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Disturbance of DKK1 level is partly involved in survival of lung cancer cells via regulation of ROMO1 and γ-radiation sensitivity

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ABSTRACT

Dickkopf1 (DKK1), a secreted protein involved in embryonic development, is a potent inhibitor of the Wnt signaling pathway and has been postulated to be a tumor suppressor or tumor promoter depending on the tumor type. In this study, we showed that *DKK1* was expressed differently among non-small-cell lung cancer cell lines. The *DKK1* expression level was much higher in A549 cells than in H460 cells. We revealed that blockage of *DKK1* expression by silencing RNA in A549 cells caused up-regulation of intracellular reactive oxygen species (ROS) modulator (ROMO1) protein, followed by partial cell death, cell growth inhibition, and loss of epithelial–mesenchymal transition property caused by ROS, and it also increased γ-radiation sensitivity. *DKK1* overexpression in H460 significantly inhibited cell survival with the decrease of ROMO1 level, which induced the decrease of cellular ROS. Thereafter, exogenous *N*-acetylcysteine, an antioxidant, or hydrogen peroxide, a pro-oxidant, partially rescued cells from death and growth inhibition. In each cell line, both overexpression and blockage of *DKK1* not only elevated p-RB activation, which led to cell growth arrest, but also inactivated AKT/NF-kB, which increased radiation sensitivity and inhibited cell growth. This study is the first to demonstrate that strict modulation of *DKK1* expression in different cell types partially maintains cell survival via tight regulation of the ROS-producing *ROMO1* and radiation resistance.

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1. Introduction

The Wnt/β-catenin signal is a critical and strictly regulated pathway of physiological processes underlying normal embryonic development and tissue homeostasis. This pathway is also involved in an intricate network of signaling that serves as a negative regulator of osteogenesis in adult animals [1,2]. Deregulated activation of the Wnt/β-catenin signal is an early event in many human cancer types, and it is thought to be associated with an aggressive phenotype of several cancers [3]. Dickkopf1 (DKK1) is the predominant secretory antagonist of the Wnt/β-catenin signal, suggesting that if DKK1 were not precisely regulated, it could result in tumor formation and progression. In fact, several clinical studies demonstrated that DKK1 was down-regulated in breast, melanoma, and colon cancer [4,5]. Other study showed that oncogenic Wnt/β-catenin signaling pathway is down-regulated by DKK1 [6]. Qiao et al. also showed that conditioned media from human mesenchymal stem cells, with high levels of DKK1, inhibited growth of human MCF-7 breast cancer cells *in vitro* and *in vivo* by inhibiting or down-regulating the Wnt/β-catenin signaling pathway [7]. In turn, removing DKK1 from the medium by adding a neutralizing antibody nullified these inhibitory effects. This result strongly suggests that DKK1 can function as a suppressor of tumor growth in a paracrine fashion. However, DKK1 expression levels are elevated in a wide variety of cancers. Several reports also suggest that DKK1 overexpression is associated with cancer progression and poor prognosis; for example, there have been several reports of DKK1 up-regulation in malignant cancers including multiple myeloma, hepatoblastoma, and Wilms' tumor [8,9]. DKK1 expression in myeloma cells plays a major role in osteoblastic differentiation in patients with multiple myeloma [9].

An enhanced level of reactive oxygen species (ROS) from the mitochondria has been investigated in many cancer cells [10,11]. Previous studies showed that a strictly regulated cellular level of ROS is essential for the proliferation of tumor cell growth, and *ROMO1* expression triggered ROS production in the mitochondria and thus cell growth [12,13]. Therefore, cell growth inhibition

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by *ROMO1* suppression with silenced RNA (siRNA) can be rescued by exogenous oxidant such as hydrogen peroxide (H_2O_2). In addition, ROS production caused by *ROMO1* overexpression is associated with the invasiveness of hepatic tumor cells [14]. Together, these findings suggest that *ROMO1* expression-induced ROS production plays a critical role in redox signaling and thus cell proliferation in cancer cells.

In this study, we revealed that DKK1 negatively regulates the expression of *ROMO1* and thus modulates cellular ROS in non-small-cell lung cancer (NSCLC) cell lines. Disturbance of cellular DKK1 levels is partially involved in cell death, cell growth inhibition, and loss of the epithelial–mesenchymal transition (EMT) property via ROMO1-induced imbalance of intracellular ROS and resistance to γ-radiation.

2. Materials and methods

2.1. Cell culture and irradiation

All NSCLC cell lines used in the study were obtained from Korea Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 95% air/5% CO_2. Cells were inoculated at a density of 1×10^5 cells in a T-25 flask, incubated for 1 day, and then irradiated with a dose of 2 Gy from a ^{60}Co γ-ray at a dose rate of 0.2 Gy/min.

2.2. Construction of DKK1 and ROMO1 expression vector and PCR amplification

An 801-bp insert of human *DKK1* and 240-bp insert of human *ROMO1* were amplified from human lung cancer cell poly(A) mRNA by RT-PCR to construct the *DKK1* and *ROMO1* gene expression vector. Total RNA was isolated from H460 or A549 cells, with the use of TRIzol reagent (Invitrogen) RNA extraction. To generate first-strand complementary DNA (cDNA) from the total RNA (1 μg) using oligo-dT, we used a cDNA synthesis kit (Intron Biotechnology). Resultant cDNAs served as templates for PCR amplification with the following forward and reverse primers (DKK1, *Eco*RI [forward]: 5′-ATATGAATTCATGATGGCTCTGGGCG-3′; *Xho*I [reverse]: 5′-ATATCTCGAGTTAGTGTCTCTGACAAG-3′; ROMO1, *Eco*RI [forward]: 5′-ATATCTCGAGATGCCGGTGGCCGTG-3′; *Xho*I [reverse]: 5′-ATATCTCGAGTTAGCATCGGATGCC-3′). The DKK1 and ROMO1 cDNA inserts were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). The DKK1 and ROMO1 gene expression vector was transfected into the cells using Lipofectamine 2000 (Invitrogen), followed by selection with 400 μg/ml G418 (Calbiochem). For PCR analysis of other gene expression, we analyzed *β-actin* (forward: 5′-CATCCTCACCCTGAAGTACCC-3′; reverse: 5′-AGCCTGGATAGCAACGTACATG-3′) and *GAPDH* (forward: 5′-ATGGGGAAGGTGAAGG-3′; reverse: 5′-TTACTCCTTGGAGGCC-3′). The PCR conditions for *DKK1*, *ROMO1*, *β-actin*, and *GAPDH* were as follows: denaturating at 94 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 10 min. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and then photographed under ultraviolet light.

2.3. Western blot analysis

Western blot analysis was performed with primary antibodies specific for human DKK1, p53, ROMO1, cyclin D1, NF-kB, and retinoblastoma protein (Santa Cruz Biotechnology), p-AKT, AKT, E-cadherin, $p21^{Waf1/cip1}$, phosphorylated retinoblastoma protein (p-RB: serine 608, 780, and 807/811), β-actin (Cell Signaling Technology), Vimentin (Thermo Fisher Scientific), N-cadherin and anti-Snail (Abcam). Protein concentration was determined with a protein assay (Bio-Rad). Equal amounts of protein were separated on a 10–15% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia). The blots were blocked for 1 h at room temperature with blocking buffer (10% nonfat milk in PBS containing 0.1% Tween 20; TBS). The membrane was incubated overnight in a cold chamber with specific antibodies. After being washed with TBS, the membrane was incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase linked secondary antibody (Cell Signaling Technology) and visualized with the Westzol enhanced chemiluminescence detection kit (Intron Biotechnology).

2.4. Silencing RNA targeting of DKK1

Cells were transfected with three different Stealth RNA targeting *DKK1* genes (Invitrogen; primer sequences: 5′-CACUAAACCAGCUAUCCAA-3′/5′-UUGGAUAGCUGGUUUAGUG-3′) or with Stealth RNAi Negative Control Medium GC at a concentration of 80 nM of Lipofectamine RNAi MAX reagent (Invitrogen). The cells were incubated for 72 h after transfection, and then *DKK1* expression was determined by reverse transcription RT-PCR.

2.5. Flow cytometry analysis

Cells (1×10^6 cells in 200 μl PBS) were collected, washed with PBS, and fixed with 70% ethanol at 4 °C for 2 h in the dark. Fixed cells were washed with PBS and stained with propidium iodide (50 μg/ml). The DNA content was measured with a FACScan (EPICS XL; Beckman Coulter Counter). A minimum of 10,000 cells was counted for each sample. The percentage of cells in each phase was determined by Phoenix Multicycler Software (Phoenix Flow System).

2.6. Colony-forming assay

For the colony-forming assay, transfected suppression and overexpression cells were plated in 35-mm culture dishes at 1×10^3 cells per plate and allowed to attach overnight. Cells were left untreated or exposed to a 2 Gy dose of radiation and then incubated for 10–14 days post-irradiation and stained with 0.5% crystal violet. Colonies (i.e., groups $\geqslant$ 50 cells) were counted. Clonogenic survival was expressed as a percentage relative to the untreated controls.

2.7. Microscopic analysis

All attached cells at the bottom of the culture plate were photographed by light microscopy (Leica Microsystems). Images were captured with a Canon Power Shot S45 digital camera system.

2.8. Detection of intracellular ROS

Intracellular ROS was measured by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes), as described previously [15].

2.9. Senescence-associated β-galactosidase (SA-β-Gal) activity

Staining for SA-β-Gal activity was performed using bromo-4-chloro-3-indolyl-β-D-galactosidase according to the manufacturer's instructions (Intron Biotechnology). Quantification of SA-β-Gal-positive cell was obtained by counting five random fields per dish and calculating the percentage.

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