RESEARCH ARTICLE

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Effect of *NQO1* Downregulation on the Migration and Invasion of HPV16-Positive Cervical Cancer Cells

Warattaya Wattanathavorn[1,2], Supranee Buranapraditkun[3,4,5], Nakarin Kitkumthorn[6], Parvapan Bhattarakosol[2,7], Arkom Chaiwongkot[2,7]*

Abstract

Objective: This study aimed to identify upregulated genes in HPV16-positive cervical cancer cells and investigate the impact of downregulating NAD(P) H:quinone oxidoreductase 1 (NQO1) on the survival of these cells. **Methods:** Transcriptomic sequencing (RNA-seq) was utilized to pinpoint upregulated genes and associated cancer-related pathways in HPV16-positive cervical cancer cells, comparing them to HPV-negative cervical cancer cells. *NQO1* gene knockdown was performed in HPV16-positive cervical cancer cell lines to assess its effect on cell survival, including parameters such as cell proliferation, migration, invasion, cell cycle progression, apoptosis, and the expression of key proteins in the PI3K/AKT pathway, p53, and RECK. **Results:** Genes with a fold change ≥4.0 in HPV16-positive cervical cancer cell lines were predominantly localized to the extracellular region and plasma membrane. These genes were involved in protein binding and cell adhesion, influencing cellular responses to stimuli and tissue development. KEGG pathway analysis identified the most significant pathways, including metabolic pathways, cancer pathways, MAPK signaling, and PI3K-AKT signaling. Knockdown of NQO1 significantly decreased cell proliferation, migration, and invasion, while increasing apoptosis in HPV16-positive cervical cancer cells ($p \leq 0.01$). Additionally, proteins associated with the PI3K-AKT pathway were downregulated, while p53 and RECK protein levels were elevated. **Conclusion:** Our findings suggest that NQO1 plays a crucial role in promoting migration and invasion in HPV16-positive cervical cancer cells, highlighting its potential as a therapeutic target.

Keywords: RNA sequencing- gene ontology- cervical cancer- *NQO1*

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Introduction

Cervical cancer is the fourth most common cancer in women worldwide [1]. High-risk HPV types (HR-HPVs) was detected more than 90% in cervical cancer cases [2]. High-risk human papillomavirus (HR-HPV) types are detected in over 90% of cervical cancer cases [2-6]. The overexpression of the E6 and E7 oncoproteins from HR-HPV contributes to genomic instability, altered cellular gene expression, and the activation of cancer-associated pathways. For instance, the E7 protein interacts with retinoblastoma protein (pRB) to release E2F transcription factors, promoting progression into the S-phase of the cell cycle, while the E6 protein targets p53, a tumor suppressor, for degradation, thus inhibiting apoptosis [7-13].

Dysregulated gene expression in cervical cancer has been implicated in several critical pathways, including viral carcinogenesis, mitotic cell cycle regulation, cancer-associated signaling pathways, focal adhesion, and PI3K-Akt signaling. These pathways are essential for the proliferation, survival, and metastasis of cancer cells [14-16]. Studies have also shown that genes related to metabolic pathways are upregulated in HPV16-positive cervical cancer cells [17]. For example, HPV16E6/E7 has been shown to regulate aerobic glycolysis in cervical cancer cells, contributing to cancer progression [18].

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme involved in quinone metabolism. It prevents oxidative stress by reducing quinones to hydroquinones

*[1]Interdisciplinary Program, Graduate school, Chulalongkorn University, Bangkok, 10330, Thailand. [2]Center of Excellence in Applied Medical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand. [3]Division of Allergy and Clinical Immunology, Department of Medicine, King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, Thai Red Cross Society, Bangkok, 10330, Thailand. [4]Center of Excellence in Thai Pediatric Gastroenterology, Hepatology and Immunology (TPGHAI), Faculty of Medicine, Chulalongkon University, Bangkok, 10330, Thailand. [5]Center of Excellence in Vaccine Research and Development (Chula Vaccine Research Center- Chula VRC), Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. [6]Department of Oral Biology, Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand. [7]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand. *For Correspondence: arkom.cha@chula.ac.th, arkomchaiwongkot@gmail.com*

using NADH or NADPH as substrates, thus preventing the formation of reactive semiquinones [19-21]. NQO1 has been found to be highly expressed in various solid tumors, including cervical, endometrial, lung, colon, pancreatic, and ovarian cancers [20, 22, 23]. Its role has been extensively studied in malignancies such as breast cancer, non-small cell lung cancer (NSCLC), glioblastoma multiforme (GBM), and lung adenocarcinoma [24-29]. In breast cancer, NQO1 promotes tumor growth, progression, and metastasis through activation of the AMPK and AKT/mTOR pathways [24]. In GBM cells, NQO1 overexpression increases the activity of the PI3K/Akt/mTOR pathway [24, 30]. Overexpression of NQO1 in GBM cancer cells increased the activity of the PI3K/Akt/mTOR signaling pathway [26]. Similarly, high NQO1 expression enhances the proliferation and migration of human lung adenocarcinoma cells, with PI3K/AKT signaling being a key pathway in cancer progression [27]. Elevated NQO1 expression has also been observed in cisplatin-resistant ovarian cancer cells [31]. Additionally, downregulation of NQO1 in HPV18-positive cervical cancer cells has been shown to reduce cell survival [32]. High NQO1 expression is associated with poor prognosis in various cancers, including cervical and ovarian cancers [23, 33, 34] . All of these studies indicated that high expression of NQO1 is associated with tumor progression in various cancers.

The upregulation of NQO1 in HPV16-positive cervical cancer cells has been confirmed by differentially expressed gene (*DEG*) analysis. However, the functional role of NQO1 in these cells remains underexplored. Therefore, this study aimed to investigate the impact of NQO1 downregulation on the survival of HPV16-positive cervical cancer cells and to assess whether NQO1 can be a crucial factor in cervical cancer progression.

Materials and Methods

Cervical cancer cell lines

HPV16-positive cervical cancer cell lines, SiHa (HTB-35, ATCC) and CaSki (CRL-1550, ATCC), along with the HPV-negative cervical cancer cell line, C33A (HTB-31, ATCC), were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM high glucose medium (cat no. SH30022.02, Hyclone) supplemented with 10% fetal bovine serum (Gibco, USA) and 100 U/mL penicillin and streptomycin (Cytiva, USA). The cultures were maintained at 37°C in a 5% CO2 incubator. Ethical approval for the study was obtained from the Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) of the Faculty of Medicine, Chulalongkorn University (COE No. 005/2022, IRB No. 027/65, MDCU-IBC026/2021).

RNA Sequencing and Pathway Analysis

RNA sequencing (RNA-seq) was conducted on HPV16-positive cell lines (CaSki and SiHa) and the HPV-negative C33A cell line. RNA was extracted using the RNeasy® Mini Kit (QIAGEN, Valencia, CA, USA) and quality assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only samples with an RNA integrity number (RIN) > 8.0 were selected for sequencing. The mRNA was prepared using the TrueSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Sequencing was performed using the Illumina HiSeq2500 system (Illumina, San Diego, CA, USA) with paired-end reads. Reads were aligned to the human genome (GRCh38, release 87), and the raw data (FASTQ) were deposited in the National Center for Biotechnology Information (NCBI) under BioProject accession PRJNA1077769 (https://www.ncbi.nlm.nih.gov/bioproject/1077769). Differentially expressed genes (DEGs) between the HPV16-positive and HPV-negative cell lines were identified. DEGs with a log2 fold change (FC) > 4 in both SiHa and CaSki cell lines were analyzed for biological processes (BP), molecular functions (MF), and cellular components (CC) using Gene Ontology (GO) based on the STRING database. Pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The GSE9750 dataset, which includes both HPV16-positive and HPV-negative cell lines and clinical samples, was retrieved from GeneBank (http://www.ncbi.nlm.nih.gov/geo) and analyzed with the GEO2R tool.

Real-Time RT-PCR Validation

Primers used for real-time RT-PCR were: NQO1 forward: 5'-AGCGAGTGTTCATAGGAGAGT-3', NQO1 reverse: 5'-GCAGAGAGTACATGGAGCCAC-3', GAPDH forward: 5'-CACCGTCAAGGCTGAGAAC-3', GAPDH reverse: 5'-ATGGTGGTGAAGACGCCAGT-3'. RNA was extracted using the RNeasy® Mini Kit (QIAGEN) and 300 ng of RNA was converted to cDNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed using SSO Advanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The delta-delta Ct method was used to analyze the expression levels, with GAPDH as the housekeeping gene.

Optimization of siRNA Transfection

SiRNA transfection was performed using Lipofectamine RNAi MAX reagent (Invitrogen, USA). NQO1 siRNA (sc-37139, Santa Cruz Biotechnology) and non-targeting control (NTC) siRNA (D-001810-01-20, Horizon) were used. Cells (1x10^5) were seeded in 24-well plates and incubated at 37°C with 5% CO2 for 16-24 hours. NQO1 siRNA was tested at concentrations of 5, 10, and 20 pmol/well, and transfected cells were incubated for 72 hours. Real-time RT-PCR and western blot analysis were used to assess NQO1 expression levels.

Cell proliferation analysis

Cell proliferation was assessed using the MTS assay. Cells were seeded in 24-well plates (1x105 cells/well) and incubated for 16-24 hours before transfection. Cells were then transfected with 20 pmol of siRNA using Lipofectamine RNAi MAX reagent. After 72 hours, 2.5x103 cells were seeded in 96-well plates and incubated for 0, 24, 48, 72, and 96 hours. MTS reagent (CellTiter96, Promega) was added and absorbance was measured at 490 nm using a microplate reader (Perkin Elmer, USA).

s*Colony Formation Assay*

For anchorage-dependent growth, cells were transfected and incubated for 72 hours, then seeded (1,000 cells/well) in 6-well plates. After 14 days of incubation, colonies were stained with 0.1% crystal violet solution (0.1% crystal violet, 10% formaldehyde, 5% isopropanol, and distilled water) and counted using ImageJ.

Wound Healing Assay

Cells were transfected and seeded in 24-well plates (2x105 cells/well). After 16-24 hours, a scratch was made using a sterile micropipette tip to create a cell-free gap. After changing the media, cell migration was monitored at 0, 12, 24, 48, and 72 hours. The wound healing percentage was calculated by measuring the gap area using Pixit Pro. A4 software.

Cell Migration and Invasion Assays

Seventy-two hours post-transfection, 1×10^5 transfected cells were resuspended in 200 µL of serum-free DMEM medium and placed in the upper chamber of a transwell with an 8.0 µm polycarbonate membrane (REF353097, Falcon), which was inserted into a 24-well plate (SKU: 353047, Falcon). The lower chamber was filled with 700 µL of 10% FBS DMEM medium as a chemoattractant. The chambers were incubated at 37°C with 5% CO_2 for 16-24 hours. After incubation, non-migratory cells on top of the membrane were removed. The migratory cells in the lower chamber were fixed with 70% ethanol, stained with 0.2% crystal violet solution, washed, and counted.

For the invasion assay, the transwell chambers were pre-coated with 35 µL of ECM at a concentration of 250 µg/mL and incubated at 37°C with 5% CO_2 for 2 hours. Then, 1×10^5 transfected cells were resuspended in 200 µL of serum-free DMEM medium and placed in the upper chamber coated with ECM gel, which was placed into a 24-well plate (SKU: 353047, Falcon). The lower chamber contained 700 µL of 10% FBS DMEM medium as a chemoattractant and was incubated at 37 °C with 5% CO_2 for 16-24 hours. Non-invasive cells on top of the membrane were removed, and the invasive cells were fixed with 70% ethanol, stained with 0.2% crystal violet solution, washed, and counted.

The percentage of migration and invasion was calculated by comparing the number of migratory/invasive cells in the experimental groups to those in the negative control group (NTC siRNA transfected cells).

Cell Cycle Progression Analysis by Flow Cytometry

Seventy-two hours post-transfection, 1×10^6 transfected cells were washed with cold PBS. The cell pellet was then fixed with 1 mL of cold 70% ethanol at 4°C for 30 minutes. After fixation, the cells were resuspended in 1 mL of PBS. To treat the fixed cells, 50 µL of 100 µg/mL RNase A (Thermo Scientific, USA) was added, followed by 425 µL of cell staining buffer (420201, BioLegend) and 25 µL of Propidium Iodide solution (421301, BioLegend). Finally, cell cycle progression was analyzed using a BD FACSCalibur™ Flow Cytometer (BD Biosciences).

Apoptosis Analysis by Flow Cytometry

Seventy-two hours post-transfection, 1×10^6 transfected cells were washed with cold BioLegend cell staining buffer (420201, BioLegend) and resuspended in 100 µL of Annexin V Binding buffer (42220, BioLegend). The suspension was then treated with 5 µL of APC Annexin V (640920, BioLegend) and 10 µL of Propidium Iodide solution (421301, BioLegend) and incubated in the dark for 15 minutes. After incubation, 400 µL of Annexin V Binding buffer (42220, BioLegend) was added. Apoptotic cells were analyzed using a BD FACSCalibur™ Flow Cytometer (BD Biosciences).

Western Blot Analysis

The expression of p53, RECK, and proteins involved in the PI3K/AKT signaling pathway, including AKT, p-AKT (Ser473), PI3K, and p-PI3K, was analyzed by Western blot. Protein extraction was performed on NQO1 siRNA-transfected SiHa and CaSki cervical cancer cell lines using RIPA Lysis and Extraction Buffer (89900, Thermo Scientific). Protein concentration was determined using the BCA Protein Assay Kit (23227, Thermo Scientific). For each sample, 200 µg of protein was loaded and separated using a 10% SDS-PAGE gel, followed by transfer to a nitrocellulose membrane (Bio-Rad). The membranes were incubated with primary antibodies: NQO1 (ab805588, 1:10,000, Abcam), RECK (sc-373929, 1:1,000, Santa Cruz Biotechnology), PI3K (p110, 4255, 1:1,000, Cell Signaling Technology), p-PI3K (p-p85, 4228, 1:1,000, Cell Signaling Technology), p53 (9282, 1:1,000, Cell Signaling Technology), and GAPDH (sc-47724, 1:200, Santa Cruz Biotechnology). Following primary antibody incubation, the membranes were incubated with secondary HRP-conjugated antibodies: rabbit anti-mouse (ab205719) and anti-rabbit IgG (ab205718) antibodies (Abcam). Protein detection was carried out using a chemiluminescent detection method on the ChemiDoc XRS+ System (Bio-Rad). The intensity of the protein bands was quantified using Image Lab™ 6.0 Software.

Statistical Analysis

All in vitro assays were performed in duplicate or triplicate across three independent experiments. Statistical differences between groups were assessed using an unpaired t-test. Data are presented as mean ± standard deviation (SD), and a p-value ≤ 0.05 was considered statistically significant. Statistical analyses were conducted using GraphPad Prism 9 (Dotmatics, San Diego, CA, USA).

Results

Analysis of Cellular Gene Expression, Gene Ontology, and KEGG Pathways

Transcriptomic analysis was performed to compare the HPV16-positive cervical cancer cell lines (SiHa and CaSki) with the HPV-negative cervical cancer cell line (C33A). Genes with a log2 fold change greater than 4 were selected for analysis. In total, 1,085 upregulated genes were identified in CaSki, and 854 upregulated genes were identified in SiHa (Supplementary Figures 1 and 2).

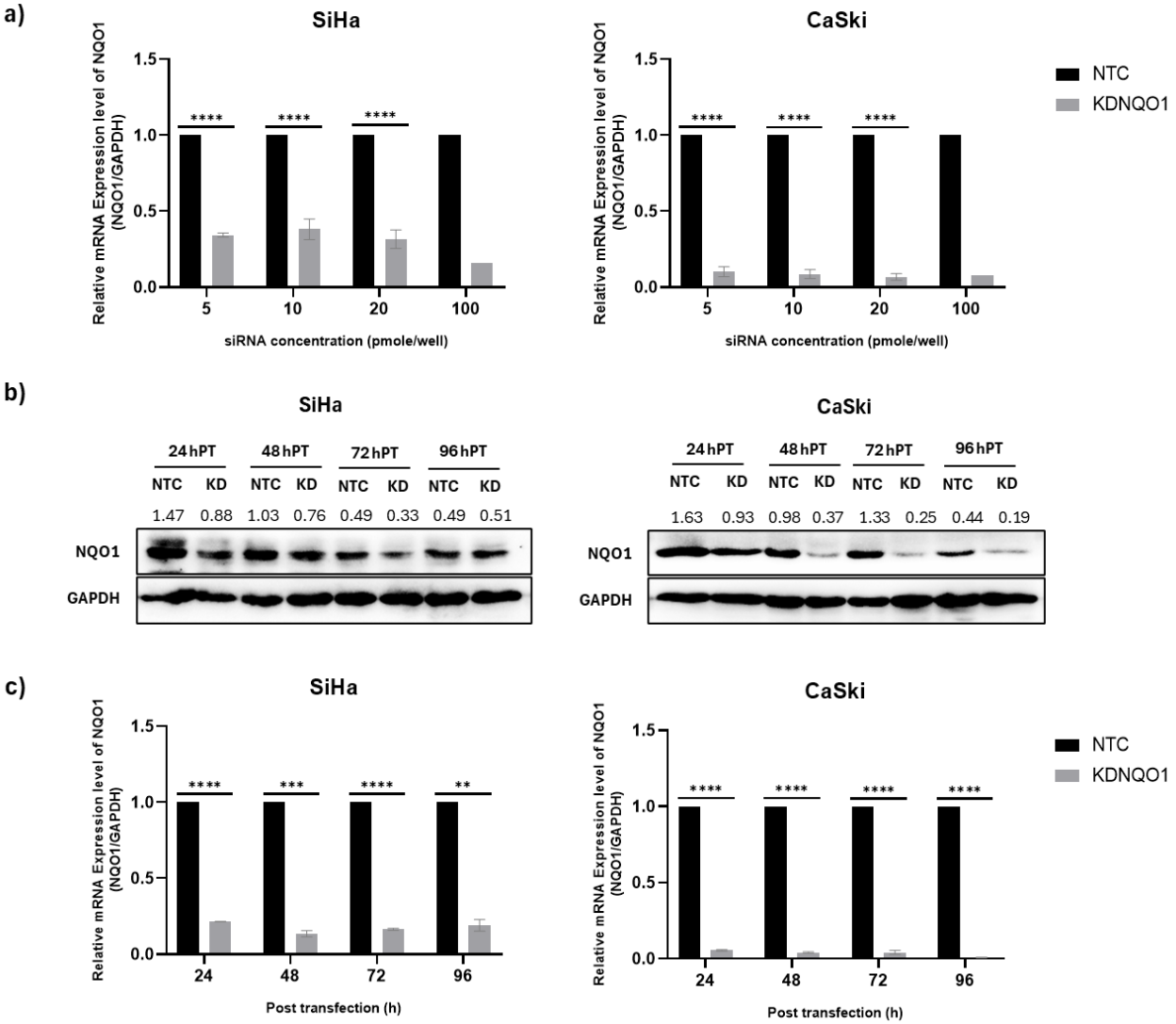

Figure 1. The Optimization of siRNA Concentration and Time Point for NQO1 Knockdown in SiHa and CaSki Cell Lines. (a) NQO1 mRNA expression was detected by real-time RT-PCR at different NQO1 siRNA concentrations (5, 10 and 20 pmol/well) (b) protein expression at different time points detected by western blot (c) NQO1 NQO1 mRNA expression at different time points detected by real-time RT-PCR. GAPDH was used as a housekeeping gene. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

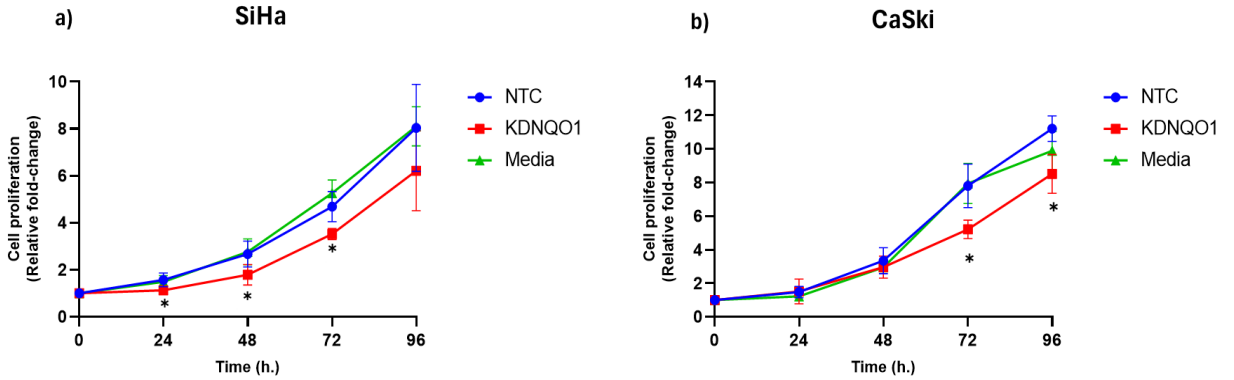

Figure 2. Effect of NQO1 Knockdown on Cervical Cancer Cell Proliferation. Cell proliferation was measured at 0, 24, 48, 72 and 96 hours by MTS assay. Relative fold change of cervical cell proliferation of SiHa (a) and CaSki (b) with and without NQO1 knockdown was calculated. An experiment was performed in triplicate on three independent experiments. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

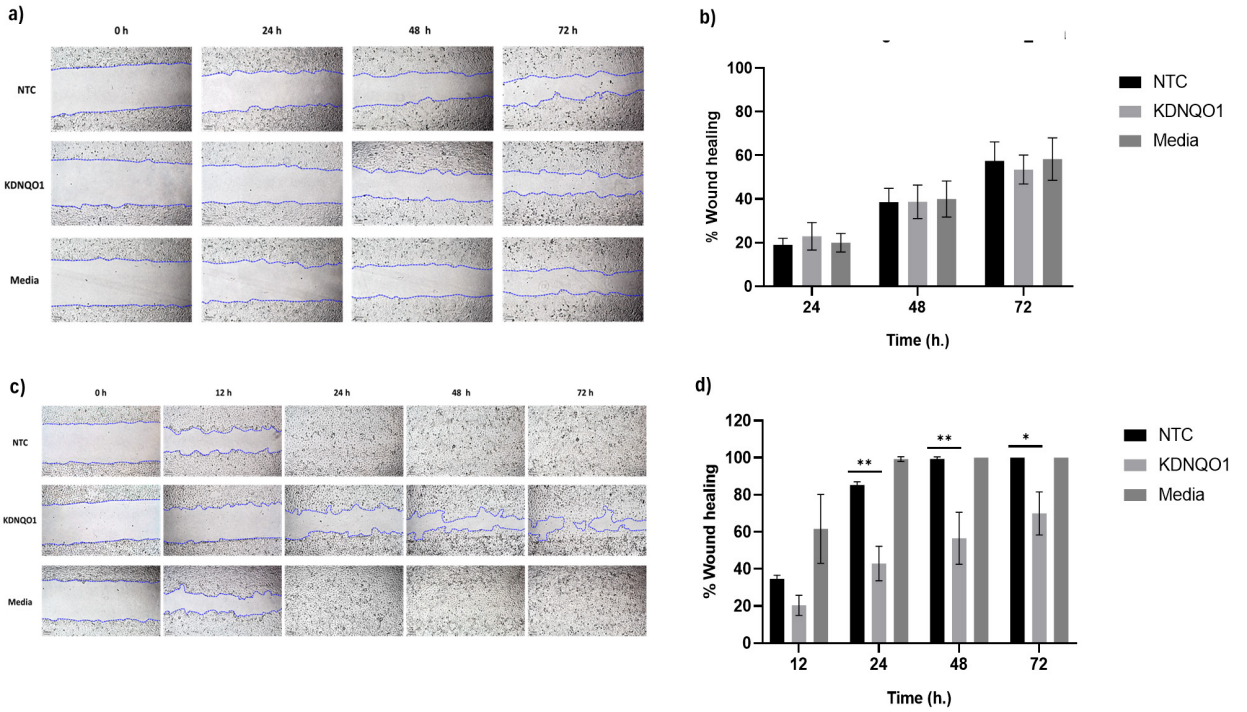

Figure 3. Effect of NQO1 Knockdown on Cervical Cancer Cell Migration Ability. Time course of scratch wound healing assay in NQO1 knockdown SiHa (a) and CaSki (c) cells. The percentage of wound healing of NQO1 knockdown SiHa (b) and CaSki (d) cells was measured by calculating the cell free surface area. An experiment was performed in triplicate on three independent experiments. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

A total of 593 differentially expressed genes (*DEGs*) were found to be upregulated in both HPV16-positive cell lines (SiHa and CaSki) (Supplementary Table 1). Gene ontology (GO) analysis was performed using the STRING database, and KEGG pathway analysis was conducted to explore the biological relevance of these DEGs. The most significant biological processes (BP) included tissue development, immune system processes, and responses to stimuli. The most notable molecular functions (MF) included protein binding, cell adhesion molecule binding, and signaling receptor binding. Cellular components (CCs) predominantly involved the extracellular space, extracellular region, and extracellular exosome. The top KEGG pathways enriched in these DEGs included metabolic pathways, pathways in cancer, and human papillomavirus (HPV) infection. Key cancer-related pathways, such as the MAPK and PI3K-Akt signaling pathways, were also identified. Supplementary Table 2 presents the Gene Ontology and KEGG pathways of genes with a log2 fold change greater than 4 obtained from the GSE9750 dataset, showing consistent results with our RNA sequencing findings. NQO1, which is involved in both cancer pathways and metabolic pathways, was selected for further functional analysis. Real-time RT-PCR was conducted to confirm *NQO1* gene expression. The results demonstrated significantly higher levels of NQO1 expression in the HPV16-positive cervical cancer cell lines (SiHa and CaSki) compared to C33A (Supplementary Figure 3a). Further validation using the GSE9750 dataset revealed that HPV16-positive cervical cancer samples and cell lines (CaSki and SiHa) expressed higher levels of NQO1 than HPV-negative cervical cancer samples, normal cervical cells, and cell lines (C33A) (Supplementary Figures 3b and 3c).

Effect of NQO1 siRNA Knockdown on Cell Proliferation

The optimization of siRNA concentration for NQO1 knockdown in HPV16 positive cervical cancer cell lines was performed by using different siRNA concentration and time periods. The siRNA concentration of 20 pmol/well of a 24 well-plate transfected for 72 hours, which could inhibit *NQO1* gene expression approximately 70% in both CaSki and SiHa cells was further employed for functional investigation (Figure 1). When comparing NQO1 knockdown SiHa and CaSki cells to cells transfected with non-targeting siRNAs (NTC), cervical cancer cell proliferation was significantly reduced at timepoint 72 hours ($P<0.05$) (Figure 2). This indicates that NQO1 may have a significant impact on cervical cancer cell proliferation.

Effect of NQO1 siRNA Knockdown on Wound Healing Ability

To study cell migration, an in vitro wound healing assay was conducted to measure the percentage of open wound area following NQO1 knockdown at 0, 24, 48, and 72 hours for SiHa cells and at 0, 12, 24, 48, and 72 hours for CaSki cells. The results indicated a slight decrease in migratory ability in SiHa cells with NQO1 knockdown, most evident at 72 hours post-transfection

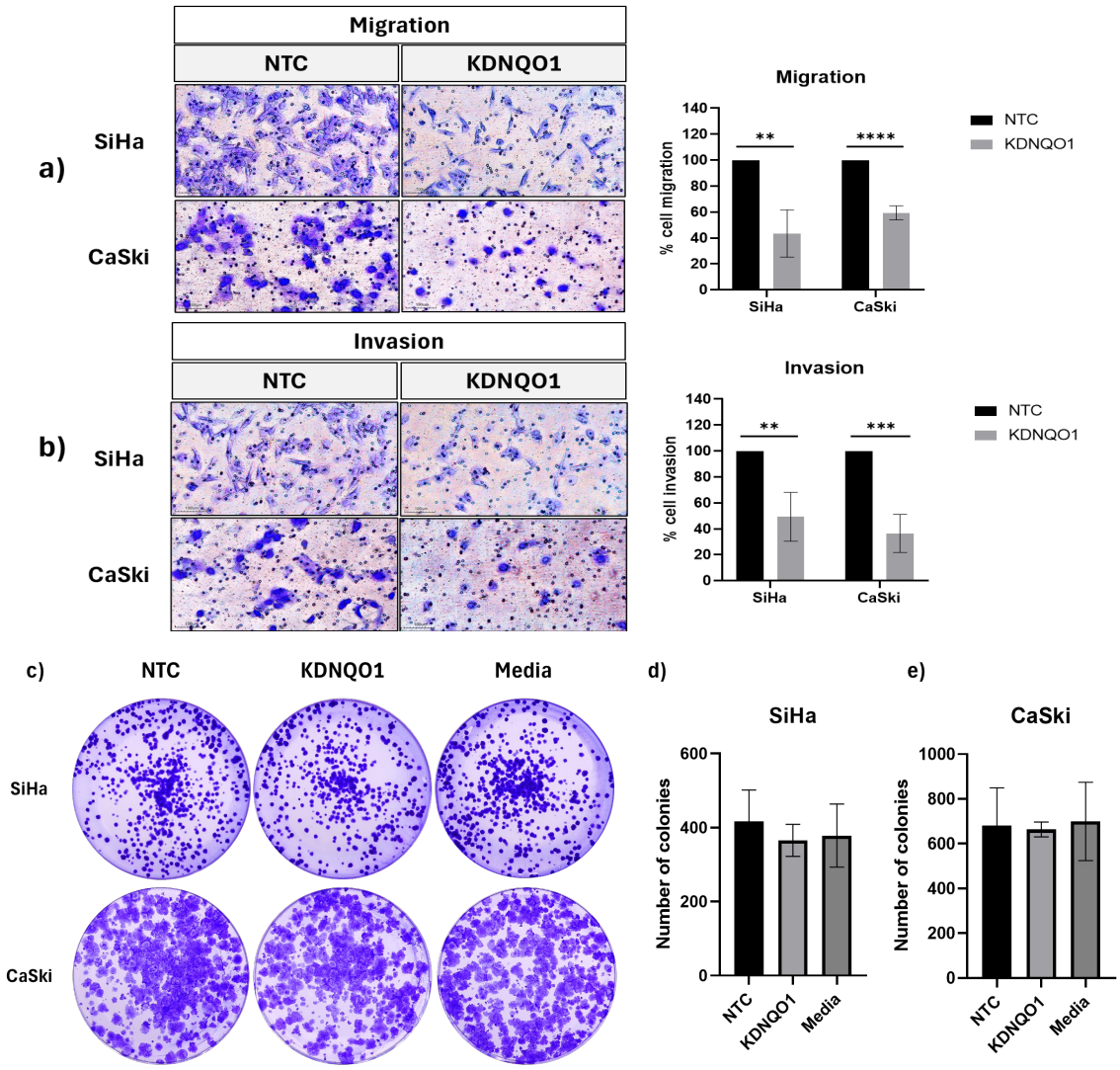

Figure 4. Effect of NQO1 siRNA Knockdown on Cell Migration, Invasion and Anchorage Dependent Cell Growth of Cervical Cancer Cell Lines. The percentage of migration and invasion (a and b) in NQO1 siRNA knockdown SiHa and CaSki cells were compared to NTC detected by Boyden chamber assay. The number of SiHa and CaSki (c, d and e) colonies with NQO1 siRNA knockdown was compared to NTC. An experiment was performed in triplicate on three independent experiments. NTC; Non-Target Control (non-targeting siRNAs), KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

but not significant between 24 and 48 hours (Figures 3a and 3b). In contrast, NQO1 knockdown in CaSki cells significantly reduced migratory ability compared to the non-targeting control (NTC) at all time points ($P < 0.05$) (Figures 3c and 3d).

Effect of NQO1 siRNA Knockdown on Cell Migration, Invasion, and Anchorage Dependent Cell Growth

The impact of NQO1 siRNA knockdown on cell migration and invasion was assessed in SiHa and CaSki cells using the Boyden chamber assay. Migration of NQO1 siRNA knockdown cells was significantly reduced compared to the non-targeting control (NTC) ($P < 0.05$) (Figure 4a). Similarly, invasion capacity was significantly decreased in NQO1 siRNA knockdown cells compared to NTC ($P < 0.05$) (Figure 4b). Thus, NQO1 knockdown inhibited both migration and invasion in these cervical cancer cell lines. However, there was no significant difference in colony formation ability between NQO1 siRNA knockdown cells and NTC (Figures 4c-e), suggesting that NQO1 may not play a role in anchorage-dependent growth of SiHa and CaSki cells.

Effect of NQO1 siRNA Knockdown on Cell Apoptosis and Cell Cycle Progression

The results demonstrated that NQO1 siRNA knockdown in SiHa cells significantly decreased the number of cells in the G1 phase and slightly increased the number of cells in the subG1 phase compared to the negative control (NTC) ($P<0.05$). Additionally, the NQO1 siRNA knockdown in SiHa cells significantly accumulated in the S and G2/M phases compared to the NTC ($P<0.05$) (Figure 5a, Supplementary Table 3). NQO1 knockdown in CaSki cells showed a slight increase in the S and G2/M phases and a slight decrease in the G1 phase, but this change was not statistically significant (Figure 5b, Supplementary Table 3). NQO1 siRNA knockdown in both SiHa and CaSki cells also resulted in

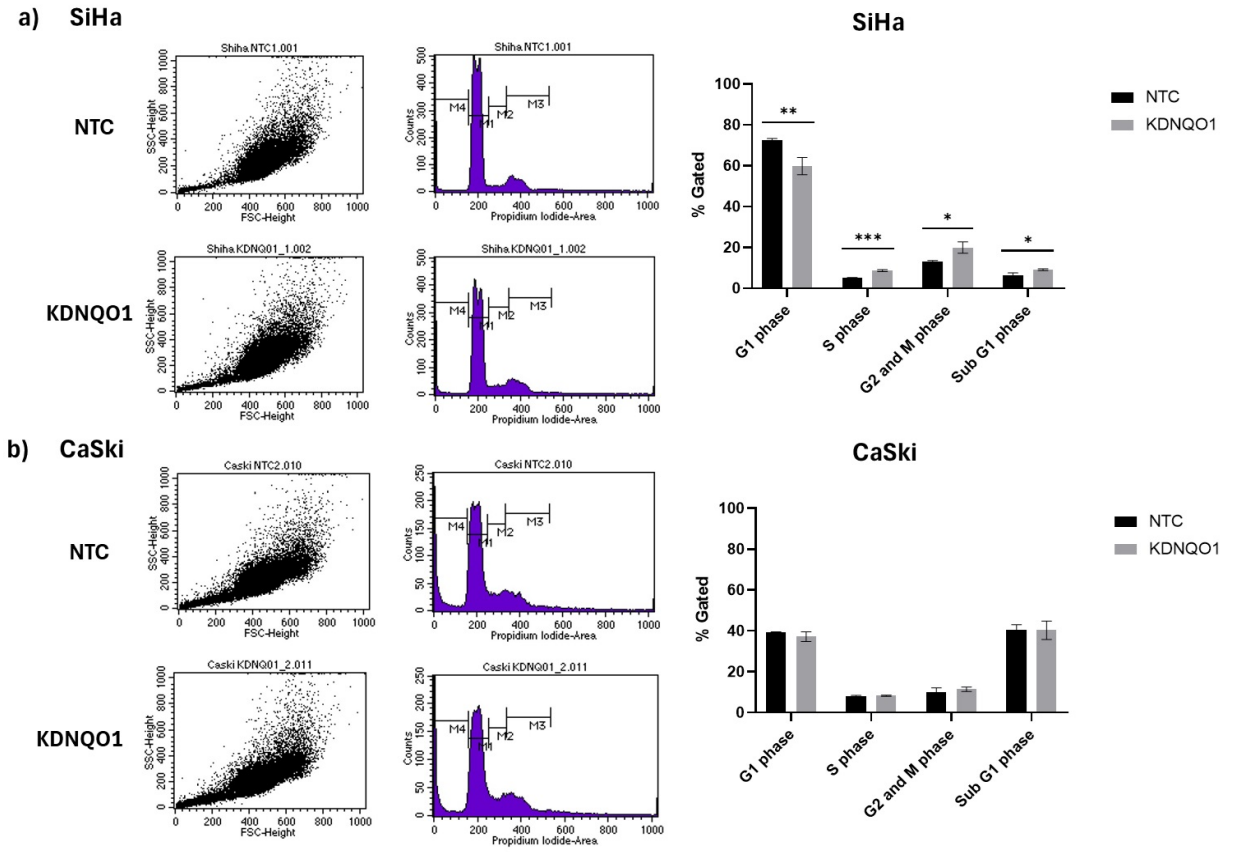

Figure 5. Effect of NQO1 siRNA Knockdown on the Cell Cycle Profile in HPV16 Positive Cervical Cell Lines. The flow cytometry results after 72 hours post transfection. The percentage of cell cycle population in cell cycle profile of SiHa (a) and CaSki (b) with and without NQO1 siRNA knockdown was compared to NTC siRNA control cells. An experiment was performed in triplicate on three independent experiments. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

a significant increase in apoptotic cells (Figures 6a and 6b, Supplementary Table 4). This indicated that NQO1 knockdown affected the survival of HPV16 positive cervical cancer cells.

Effect of NQO1 siRNA Knockdown on Proteins Involved in PI3K-AKT Signaling Pathway, p53, and RECK Protein

The PI3K-AKT signaling pathway is crucial for regulating cell proliferation, migration, and survival. To investigate the molecular mechanisms underlying the effects of NQO1 knockdown, we assessed the protein levels of key components in SiHa and CaSki cells following NQO1 siRNA transfection. As shown in Figure 7, NQO1 siRNA knockdown resulted in significantly lower levels of pAKT, PI3K, and pPI3K compared to control cells transfected with non-targeting siRNA (NTC). In contrast, protein levels of p53 and RECK were upregulated in NQO1 siRNA knockdown cells. The increased p53 protein levels were associated with enhanced cell apoptosis, while the upregulation of RECK protein correlated with reduced cancer cell invasion. These results suggest that NQO1 knockdown may exert its effects through modulation of both the PI3K-AKT signaling pathway and the regulation of apoptosis and cell migration.

Discussion

Persistent infection with HR-HPV, accompanied by the overexpression of viral oncogenes, is a critical precondition for the development of cervical cancer and its precursor lesions. This infection leads to both genetic and epigenetic alterations, which play a pivotal role in the pathogenesis of cervical cancer [35-37]. The pathway analysis of both datasets in this study revealed similarities, indicating the reliability of RNA microarray and short-read sequencing methods. The altered cellular genes in HPV16-positive cervical cancer cell lines are involved in several significant pathways. These include genes located in the extracellular region and plasma membrane, which play roles in protein binding, cell adhesion molecule binding, signaling receptor binding, enzyme binding, and protease binding. These changes contribute to key cancer-related pathways, such as the MAPK and PI3K-AKT signaling pathways, consistent with previous reports [17, 38]. Previous research has shown that HPV16 is involved in several critical pathways, including focal adhesion, MAPK signaling, and WNT signaling. Additionally, genes associated with various metabolic pathways are altered in HPV16-positive cervical cancer [17]. Upregulated cellular proteins involved in the Ras signaling pathway, cell cycle regulation, MAPK signaling, epidermal growth factor receptor (EGFR) signaling, and p53 signaling pathways

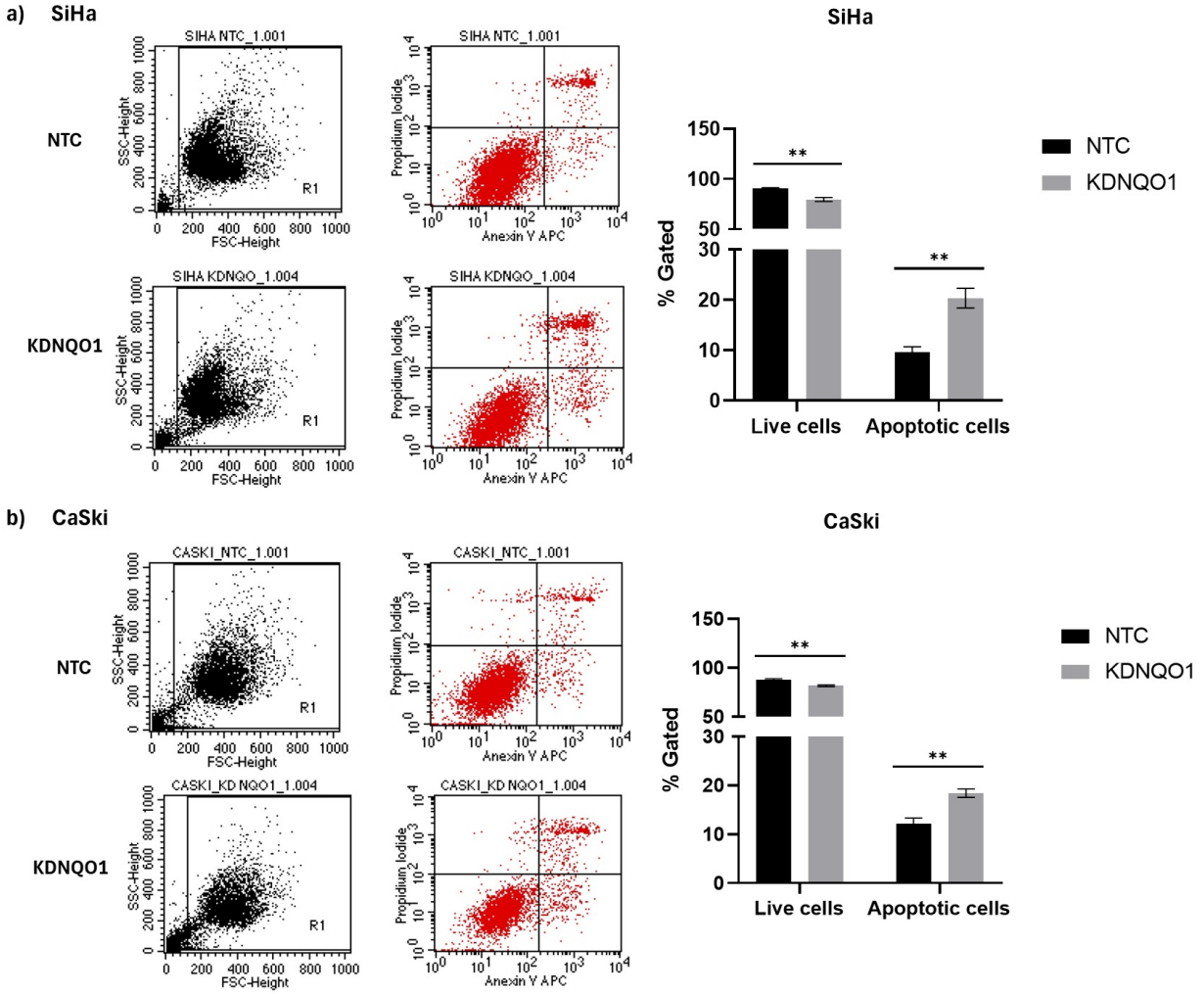

Figure 6. Effect of NQO1 siRNA Knockdown on the Apoptosis in HPV16 Positive Cervical Cell Lines. The flow cytometry results after 72 hours post transfection. The percentage of apoptosis of SiHa (a) and CaSki cells (b) with NQO1 siRNA knockdown was compared to NTC. An experiment was performed in triplicate on three independent experiments. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

are linked to metabolic enzymes in cervical cancer [39]. HPV16 E6/E7 oncoproteins play a role in regulating aerobic glycolysis in cervical cancer cells through the HPV E6/E7/IGF2BP2/m6A-MYC/glycolysis axis, which may be crucial for cancer progression [18]. Furthermore, HPV16 E7 can modify host tryptophan metabolism, enabling cancer cells to evade the immune system by upregulating indoleamine 2,3-dioxygenase (IDO1), an immunoregulatory enzyme [40].

NQO1 has been shown to be overexpressed in various solid tumors, including cervical cancer [23]. The present study found that NQO1 downregulation resulted in decreased cell proliferation, migration, and invasion of HPV16-positive cervical cancer cells, which is consistent with previous reports on other cancer cell types. Specifically, NQO1 knockdown inhibits the growth of human colorectal cancer cell lines, breast cancer cell lines, and glioma cells [41, 42].

As previously reported, PI3K/AKT pathways are deregulated in various cancers, including HPV-induced cancers. PI3K/AKT plays an important role in the regulation of cell motility, growth, proliferation, survival, autophagy, protein synthesis, transcription, and angiogenesis [13, 37]. The present study showed that proteins involved in the PI3K/AKT pathway were downregulated in NQO1 knockdown HPV16-positive cancer cells, which is consistent with a previous study showing that downregulation of NQO1 expression inhibited the activity of the PI3K/Akt/mTOR signaling pathway in malignant glioma cell lines [26]. One study found that NQO1 acts as an upstream activator of both the PI3K/AKT and MAPK/ERK pathways in liver cancer cells, and that NQO1 downregulation decreased metabolic activity and liver cancer cell proliferation [43].

RECK protein, an inhibitor of angiogenesis, was found to be expressed more in *NQO1* knockdown HPV16-positive cervical cancer cells, which could explain the decreased cell migration and invasion. RECK has also been demonstrated to prevent pancreatic ductal adenocarcinoma (PDAC) metastasis [44]. RECK was found to be downregulated in HPV18-positive cervical cancer cell lines, including HeLa [45]. The results of the present study in HPV16-positive cervical cancer cell lines (SiHa and CaSki) were consistent with those

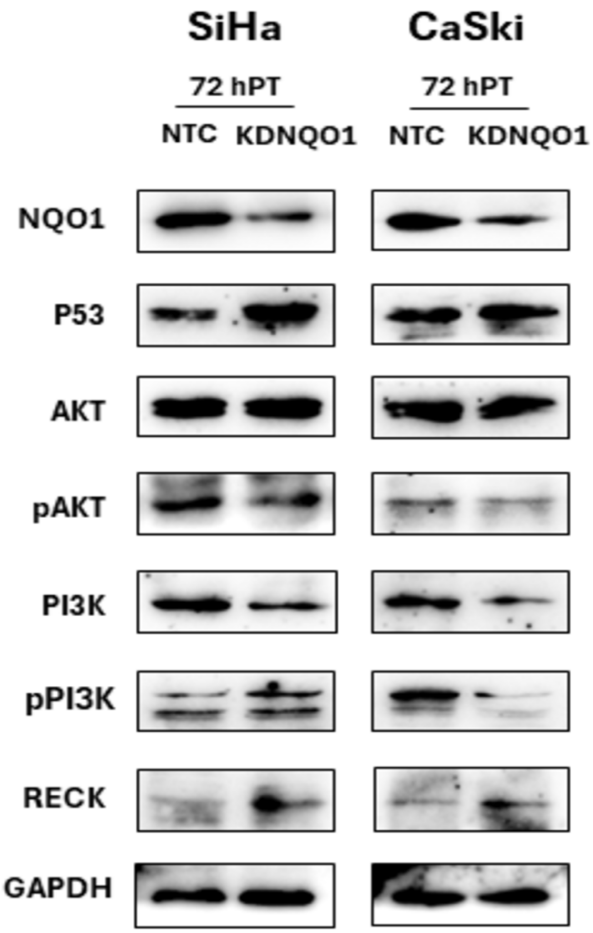

Figure 7. Effect of NQO1 siRNA Knockdown in Expression Levels of Proteins Involved in the PI3K-AKT Signaling Pathway, p53 Protein and RECK Protein in HPV16 Positive Cervical Cancer Cell Lines. The western blot results after 72 hours post transfection, an experiment was performed in duplicate on three independent experiments. NTC; Cells transfected with non-targeting siRNAs, KDNQO1; NQO1 siRNA knockdown. SD was shown as error bars. Ns, non-significant: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

in HPV18-positive cervical cancer cell line (HeLa), which showed that RECK overexpression in HeLa cells inhibited cervical cancer cell migration and invasion while increasing the expression of p53 protein [45]. It was also observed that NQO1 downregulation caused apoptosis in HPV18-positive cancer cell line (HeLa) via the production of reactive oxygen species (ROS) [46]. NQO1 knockdown increased apoptosis in HPV16-positive cancer cells, which was associated with an increase in p53 protein, as demonstrated in the present study. Other studies showed that inhibiting NQO1 led to increased apoptosis in ovarian cancer cell line (SKOV3) and A549 and H292 lung adenocarcinoma cells [20, 47, 48]. NQO1 knockdown and chemotherapeutic drug therapy in cholangiocarcinoma induced p53 expression [49].

In contrast to solid tumors, one study found that NQO1 knockdown increases the protein expression of the TOP2A and MCM complex, promoting lymphoblast (K562) cell growth [50]. A study in human breast cancer cells and human colon cancer cells found that NQO1 is involved in the mechanism of cancer cell cycle progression at the G2/M phase through NQO1/c-FOS/CKS1 signaling, and that NQO1 silencing delayed cell cycle progression at the G2/M phase [51], , which was consistent with the present study. However, there was a slight increase in the S and G2/M phases and a slight decrease in the G1 phase in CaSki cells compared to SiHa cells, which could be due to some genes that were more highly expressed in CaSki than SiHa cells. These genes were involved in cancer cell proliferation and cell cycle progression, such as one study showing that there were high expressions of carbonic anhydrase IX (CAIX) in CaSki cells but lowest in SiHa cells. The cell cycle result showed that CAIX knockdown in CaSki cells slightly accumulated the cell cycle in the S and G2/M phases compared to the NTC [52]. Another study showed that Pyrroline-5-Carboxylate Reductase 1 (PYCR1) was more expressed in CaSki than SiHa cells [53], and loss of PYCR1 results in the arrest of the cell cycle [54].

Our findings provide a new perspective on a potential cervical cancer drug target and ways to prevent cancer cell migration. However, the limitation of the present study was the lack of animal experiments.

In conclusion, the present study demonstrated that NQO1 down-regulation affects the important properties of HPV16-positive cervical cancer cells, including decreased migration, invasion, and increased apoptosis-related protein levels.

Author Contribution Statement

AC designed the study, WW performed the experiments, AC and WW analyzed, and interpreted data. AC and WW drafted the manuscript. AC, NK, PB, reviewed and edited the manuscript. PB, NK and SB supervised the study. All authors have read and agreed to the published version of the manuscript.

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Ethical Declaration

The present study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (COE No. 005/2022, IRB No. 027/65), and the Institutional Biosafety Committee (IBC) of the Faculty of Medicine, Chulalongkorn University (MDCU-IBC026/2021). This study was conducted independently and is not part of any approved student thesis.

Data Availability

The data presented in this study are available upon reasonable request to the corresponding author. Raw data (FASTQ) files have been deposited in the National Center for Biotechnology Information (NCBI) under the BioProject database: https://www.ncbi.nlm.nih.gov/bioproject/1077769.

Conflict of Interest

The authors have no conflict of interests related to this publication.

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