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Research paper

Nuclear factor erythroid-2-related factor regulates *LRWD1* expression and cellular adaptation to oxidative stress in human embryonal carcinoma cells

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ABSTRACT

Leucine-rich repeats and WD repeat domain-containing protein 1 (LRWD1) is implicated in the regulation of signal transduction, transcription, RNA processing and tumor development. However, LRWD1 transcriptional regulation is not fully understood. This study aimed to investigate the relationship between LRWD1 expression and reactive oxygen species (ROS) level in human embryonal carcinoma cell line, NT2/D1 cells, which will help in understanding the transcriptional regulatory role of ROS in cells. Results showed that the exposure of NT2/D1 cells to various concentrations of hydrogen peroxide (H_2O_2) and the nitric oxide (NO) donor sodium nitroprusside (SNP) caused a significant increase in the mRNA and protein expression of LRWD1. In addition, LRWD1 promoter luciferase reporter assay, and Chromatin Immunoprecipitation assay (CHIP assay) showed that nuclear factor erythroid-2-related factor (Nrf2) was involved in the regulation of LRWD1 expression in response to oxidative stress. The involvement of Nrf2 was confirmed by shRNA-mediated knockdown of Nrf2 in NT2/D1 cells, which caused a significant decrease in LRWD1 expression in response to oxidative stress. Similarly, LRWD1 knockdown resulted in the accumulation of H_2O_2 and superoxide anion radical (O2-). Blocking ROS production by N-acetyl cysteine (NAC) protected NT2/D1 shLRWD1cells from H_2O_2-induced cell death. Collectively, oxidative stress increased LRWD1 expression through a Nrf2-dependent mechanism, which plays an important role in cellular adaptation to oxidative stress. These results highlight an evidence, on the molecular level, about LRWD1 transcriptional regulation under oxidative stress.

1. Introduction

Leucine-rich repeats and WD repeat domain containing 1 (*LRWD1*) gene is highly expressed in different human and mouse tissues [1,2]. WD family regulates signal transduction, transcription, RNA processing, protein-protein interaction and tumor development [3–5]. In our previous study, we demonstrated that LRWD1 interacts with γ-tubulin in the centrosome of mouse testis germ cells [2]. In somatic cells, LRWD1 plays a critical role in the initiation of DNA replication and cell-cycle progression [6]. In addition, LRWD1 was found to co-localize with Orc2 (origin recognition complex subunit 2) at pericentric heterochromatin [7]. Such association between LRWD1 and pericentric heterochromatin was required for heterochromatin silencing and maintenance [7]. Recent studies demonstrated that *LRWD1* is required for the initiation of DNA replication and plays a major role in heterochromatin organization through binding to multiple repressive histone methyl transferases at heterochromatic sites [8]. In our previous study, we

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found that *LRWD1 core* promoter region was located at −198/+1 upstream region from the transcription start site [9]. The transcription activity of *LRWD1* requires the conserved NF-κB binding site and NF-κB transcription factor is essential for LRWD1 promoter activity [9].

The nuclear factor erythroid 2–related factor 2 (Nrf2) is a member of the subfamily of basic region leucine zipper (bZip) transcription factors. These factors regulate the expression of antioxidant proteins and protect against cellular oxidative damage [10,11]. In normal conditions, Nrf2 is kept in the cytoplasm by a cluster of proteins, Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3 (Cul3) [12]. The degradation of Nrf2 is initiated by poly-ubiquitination through the Keap1/Cul3 ubiquitin ligase [13]. The knockout of Keap1 in mice caused constitutive activation of Nrf2 [14].

Under oxidative stress conditions, Nrf2 gets phosphorylated then moves to the nucleus where it binds to a specific DNA promoter to activate the transcription of antioxidant genes [15]. These target genes include those involved in the antioxidant defense, oxidant signaling and drug metabolism [15–17]. Nrf2 is involved in germ cells defense against oxidative stress [18] and has a major role in oxidative stress-induced neurodegenerative diseases [19], gastrointestinal pathophysiology [20,21], and cancer cell resistance [22,23].

Oxidative stress caused by the accumulation of reactive oxygen species (ROS) is tightly linked to many physiological and pathophysiological conditions. Excessive ROS production can cause DNA damage, lipid oxidation, and cell death [24–26]. The accumulation of ROS usually results in the activation of the downstream signaling pathway leading to apoptosis or inflammatory responses [27]. In addition, the level of ROS plays a key role in the physiological function and pathological conditions affecting different types of cells [28,29]. Severe oxidative stress is usually observed in sperm cells of infertile patients, which could be due to the ROS damage that results in shorter telomeres [30]. Many clinical conditions are strongly linked to oxidative damage such as mental disorders [31–33], gastrointestinal diseases [34,35], different types of cancers [36,37].

While LRWD1 is abundantly expressed in different types of cells and has a role in chromatin modifications, DNA expression and histone methylation [38,39], which are all regulated through ROS signaling [33,40,41], yet LRWD1 transcriptional regulation under oxidative stress is not fully understood. The current study aimed at investigating the relationship between LRWD1 expression and ROS and the modulatory role of Nrf2.

2. Material and methods

2.1. Cell culture

NT2/D1 cell line (human embryonal carcinoma, American Type Culture Collection). The cells were maintained at 37 °C in a 5% CO_2 incubator in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen, Ground Island, NY, USA).

2.2. Chemicals and material

Antibodies against LRWD1, Nrf2, *p*-Nrf2, β-actin, and rabbit IgG were from Santa Cruz Biotechnology (Dallas, TX, USA). Hydrogen peroxide (H_2O_2), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium nitroprusside (SNP), L-N^G-Nitroarginine methyl ester (L-NAME) and N-acetylcysteine (NAC) were from Sigma-Aldrich (St. Louis, MO, USA). Luciferase reporter assay kit was from Promega (Madison, WI, USA). Enhanced chemiluminescent (ECL) reagent for Western blot detection was from Millipore (Billerica, MA, USA). Anti-rabbit and anti-mouse IgG-HRP conjugates antibodies were from Cell Signaling (Beverly, MA, USA). The SYBR Green PCR Master Mix kit was obtained from Thermo Scientific (Waltham, MA, USA).

2.3. Cell viability assay

MTT assay was used for cell viability analysis. NT2/D1 and NT2/D1-shLRWD1 cells were seeded in 96-well plates and incubated in DMEM for 24 h. Cells were treated with 200 μM H_2O_2 with or without NAC for 24 h while controls received the vehicle (DMSO). After incubation, the media were replaced with DMEM containing MTT solution and incubated at 37 °C for 4 h. The reduced MTT crystals were solubilized in 100 μl DMSO, and the absorbance was measured at 570 nm.

2.4. Western blots

The total cell lysates were extracted using RIPA lysis buffer and Western blotting was performed as detailed before [42] using PVDF members. The membranes were probed with primary antibodies against LRWD1, Nrf2, *p*-Nrf2, and β-actin in 1% TBST nonfat milk at 4 °C overnight, then suitable secondary antibodies were used for 1 h at room temperature and the bands were detected by enhanced chemiluminescence reagent.

2.5. Quantitative reverse transcription PCR (RT-qPCR)

The process of RT-qPCR was performed as detailed before [43]. Briefly, the RNA was extracted from treated cells using TRIzol (Life Technologies, NY, USA) and the cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase. The used primers were as follows: *LRWD1* gene (F) 5′-GGGGATTGTGCTCTGTGG-3'/(R) 5′- GGGGCCACTTCAGGATCT -3'; *Nrf2* gene (F) 5′- CATTTGCAACAGGAGCTGAA -3'/(R) 5′-ACCAGCCCTTACGGAAAAGT -3'; *GAPDH* gene (F) 5′- GAGTC-CACTGGCGTCTTCAC-3'/(R) 5′- TTCACACCCATGACGAACAT-3'. RT-qPCR results were analyzed by StepOne Software v2.1 (Thermo Fisher Scientific).

2.6. Promoter analysis and plasmid construction

The transcription factor binding sites on *LRWD1* promoter were analyzed by PROMO 3.0 software and reported before [33]. The pGL3-hLRWD1-198/+1 plasmid containing *LRWD1* promoter spanning nucleotides −198 to +1 (−198/+1) was cloned into the pGL3-Basic vector as mentioned previously [9,44]. In pGL3-hLRWD1-198/+$1^{\triangle Nrf2}$ plasmid, the Nrf2 binding site was disrupted using QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The Renilla luciferase gene in the pRL-TK vector was used as a control. All the produced constructs were verified by nucleotide sequence analysis.

2.7. Luciferase reporter gene assay

The transcriptional activity of *LRWD1* was measured using a luciferase reporter gene assay as detailed before [45]. In brief, NT2/D1 cells were transfected with pGL3, pGL3-LRWD1-198/+1, pGL3-hLRWD1-198/+$1^{\Delta Nrf2}$ and pRL-TK vector plasmid using Invitrogen LipofectAMINE 2000 (Invitrogen, Eugene, OR, USA) according to the manufacturer's manual. After 48 h incubation, the cells were treated with or without H_2O_2 or SNP and then were washed with PBS. The cells were then lysed with Promega lysis buffer and