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Adenovirus-mediated NDRG2 inhibits the proliferation of human renal cell carcinoma cell line OS-RC-2 *in vitro*

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ABSTRACT

Objective: To investigate the inhibitory effects of adenovirus-mediated NDRG2 on the proliferation of human renal cell carcinoma cell line OS-RC-2 *in vitro*. **Methods:** NDRG2 was harvested by RT-PCR, confirmed by DNA sequencing, and then cloned into the eukaryotic expression vector pIRES2-EGFP, which encodes green fluorescent protein (GFP), to construct pIRES2-EGFP-NDRG2 plasmid. OS-RC-2 cells with NDRG2 negative expression were transfected with pIRES2-EGFP-NDRG2 plasmid. The growth of transfected OS-RC-2 cells was observed under light and fluorescence microscopes. After colony-forming cell assays, cell proliferation detection and MTT assays, the growth curves of cells in each group were plotted to investigate the inhibitory effects of adenovirus-mediated NDRG2 on the proliferation of OS-RC-2 cells. Cell cycle was determined by flow cytometry. Confocal laser scanning microscopy showed that NDRG2 protein was specifically located on subcellular organelle. **Results:** A eukaryotic expression vector pIRES2-EGFP-NDRG2 was successfully constructed. After NDRG2 transfection, the growth of OS-RC-2 cells was inhibited. Flow cytometry showed that cells were arrested in S phase but the peak of cell apoptosis was not present, and confocal laser scanning microscopy showed that NDRG2 protein was located in mitochondrion. **Conclusions:** NDRG2 can significantly inhibit the proliferation of OS-RC-2 cells *in vitro* and its protein is specifically expressed in the mitochondrion.

1. Introduction

NDGR2 is a newly discovered gene that can be harvested from human brain tissue by clone technology and contains an acyl carrier protein-like domain[1]. NDRG2 expression differs between tumor and normal tissues. Generally, its expression level is positively correlated with the degree of organ development and its expression state is negatively correlated with the proliferative capacity of cells[2-4]. NDRG2 is related to cell proliferation and differentiation and plays a remarkable inhibitory role in tumor occurrence and development in diverse systems including aspiratory, digestive and nervous systems[5-8]. The occurrence and development of renal cell carcinoma correlate various factors, and activation of anti-oncogene is one of the most important factors[9-11]. This study established NDRG2-transfected OS-RC-2-GFP-NDRG2 cell strains and investigated the inhibitory effects of NDRG2 on OS-RC-2 cell growth and proliferation and the possible underlying mechanism.

2. Materials and methods

2.1. Materials

1640 culture medium was purchased from Gibco (New York, NY, USA). Trypsin-ethylenediamine tetraacetic acid (EDTA), MTT, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Zhejiang Tianhang Biological

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Technology Co., Ltd., China (Hangzhou, Zhejiang Province, China). Human renal cell carcinoma cell line OS-RC-2 was purchased from Cell Bank, Chinese Academy of Sciences, China. Recombinant human NDRG2 adenovirus was packed and identified by Cyagen Biosciences Co., Ltd., (Guangzhou, Guangdong Province, China). Mouse anti-human NDRG2 antibody was purchased from Abcam(HK)Ltd (Hong Kong, China). Rabbit anti-mouse antibody was from Kangchen Biological Co., Ltd., (Shanghai, China).

2.2. Construction of pAV.EX1d-NDRG2 /IRES/eGFP adenovirus plasmid

The gene product attB1-Kozak-SYLD/IRES/eGFP-attB2 was amplified by PCR. The primer and sequence used were as follows.

Primer	Sequence (5′-3′)
attB1-K-SYLD	GGGGACAAGTTTGTACAAAAAAGCAGGCT GCCACCATGGCGGAGCTGCAGGAG
SYLD-IRES-R	GGGGGAGGGAGAGGGGCTCAACAGGAGAC CTCCATGG
IRES/EGFP-F	GCCCCTCTCCCTCCCCC
attB2-EGFP-R	GGGGACCACTTTGTACAAGAAAGCTGGGTT TACTTGTACAGCTCGTCCATG

pDown-SYLD/IRES/eGFP and pAV.EX1d-SYLD/IRES/eGFP plasmids were constructed by Gateway Technology. pDown-SYLD/IRES/eGFP and pAV. Des1d plasmid DNA was extracted. After sequencing, adenovirus plasmids with correct sequences were packaged, amplified and condensed, and finally virus titer was determined.

2.3. Adenovirus transfection

On day 2 after cell inoculation, adenovirus transfection was performed when cells reached 30%-40% confluence. Before transfection, cells in each well were treated with 1 mL fresh 1640 culture medium plus 10% FBS. A 10-mL aliquot of adenovirus I carrying NDRG2 and GFP gene was separately added to one well from each of two 6-well plates and a 10-mL aliquot of adenovirus II carrying GFP gene was added to another well from each plate. After fully mixed, the resultant sample was incubated in a 5% CO_2, 95% relative humidity environment at 37 ℃ for 16 hours. After getting rid of adenovirus-containing complete medium, cells were washed twice with PBS and treated with fresh 1640 culture medium plus 10% FBS in a 5% CO_2, 95% relative humidity environment at 37 ℃ for 48 hours. Finally, cells were observed under a fluorescence microscope and photographed.

2.4. Western blot analysis[12]

After 48 hour transfection, total protein was extracted from cells in each group and quantified according to the method recommended by protein quantification kit. Briefly, 100 μg protein sample was subjected to SDS-PAGE and electrically transferred onto a nitrocellulose membrane at 300 mA. Seventy minutes later, the sample was blocked with 5% defatted milk for 1 hour at room temperature and treated with mouse anti-human NDRG2 (at 1:1 200 dilution) at 4 ℃ overnight, washed with TBST, blocked with rabbit anti-mouse antibody (at proper dilution) for 1 hour at room temperature, and developed by enhanced chemiluminescence method. Finally, it was observed through the use of FluorChem HD2 gel image system and photographed.

2.5. Cell proliferation detection[13,14]

When cells reached 80%-90% confluence, they were trypsinized. Cell suspension was diluted by gradient method and cells were counted. 500 OS-RC-2-GFP-NDRG2, OS-RC-2-GFP or OS-RC-2 cells were seeded into a 100-mm-sized culture dish, treated with 15 mL of 1640 culture medium plus 10% FBS in a 5% CO_2 incubator at 37 ℃. The culture medium was renewed every 3 days. Thirteen days later, cells were stained with Giemsa dye, fixed, cloned and counted.

When cells were 80%-90% confluent, they were trypsinized, counted and seeded into a 96-well plate at a density of 2×10^3 cells/well. Five parallel wells were designated for each cell strain. Each cell strain was seeded into 35 wells. Seven 96-well plates were used. After addition of 200 μL 1640 culture medium plus 10% FBS to each well, cells were incubated in a 5% CO_2 incubator at 37 ℃. Day 1 was designated for 24 hours after inoculation. On each day after inoculation (total 7 days), one 96-well plate was taken for determination of absorbance value using MTT assay.

2.6. Cell cycle analysis[15]

Cells were trypsinized when they reached 80%-90% confluence. After centrifugation at 244 × *g* for 4 minutes, the supernatant was discarded. After one wash with ice-cold PBS, cells were re-suspended with 200 μL ice-cold PBS, and ice-cold 70% ethanol was added in a drop-wise manner while shaking gently. Samples were fixed at 4 ℃ for a minimum of 6 hours or at -20 ℃ till use. Following centrifugation at 250 × *g* for 4 minutes, the supernatant was carefully discarded and cells were re-suspended with 1-2 mL ice-cold PBS to get rid of the fixing solution. After addition of 500 μL of PBS containing 100 μg/mL RNase, cells were incubated at 37 ℃ for 0.5-1 hour. After addition of 500 μL of PBS containing 50 μg/mL PI and 0.2% Triton X-100, cells were incubated in the dark at 4 ℃ for 20-30 minutes. Centrifugation and supernatant removal were performed before incubation. Finally, cells were re-suspended with 300-400 μL PBS. Cell cycle was determined by flow cytometry. The percentage of cells in G_1, G_2 and S phases was calculated.

2.7. Observation of subcellular localization of NDRG2 protein by confocal laser scanning microscopy

Cells were trypsinized when they reached 80%-90% confluence. The cells were seeded onto the slide placed in the well of 96-well plate at a density of 5×10^4-5×10^5 cells/mL and incubated with culture medium. After 6, 16, 24, 48 and 72 hours of incubation, culture medium was discarded and cells were further incubated for 30-60 minutes with Rhodamine 123 working solution (probe for mitochondrion). The slide was taken out, enveloped with glycerine, observed under confocal laser scanning microscope, and photographed. Subcellular localization of NDRG2 protein was observed using the same method with boron dipyrromethene (probe for Golgi apparatus) and Lucifer yellow (probe for lysosome).

3. Results

3.1. Specifically expression of NDRG2 protein in OS-RC-2-GFP-NDRG2 cells after adenovirus transfection

After adenovirus transfection for 48 hours, NDRG2 protein expression in cells was detected by western blot analysis. NDRG2 protein expression was present in neither OS-RC-2 nor OS-RC-2-GFP cells. While significant NDRG2 protein expression was detected in OS-RC-2-GFP-NDRG2 cells (Figure 1).

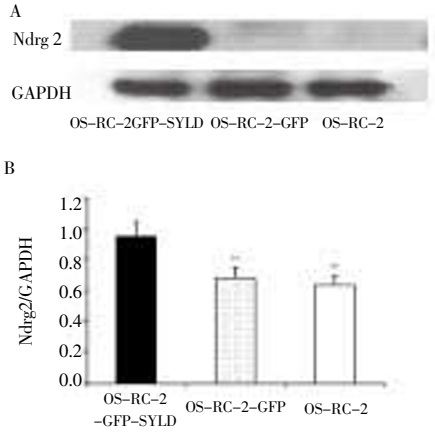


Figure 1. NDRG2 protein expression in OS-RC-2-GFP-NDRG2, OS-RC-2-GFP and OS-RC-2 cells.
(A) Western blot analysis. (B) Quantification results by analysis software. Values are expressed as mean±SEM. $^{**}P<0.01$, *vs.* OS-RC-2-GFP-NDRG2 cells.

3.2. Biological characteristics of OS-RC-2-GFP-NDRG2 cells

After adenovirus transfection for 48 hours, cell growth was observed through the use of light and fluorescence microscopes[16,17]. After transfection, approximately 100% of OS-RC-2-GFP cells exhibited green fluorescence, with slight atrophy symptom, and approximately 60% of OS-RC-2-GFP-NDRG2 cells exhibited green fluorescence, with more obvious atrophy symptom (Figure 2).

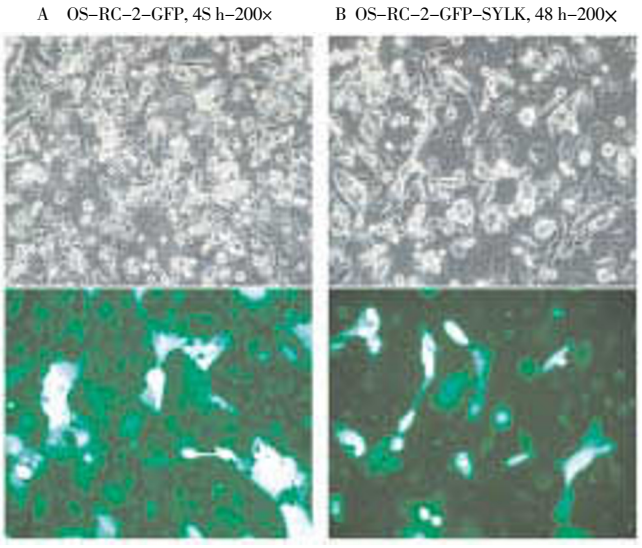
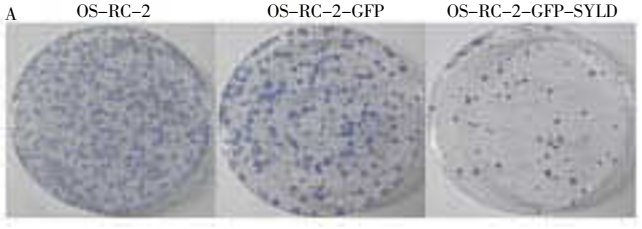


Figure 2. Effects of adenovirus-mediated GFP gene (A) and GFP + NDRG2 gene transfection for 48 hours on the growth state of OS-RC-2 cells (B) as shown by light and fluorescence microscopes.

3.3. Proliferation of OS-RC-2-GFP-NDRG2 cells after adenovirus transfection

Colony-forming cell assay results showed that the colony-forming efficiency of OS-RC-2-GFP-NDRG2 cells was significantly lower than that of OS-RC-2-GFP cells or OS-RC-2 cells (24.8% *vs.* 51.65% or 76.4%). MTT assay showed that the proliferative capacity of OS-RC-2-GFP-NDRG2 cells was significantly lower than that of OS-RC-2-GFP cells or OS-RC-2 cells (Figures 3, 4).



B

Cloning efficiency of cells

Cells	Numbers of cloning cells (*n*)	Cloning efficiency (numbers/500 cells)
OS-RC-2	382	76.4%
OS-RC-2-GFP	258	51.6%
OS-RC-2-GFP-SYLD	124	24.8%*

Figure 3. Effect of NDRG2 transfection on proliferation of OS-RC-2 cells.
(A) Colony-forming cell assay results. (B) Calculation of colony-forming efficiency of OS-RC-2, OS-RC-2-GFP and OS-RC-2-GFP-NDRG2 cells. The colony-forming efficiency=number of cloned cells /500 cells ×100%. $^{*}P<0.01$, *vs.* OS-RC-2 cells.

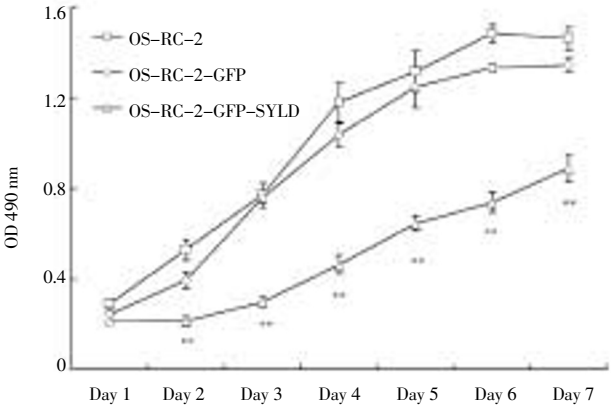


Figure 4. Growth curves of OS-RC-2, OS-RC-2-GFP and OS-RC-2-GFP-NDRG2 cells.

Absorbance (OD) value at 490 nm of each kind of cells at different time points was determined by MTT assay. Cell growth curves were plotted. Values are expressed as mean±SEM. $^{**}P<0.01$, *vs.* OS-RC-2 cells.

3.4. Determination of cell cycle by flow cytometry

Flow cytometry results showed that after adenovirus transfection, the number of cells in S phase was significantly increased ($P<0.05$), while the number of cells in G_2M and G_0G_1 phases was significantly decreased in the OS-RC-2-GFP-NDRG2 group than in the OS-RC-2 group, and cells were arrested in S phase, but peak of cell apoptosis was not observed (Figure 5).

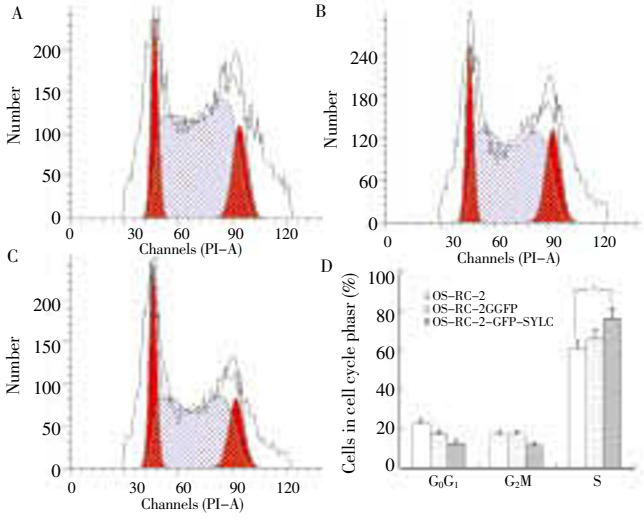


Figure 5. Effects of adenovirus-mediated NDRG2 transfection on cell cycle.

(A) Cell cycle of OS-RC-2-GFP-NDRG2 cells. (B) Cell cycle of OS-RC-2-GFP cells. (C) Cell cycle of OS-RC-2 cells. (D) Cell cycle analysis by ModFit software. All measurements were repeated three times. Data were expressed as mean±SEM. $^{*}P<0.05$, *vs.* OS-RC-2 ells.

3.5. NDRG2 protein was specifically expressed in the mitochondrion of OS-RC-2-GFP-NDRG2 cells

Confocal laser scanning microscopy results showed that NDRG2 protein was specifically expressed in the mitochondrion of OS-RC-2-GFP-NDRG2 cells and its expression was gradually increased with time (Figure 6), and this protein was not detected in the lysosome or Golgi apparatus.

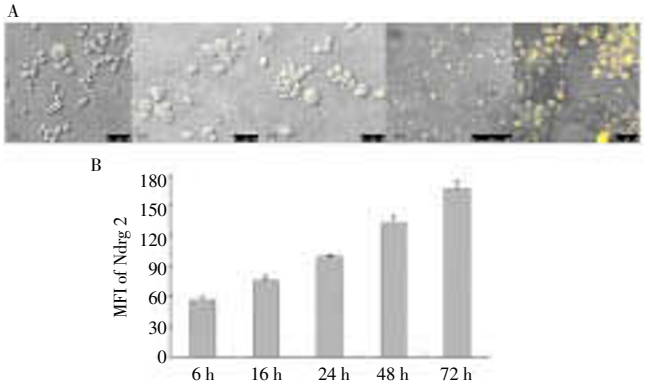


Figure 6. Subcellular location of immunofluorescence in OS-RC-2-GFP-NDRG2 cells.

(A) Mitochondrial location of immunofluorescence at different time points. (B) Mean fluorescent intensity (MFI) value analysis using image process software. Data are expressed as mean±SEM.

4. Discussion

There is strong evidence that NDRG2 gene is related to cell growth, differentiation, apoptosis, stress reaction, histogenesis and organ formation[18-21]. Previous histological findings have demonstrated that NDRG2 expression differs in diverse tumor tissues, including liver cancer, breast cancer, colon cancer and prostate cancer tissues[22-24]. All these suggest that NDRG2, likely as an anti-oncogene, participates in tumor occurrence, development and turnover. This study investigated the effects of adenovirus-mediated exogenous NDRG2 transfection on the biological characteristics of OS-RC-2 cells. Results showed that after transfection, NDRG2 protein was specifically expressed in OS-RC-2-GFP-NDRG2 cells rather than in OS-RC-2 cells or OS-RC-2-GFP cells. These findings demonstrate that NDRG2 gene can be highly transfected into OS-RC-2 cells by adenovirus mediation. Results also showed that after transfection, approximately 100% of OS-RC-2-GFP cells exhibited fluorescence, with nearly unchangeable morphology, however, about 60% of OS-RC-2-GFP-NDRG2 cells exhibited fluorescence, with obvious atrophic morphology. This suggests that NDRG2 gene transfection significantly inhibits the growth of OS-RC-2 cells.

This study investigated the proliferation and growth of

NDRG2-expressing cells by colony-forming cell assay and MTT assay. Colony-forming cell assay showed that the colony-forming efficiency of OS-RC-2-GFP-NDRG2 cells was significantly decreased than that of OS-RC-2 and OS-RC-2-GFP cells. MTT assay showed that the growth of OS-RC-2-GFP-NDRG2 cells was significantly inhibited. These findings suggest that NDRG2 transfection can significantly inhibit the proliferation and growth of OS-RC-2 cells. This study determined cell cycle using flow cytometry to investigate the inhibitory effects of NDRG2 transfection on cell proliferation. The number of cells in S phase was significantly increased, while that in G_2M and G_0G_1 phases was significantly decreased in the OS-RC-2-GFP-NDRG2 group than in the OS-RC-2 group. But the peak of cell apoptosis was not observed in S phase in the OS-RC-2-GFP-NDRG2 group. S phase of cell cycle is an important period for cellular DNA replication and enzyme synthesis. Cells were arrested in S phase, which influences cells entry into G_2 phase, subsequent DNA replication and mitosis, and final cell proliferation

To preliminarily investigate the possible mechanism by which NDRG2 gene inhibits cell proliferation, we performed subcellular location of NDGR2 protein by immunofluorescence staining. Confocal laser scanning microscopy results showed that NDRG2 protein was mainly expressed, and gradually increased with time, in the mitochondrion of OS-RC-2-GFP-NDRG2 cells, however, its expression was not present in the lysosome or Golgi apparatus. The mitochondrion is a special organelle that exists in the cytoplasm of the eukaryocytes and plays an important role in cell energy metabolism, oxygen free radical generation, aging, and apoptosis[25-28]. Most ATP production relies on oxidative phosphorylation in normal cells and on glycolytic pathway in tumor cells.

ATP-synthesizing enzyme subunit B expression is significantly decreased in the mitochondrial inner membrane in the majority of tumor cells. Any factors that can decease mitochondrial oxidative phosphorylation are related to the rapid growth and the increasing invasion of tumor cells[29]. Therefore, interfering mitochondrial function in tumor cells is likely to play an inhibitory effect on tumor cell growth and invasion. In addition, an important pathway by which mitochondrion participants in tumor cell toxicity is to increase intracellular oxygen free radical generation, because which can injury or decrease mitochondrial membrane potential and lead to release of cytochrome C and apoptosis inducing factors[30,31]. Results from this study showed that NDRG2 is specifically expressed in the mitochondrion of OS-RC-2-GFP-NDRG2 cells. This suggests that the inhibitory effect of NDRG2 on cell proliferation is accomplished via intracellular mitochondrion. However, no obvious apoptosis was detected by flow cytometry. This suggests that NDRG2 inhibits OS-RC-2 cell proliferation not through initiating apoptosis, which is different from previous findings[32,33]. Thus, it is presumed that NDRG2 inhibits the proliferation of OS-RC-2 cells possibly through inhibiting mitochondrial ATP-synthesizing enzyme generation and interfering the activity of mitochondrial NADH CoQ reductase or through inhibiting the function of mitochondrial inner membrane-bound protein and inhibiting mitochondrial respiratory function and energy metabolism. The precise mechanism needs to be further investigated.

Taken together, NDRG2 has an obvious inhibitory effect on the proliferation of human renal cancer OS-RC-2 cells and its protein is specifically expressed in the mitochondrion of OS-RC-2 cells. This provides a novel clue for studying the pathological mechanism underlying renal cancer and also suggests that NDRG2 likely regulates the growth of renal cancer cells and becomes a novel target for gene therapy of renal cancer. Nevertheless, some uncertainties need to be further investigated, including the precise mitochondrion related mechanism by which NDGR2 inhibits the proliferation of OS-RC-2 cells and the effect of NDGR2 gene on biological characteristics of OS-RC-2 cells *in vivo*.

Conflict of interest statement

We declare that we have no conflict of interest.

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