이학석사 학위논문

Regulatory Mechanism of Quinone Oxidoreductase 2 Associated with Aggressiveness of Breast Cancer

유방암 세포의 악성화 관련 Quinone Oxidoreductase 2 기전 연구

2021 년 8 월

서울대학교 대학원
협동과정 종양생물학과

권 순 규

Master' s Thesis of Science

Regulatory Mechanism of Quinone Oxidoreductase 2 Associated with Aggressiveness of Breast Cancer

August 2021

Graduate School of Medicine
Seoul National University
Cancer Biology Major

Sunkyu Kwon

Regulatory Mechanism of Quinone Oxidoreductase 2 Associated with Aggressiveness of Breast Cancer

유방암 세포의 악성화 관련 Quinone Oxidoreductase 2 기전 연구

지도 교수 한 원 식

이 논문을 이학석사 학위 논문으로 제출함
2021년 4월

서울대학교 대학원
협동과정 종양생물학과
권 순 규

권순규의 이학석사 학위논문을 인준함
2021년 7월

위 원 장 _____ 임 석 아 _____

부위원장 _____ 한 원 식 _____

위 원 _____ 예 상 규 _____

Abstract

Background

NRH:Quinone Oxidoreductase 2 (NQO2) is one of the key flavoproteins that forms hydroquinone by catalyzing the two-electron reduction of quinone substrates and uses dihydronicotinamide riboside as a reducing coenzyme. [3] Despite the high level of NQO2 expression in breast cancer, its role in breast cancer is not adequately understood. The aim of this study is to investigate the biological role of NQO2 in cell survival, growth, migration, and invasion, and which are thought to be fundamental to tumor aggressiveness in several breast cancer cells.

Methods

Nine human breast cancer cell lines (MCF-7, ZR75-1, T47D, BT-474, SK-BR-3, BT20, MDA-MB-468, MDA-MB-231 and HS578T) and one normal breast epithelial cell line (MCF10A) were used. To analyze the prognostic significance of NQO2 gene, hazard ratios was analyzed by public database (KM plotter). To explore the biological function of NQO2, knockdown of NQO2 was assessed using specific siRNA. Scrambled siRNA was used for control. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to compare NQO2 mRNA

and protein expression level among cell lines respectively. Cell growth was evaluated by MTT assay. Transwell migration and invasion assay was performed and the migrated cells in the bottom chamber were analyzed crystal violet staining. Cell cycle analysis by flow cytometry was performed based on measurement of DNA content by stain with propidium iodide. To explore the impact of NOQ2 on the epithelial-mesenchymal transition (EMT), the expressions of E-cadherin, N-cadherin, Vimentin, and Slug were evaluated by Western blot in NQO2 knockdown cells. Since TGF-β/Smad pathway is a well-known EMT-inducer, TGF-β/Smad representative molecules are detected by Western blot to investigate whether the silencing of NQO2 effects the signaling pathway.

Result

Among nine different breast cancer cells, ZR75-1, SKBR3, BT-20 and HS578T cells exhibited significantly high mRNA and protein expression levels of NQO2 as compared with normal epithelial cells MCF10A. Therefore, the biological function of NQO2 was studied in ZR75-1, SK-BR-3, BT-20 and HS578T cells. As compared with scrambled siRNA, the transfection of NQO2 siRNA for 48 h resulted in 80-90 % decrease in NQO2 protein levels of ZR75-1, SK-BR-3, BT-20 and HS578T cells. NQO2 knockdown

ZR75-1, SK-BR-3, BT-20 and HS578T cells displayed a significant decrease of migration (~30 %) and invasion (~50%) cell growth (~80 %) capacities compared with control cells. Moreover, G0 and G1 cell cycle arrest in SK-BR-3, BT-20 and HS578T and apoptosis in ZR75-1 were caused by NQO2 knockdown. In analysis of biomarkers of EMT, NQO2 knockdown increased E-cadherin, but decreased the level of N-cadherin, Vimentin and Slug in ZR75-1 and HS578T cells. Additionally, NQO2 knockdown decreased not only the expression of TGF-β1 but also the expression levels of Smad2, Smad3, P-Smad2, and P-Smad3 were decreased indicating that NQO2 knockdown suppresses TGF-β-driven EMT in ZR75-1 and HS578T cells.

Conclusion

These data indicated that NQO2 has a functional role in the migration, invasion, cell survival, growth, and EMT which is associated with aggressive behaviors of breast cancer cells. Our results suggest the potential of NQO2 as a therapeutic target for the breast cancer therapy.

Keyword: Breast cancer, NQO2, Cell Migration, EMT (Epithelial-Mesenchymal Transition), TGF-β/Smad, Cancer Aggressiveness

Table of Contents

Abstract i

List of Tables.....vi

List of Figures vi

Abbreviations.....viii
1. Background 1

1.1 Study Background

1.2 Purpose of Research

2. Materials and Methods 10

3. Results 21

4. Discussion..... 40

5. Conclusion..... 45

Author Contribution 48

Funding..... 48

Reference..... 50

Abstract in Korean..... 55

List of Tables

Table 1 – Sequence of the primers used in NQO1, NQO2 and GAPDH gene expression

Table 2 – Clinical and prognostic implication of NQO2 gene in breast cancer patients using Kaplan–Meier Plotter (https://kmplot.com/analysis/)

List of Figures

Figure 1 – NQO2 expression analysis in the subtypes of breast cancer using RNA–seq in TCGA database.

Figure 2 – Recurrent free survival (RFS) analysis in all breast cancer patient.

Figure 3 – Distant disease–free survival (DDFS) analysis in Chemotherapy–received breast cancer patient (Tumor grade III)

Figure 4A – Comparison of NQO2 mRNA expression levels in multiple breast cancer cell lines detected by qRT–PCR.

Figure 4B – Comparison of NQO2 protein expression levels in multiple breast cancer cell lines detected by western blot and statistical analysis of western blots intensity relative to β-actin.

Figure 5A - NQO2 siRNA transfection in ZR75-1, SK-BR-3, BT-20 and HS578T cells detected by western blot and statistical analysis of western blots intensity relative to β-actin.

Figure 5B - Representative cell Images after si-transfection in ZR75-1, SK-BR-3, BT-20 and HS578T.

Figure 6 – Representative cell images of ZR75-1, SK-BR-3, BT-20 and HS578T in migration assay. Statistical analysis of cell migration.

Figure 7 - Representative cell images of ZR75-1, SK-BR-3, BT-20 and HS578T in invasion assay. Statistical analysis of cell invasion.

Figure 8 – Statistical analysis of ZR75−1, SK−BR−3, BT−20 and HS578T proliferation and cell viability.

Figure 9 – Flow cytometric analysis of the cell cycle distribution in ZR75−1, SK−BR−3, BT−20 and HS578T. Statistical analysis for percentage of cells in apoptosis, G0, G1, S and G2 phases.

Figure 10 – Intracellular ROS level in HS578T breast cancer cell.

Figure 11 – Western blot analysis of EMT markers in ZR75−1 and HS578T. Statistical analysis of western blots intensity relative to β−actin.

Figure 12 – Western blot analysis of TGF−β/Smad signaling markers in ZR75−1 and HS578T. Statistical analysis of western blots intensity relative to β−actin.

Figure 13 – A schematic model of TGF−β−induced EMT transition in NOQ2 Knockdown HS578T cells.

1. Background

1.1. Study Background

Breast cancer is one of the most common diagnosed cancers leading to a significant cause of death among in women worldwide. According to the International Agency for Research on Cancer (IARC) in December 2020, it has now become the first leading cause of cancer over lung cancer among women. The estimated incidence of new cases of breast cancer nearly doubled in the past two decades, from an estimated 10 million patients in 2000 to 19.3 million patients in 2020. [2] Studies over the previous decades have found that breast cancer is highly dependent on types of receptor and its subtypes. They have been investigated to develop targeted therapy and prognosis for breast cancer. Although numerous studies were performed, the survival rates for patients with breast cancer have not meaningfully improved. Consequently, the finding of new therapeutic target related invasion and metastasis of breast cancer is of a great value.

NRH:Quinone Oxidoreductase 2 (NQO2), known as quinone reductase 2 (QR2) is one of the key flavoprotein encoded by a gene located on chromosome 6p25.2. It forms hydroquinone by catalyzing the two-electron reduction of quinone substrates and uses

dihydronicotinamide riboside as a reducing coenzyme. [3] NQO2 is reported to have two functions: detoxification of chemical stressors and oxidation of NRH and NAD(P)H respectively, providing cellular protection against toxic agents in cancer cells.[6] Furthermore, high-level of NQO2 expression has been reported to be associated with many human cancers such as breast, pancreatic. ovarian and neurological diseases. However, the biological significance of NQO2 expression is still unclear. Therefore, understanding NQO2 and mechanisms underlying breast cancer progression is critical for prevention and treatment of this disease.

1.2. Purpose of Research

In present study, it investigates the biological activity of NQO2 related to tumor aggressiveness in breast cancer. To test this hypothesis, biochemical and cellular assays were performed in vitro. The result revealed that tumor activity is suppressed when NQO2 is downregulated in breast cancer cells. These findings indicate that NQO2 may be a good prognostic indicator for patients with breast cancer.

2. Materials and Methods

Kaplan-Meier plots and TCGA expression data

To assess the prognostic meaning of NQO2, Kaplan-Meier Plotter (https://kmplot.com/ analysis/) was operated. It allows a meta-analysis of NQO2 expression in relation to clinical and prognostic implication of NQO2 gene in breast cancer patients. Using the comprehensive survival analysis of Kaplan-Meier plotter, clinical datasets and RNA-seq datasets categorized by subtypes for 3455 breast cancer patients were obtained. Table 2 and Figure 1 are derived from Kaplan-Meier Plotter (https://kmplot.com/ analysis/) and The Cancer Genome Atlas. (TCGA, https://www.cancer.gov/tcga)

Cell culture

Human breast carcinoma cell lines (MCF-10A, MDA-MB-468, MCF-7, T-47D, ZR-751, BT-474, SK-BR-3, BT-20, MDA-MB-231, HS578T) were provided by ATCC (Manassas, VA, USA) and Korean cell line bank (Seoul, South Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institute medium (RPMI-1640) with 10%

fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S, Gibco) at a temperature of 37 ° C under 5% CO2.

Quantitative real-time PCR (qRT-PCR)

Cells were harvested and total RNA was extracted by using TRIzol (300-500ul) according to the manufacturer' s instructions (Invitrogen, CA, USA). Isolated RNA for each cell line was converted into cDNA using cDNA kit (Applied Biosystems) and Power SYBRTM Green PCR Master Mix (Applied Biosystems) according to the manufacturer' s instructions. (Invitrogen, CA, USA). Reverse transcription was performed by PrimeScript™ Reverse Transcriptase for RT-qPCR (Takara). Reactions were performed by Real time PCR System (Light Cycler 480 Ⅱ, Roche). Relative mRNA expression was quantified by using the comparative Ct method using 2^(-⊿⊿Ct) and GAPDH was used as an internal control. The results were expressed as fold change.

The primers used were as follows:

Gene	Forward 5’ -3’	Reverse 5’ -3’
NQO1	TCTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG
NQO2	GCTGGAGAGCTACAAGAGGATCA	ACAGACCTCTCTCTTGAGCTTGGT
GAPDH	GTGGACTCCCATTGAGCCTA	CTCCTCTTCACCCTCACTCG

Table1: Sequence of the primers used in the gene expression.

(Bionics, Seoul, Republic of Korea). The quantitative real-time PCR was performed and analyzed by Real-Time PCR Detection System (Bio-Rad). All assays were performed at least three times.

siRNA-Transfection assay

BT-20, SK-BR-3, MCF-7, HS578T cells were seeded (1×10^5 and 5×10^5) into 6-cm plates and allowed to adhere for 24 hours. Human NQO2 short interfering RNA (siRNA; sc-41575 AUACAAAUCAGACACUGUG, Santa Cruz Biotechnology) was transiently transfected (50nM) using oligofectaminutese transfection reagent diluted in Opti-MEM media. After 5 hours of incubation at 37C, 5% CO2 the medium was replaced with RPMI and

DMEM supplemented with 10% FCS. Maximum reduction of NQO2 was seen at 48 hours post-transfection.

Western blot analysis

Breast cancer cells (5 × 10^5) in 6-well plate were lysed with RIPA Lysis and Extraction Buffer containing 25mM Tris-HCI pH 7.6, 150mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and proteinase inhibitor cocktail (Thermo Scientific, USA). Cell lysates were centrifuged at 13,000 rpm for 15 minutes at 4° C, and then the supernatant was collected. Protein concentration was measured by constructing a standard curve based using the bovine serum albumin (BSA) standard solution. The absorbance at 562 nm (A562nm) was measured immediately to measure the concentration of these protein samples. Equal amounts (25ug) of protein per lane were electrophoresed on 12% SDS-polyacrylamide gels, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). Membranes were blocked with 5% BSA for 1 hour, then incubated in 5% BSA with a specific primary antibody to each target protein overnight at 4 ° C. The following primary antibodies were used in this study: NQO1, NQO2, β-actin, (Santa Cruz Biotechnologies, Santa Cruz, CA) E-cadherin, N-cadherin,

Vimentin, Slug, Snail and Twist (Cell Signaling Technology, USA). After the incubation for primary antibody, membranes were washed in 1X TBST (10 minutes x 3 times) to remove unbound antibodies then membranes were further incubated in 5% BSA with a species-specific HRP-conjugated secondary antibody for 1 hour at room temperature. After membranes were probed by secondary antibodies, membranes were washed in 1X TBST (20 minutes x 3 times). Immunoblot analysis was visualized with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology Inc.) for 1 hour at room temperature. The membranes were incubated with enhanced chemiluminutesescence (ECL) substrate solution (Amersham™ ECL™ Prime Western Blotting detection reagent; GE Healthcare Life Sciences, Chalfont, UK) for 1 minute at room temperature (25° C). The optical densities of the protein bands were determined using ImageJ (National Institutes of Health).

Migration and Invasion assay

To investigate breast cancer cell migration activity under the effect of siRNA-NQO2 transfection, Transwell migration assay was conducted in a 24-well plate, 8.0 μm-pore transwell chambers (Corning Incorporated, Corning, NY). For the transwell migration activity, breast cancer cells transfected with NQO2 siRNA were plated into the upper compartment of each chamber at a cell number of 1 × 10^5 cells/well in serum-free DMEM and RPMI medium. While the lower parts of the chambers were filled with complete medium. Following 24 hours incubation at 37° C with 5% CO2, the migrated cells on the bottom surface of the chambers were fixed with 4% paraformaldehyde (PFA) for 30 minutes and stained with 0.01% crystal violet solution for another 30 minutes at room temperature. The cells were washed with deionized water. The cells in the upper chamber were removed with a cotton swab. The migrated cells were taken pictures under a light microscope at × 5 magnification. The pictures were taken in 3 randomly selected fields for each chamber. Each assay was performed in duplicate and repeated three times. The crystal violet stain solution in each chamber were extracted by acetic acid and quantified by optical density (OD) reading at 570 nm.

To investigate breast cancer cell invasion activity under the effect of NQO2 knockdown, invasion assay was performed in a 24-well plate, 8.0 μm-pore chambers (Corning Incorporated, Corning, NY) with 20% Matrigel (BD Falcon). The cells were added to the upper chambers at a density of 1 × 10^5 cells/well in serum-free DMEM and RPMI medium. The lower part of the chambers was filled with complete medium. After 24 hours of incubation at 37° C with 5% CO2, the cells on the bottom surface of the chambers were fixed with 4% paraformaldehyde (PFA) for 30 minutes and stained with 0.01% crystal violet solution for another 30 minutes at room temperature. The cells with Matrigel in the upper chambers were removed with a cotton swab. The moved cells to lower surface of the chambers through Matrigel were taken pictures under light microscope at × 5 magnification. Three randomly selected fields in each membrane were taken pictures under a light microscope.

Proliferation assay (MTT assay)

To analyze the proliferation of human breast cancer cells under the effect of transfection, cells were collected after 48hour transfection. The cells were seeded at 2000, 4000, 6000, 8000, 10000, 12000 and 14000 cells/well into a 96-well plate in triplicate and incubated for 24, 48, 72 and 96 hours respectively at a

temperature of 37 ° C under 5% CO2. After incubation, 10μL MTT (5 mg/mL) was added to each well when the time was up and the plate was incubated at 37 ° C for another 1 hour. MTT solution was removed and 200μL dimethyl sulfoxide (DMSO) was added into each well and mixed thoroughly then the optical density (OD) at 570 nm was determined the cell viability assay was repeated at least three times.

Cell cycle analysis

ZR75-1, SK-BR-3, BT-20, HS578T cells (5 x 10^5) were cultured in 6-well plates for post-transfection 48hours. They were harvested and washed twice with PBS, then fixed in cold ethanol (70%) while voltexing. The cells were stained with propidium iodide (10 ug/mL) and RNase A (2 mg/mL) for 1 hour. The stained cells (at least 1x10^5) were analyzed by flow cytometry (FACSCalibur flow cytometer) and the DNA content were analyzed with FlowJo software. All cell cycle analysis was performed in triplicate and repeated at least three times.

Intracellular ROS measurement

The level of ROS in HS578T cells seeded on 6-well pates was measured by the oxidation-sensitive florescent probes DCF-DA. It was used to monitor the production of hydrogen peroxide. Cells in each well treated with si-RNA (Si-Ctrl and Si-NQO2) at 50uM for 48 hours, then incubated with 5uM of DCF-DA (Cellular Ros assay kit by Abcam) for the detection of cellular ROS for 30min at 37C in the dark. 0.005% H2O2 was used as positive control. Fluorescence was read with excitation at 485nm and emission at 535nm (FITC).

Statistical analysis

All experiments were repeated at least three times using a different cell preparation. Statistical analyses were performed using Microsoft Office Excel 2019, ImageJ (National Institutes of Health) and GraphPad Prism 9.1.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean ± SEM. Data were considered statistically significant when * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$.

Antibodies

1. Santa Cruz Biotechnology NQO2 Antibody (A−5) sc−271665

2. Santa Cruz Biotechnology m−IgGλ BP−HRP sc−516132

3. Dharmacon ON−TARGETplus Human NQO2 (4835) siRNA − SMARTpool L−006334−00−0005

4. Dharmacon ON−TARGETplus Non−targeting Pool D−001810−10−05

5. Santa Cruz Biotechnology NQO1 Antibody (A180) sc−32793

6. Santa Cruz Biotechnology m−IgGκ BP−HRP sc−516102

7. Santa Cruz Biotechnology NQO2 siRNA (m) sc−41576

8. Santa Cruz Biotechnology Control siRNA−A sc−37007

9. Cell Signaling Technology Vimentin (D21H3) XP® Rabbit mAb 5741

10. Cell Signaling Technology N−Cadherin (D4R1H) XP® Rabbit mAb 13116

11. Cell Signaling Technology Slug (C19G7) Rabbit mAb 9585

12. Cell Signaling Technology E−Cadherin (24E10) Rabbit mAb 3195

13. Cell Signaling Technology Anti−rabbit IgG, HRP−linked Antibody 7074

14. Santa Cruz Biotechnology TGF−β1 Antibody (3C11) sc−130348

15. Cell Signaling Technology Smad2 (D43B4) XP® Rabbit mAb 5339

16. Cell Signaling Technology Smad3 (C67H9) Rabbit mAb 9523

17. Cell Signaling Technology Smad2/3 (D7G7) XP® Rabbit mAb 8685

18. Cell Signaling Technology Phospho-Smad3 (Ser423/425) (C25A9) Rabbit mAb 9520

19. Cell Signaling Technology Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb 3108

20. Cell Signaling Technology Smad4 (D3M6U) Rabbit mAb 38454

3. Results

		95.0% CI for Exp(B)		
	HR	Lower	Upper	*P*-value
Total (N=3455)	1.17	1.04	1.31	0.008
Subtypes				
Luminal A (N=1678)	1.15	0.96	1.38	0.130
Luminal B (N=989)	**1.35**	1.1	**1.65**	**0.004**
HER2 (N=207)	1.44	0.95	2.54	0.086
Basal (N=581)	**1.34**	1.04	1.74	**0.025**

Table 2. Clinical and prognostic implication of NQO2 gene in breast cancer patients using Kaplan−Meier Plotter. (https://kmplot.com) B, coefficient of regression; HR, hazard ratio; CI, confidence interval; HER2, human epidermal receptor 2.

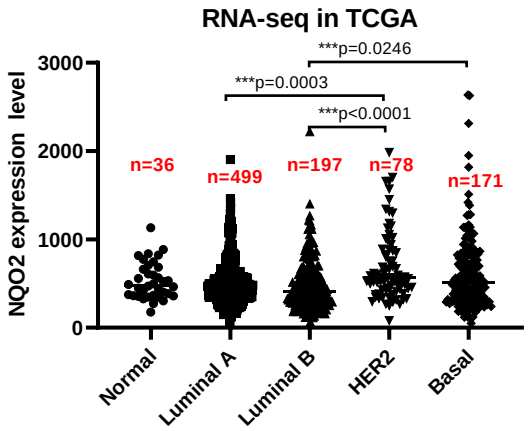

Figure 1. NQO2 expression analysis in the subtypes of breast cancer using RNA−seq in TCGA database. Statistical analysis was done using Ordinary one−way ANOVA (*** p≤0.0001)

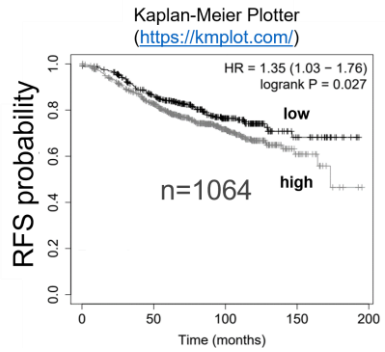

Figure 2. Recurrent free survival (RFS) analysis in all breast cancer patient with low or high NQO2 expression based on Kaplan−Meier plotter. Recurrent free survival was compared between higher and lower expression of NQO2 through the Kaplan−Meier Plotter database, and a difference of P<0.05 was deemed as statistically significant. ***, p≤ 0.001. TCGA, The Cancer Genome Atlas. n = 1064. HR, hazard ratio.

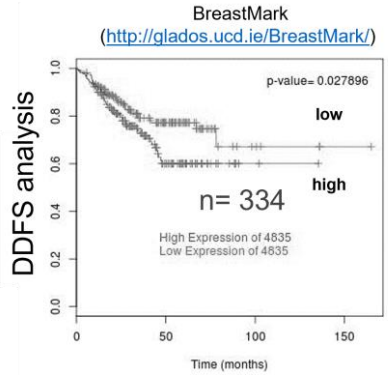

Figure 3. Distant disease−free survival (DDFS) analysis in Chemotherapy−received breast cancer patient (Tumor grade III) with low or high NQO2 expression based on BreastMark. This figure was generated using BreastMark. Kaplan−Meier plot of breast cancer patient survival based on NQO2 expression. Plots represent the DDFS. n = 334, p = 0.027 (* p≤0.05, ** p≤0.01)

Clinical significance of high expression of NOQ2 in breast cancer cells.

To have an overview of NQO2 expression in breast cancer cells, it was analyzed using the Kaplan−Meier plotter database. It provides information about the gene expression data over three thousand breast cancer tumor samples to identify the correlation of NQO2 expression to the survival of breast patients ranked by different clinical subtypes. The results of the present study showed that patients with breast cancer Luminal B and Basal type that had NQO2 expression level had a poor outcome compared to other breast cancer subtypes. (*p−value* 0.004 and 0.025, respectively) (Table 2) In Figure 1, the mRNA−seq data from TCGA were analyzed to verify these findings. It indicated that the differential expression of NQO2 between the adjacent normal tissues and tumor. The data illustrated that NQO2 expression is higher in Luminal B and Basal relatively to other subtypes of breast cancer. Recurrent free survival and disease−free survival curve was shown in Figure 2 and 3 respectively. They show that NQO2 gene expression is correlated with survival of patient with breast cancer. They demonstrated that the patients with poor survival rate tend to have higher NQO2 gene expression. (Figure 2 and 3)

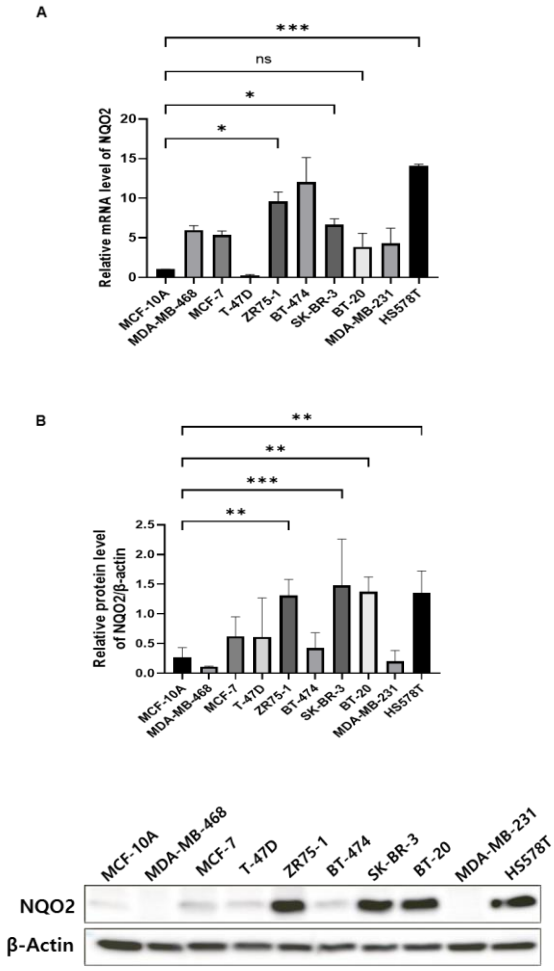

Figure 4. Comparison of NQO2 mRNA and protein expression levels in multiple breast cancer cell lines. (A) NQO2 mRNA levels were measured by qRT−PCR analysis. GAPDH was used as a housekeeping gene. (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$) (B) NQO2 protein levels were measured by Western blot. Relative protein levels are compared to β−actin using Image J analysis National Institute of Health (NIH) software. Bars, SD. Statistical analysis was done using ANOVA and Dunn's Multiple Comparison (* $p \leq 0.05$, ** $p \leq 0.01$) vs MCF−10A. The error bar represents three independent experiments, and each experiment was repeated three times.

NQO2 is expressed in breast cancers.

NQO2 mRNA and protein expression levels were examined in ten different breast cancer cell lines. Those cell lines include MCF10A, two Luminal A types (MCF-7 and T-47D), two Luminal B types (ZR75-1 and BT-474), HER2+ type (SK-BR-3), and four TNBC types (MDA-MB-468, BT-20, MDA-MB-231, and HS578T). The result showed that NQO2 mRNA and protein are up-regulated in breast cancers. (Figure 4)

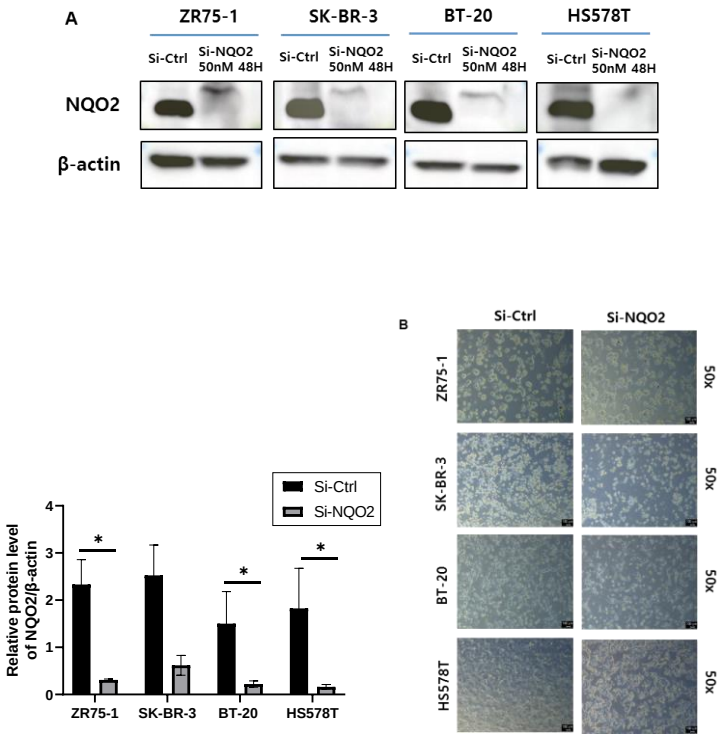

Figure 5. Silencing of NQO2 by siRNA transfection in breast cancer cells. (A) ZR75-1, SK-BR-3, BT-20 and HS578T cells were transfected with scrambled siRNA or NQO2 siRNA at 50 nM for 48 hours. NQO2 expression was detected by Western blot analysis. The error bars represent three independent experiments, and each experiment was repeated three times. Relative protein levels are compared to β-actin using Image J analysis National Institute of Health (NIH) software. Bars, SD. Statistical analysis was done using Student t-test (* $p \leq 0.05$, ** $p \leq 0.01$) (B) Representative images of post-transfection cells acquired by a microscope Magnification: 50x; scale bar: 100 μM.

NQO2 is silenced by short interfering RNA transfection.

Among ten breast cancer cell lines, four cell lines were selected, ZR75-1, SK-BR-3, BT-20 and HS578T that most highly expressed NQO2 compared to MCF10A cell. To determine the function of NQO2, NQO2 siRNA was transfected in these four cell lines. The efficiency of NQO2 siRNA transfection was established by Western blot analysis. (Figure 5A) NQO2-knockdown cells at 48 hours transfection exhibited a decrease NQO2 expression at the protein level by more than 60% respectively compared with scramble (control) siRNA.

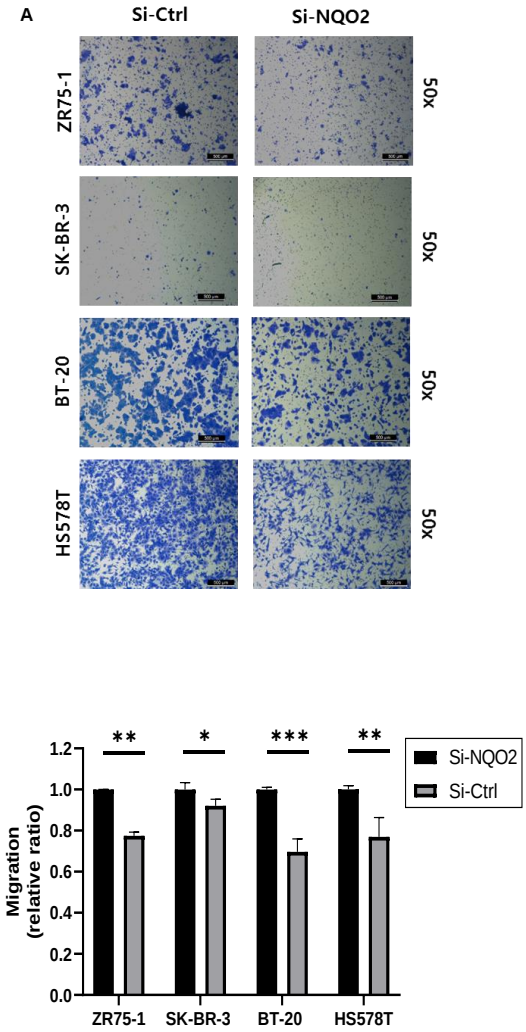

Figure 6. Down-regulation of NQO2 inhibited migration and invasion of breast cancer cells. (A) Transwell migration assays of ZR75-1, SK-BR-3, BT-20 and HS578T cells migrated for 24 hours upon siRNA transfection of NQO2 siRNA. Statistical analysis was done using Student t-test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs scrambled siRNA groups) Representative images of post-transfection cells in migration and invasion assays acquired by a microscope Magnification: 50x; scale bar: 100 μM.

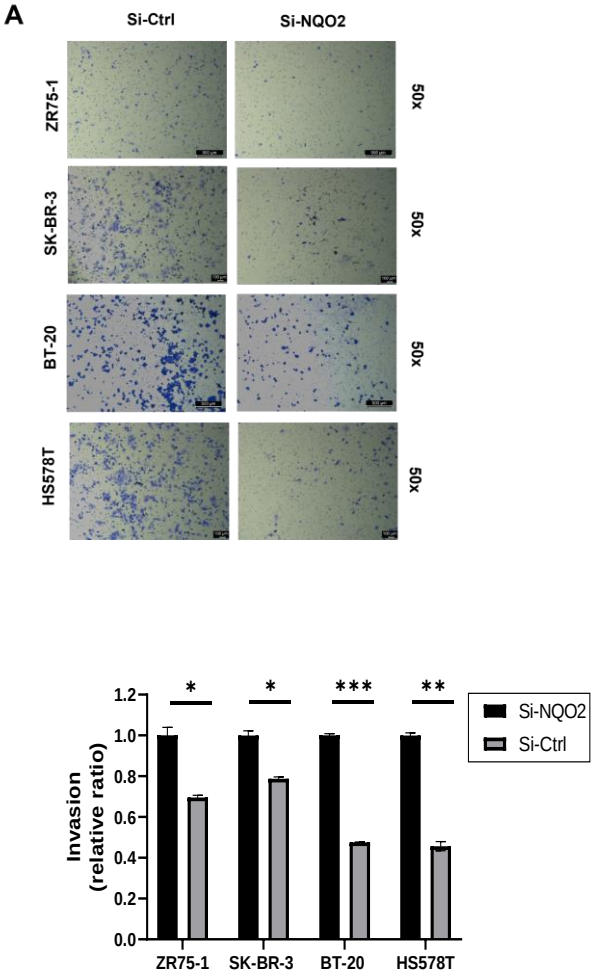

Figure 7. Down−regulation of NQO2 inhibited migration and invasion of breast cancer cells. (A) Transwell invasion assays of ZR75−1, SK−BR−3, BT−20 and HS578T cells for 24 hours upon siRNA transfection of NQO2 siRNA. Statistical analysis was done using Student t−test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs scrambled siRNA groups) Representative images of post−transfection cells in migration and invasion assays acquired by a microscope Magnification: 50x; scale bar: 100 μM.

NQO2 siRNA decreased the migratory and invasion ability in breast cancer cells.

To demonstrate the role of NQO2 in breast cancer cell activity, the migration of four cell lines in Transwell migration assay was performed at 24 h time-point. The result revealed that the migrated cells were decreased when NQO2 knocked down by siRNA transfection. In the presence of NQO2 siRNA, the number of migrated BT-20 cells decreased by 37.23%±0.55 and HS578T cells decreased by 35.98%±1.71 respectively. (Figure 6) To determine whether knockdown of NQO2 is associated with decreased cell migration, cell invasion was also assessed using transwell inserts. The result showed that cell invasion was suppressed in NQO2 siRNA transfected cells. (Figure 7) Therefore, these results implied that NQO2 plays a role in breast cancer cell migration and invasion.

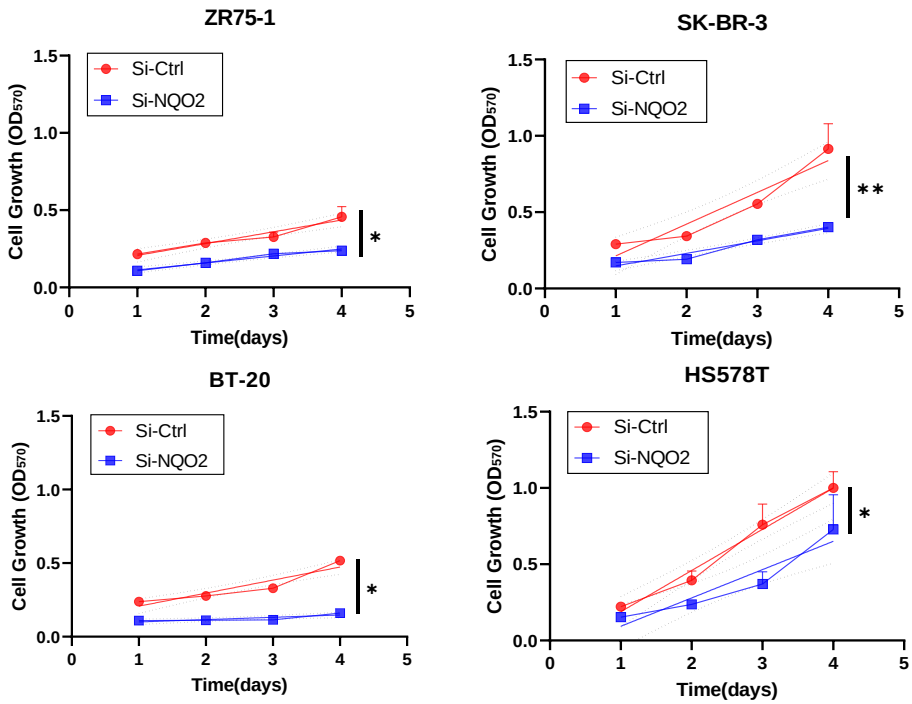

Figure 8. Deficiency of NQO2 decreased proliferation of breast cancer cells. (A) MTT assay was performed to detect cell viability of ZR75-1, SK-BR-3, BT-20 and HS578T cells for 24, 48, 72, 96 hours upon siRNA transfection of NQO2 siRNA. Statistical analysis was done using Student t-test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs scrambled siRNA groups) The error bars represent three independent experiments and each experiment was repeated three times.

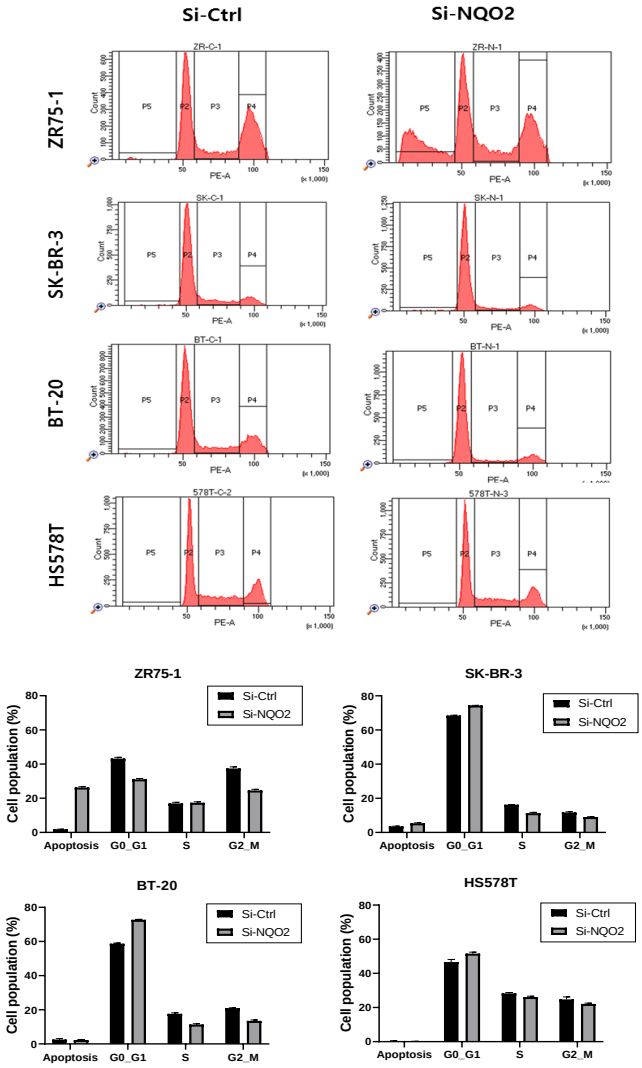

Figure 9. Silencing of NQO2 suppressed cell cycle progression of breast cancer cells. (A) Flow cytometric analysis of the cell cycle distribution in ZR75-1, SK-BR-3, BT-20 and HS578T after NQO2 si-transfection. Statistical analysis for percentage of cells in apoptosis, G1, S and G2 calculated using Student t-test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs scrambled siRNA groups) The error bars represent three independent experiments and each experiment was repeated three times.

NQO2 siRNA decreased the proliferation and cell cycle progression in breast cancer cells.

Having seen the expression of NQO2 in breast cancer cells, MTT assay was performed to further investigate the cell proliferation and motility after cells were transfected with NQO2 siRNA. As shown in Figure 8, the result displayed that siRNA transfection of cells inhibited the proliferation activity by 49.16%±1.19 in ZR75-1 cells and 35.32%±0.23 in HS578T cells at 48 h time-point. (Figure 8) These results indicated that there is a biological role of NQO2 in proliferation and motility in breast cancer cells. Moreover, the transfection of NQO2 siRNA significantly decrease the number of cells in S, G2/M phase in four cell lines and increase the number of cells in G0/G1 phase in SK-BR-3, BT-20 and HS578T cells. G0 and G1 cell cycle arrest in SK-BR-3 (siCtrl vs siNQO2: 68.4±0.2 vs 74.3±0.1), BT-20 (siCtrl vs siNQO2: 58.7±0.4 vs 72.7±0.3) and HS578T. (siCtrl vs siNQO2: 46.6±1.6 vs 51.5±0.9) Interestingly, NQO2 knockdown with siRNA transfection triggered apoptosis (siCtrl vs siNQO2: 1.8±0.06 vs 26.3±0.5) in ZR75-1 cells. (Figure 9) This result reported that there was induction of G1 phase cell cycle arrest and apoptosis in NQO2 siRNA transfection cells.

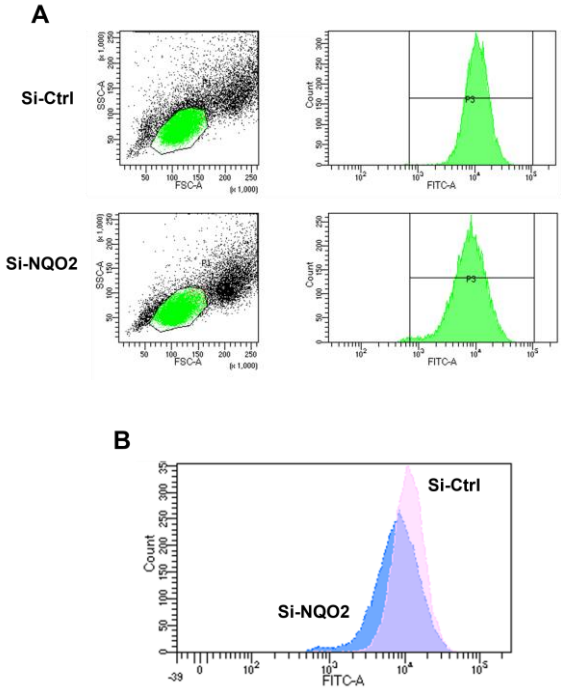

Figure 10. Intracellular ROS level in HS578T breast cancer cell was measured by flow cytometry analysis using DCFDA cellular ROS detection assay kit. (A) Detection of ROS by flow cytometry analysis after incubation with DCF-DA and 0.05% H2O2 in HS578T cells (2x10^5/ml/well). They were divided into two three group, which were Si-control group, Si-NQO2 group and parental. (B) Detection of ROS by flow cytometry analysis after incubation with DCF-DA and 0.05% H2O2 in post-Si-RNA-Hs578T cells.

Intracellular ROS level was reduced in HS578T cells with post- NQO2 siRNA.

Since NQO2 expression is highly related to intracellular ROS and NQO2 is well-known ROS generator, intracellular ROS level was determined in human breast cancer HS578T. ROS level was monitored using DCF-DA, the oxidation-sensitive florescent probes. As a result, ROS level in HS578T cells transfected with Si-NQO2 were lower than those in cells transfected with Si-control. (Figure 10 B). There findings indicate that the expression of NQO2 effects ROS level and they raise the possibility that the NQO2 gene may increase ROS levels.

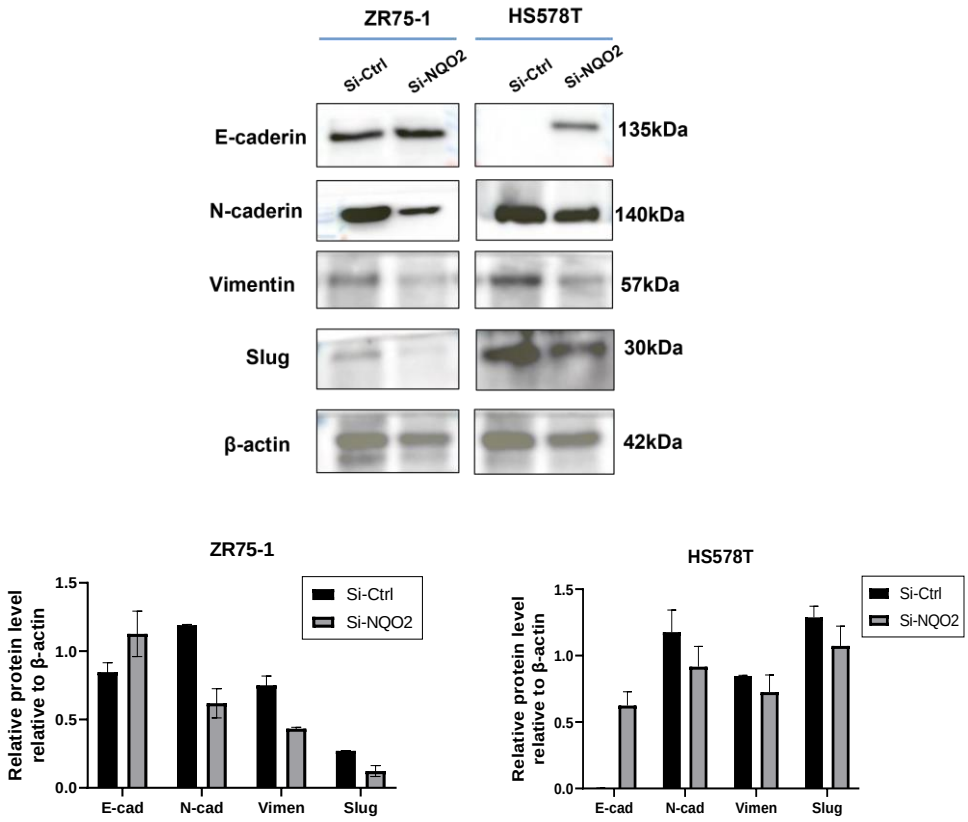

Figure 11. NQO2 siRNA suppressed EMT progression in breast cancer cells. (A) The expression of EMT markers, E-cadherin, N-cadherin, Vimentin and Slug were examined in ZR75-1 and HS578T cells by western blot analysis. The error bars represent three independent experiments, and each experiment was repeated three times. Relative protein levels are compared to β-actin using Image J analysis National Institute of Health (NIH) software. Bars, SD. Statistical analysis was done using Student t-test. (* $p \leq 0.05$, ** $p \leq 0.01$ vs β-actin)

NQO2 siRNA suppressed EMT progression in breast cancer cells.

To investigate whether NQO2 effects breast cancer cell aggressiveness through regulating EMT. Expression of representative molecules, E-cadherin, N-cadherin, Vimentin and Slug, are assessed by Western blot analysis after NQO2 knockdown. The result demonstrated that E-cadherin was increased in NQO2 siRNA transfected cells and N-cadherin, Vimentin and Slug were inhibited in NQO2-silenced cells. These findings suggested that EMT progression is suppressed in cells transfected with NQO2 siRNA. (Figure 11)

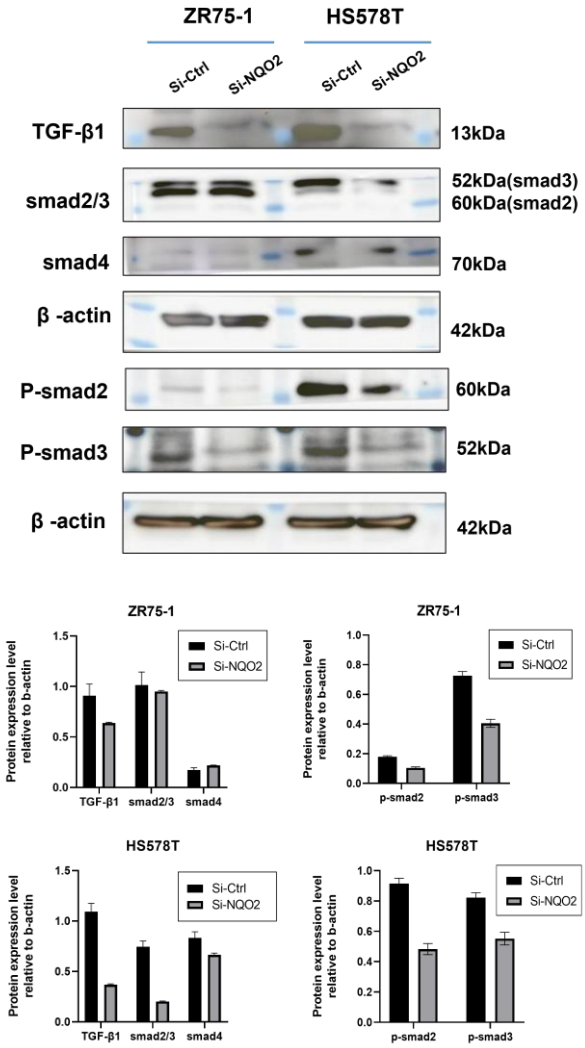

Figure 12. TGF−β/Smad signaling is deactivated in NQO2 knockdown breast cancer cells. (A) The expression of TGF−β/Smad signaling molecules, TGF−β1, Smad2/3, Smad4, P−Smad2 and P−Smad3 were detected in ZR75−1 and HS578T cells by western blot analysis. The error bars represent three independent experiments, and each experiment was repeated three times. Relative protein levels are compared to β−actin using Image J analysis National Institute of Health (NIH) software. Bars, SD. Statistical analysis was done using Student t−test. (* p≤0.05, ** p≤0.01 vs β−actin)

NQO2 siRNA inhibited TGF-β/Smad signaling pathway in breast cancer cell.

To further explore the effect of NQO2 in EMT, TGF-β-/Smad signaling in breast cancer was observed in vitro. Expression levels of TGF-β1, Smad2/3, Smad4, Phospho-Smad2 and Phospho-Smad3 were detected by Western blot analysis in NQO2-siRNA ZR75-1 and HS578T cells. The result indicated that silencing of NQO2 induced down-regulation of these TGF-β/Smad signaling representative molecules. This correlation was confirmed by both cell lines, in which the expression of TGF-β1, Smad2/3, Smad4, Phospho-Smad2 and Phospho-Smad3 were decreased. (Figure 12) The result suggested that inhibition of NQO2 leads to suppression of EMT-mediated TGF-β/Smad signaling.

4. Discussion

NQO2 is highly expressed in multiple types of breast cancer cells. In the present study, it focused to the utility of NQO2 in aggressiveness of breast cancer cells. It was found that NQO2 is associated with migration, invasion, proliferation, and cell cycle progression. Previous studies have shown that resveratrol (NQO2 inhibitor) was found to decrease proliferation of both hormone-dependent and hormone-refractory cells. [10] According to the previous study, it was demonstrated that the NQO2 knockdown cells by shRNA leaded to slower proliferation and increased G1 phase in CWR22Rv1 prostate cancer cells. [18] However, there was no further study on its anti-migratory and invasive capacities in post-transfection breast cancer cells. In this work, it was established that the downregulation of NQO2 expression weakened the migratory and invasive abilities of breast cancer cells. (Figure 6 and 7) The absent of NQO2 influenced reducing cell proliferation and caused cell cycle arrest. (Figure 8 and 9)

To further confirm this observation, the biological importance of NQO2 in metastasis was investigated by observing EMT development. It therefore comes as no surprise that the silencing of NQO2 caused suppression the EMT process. As shown

in Figure 10, E-cadherin, one of epithelia phenotypical markers and supposed to be down-regulated during EMT, was increased in ZR75-1 and HS578T cells transfected with NQO2 silencing. In contrast, N-cadherin, Vimentin, which are mesenchymal phenotypical markers and usually upregulated during EMT, were decreased in NQO2 down-regulated cells. There findings suggested that silencing of NQO2 might inhibit cell migration and invasion through suppressing EMT and resulted in a MET (reversed to epithelial morphology) in breast cancer cells.

Since TGF-β/Smad signaling is a prominent EMT-inducer, it was examined whether the silencing of NQO2 effects on TGF-β-induced EMT through the TGF-β/Smad pathway. Previous studies have shown that TGF-β-induced migration in breast cancer cell was inhibited when PI3K/AKT pathway was inhibited. [22] The PI3K inhibitor LY290042 was used combined with TGF-β1 and it reduced cell migration and restored the epithelial phenotype of breast cancer cells. [22] The present study is dedicated to the Smad-dependent signaling pathway in the EMT process. Previous researches showed that Smad2/3, and Smad4 play a significant role in the process of breast cancer EMT, which are second messengers of TGF-β1/2 tyrosine kinase pathways. The overexpression of Smads encourage EMT in breast cancer

cells. In Figure 11, the result indicated that when NQO2 is down-regulated, TGF-β/Smad-dependent signaling pathway is suppressed. Additionally, silencing of NQO2 decreased the expression levels of Phospho-Smad2 and Phospho-Smad3, which are important in TGF-β/Smad pathway. The results revealed that inhibition of NQO2 represses TGF-β/Smad -induced EMT.

Another interesting finding in these data in vitro is that the result was not seen consistent across breast cancer cell lines. Notably, cell growth was mostly decreased in SK-BR-3 cells. Cell migration and invasion was more inhibited in BT-20 and HS578T cells compared to other cell lines. Cell toxicity induced highest in ZR75-1. It will be a remarkable area of future research to determine what causes different result depending on type of cell lines. Nevertheless, these results nicely fit the hypothesis that the functionality of NQO2 influences aggressiveness of breast cancer cells.

As well known, multiple metabolic pathways are changed simultaneously in progression of tumor rather than a single metabolite. Speaking of multiple metabolic pathways in this study, ROS (reactive oxygen species) production could be associated with metastatic genes in malignant breast cancer cell lines. [15] Since ROS generation is strongly involved in NQO2 expression and EMT

progression, its inhibition could repress the TGF−β/Smad and ROS−induced EMT. Therefore, it will be very interesting in the future to further investigate the role of ROS in TGF−β−induced EMT via the TGF−β1/2 tyrosine kinase pathways.

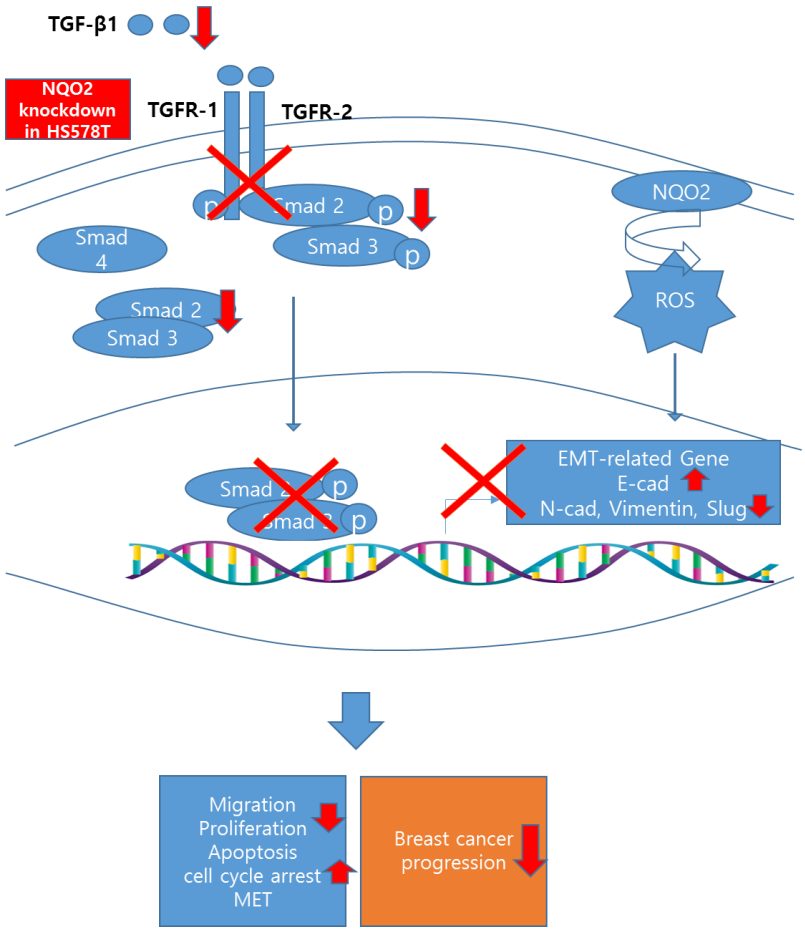

Figure 13. A schematic model of TGFβ−induced EMT transition in NOQ2 Knockdown HS578T cells. Silencing of NQO2 downregulates TGF−b/Smad representative molecules such as TGF−b1, Smad2/3, Smad4, P−smad2 and P−smad3 eventually suppressed EMT progression. It is a good indicator of breast cancer suppressor through repressing the signaling pathway.

5. Conclusion

Taken together, elucidating the role of NQO2 in breast cancer cells is critical for the understanding of their participation in migration, invasion, proliferation, cell cycle and tumor metastasis. Importantly, these findings illustrates that the understating and targeting of TGF-β/Smad mediated changes during EMT is essential for treating of metastatic capable cells and could eventually discover potential novel cancer treatment in future therapeutics for breast cancer patients. (Figure 9)

Abbreviations

TCGA; The Cancer Genome Atlas

K-M; plotter Kaplan-Meier Plotter

NQO1; NRH:Quinone Oxidoreductase 1

NQO2; NRH:Quinone Oxidoreductase 2

GAPDH; glyceraldehyde-3-phosphate dehydrogenase

ROS; Reactive oxygen species

ER; estrogen receptor

TNBC; triple negative breast cancer

HER-2; human epidermal growth factor 2,

CI; confidential interval,

HR; hazard ratio

ATCC; American Type Culture Collection

KCLB; Korean Cell Line Bank

FBS; Fetal Bovine Serum

PBS; phosphate buffered saline

PCR; Polymerase Chain Reaction

qRT-PCR; Quantitative Real-Time Polymerase Chain Reaction

mRNA; messenger RNA

siRNA; small interfering RNA

EMT; Epithelial–mesenchymal transition

MET; mesenchymal−to−epithelial transition

TGF−β1; Transforming growth factor beta1

MTT; 3−(4,5−dimethylthiazol−2−yl)−2,5−diphenyl tetrazolium bromide

Ethics approval and consent to participate: The present study was approved by the Ethics Committee of Seoul National University and was conducted previously in accordance with the Ethics Committee of Korea Cancer Research Foundation.

Consent for publication: All patients provided consent for the publication of this study.

Availability of data and material: The data presented in this study are available upon request via email to the corresponding author (sunkyu490@snu.ac.kr).

Competing interests: The authors declare that they have no competing interests.

Funding: This research was supported by Korea Cancer Research Foundation Tel. 02-747-0224 http://Koreanfcr.or.kr and Seoul National University of Hospital Dept. of Surgery and Cancer Research Institute Tel. 02-2072-1958 http://snuh.org

Author Contributions: Conceptualization, S.K.K., H.S.K., H.B.L and H.W.S.; Data curation, S.K.K., H.S.K., H.B.L and H.W.S.; Writing-original draft, S.K.K.; Writing-review and edition, , S.K.K. and H.S.K. All authors have read and approved the manuscript.

Acknowledgements: I would like to thank my dissertation advisors, Wonshik Han, Hoe Suk Kim, and Han-byoel Lee who have been invaluable mentors. Han's gift for conceptualization, Kim's enduring encouragement and Lee's practical advice have been a great source of support for me

during this research process. I would also like to thank graduate students in our lab for their help and feedback. Finally, thank you to all the students and mentors who gave their time and provided me with insight into their experiment with Laboratory of Breast Cancer Biology.

Reference

1. Thierry Cresteil and Anil K. Jaiswal, ‘High Levels of Expression of the NAD(P)H:Quinone Oxidoreductase (NQO1) Gene in Tumor Cells Compared to Normal Cells of the Same Origin’, *Biochemical Pharmacology*, 42.5 (1991), 1021-27 <https://doi.org/10.1016/0006-2952(91)90284-C>.

2. WHO, ‘Source: Globocan 2020’, Globocan 2020, 419 (2020), 3-4 <https://ascopost.com/news/december-2020/globocan-2020-database-provides-latest-global-data-on-cancer-burden-cancer-deaths/#:~:text=Female breast cancer has now, with 685%2C000 deaths in 2020.>.

3. Elham Santina, ‘The Potential Role of the Oxidoreductase , NQO2 in Breast Cancer A Thesis Submitted to The University of Manchester for the Degree of Elham Santina School of Pharmacy and Pharmaceutical Sciences’, 2015.

4. Yang Yang, Yan Zhang, and others, ‘Clinical Implications of High NQO1 Expression in Breast Cancers’, *Journal of Experimental and Clinical Cancer Research*, 33.1 (2014), 1-9 <https://doi.org/10.1186/1756-9966-33-14>.
5. Balqis A J Ikhmais, ‘The Role of NQO2 in Tumour Growth and Response to Therapeutic Drugs’, 2017.

6. Elham Santina, ‘The Potential Role of the Oxidoreductase , NQO2 in Breast Cancer A Thesis Submitted to The University of Manchester for the Degree of Elham Santina School of Pharmacy and Pharmaceutical Sciences’, 2015.

7. H. J. Menzel and others, ‘Association of NQOI Polymorphism with Spontaneous Breast Cancer in Two Independent Populations’, *British Journal of Cancer*,

90.10 (2004), 1989-94 <https://doi.org/10.1038/sj.bjc.6601779>.

8. Martin Belinsky and Anil K. Jaiswal, 'NAD(P)H:Quinone Oxidoreductase1 (DT-Diaphorase) Expression in Normal and Tumor Tissues', *Cancer and Metastasis Reviews*, 12.2 (1993), 103-17 <https://doi.org/10.1007/BF00689804>.

9. Taishan Hu and others, 'Reactive Oxygen Species Production via NADPH Oxidase Mediates TGF-β-Induced Cytoskeletal Alterations in Endothelial Cells', *American Journal of Physiology - Renal Physiology*, 289.4 58-4 (2005), 1-20 <https://doi.org/10.1152/ajprenal.00024.2005>.

10. Hendrik Ungefroren and others, 'Rac1b Regulation of Tgfb1 Reveals an Unexpected Role of Autocrine Tgfβ1 in the Suppression of Cell Motility', *Cancers*, 2020, 1-17 <https://doi.org/10.3390/cancers12123570>.

11. Xiwen Zhang and others, 'Resveratrol Inhibits Angiotensin II-Induced ERK1/2 Activation by Downregulating Quinone Reductase 2 in Rat Vascular Smooth Muscle Cells', *Journal of Biomedical Research*, 26.2 (2012), 103-9 <https://doi.org/10.1016/S1674-8301(12)60019-0>.

12. Elzbieta Janda and others, 'Parkinsonian Toxin-Induced Oxidative Stress Inhibits Basal Autophagy in Astrocytes via NQO2/Quinone Oxidoreductase 2: Implications for Neuroprotection', *Autophagy*, 11.7 (2015), 1063-80 <https://doi.org/10.1080/15548627.2015.1058683>.

13. David Jamieson and others, 'Two Minor NQO1 and NQO2 Alleles Predict Poor Response of Breast Cancer Patients to Adjuvant Doxorubicin and Cyclophosphamide Therapy', *Pharmacogenetics and*

Genomics, 21.12 (2011), 808-19 <https://doi.org/10.1097/FPC.0b013e32834b6918>.

14. M. M. Montano and others, 'Protective Roles of Quinone Reductase and Tamoxifen against Estrogen-Induced Mammary Tumorigenesis', *Oncogene*, 26.24 (2007), 3587-90 <https://doi.org/10.1038/sj.onc.1210144>.

15. Maria Peiris-Pagès and others, 'Metastasis and Oxidative Stress: Are Antioxidants a Metabolic Driver of Progression?', Cell Metabolism, 22.6 (2015), 956-58 <https://doi.org/10.1016/j.cmet.2015.11.008>.

16. Kristina Subik and others, 'The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, KI-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines', *Breast Cancer: Basic and Clinical Research*, 4.1 (2010), 35-41 <https://doi.org/10.1177/117822341000400004>.

17. Yang Yang, Xianchun Zhou, and others, 'β-Lapachone Suppresses Tumour Progression by Inhibiting Epithelial-to-Mesenchymal Transition in NQO1-Positive Breast Cancers', *Scientific Reports*, 7.1 (2017), 1-13 <https://doi.org/10.1038/s41598-017-02937-0>.

18. Qiang Ma, 'Role of Nrf2 in Oxidative Stress and Toxicity', *Annual Review of Pharmacology and Toxicology*, 53.1 (2013), 401-26 <https://doi.org/10.1146/annurev-pharmtox-011112-140320>.

19. Yulong Liang and others, 'SiRNA-Based Targeting of Cyclin E Overexpression Inhibits Breast Cancer Cell Growth and Suppresses Tumor Development in Breast Cancer Mouse Model', *PLoS ONE*, 5.9 (2010), 1-10 <https://doi.org/10.1371/journal.pone.0012860>.

20. Tze Chen Hsieh, ‘Antiproliferative Effects of Resveratrol and the Mediating Role of Resveratrol Targeting Protein NQO2 in Androgen Receptor-Positive, Hormone-Non-Responsive CWR22Rv1 Cells’, *Anticancer Research*, 29.8 (2009), 3011-17.

21. Aniruddha Pawar, ‘Classification of Breast Cancer Cell Lines Into Subtypes Based on Genetic Profiles’, May, 2015 <https://scholarworks.iupui.edu/handle/1805/7912>.

22. Long Zhang, Fangfang Zhou, and Peter ten Dijke, ‘Signaling Interplay between Transforming Growth Factor-β Receptor and PI3K/AKT Pathways in Cancer’, Trends in Biochemical Sciences, 38.12 (2013), 612-20 <https://doi.org/10.1016/j.tibs.2013.10.001>.

23. Yang Sun and others, ‘Resveratrol Inhibits the Migration and Metastasis of MDA-MB-231 Human Breast Cancer by Reversing TGF-B1-Induced Epithelial-Mesenchymal Transition’, Molecules, 24.6 (2019), 1-16 <https://doi.org/10.3390/molecules24061131>.

24. Ke Da Yu and others, ‘Genetic Variants in Oxidative Stress-Related Genes Predict Chemoresistance in Primary Breast Cancer: A Prospective Observational Study and Validation’, *Cancer Research*, 72.2 (2012), 408-19 <https://doi.org/10.1158/0008-5472.CAN-11-2998>.

25. Mark Gray and others, ‘Development and Characterisation of Acquired Radioresistant Breast Cancer Cell Lines’, Radiation Oncology, 14.1 (2019), 1-20 <https://doi.org/10.1186/s13014-019-1268-2>.

26. Sonia Ciotti and others, 'GSK3β Is a Key Regulator of the ROS-Dependent Necrotic Death Induced by the Quinone DMNQ', *Cell Death and Disease*, 11.1 (2020) <https://doi.org/10.1038/s41419-019-2202-0>.

27. Seok Ahn Kwang and others, 'Deficiency of NRH:Quinone Oxidoreductase 2 Differentially Regulates TNF Signaling in Keratinocytes: Up-Regulation of Apoptosis Correlates with down-Regulation of Cell Survival Kinases', *Cancer Research*, 67.20 (2007), 10004-11 <https://doi.org/10.1158/0008-5472.CAN-07-2213>.

28. Dong Wook Kim and Je Yoel Cho, 'NQO1 Is Required for β-Lapachone-Mediated Downregulation of Breast-Cancer Stem-Cell Activity', *International Journal of Molecular Sciences*, 19.12 (2018) <https://doi.org/10.3390/ijms19123813>.

29. Clare F. Megarity and David J. Timson, 'Cancer-Associated Variants of Human NQO1: Impacts on Inhibitor Binding and Cooperativity', *Bioscience Reports*, 39.9 (2019), 1-10 <https://doi.org/10.1042/BSR20191874>.

30. Dandan Chen and others, 'NQO2 Inhibition Relieves Reactive Oxygen Species Effects on Mouse Oocyte Meiotic Maturation and Embryo Development', *Biology of Reproduction*, 97.4 (2017), 598-611 <https://doi.org/10.1093/biolre/iox098>.

31. Cedric O. Renaud and others, 'Keap1/Nrf2 Signaling: A New Player in Thyroid Pathophysiology and Thyroid Cancer', *Frontiers in Endocrinology*, 10.August (2019) <https://doi.org/10.3389/fendo.2019.00510>.

Abstract (Korean)

국문초록

1. 연구 배경 및 필요성

-Quinone oxidoreductase2 (NQO2)는 플라보 단백질 계통의 전자환원 효소 이며 NADH나 NADPH를 보조인자로 세포 내 퀴논 화합물을 환원하여 더 불안정한 구조인 하이드로퀴논을 생성한다. 그 결과로 세포 내 산화적 스트레스가 야기되고 암세포 발생, 증식, 전이와 더불어 항암에 대한 저항을 가져온다. NQO2는 다양한 암종의 암세포에서 발현이 높고 암뿐만 아니라 신경적 질환에도 영향이 있다.

-본 연구는 생물학적 기전 분석을 통해 분자 진단 및 치료 바이오 마커를 이용한 키드 개발에 이용될 것이며 유방암 발생 및 악성화 예측 모델을 수립하기 위한 자료로 이용될 수 있다. 따라서 본 연구에서 NQO2 유전자를 억제시킨 유방암 세포와 유방암 동물 모델에서 NQO2에 의한 유방암세포의 악성화 관련 분자 조절 기전을 규명하고자 한다.

2. 연구 방법 및 연구 대상

-유방암 서브타입 에 따른 NQO2 발현 분석

본 연구자는 The Cancer Genome Atlas Program (www.cancer.gov/nci)에서 제공하는 유방 정상 조직과 유방암 환자의 서브타임별 (Lunmial A, Lumial B, HER2, Basal) 종양조직의 RNA-seq 데이터를 활요하여 NQO2의 발현을 비교 분석하였다.

-NQO2 발현에 따른 유방암 환자의 예후 분석

본 연구자는 공개 데이터 베이스 (www.glados.ucd.ie/BreastMark)를 활용하여 종양 등급III 이며 항암치료를 받은 334명의 유방암 환자를 대상으로 DDFS (Distant Disease Free Survival)을 분석 하였다.

-qRT-PCR 과 Western blot 분석으로 유방암 세포주 에서 NQO2 발현 확인

본 연구에서는 10가지 유방암 세포주를 이용하여 NQO2 발현을 확인하였다. qRT-PCR 과 Western blot 분석을 이용하여 NQO2 mRNA 과 protein 발현량을 확인하였다.

-siRNA를 이용하여 NQO2 유전자 넉다운

여러 유방암 세포주에서 NQO2의 발현을 확인한 후 (정상 유방암 세포주 MCF-10A와 비교하여) 상대적으로 NQO2의 발현이 높은 세포주를 선별하였다. NQO2의 생물학적 기능을 알기 위해서 선별된 세포주에서 siRNA를 형질주입하여 NQO2 넉다운 세포를 형성하였다. 50uM로 48시간 동안 NQO2를 타겟으로 하는 siRNA를 유방암세포에 처리해주었다. Western blot 분석법을 이용하여 NQO2가 넉다운률(%) 정도를 확인하였다.

-Transwell migration, Transwell invasion 분석으로 유방암세포 이동성과 침윤성 확인

siRNA 형질주입 하여 NQO2 발현이 억제된 세포에서 Transwell migration 과 Transwell invasion assay를 통해서 유방암 세포의 이동성과 침윤성을 24, 48시간 간격으로 확인하였다. 이동한 세포를 paraformaldehyde로 고정 후 crystalviolet으로 염색하여 현미경 하에서 관찰하였다. Graphpad prism9을 이용하여 컨트롤 그룹(siRNA-control) 과 비교 그룹(siRNA-NQO2) 간에 통계적 분석을 진행하였다.

-MTT assay 와 유세포 분석기를 통해 유방암세포의 분화성과 세포주기 확인

NQO2 발현이 억제된 세포에서 세포 증식과, 분화력을 확인하기 위해서 proliferation assay를 진행하였다. MTT solution (Amresco, Solon. Ohio, USA)을 첨가하여 37’C에서 2시간 반응시켰다. DMSO(Sigma) 원액을 첨가한 후 보라색으로 발색 하면 상층액을 96well-plate에 넣어주었다. 그 후 570nm에서 흡광도를 측정하였다. NQO2 발현 유무에 따른 세포고사와 세포주기를 알아보기 위해 유세포 분석법(Fluorescence-activated cell sorting)을 사용하였다. 배양된 세포를 차가운 PBS로 2회 세척 후 분석에 사용될 세 포수를 1x10^6/mL으로 하였다. PI (propidium iodide) 용액을 이용하여 상온에서 1시간 염색한 후 유세포 분석기를 이용하여 분석하였다. 통계적 분석은 Graphpad prism9를 사용하였다.

-유방암세포의 EMT 관련 악성도 확인

NQO2 발현과 유방암세포의 악성도를 알아보기 위해 EMT 현상을 Western blot 분석을 통해 확인하였다. siRNA-NQO2 유방암 세포에서 EMT 마커로 알려진 E-caderin, N-caderin, Vimentin, Slug 발현을 확인하였고 Graphpad prism9을 사용하여 통계적 유의성을 측정하였다.

-유방암세포의 TGF-b1/Smad 신호과정 확인

EMT 현상에 중요한 역할을 하는 것으로 알려진 TGF-b1/Smad 신호 과정을 확인하였다. siRNA-NQO2 세포에서 TGF-b1/Smad 신호전달 활성 변화를 Western blot 기법으로

분석하였다. TGF-b1/Smad 신호전달 과정 중 주요 기능을 하는 TGF-b1, Smad2/3, Smad4, p-Smad2. p-Smad3의 발현정도를 NQO2 넉다운 유 방암세포에서 확인하였다. 통계적 분석은 Graphpad prism9를 사용하였다.

3. 연구 수행 결과

-임상적으로 NQO2의 발현은 유방암 환자들에게 나쁜 예후 인자임을 확인

NQO2 의 발현에 대한 임상적 의미를 찾기 위해 공개데이터 베이스 KM-plotter 와 TCGA를 기반으로 유방암 세포에서 NQO2 발현을 확인하였다. 정상상피조직에서 보다는 암 조직에서 NQO2의 발현이 높았고 그중에서 Luminal B와 Basal 타입 유방암에서 NQO2의 발현이 높았다. 이를 통해 통계적으로 유의하게 NQO2의 나쁜 예후를 예측하였다.

-유방암 세포주 에서 NQO2의 높은 발현도 확인

유방암 세포에서 NQO2 발현도를 알아보기 위해 9 가지 유방암세포와 1가지 정상유방암세포에서 NQO2의 mRNA와 protein 발현 정도를 알아보았다. qRT-PCR 과 Western blot 기법으로 NQO2 의 발현을 알아보았을 때 대부분의 유방암세포에서 NQO2의 발현이 확인 되었다. MCF10A 와 상대비교를 해보았을 때 ZR-751(Lumial), SK-BR-3(HER2), BT-20(TNBC), HS578T(TNBC) 세포에서 NQO2의 발현이 높았고 통계적으로 유의한 차이를 확인하였다.

-siRNA를 이용하여 NQO2 유전자 넉다운 세포주 형성

NQO2의 생물학적 기능을 알아보기 위해 siRNA를 위 네 가지 세포(ZR-751, SK-BR-3, BT-20, HS578T) 에 형질 주입하여 NQO2

의 기능이 억제된 세포를 형성하였다. NQO2를 타겟으로 하는 siRNA를 50uM, 48시간 처리하였을 때 네 가지 유방암세포 모두에서 60% 이상의 억제률을 확인 하였다.

-NQO2 발현 억제된 유방암세포의 이동성과 침윤성 감소

NQO2 유전자 발현 유무에 따른 유방암세포의 성장 및 악성화 과정을 관찰하기 위해서 세포 이동과 침윤 능력을 확인하였다. Transwell migration 과 Transwell invasion assay를 통해서 세포 이동성과 침윤성을 확인한 결과, NQO2의 발현을 억제한 세포에서 세포 이동성이 감소하였다. 특히, control 그룹과 비교하였을 때 BT-20와 HS578T 세포에서 각 37.23%±0.55 와 35.98%±1.71 감소하였다. 이를 통해 NQO2의 발현 억제을 통해 세포 이동성과 침윤성 감소를 확인하였다.

-NQO2 발현 억제된 유방암세포의 분화성과 세포주기 감소

NQO2 유전자 발현이 증식능에 미치는 영향을 측정하기 위해서 MTT assay를 이용하여 24, 48, 72시간 동안 관찰하였다. 그 결과, NQO2 의 발현이 억제된 세포에서 세포 증식능력이 감소하였다. 대조군과 비교하였을 때 ZR-571 세포와 HS578T 세포에서 49.16%±1.19 와 35.32%±0.23 의 유의한 감소를 보였다. 분화력 뿐만 아닌 세포주기에 NQO2의 영향을 알아보기 위해서 유세포 분석법을 시행하였다. S 분획과 G2/M 분획은 세포주 모두에서 대조근에 비해 유의한 감소를 나타내었고 SK-BR-3, BT-20, HS-578T 세포에서는 G0/G1 분획이 대조군에 비해서 증가하였다. 대조군과 비교하였을 때, G0/G1 분획에서 SK-BR-3 세포는 siCtrl vs siNQO2: 68.4±0.2 vs 74.3±0.1, BT-20 세포는 siCtrl vs siNQO2: 58.7±0.4 vs 72.7±0.3, HS578T 세포는 siCtrl vs siNQO2: 46.6±1.6 vs 51.5±0.9 의

차이를 보였다. 흥미롭게도 ZR-751 세포에서는 대조군에 비해 NQO2가 억제된 세포에서 세포자살이 유발되었다. 이를 통해 NQO2의 넉다운이 유방암세포의 세포 주기 정지 및 세포 사멸에 관여함을 알 수 있다.

-NQO2 발현 억제된 유방암세포의 EMT 관련 악성도 감소

유방암세포의 악성화 형질을 관찰하기 위해서 유방암세포 특이적인 EMT 신호 전달 기전을 규명하고자 하였다. 이를 위하여, ZR-751과 HS578T 세포에 siRNA를 이용하여 NQO2의 발현을 감소시킨 후 상피성 세포의 마커로 알려진 E-cadherin 과 중배엽성 세포의 마커로 알려진 N-cadherin, Vimentin 발현, 그리고 EMT의 주요 전사인자로 알려진 Slug의 발현 변화를 분석하였다. Western blot 분석법으로 확인한 결과, NQO2의 발현을 억세시킨 세포에서 N-cadherin, Vimentin, Slug 발현이 감소하였으며 E-cadherin의 발현은 두 세포 모두에서 증가하였다. 이러한 결과는 NQO2에 의해서 암세포의 EMT가 유도되며 NQO2의 발현이 되지 않을 때는 유방암세포의 상피성 세포형질 유지에 기여하는 것으로 확인된다.

-NQO2 발현 억제된 유방암세포의 TGF-b1/Smad 신호과정 감소

TGFβ/smad 신호전달은 EMT의 활성화에 중요한 역할을 하고 있는 것으로 알려져 있다. 이에 따라 NQO2의 발현이 TGFβ/smad 신호전달에 미치는 영향을 관찰하고자 하였다. ZR-75-1 과 HS578T 세포에서 TGFβ/smad 신호전달에 관여하는 마커들의 발현을 Western blot 기법으로 확인하였다. TGFβ1, Smad2/3, Smad4, P-Smad2, P-Smad3의 발현을 NQO2 넉다운 세포에서 확인하였을 때, 관찰한 모든 마커에서 발현의 감소를 확인하였다. 대조군과 비교하였을 때 ZR-75-

1 세포에서는 TGF-b1, P-Smad2, P-Smad3의 발현에 유의한 차이를 보였고 HS578T 세포에서는 TGFβ1, Smad2/3, P-Smad2, P-Smad3의 발현에 통계적으로 유의한 차이를 확인하였다. 이러한 결과를 바탕으로 NQO2의 발현은 유방암세포의 TGFβ/smad 신호전달에 기여함을 규명하였다.

4. 고찰

공개 데이터를 기반으로 NQO2의 발현은 임상적으로 나쁜 예후 인자임을 확인하였지만, 이를 보완하기 위한 NQO2의 생물학적 기전 연구는 현재까지도 절실히 필요한 상황이다. 이러한 관점에서 본 연구는 암세포의 악성화와 NQO2 발현이 관계가 있음을 증명하였다. 여러 유방암세포에서 NQO2의 발현을 확인하였다. 내강형(luminal type)과 기저형(basal type) 유방암세포주 대부분에서 NQO2의 높은 발현수준을 확인하였다. 그 중에서도 MCF-10A를 대조군으로 비교하였을 때 ZR-751, SK-BR-3, BT-20, HS578T 세포에서 유의한 차이를 보였고 이는 내강형(luminal type) 2가지, 기저형(basal type) 2가지로 확인되었다. NQO2 발현의 유무에 따른 생물학적 기전 변화를 알아보기 위해 위 4가지 세포에서 siRNA를 사용하여 NQO2의 발현을 감소한 세포를 형성하였다. Western blot 기법으로 확인하였을 때 사용된 모든 세포(ZR-751, SK-BR-3, BT-20, HS578T)에서 60% 이상의 억제률을 확인하였다. 암세포의 이동과 침윤은 암세포 악성화와 관련이 있으며 이동과 침윤의 억제는 암세포의 치료에 필수적은 요건이다. 본 연구에서는 NQO2 발현에 따른 ZR-751, SK-BR-3, BT-20, HS578T 암세포 이동 및 침윤 억제 가능성에 대한 연구를 진행하였다. Transwell migration 과 invasion assay의 결과에 따라 NQO2의 발현이 억제되었을 때 시간 의존적으로 유방암 세포의 이동과 침윤성도 함께 감소하였다. 24시간 이후 이동된 세포를 crystalviolet으로 염색하여 측정하였을 때 NQO2 넉다운 세포에서 이동성과 침윤성이 감소하는 경향을 확인하였다. 유방암 타입별로 비교해보았을 때 삼중음성유방암(Triple negative breast

cancer)에서 상대적으로 유의한 차이를 확인하였다.

이와 관련하여 NQO2가 유방암세포의 증식 억제와 연관된 세포주기 진행 연구를 위해서 NQO2 특이적 siRNA를 50uM로 48시간 처리 후 NQO2 발현이 억제된 유방암 세포에서 MTT assay를 진행하였다. 결과에 의하면 이동 및 침윤의 경향성과 동일하게 NQO2 넉다운 세포에서 시간 의존적으로 세포 분화 능력이 감소하였다. ZR-751, SK-BR-3, BT-20, HS578T 세포 모두에서 대조군에 비해 시간 의존적으로 유의한 차이를 확인하였다. 이를 뒷받침하여 NQO2의 유무에 따른 유방암세포 세포주기 진행 변화를 유세포 분석법을 통해 관찰하였다. SK-BR-3, BT-20, HS578T 세포에서 S분획과 G0/G1 분획에 해당하는 세포가 감소하였고 G1 분획 세포군은 증가하였다. 따라서 유방암세포에서 NQO2 넉다운에 따른 암세포 증식억제 효과는 세포주기 G1 arrest와 연관성 있으며 G1 arrest 유도 효과는 상대적으로 다른 세포주에 비해 BT-20에서 더 높았다. 아울러 ZR-751세포에서는 apoptosis 세포군이 대폭 증가하였다. 이를 통해 ZR-751 세포 에서는 NQO2의 넉다운이 세포 사멸을 유도한 것으로 추청된다. NQO2의 발현 정도가 세포주기에 조절에 다소 영향을 미치는 것으로 확인되므로 NQO2 발현에 따른 Cyclins 나 cyclin-dependent kinases (Cdks) 와 같은 세포주기 조절인자들의 발현의 부가적인 연구도 필요하다. 아울러 세포 이동 및 증식의 억제만으로 전이억제능력 유무를 논하는 것은 무리가 있으므로 이와 관련된 여러 가지 유전자들의 발현정도와 전이억제능력을 측정하는 추가 실험이 필요하다.

EMT는 세포 이동과 침윤뿐 아니라 암의 악성화로 암전이 까지 관여한다고 알려져 있다. NQO2 발현 유무에 따라 EMT marker들의 발현을 Western blot 분석법으로 확인하여 EMT 과정의 변화를 관찰하였다. siRNA-NQO2 ZR-751 과 HS578T 세포를 형성하고 상피성 세포의 마커로 알려진 E-cadherin 과 중배엽성 세포의 마커로 알려진 N-cadherin, Vimentin 발현, 그리고 EMT의 주요 전사인자로 알려진 Slug의 발현을 분석하였다.

EMT marker 들의 발현을 Western blot 분석으로 확인한 결과, NQO2의 발현이 억제된 세포에서 N-cadherin, Vimentin, Slug 발현이 감소하였으며, E-cadherin의 발현은 증가하였다. N-cadherin, Vimentin, Slug 발현의 감소 정도는 ZR-751과 HS578T 세포에서 비슷하였으나 E-cadherin 발현의 증가는 대조군 HS578T 세포에 비해서 siNQO2-HS578T 세포에서 더욱 유의한 차이를 보였다. HS578T 세포에서 NQO2가 유방암세포의 상피성 세포형질 유지에 기여하는 것으로 확인된다. 내강형(luminal) 타입인 ZR-751 세포에서 보다 삼중음성유방암(Triple negative) 타입인 HS578T 세포에서 더욱 유의한 차이를 보이는 것으로 보아 세포 타입 별로 NQO2의 발현에 따라 EMT의 진행에 변화를 확인하는 연구가 필요하다.

본 연구에서는 유방암세포의 이동성과 EMT 과정을 통해 암세포 악성화에 대해 알아보았다. 결과를 보았을 때 NQO2의 발현 유무는 EMT 과정에서 중요함을 확인하였다. 그리하여 EMT 과정 중에서 핵심적인 역할을 한다고 알려진 TGF-β-Smad 신호 변화를 확인하였다. [1] TGF-β1 단백질이 TGF-β1/2 이량체 수용체에 결합하면 수용체의 인산화 및 Smad complex를 활성화시켜 이어져 EMT를 활성화시킨다. [1] 이러한 사전 연구를 기반으로 ZR-75-1 과 HS578T 세포에서 TGFβ/smad 신호전달에 관여하는 마커들의 발현을 Western blot 기법으로 확인하였다. TGFβ1, Smad2/3, Smad4, P-Smad2, P-Smad3의 발현을 NQO2 넉다운 세포에서 확인하였을 때, 관찰한 마커에서 발현이 감소하였다. 대조군과 비교하였을 때 ZR-75-1 세포에서는 TGF-β1, P-Smad2, P-Smad3의 발현에 유의한 차이를 보였고 HS578T 세포에서는 TGFβ1, Smad2/3, P-Smad2, P-Smad3의 발현에 통계적으로 유의한 차이를 확인하였다. NQO2 넉다운은 TGFβ1 과 Smad 활성화를 억제하고 결과적으로 EMT 억제를 동반하는 것을 의미한다. NQO2 발현에 따른 PI3K, JNK, NF-κB, ERK 와 같은 다른 선호전달경로 marker 들의 변화를 관찰하고 EMT 과정에서 가장 영향력 있는 선호전달 경로

를 확인하는 연구가 필요할 것이다.

5. 요약

-연구 목적-

본 연구는 NQO2 의 발현이 유방암 세포의 세포증식 및 전이과정에 미치는 영향을 규명 하고자 한다. NQO2는 플라보 단백질 계통의 전자환원 효소이며 NADH나 NADPH를 보조인자로 세포 내에 자연적으로 만들어지는 퀴논 화합물을 환원하여 더 불안정한 구조인 하이드로퀴논을 생성한다. 그 결과로 세포 내 산화적 스트레스가 야기되고 암세포 발생, 증식, 전이와 더불어 항암에 대한 저항을 가져온다. 여러 임상적 연구들은 NQO2의 발현이 암 뿐 만 아니라 신경적 질환도 영향이 있다고 말한다. 따라서, NQO2를 표적으로 작용하는 치료법은 암세포 사멸의 가능성을 부여 줌으로 본 연구의 의미가 있다.

-연구 방법-

NQO2 의 발현에 대한 임상적 의미를 찾기 위해 공개데이터 베이스 (KM-plotter) 와 TCGA를 기반으로 유방암 세포에서의 NQO2의 발현을 확인해보았다. NQO2의 생물학적 기능을 알아보기 위해 다양한 in vitro 실험을 수행하였다. 유방암 세포에서 NQO2 발현을 확인하기 위해 qRT-PCR 과 Western blot을 진행하였다. NQO2 발현의 유무에 따른 변화를 알아보기 위해 siRNA를 형질주입하여 NQO2 넉다운 세포를 형성하였다. 뒤를 이어, NQO2 넉다운 유방암 세포의 이동, 침윤, 분화, 주기 실험 및 분석을 수행하였다. NQO2 와 암세포의 악성도를 확인하기 위해서 EMT와 TGF-b/SMAD 관련 유전자들을 Western blot 으로 확인 하였다.

-연구 결과-

공개데이터 베이스 (KM-plotter) 와 TCGA를 기반으로 NQO2의 발현이 유방암에서 나쁜 예후 인자임을 확인하였다. 정상상피조직에

서 보다는 암 조직에서 NQO2의 발현이 높았고 그 중에서 Luminal B와 Basal type 유방암에서 통계적으로 유의하게 NQO2로 인한 나쁜 예후를 예측하였다. NQO2 넉다운 유방암 세포에서 세포의 이동, 침윤, 분화, 주기 가 비교군 유방암 세포에서 보다 감소하였다. Western blot을 통해 EMT 와 TGF-b/SMAD 신호에 관련된 유전자들을 확인 한 결과를 볼 때, NQO2 넉다운 세포에서 TGF-b/SMAD 신호 억제를 통한 EMT 감소를 확인 하였다.

-연구 결론-

본 연구를 통해 NQO2는 유방암에서 높게 발현되며 세포의 증식, 침윤, 주기, 분화 및 전이 과정에 관여함을 확인하였다. 특히 전이와 관련된 TGF-b1/SMAD-induced EMT에 중요한 기능을 하는 것으로 확인된다. 결과를 종합해보면 NQO2 발현의 억제는 유방암 세포의 증식, 분화 억제와 사멸 유도, 전이과정을 억제시키는 효과가 있을 것이다. 따라서 본 연구 결과를 토대로 유방암 표적 치료제가 개발될 수 있을 것으로 사료된다. NQO2의 발현에 따른 여러 유방암 세포 주에서 증식억제 및 전이과정에 미치는 영향에 대한 더 많은 연구가 필요하다.

주요어 : 유방암, NQO2, 세포 이동성, 상피-중간엽 전환(EMT, epithelial-mesenchymal transition), TGF-β/Smad 신호전달, 암 악성도

학 번 : 2018-26145