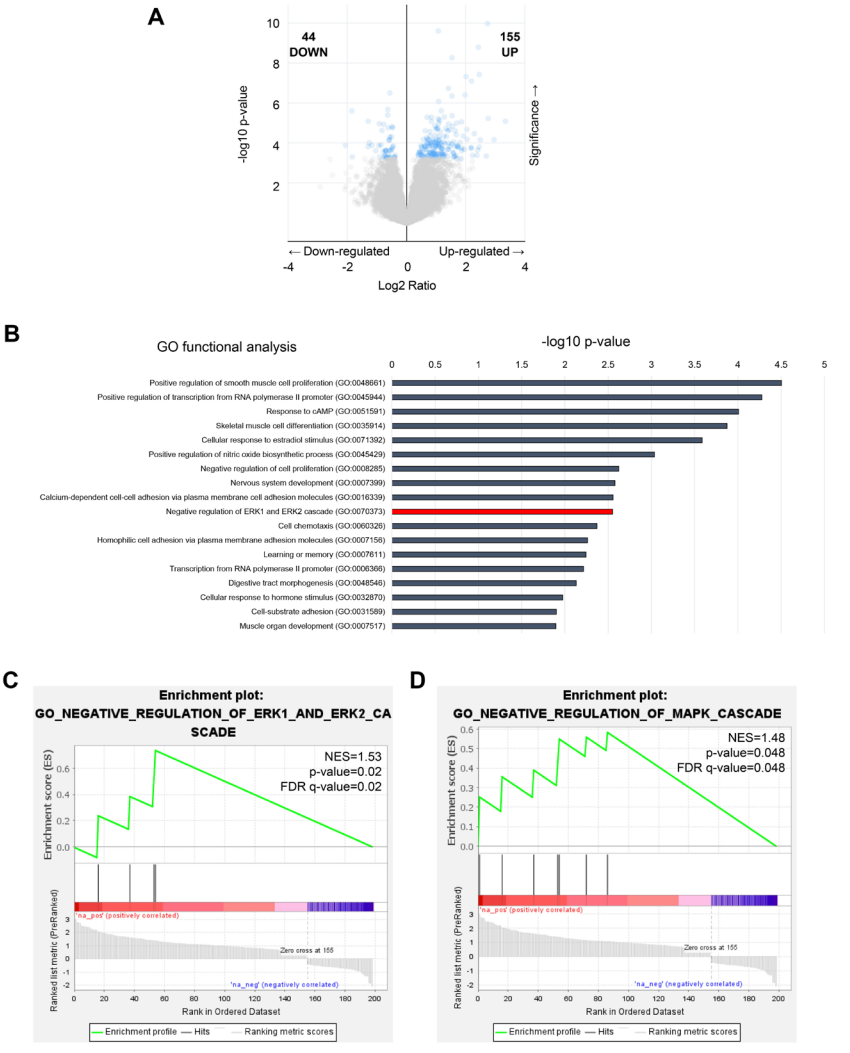

Figure 8. NR4A1 downstream genes are related to the negative regulation of ERK signaling cascade. (A) The volcano plot of significantly up- and down-regulated genes according to mRNA expression profile of NR4A1 gene (Student's t-test $p < 0.05$ and Benjamini-Hochberg procedure $q < 0.05$). **(B)** Gene ontology functional enrichment analysis showing biological processes enriched

in 199 genes regulated by NR4A1. Gene set enrichment analysis plots showing a significant correlation between high mRNA levels of NR4A1 and negative regulation of ERK1, ERK2 **(C)**, and MAPK cascade **(D)** gene sets in a breast cancer patient dataset derived from the cBioportal. NES, normalized enrichment score; FDR, false discovery rate.

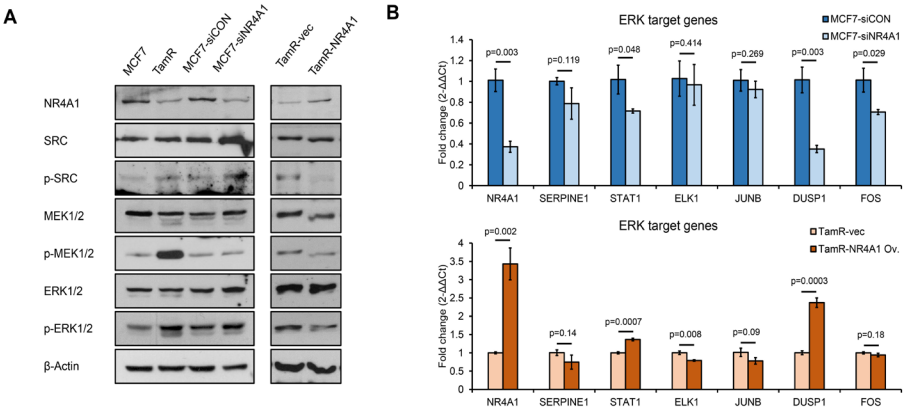

Figure 9. NR4A1 downregulates the ERK signaling pathway in ER-positive breast cancer cells. (A) Western blot analysis for the ERK signaling pathway-related protein in MCF7, TamR, MCF7 cells transfected with scramble (negative) control (siCon), NR4A1 siRNA (siNR4A1), and TamR cells transfected with pcDNA3 and pcDNA3-NR4A1. **(B)** mRNA expression levels of ERK target genes in MCF7 cells transfected with scramble (negative) control (siCon) and NR4A1 siRNA (siNR4A1), and TamR cells transfected with pcDNA3 and pcDNA3-NR4A1.

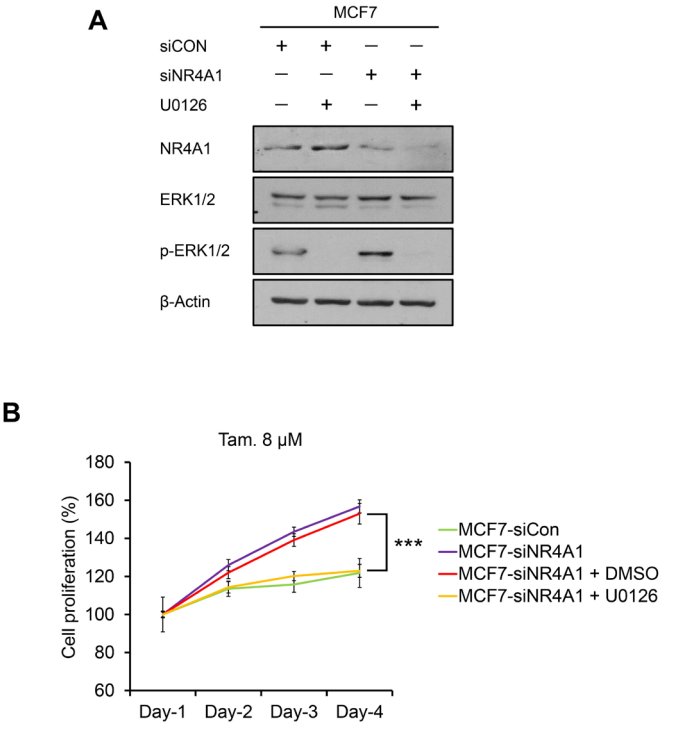

Figure 10. NR4A1 alters tamoxifen sensitivity in ER-positive breast cancer cells through suppressing the ERK signaling pathway. (A) Western blot analysis for NR4A1, ERK1/2, and p-ERK1/2 proteins in MCF7-siCon and siNR4A1 cells after treatment with U0126 or DMSO (control) **(B)** Cell proliferation curves of MCF7-siCon and siNR4A1 cells treated with U0126 or DMSO (control) under the presence of 8 μM tamoxifen (Tam.) for 4 days. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

IV. DISCUSSION

Herein, we demonstrated that NR4A1, whose expression was downregulated in TamR cells and recurrent breast cancer patients treated with tamoxifen, may be a new prognostic factor and therapeutic target for tamoxifen resistance. In our Kaplan-Meier analysis, we found that high expression of NR4A1 in ER-positive breast cancer patients treated with tamoxifen associates with a good prognosis. Through conducting gain- and loss-of-function study, the subsequent upregulation of NR4A1 expression enhanced tamoxifen sensitivity, thereby regulating cell proliferation, migration, invasion, and apoptosis in tamoxifen-resistant cells. More importantly, high cytoplasmic NR4A1 contributes to apoptosis by inducing the expression of pro-apoptotic factors in breast cancer cells. Mechanistically, we revealed that NR4A1 restored tamoxifen sensitivity by inhibiting the ERK signaling pathway in tamoxifen-resistant breast cancer cells.

NR4A1 is an early response gene promptly induced by various cellular stimuli and plays an important role in various physiological processes and diseases, including cancer[9]. Several evidences show that NR4A1 functions to promote or inhibit tumor progression depending on the tumor type, cell-specific context, and various external stimuli. NR4A1 expression has been previously reported to be upregulated in non-small-cell lung carcinoma tissues compared to that in normal tissues, and high NR4A1 level has shown an oncogenic role as a

prognostic marker for predicting adverse clinical outcomes in non-small-cell lung carcinoma[34]. Additionally, it was reported that NR4A1 expression suppresses cancer growth and progression in triple-negative breast cancer (TNBC) *in vitro* and *in vivo*, showing its role as a tumor suppressor[18]. Moreover, recent works have represented that NR4A1 induces TNFα-mediated apoptosis sensitivity in human gastric cancer[35] and improves cisplatin resistance in ovarian cancer[36], supporting that NR4A1 plays a crucial role in drug therapy sensitivity in a variety of tumor models. Nevertheless, the exact role of NR4A1 in contributing to tamoxifen resistance in breast cancer has not been elucidated. In the present study, high levels of NR4A1 were found to be associated with a favorable prognosis and tamoxifen sensitivity in ER-positive breast cancer patients treated with tamoxifen. Moreover, tamoxifen-resistant cell line (TamR) used in this study showed downregulated NR4A1 expression, whereas other NR4A factors, NR4A2 and NR4A3, did not differ in expression between MCF7 and TamR cells. Overexpression of NR4A1 restored the sensitivity to tamoxifen and attenuated proliferation, migration, and invasion; however, it enhanced tamoxifen-induced apoptosis in TamR cells. This phenomenon was also observed in previous studies that NR4A1 reduces migration in both normal and breast cancer cells[37] and mediates apoptosis in aggressive B-cell lymphoma[30], indicating that NR4A1, which regulates cellular processes in response to tamoxifen could suppress the acquisition of a tamoxifen-resistant phenotype. Thus, NR4A1 could be used as a predictor of tamoxifen responsiveness and new therapeutic target to overcome

tamoxifen resistance in ER-positive breast cancer.

Localization of the protein in the cell is crucial for understanding its biological effects, and subcellular mislocalization of proteins can work as a molecular therapeutic intervention[38,39]. The function of NR4A1 is influenced not only by expression but also by its localization within the cell. NR4A1 present in the nucleus is translocated to the cytoplasm in response to several apoptosis-inducing agents, where it binds to Bcl-2 and forms a pro-apoptotic complex, then inducing cytochrome c release and cell apoptosis through a p53-independent intrinsic pathway[32]. In this study, surprisingly, we found that NR4A1 is mainly located in the cytoplasm in both MCF7 and TamR breast cancer cells and promoted the expression of apoptosis-related molecules, including caspase-7, caspase-9, and PARP. Similarly, several reports also showed that NR4A1 existed in the cytoplasm induces cell apoptosis mediated by apoptotic-associated molecules[35,40]. Hence, its reduced expression in tamoxifen-resistant tumor cells may possibly contribute to apoptosis and cell survival, and translocation of NR4A1 to the cytoplasm from the nucleus by various agents including cytosporone B and C-DIM[41,42] could serve as a unique therapeutic strategy. However, it is still limited whether nuclear localization of NR4A1 through drugs that block nuclear export of NR4A1 confers tamoxifen resistance or tumor progression, which needs further investigation.

According to previous reports, it is known that cytoplasmic NR4A1 is associated with the ERK signaling pathway[33]. ERK signaling is a pathway that

plays a crucial role in tamoxifen resistance as well as cellular signaling in various cancers, including breast cancer[24]. In addition, activated ERK signaling reduces responsiveness and effectiveness to tamoxifen treatment in ER-positive breast cancer patients and affects poor prognosis[25,26]. These studies represent that ERK acts as a prognostic marker and can serve as an indicator of the effectiveness of tamoxifen treatment in breast cancer patients. In the present study, through *in silico* gene functional analysis and *in vitro* experiments, our results showed that NR4A1 not only reduces the expression of factors related to ERK signaling but also regulates the mRNA expression of ERK downstream target genes (STAT1 and DUSP1), implying that ERK signaling may represent a general target pathway of NR4A1. Also, we further confirmed that the enhanced proliferation ability induced by NR4A1 knockdown is greatly impaired after treatment with an ERK inhibitor. Similarly, a previous research reported that NR4A1 mitigates the level of active ERK1/2 and ERK inhibition following U0126 treatment in NR4A1-overexpressing normal breast epithelial cells suppresses cell migration[37]. These evidences indicate that deactivation of the ERK signaling pathway is responsible for NR4A1-induced tamoxifen sensitivity. Accordingly, the regulation of ERK signaling by NR4A1 revealed in this study suggests as a potential therapeutic target as well as a biomarker for treating tamoxifen resistance.

Our study firstly revealed that NR4A1 is a marker associated with tamoxifen sensitivity. In this study, NR4A1 induced sensitivity to tamoxifen by

regulating cell proliferation, migration, invasion, and apoptosis in ER-positive breast cancer cells. In addition, NR4A1 localized in the cytoplasm activated apoptotic molecules. Also, NR4A1 inhibited the ERK signaling cascade, which in turn regulated ERK target genes, resulting in attenuated tamoxifen resistance in TamR cells. Collectively, our study suggests that the NR4A1/ERK signaling axis plays an important role in overcoming tamoxifen resistance and could be a novel therapeutic target for attenuating tamoxifen resistance in ER-positive breast cancer patients.

V. CONCLUSION

The purpose of this thesis was to demonstrate the functional role of NR4A1 in ER-positive breast cancer and discover its regulatory mechanism involved in tamoxifen resistance.

In conclusion, this study emphasizes the critical role of NR4A1 in tamoxifen resistance breast cancer by suppressing oncogenic properties and enhancing apoptosis. The downregulation of NR4A1 could be a potential biomarker for tamoxifen resistance in patients receiving or that have received tamoxifen therapy. In addition, the inhibition of NR4A1 could serve as a possible therapeutic strategy to restore sensitivity in tamoxifen resistant breast cancer patients.

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ABSTRACT (IN KOREAN)

에스트로겐 수용체 양성 유방암에서 NR4A1의 ERK 신호전달 경로 억제를 통한 타목시펜 저항성 조절

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김 유 천

유방암은 전 세계에서 가장 흔한 암 중 하나이다. 에스트로겐 수용체 양성 (ER-positive) 유방암은 전체 유방암 환자 중 약 70%를 차지하며, 내분비 치료 (endocrine therapy)를 받는다. 내분비 치료 중 흔히 사용되는 치료 약물인 타목시펜은 이러한 환자들에 효과적이다. 그럼에도 불구하고, 타목시펜 치료를 받은 환자의 약 3분의 1은 타목시펜 저항성이 생기며, 이는 결국 치료 효과를 감소시킨다. 또한, 타목시펜 저항성에 관여하는 분자적 기전은 여전히 불분명한 실정이다.

NR4A1은 암화 과정, 세포 사멸, DNA 수선, 세포 증식, 염증과 같은 과정에 중요한 역할을 한다고 알려져 있다. 최근 NR4A1이 유방암을 포함한 다양한 암 모델에서 종양 유전자 혹은 종양 억제 유전자로의 기능이 보고된 바 있으나, 타목시펜 저항성 유방암에서의 역할은 알려진 바가 없다.

본 학위 논문에서는 타목시펜 저항성 에스트로겐 수용체 양성 유방암에서 NR4A1의 임상적 중요성과 기능적 역할 뿐만 아니라 분자적 조절 기전 또한 확인했다. NR4A1의 유전자

발현이 타목시펜 비저항성 유방암 세포주 (MCF7, T47D)에 비해 저항성 세포주 (TamR, T47D-TamR)에서 감소되어 있음을 확인했으며, Kaplan-Meier 생존 분석을 통해 높은 NR4A1의 발현이 타목시펜 치료를 받은 에스트로겐 수용체 양성 유방암 환자의 높은 생존률과 관련이 있음을 검증하였다. 기능적인 연구로 과발현 및 발현 저해 실험을 통해, NR4A1이 세포 증식, 이동, 침입 및 세포 사멸 능력을 조절함으로써 타목시펜에 대한 민감성을 회복시킴을 보여주었다. 또한 세포질에 위치한 NR4A1은 세포 사멸 인자의 발현을 증가시킴을 발견하였다. *In silico* 분석 및 *in vitro* 실험을 이용한 메커니즘 연구를 통해, NR4A1이 에스트로겐 수용체 양성 유방암에서 ERK 신호 전달 경로를 억제함으로써 타목시펜에 대한 반응성을 향상시킴을 확인했으며, 이 결과는 NR4A1/ERK 신호 전달 경로가 타목시펜 저항성을 조절함을 의미한다.

본 학위논문의 결과들을 종합적으로 미루어 볼 때, NR4A1이 에스트로겐 수용체 양성 유방암에서 타목시펜 저항성을 극복하는 잠재적인 치료적 타겟이 될 수 있음을 시사한다.

핵심되는 말 : NR4A1, ERK 신호전달, 타목시펜 저항성, 유방암

PUBLICATION LIST

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