Biomaterials 35 (2014) 993–1003

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of adenovirus-conjugated *NDRG2* on *p53*-mediated apoptosis of hepatocarcinoma cells through attenuation of nucleotide excision repair capacity

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ARTICLE INFO

Article history:
Received 22 August 2013
Accepted 24 September 2013
Available online 25 October 2013

Keywords:
NDRG2
p53-Mediated apoptosis
Hepatocarcinoma
The nucleotide excision repair capacity

ABSTRACT

NDRG2 mRNA and protein levels can be upregulated in a *p53*-dependent manner. *NDRG2* enhances *p53*-mediated apoptosis, whereas overexpression of *NDRG2* suppresses tumor cell growth, regardless of whether *p53* is mutated. However, the complicated mechanism by which *NDRG2* suppresses tumor cell growth and enhances apoptosis mediated by *p53* is not fully understood. Here, we demonstrated that Ad-*NDRG2* enhanced the apoptosis of HepG2 cells (wild-type *p53*). Additionally, Ad-*NDRG2* combined with rAd-*p53* enhanced the apoptosis of Huh7 cells (mutant *p53*) after chemotherapy, and the expression of the *ERCC6* gene (Cockayne syndrome group B protein gene) was suppressed in this process. Ad-*NDRG2* combined with rAd-*p53* induced the apoptosis of tumor cells (HepG2 and Huh7 cells); however, apoptosis was attenuated after transfection with *ERCC6*. Our results indicate that Ad-*NDRG2* enhances the *p53*-mediated apoptosis of hepatocarcinoma cells (HepG2 and Huh7) by attenuating the nucleotide excision repair capacity (i.e., by downregulating *ERCC6*), and *ERCC6* is a *NDRG2*-inducible target gene that is involved in the *p53*-mediated apoptosis pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, accounting for approximately 500,000 deaths annually [1]. Although significant efforts have been made to improve the efficacy of treatments, there is no effective therapeutic strategy for non-resectable HCC [1]. Therefore, therapeutic targets and new modalities of treatment are urgently needed.

It was reported that the incidence of *p53* mutation is 61% in HCC [2], and mutant *p53* is associated with resistance to radiotherapy and apoptosis-inducing chemotherapy [3]. Gene therapy is a new potential treatment modality for cancer patients, and engineered recombinant replication-defective adenovirus can express the tumor suppressor gene with encouraging clinical responses [4,5]. It has been shown that *p53* gene therapy, in combination with radiotherapy or chemotherapy, can control local tumors, suggesting that it is superior to either radiotherapy or chemotherapy alone [6,7]. However, the role of the *p53* gene in combination therapy remains unclear.

The *p53* tumor suppressor gene is often referred to as the "guardian of the genome"; loss of *p53* is responsible for the lack of apoptotic signals in tumor cells, thus leading to their uncontrolled proliferation, and for cancer recurrence [8]. Under normal circumstances, *p53* is maintained at low levels; however, upon treatment with chemotherapeutic agents, *p53* protein levels rise dramatically [9], and in response to cellular stresses, *p53* can induce diverse biological responses, including cell-cycle arrest, DNA repair and apoptosis [10]. Increasing evidence has shown that *p53* can induce apoptosis via its actions as a transcription factor by transactivating numerous target genes in the apoptotic pathway [11].

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http://dx.doi.org/10.1016/j.biomaterials.2013.09.096

Activated *p53* induces the expression of its downstream target genes, and to date, more than 100 *p53* target genes have been identified [12]. Nevertheless, none of these downstream targets fully contribute to the *p53*-mediated apoptotic response, indicating that *p53* may activate a subset of apoptosis-related genes in a cell context- or stress-dependent fashion [13]. Recent studies have reported that N-Myc downstream-regulated gene 2 (*NDRG2*) is a new target gene that is transcriptionally regulated by *p53* [14]. It has been reported that *NDRG2* expression is downregulated in a variety of carcinomas, including liver cancer, pancreatic cancer, thyroid cancer and meningioma, suggesting a possible role for *NDRG2* in tumor suppression [15–18].

NDRG2 mRNA and protein synthesis were induced by the overexpression of *p53* via adenovirus infection or stabilization of *p53* after DNA damage [14]. It has been demonstrated that *NDRG2* inhibits tumor cell proliferation and increases *p53*-mediated apoptosis, and its expression is correlated with patient survival and prognosis [14,19–21]. In addition, experiments using RNA interference suggest that *NDRG2* is involved in the *p53*-mediated apoptosis pathway. However, the role of *NDRG2* in regulating *p53*-mediated apoptosis remains unclear.

Loss of DNA mismatch repair proteins (e.g., Cockayne syndrome complementation group B [CSB]) are among the most common genetic alterations in cancer, and these alterations have been associated with resistance to chemotherapy [22–24]. Furthermore, defects in the DNA mismatch repair system can result in low resistance/high sensitivity of tumor cells to chemotherapy [25], CSB-deficient fibroblasts were found to be extremely sensitive to apoptosis induced by chemotherapy [26]. It has been reported that silencing CSB by RNA interference (RNAi) reduces the nucleotide excision repair capacity of several cancer cell lines and could significantly increase the sensitivity of *p53*-deficient tumor cells to apoptosis [27]. These results suggest that CSB plays a major role in determining the sensitivity of these *p53*-deficient tumor cells to chemotherapy.

In this study, we report that adenovirus-mediated transfection of *NDRG2* enhances the apoptosis of HepG2 cells (wild-type *p53*). Additionally, adenovirus-mediated transfection of *NDRG2* combined with *p53* enhances the apoptosis of Huh7 cells (mutant *p53*) after Adriamycin-based chemotherapy and suppresses the expression of the *ERCC6* gene (which encodes CSB [28] and is involved in a subpathway of nucleotide excision repair that preferentially repairs damage to the transcribed strand of active genes [29]). We found that *ERCC6* mRNA and protein synthesis were inhibited by the overexpression of *NDRG2* via either adenovirus infection or stabilization of *p53* after DNA damage, and the apoptosis of tumor cells (HepG2 and Huh7 cells) overexpressing *ERCC6* was attenuated by transfecting Ad-*NDRG2* combined with rAd-*p53* after DNA damage. Our results suggest that *NDRG2* enhances the *p53*-mediated apoptosis of hepatocarcinoma cells by attenuating the function of CSB after chemotherapy, and we show that *ERCC6* is a *NDRG2*-inducible target gene that is involved in the *p53*-mediated apoptosis pathway. This led us to elucidate the biological function of *NDRG2* and better understand the role of the *p53* gene in tumor cell responses to combination therapy.

In this study, we probed the underlying molecular mechanisms of the effects of *NDRG2* overexpression on the apoptosis of hepatocarcinoma cells, and we evaluated the potential of *NDRG2* as a therapeutic target in HCC treatment strategies.

2. Materials and methods

2.1. Cell lines, cell culture and chemotherapy

The human tumor cell lines HepG2 (wild-type *p53*) and Huh7 (mutant *p53*) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). The cells were incubated at 37 °C in the presence of 5% CO_2-balanced air.

The cells were treated with 0.2 μg/mL Adriamycin (ADM, Sigma Chemical) for 24 h. After removal of the culture medium, the attached cells were scraped into ice-cold PBS, centrifuged at 4000× *g*, resuspended in PBS and sonicated with an ultrasonic cell disrupter. The cellular debris was pelleted by centrifugation, and the supernatants were transferred to new tubes and stored at 70 °C until measurement.

2.2. Adenovirus infection

Adenoviruses carrying *NDRG2* (Ad-*NDRG2*) or the negative control LacZ (Ad-LacZ) were purchased from Benyuan Zhengyang Company (Beijing, China). The adenovirus titers were 1×10^9 PFU and 1.5×10^9 PFU, respectively. rAd-*p53* is a recombinant human serotype 5 adenovirus in which the E1 region has been replaced by a human wild-type *p53* expression cassette. The genes are driven by a Rous sarcoma virus promoter with a bovine growth hormone poly(A) tail. The recombinant adenoviruses were produced in human embryonic kidney 293 cells and manufactured by Shenzhen SiBiono GenTech Co., Ltd. (Shenzhen, China). The adenovirus titers were 1×10^9 PFU. Before gene therapy, a vial of rAd-*p53* or Ad-*NDRG2* was removed from a −20 °C freezer. After thawing, the solution was diluted with 1 mL of NS and loaded into a 1 mL syringe for intra-tumor injection.

2.3. Construction of plasmids and gene transfection

pcDNA3.1(+)-*ERCC6* was purchased from Takara corporation (Japan). The full-length cDNA of *ERCC6* was inserted into the pcDNA3.1(+) vector (Clontech, Palo Alto, CA), and the newly constructed plasmids were confirmed by sequencing. The cells were seeded in a 24-well plate at a density of 1×10^5 cells/well and transfected on the following day using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's protocol. All clones were analyzed by western blotting.

2.4. Groups and treatments

The human tumor cell lines HepG2 (wild-type *p53*) and Huh7 (mutant *p53*) were divided into groups and treated with different agents as follows:

Control group (C): treated with Ad-LacZ (1×10^9 PFU).

ADM group (A): treated with Adriamycin (0.2 μg/mL).

NDRG2 group (N): treated with Ad-*NDRG2* (1×10^9 PFU).

p53 group (P): treated with rAd-*p53* (1×10^9 PFU).

NDRG2 plus ADM group (NA): treated with Ad-*NDRG2* (1×10^9 PFU) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

p53 plus ADM group (PA): treated with rAd-*p53* (1×10^9 PFU) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

NDRG2 plus *p53* group (NP): treated with Ad-*NDRG2* (1×10^9 PFU) and rAd-*p53* (1×10^9 PFU).

NDRG2 plus *p53* plus ADM group (NPA): treated with Ad-*NDRG2* (1×10^9 PFU) and rAd-*p53* (1×10^9 PFU) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

ERCC6 plus ADM group (EA): treated with pcDNA3.1(+)-*ERCC6* (1×10^5 cells/well) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

NDRG2 plus *ERCC6* plus ADM group (NEA): treated with Ad-*NDRG2* (1×10^9 PFU) and pcDNA3.1(+)-*ERCC6* (1×10^5 cells/well) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

p53 plus *ERCC6* plus ADM group (PEA): treated with rAd-*p53* (1×10^9 PFU) and pcDNA3.1(+)-*ERCC6* (1×10^5 cells/well) for 12 h followed by treatment with Adriamycin (0.2 μg/mL).

NDRG2 plus *p53* plus *ERCC6* group (NPE): treated with rAd-*p53* (1×10^9 PFU), Ad-*NDRG2* (1×10^9 PFU) and pcDNA3.1(+)-*ERCC6* (1×10^5 cells/well).

2.5. Real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed with reverse transcriptase (Promega, WI, USA). First strand cDNA was used as the template for real-time quantitative PCR analysis. *β-actin* cDNA was used as an internal control for normalization. The following primers were designed using the ABI Primer Express software: *NDRG2*, 5′-GAGATATGCTCTTAACCACCCG-3′ (forward) and 5′-GCTGCCCAATCCATCCAA-3′ (reverse); *p53*, 5′-ACTGTACCACCATCCACTACAACT-3′ (forward) and 5′-ACAAACACGCACCTCAAAGC-3′ (reverse); *ERCC6*: 5′-CCACTCAAGTCAAACTCAGGAG-3′ (forward) and 5′-ATCTGATGTCGGTCGATGTGC-3′ (reverse); and *β-actin*, 5′-ATCATGTTTGAGACCTTCAACA-3′ (forward) and 5′-CATCTCTTGCTCGAAGTCCA-3′ (reverse). *NDRG2*, *p53*, *ERCC6* and *β-actin* mRNAs were detected with SYBR Green PCR Master Mix and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, UK) using the comparative threshold cycle (CT) method for relative quantification. The PCR reaction contained 12.5 μl of SYBR Green PCR Master Mix, 300 nM forward and reverse primers and 1.5 μl of template cDNA in a total volume of 25 μl. The thermal cycling conditions were as follows: 95 °C for 5 min followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. To verify that each primer pair produced a single product, a dissociation protocol was run following thermocycling to determine the dissociation of the PCR products from 65 °C to 95 °C.