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DNA damage down-regulates ΔNp63α and induces apoptosis independent of wild type p53

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ABSTRACT

The tumor suppressor p53 is pivotal in cell growth arrest and apoptosis upon cellular stresses including DNA damage. Mounting evidence indicates that p63 proteins, which are homologs of p53, are also involved in apoptosis under certain circumstances. In this study, we found that treatment with DNA damage agents leads to down-regulation of ΔNp63α and induces apoptosis in FaDu and HaCat cells carrying mutant p53. Further study shows that DNA damage reduces steady-state mRNA level of ΔNp63α, but has little effect on its protein stability. In addition, knockdown of endogenous ΔNp63α directly induces apoptosis and sensitizes cells to DNA damage, while exogenous expression of ΔNp63α partially confers cellular resistance to DNA damage. Together, these data suggest that DNA damage down-regulates ΔNp63α, which may directly contribute to DNA damage-induced apoptosis.

1. Introduction

p53 protein has been known to be a major tumor suppressor, which plays a central role in the cellular response to DNA damage from both endogenous and exogenous sources by inducing p53-dependent apoptosis, providing a protective effect against tumorigenesis through controlling cell proliferation or cell death under potentially oncogenic conditions [1,2]. However, p53 gene is not stable in the genome and it is mutated in more than 50% of human cancer types, including squamous cell carcinomas of head and neck [3]. The majority of tumor-derived mutations make p53 defective in regulating its target genes, which defines the classical loss of function for mutant p53. DNA damage can induce apoptosis independent of wild type p53 in this case [4,5]. Some of p53 mutants can also acquire new oncogenic properties, termed gain of function. These mutant p53 proteins function to promote invasion and metastasis of cancer cells [1,6,7].

p63 is a member of the p53 gene family [8,9]. It encodes two groups of protein isoforms: TA-isoforms, including TAp63α, TAp63β and TAp63γ; ΔN-isoforms, including ΔNp63α, ΔNp63β and ΔNp63γ [10,11]. Evidence from human genetics and animal models reveals that p63 gene is crucial for stratification of squamous epithelia, differentiation of mature keratinocytes, and epidermal morphogenesis during development [12,13]. In contrast to the high frequency of p53 mutation, p63 gene is rarely mutated in tumor cells. Recently, mounting evidence indicates that TAp63 isoforms function as tumor suppressors while ΔNp63 as oncoproteins, and they play different roles during tumorigenesis [14,15]. It has been reported that p53-dependent apoptosis can take place in either p63-required or p63-unrequired pathways [16,17]. It has been also reported that ΔNp63α can antagonize DNA damage-induced apoptosis in a p53-independent manner in some cell lines [18].

ΔNp63 proteins are overexpressed in some types of human cancer, particularly in squamous cell carcinoma of the head and neck (SCCHN). ΔNp63α is the predominant isoform of p63 expressed in FaDu cells (an SCCHN cell line) and in HaCat cells (immortalized keratinocytes cell line) [19]. Mutation of the p53 gene is the most common genetic alteration detected in SCCHN. In FaDu cells, p53 gene bears an R248L mutation and this point mutation stabilizes mutant p53 protein. It is unclear whether this p53 mutant protein has any effects on the function of ΔNp63α [20]. In another ΔNp63α-expressed cell line, HaCat cells, p53 gene carries two point mutations: H179Y and R282W. Mutant p53 in HaCat cells has been reported to have some oncogenic functions [21]. In our study, we found that treatment with DNA damage drugs (doxorubicin and cisplatin) can lead to down-regulation of ΔNp63α and induce apoptosis without affecting expression of mutant p53 in FaDu and HaCat cells. Our further study revealed that DNA damage-induced down-regulation of ΔNp63α is mainly at transcriptional level but not due to the effect on protein stability. Moreover, the knockdown of ΔNp63α directly induces apoptosis and increases cellular sensitivity to DNA damage agents, while exogenous expression of ΔNp63α confers cells resistance to DNA damage-induced apoptosis. Taken together, our data show that ΔNp63α is a major player of DNA damage-induced apoptosis in FaDu cells and HaCat cells carrying p53 mutations. This study

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would be helpful for exploring the therapeutic strategy to manage tumors that depends on ΔNp63α for survival.

2. Materials and methods

2.1. Cell culture and drug treatment

FaDu, HaCat and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin G/streptomycin sulfate at 37 °C in a humidified 5% CO_2 incubator. Doxorubicin (Dox), cisplatin (CDDP) and cycloheximide (CHX) were purchased from Sigma.

2.2. Western blot analysis

Cells were collected, washed with phosphate-buffered saline, and resuspended in EBC 250 lysis buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, and 0.5 mM Na_3VO_4). Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). An equal amount of protein (about 50 μg total protein) was loaded, separated on a 10% SDS–PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and hybridized to an appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody for subsequent detection by ECL(Millipore). Monoclonal antibody DO-1 specific for p53 (Santa Cruz Biotechnology) was used at a dilution of 1:250. Monoclonal antibody 4A4 specific for p63 (Santa Cruz Biotechnology) was used at 1:200 and goat polyclonal C-11 for actin (Santa Cruz Biotechnology) was used at 1:500. PARP1 polyclonal antibodies (Zenable, ChengDu, China) were used at 1:3000.

2.3. Constructs

Constructs for p63 shRNA were generated as described [22]. In brief, stem-loop oligomers were synthesized in sense and antisense directions corresponding to human ΔNp63α at nucleotides 808–826 (1#: 5′-GTT TCG GAC AGT ACA AAG A-3′), 566–584 (2#: 5′-GAC AGA GTG TGC TGG TAC C-3′) and 787–795 (3#: 5′-GAT AGC ATC AGA AAG CAG A-3′); the fragments were cloned into lentiviral vector pLKO.1 (Invitrogen).

For overexpression, the full-length human ΔNp63α cDNA was cloned into lentiviral vector pHAGE (Invitrogen).

2.4. Lentiviral infection

293T cells were transfected with ΔNp63α overexpression or shRNA constructs (or their vector controls) along with psPAX2/pMD2.G lentiviral packaging plasmids in Lipofectamine 2000. At 48 h after transfection, the media were collected and filtered through a 0.45 μM filter to remove debris. The lentiviral particles were then concentrated by ultra-centrifugation (20,000 rpm, 2 h at 4 °C), resuspended in fresh medium at 4 °C at least 1 h, then supplemented with polybrene (10 μg/ml) and used to infect cells. 48 h after infection, the cells were selected in growth medium supplemented with puromycin for 48 h.

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from treated cells according to manufacturer's protocol (QIAGEN). 2 μg of total RNA was reversely transcribed to cDNA using Reverse Transcription System according to manufacturer's instructions (Promega, USA). Quantitative-PCR (Q-PCR) was performed in CFX96 Real-Time PCR System (Bio-Rad) using a SoFast EvaGreen Supermix (Bio-Rad). The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s. GAPDH expression was used as an endogenous control to normalize target gene expression. Primer sequences are as follows: pan-p63 Fwd, GTTATCCGCGCCATGCCTGTCTAC; pan-p63 Rev, TCCCCTCTACTCGAATCAAATG; GAPDH Fwd, GGGGAGCCAAAAAGGGTCATCATCT; GAPDH Rev, GAGGGGCCATCCACAGTCTTCT.

2.6. Cell viability assay and protein stability assay

Cell viability assay (MTT) with CellTiter 96® kit (Promega, USA) was performed as described in the instruction.

To test protein stability of ΔNp63α, FaDu cells were incubated with 1 μM Dox and 50 μg/ml CHX for different times. Cells were collected at the indicated time points for western blot. β-actin was used as a loading control.

2.7. Flow cytometry analysis (FACS)

Cells were trypsinized, washed with cold phosphate-buffered saline, and fixed in 70% ethanol at 4 °C overnight. 1×10^6 cells were stained with 50 μg/ml propidium iodide (PI) supplemented with 80 μg/ml RNase A at room temperature in dark for 1 h. Cells were then subjected to FACS analysis by FACScan flow cytometer (Becton Dickson). Data were analyzed using the Cell Quest program.

3. Results

3.1. DNA damage induces apoptosis and down-regulates ΔNp63α without affecting expression of mutant p53

p53 is activated in response to DNA damage. Activation of wild type p53 plays important roles in regulating cell cycle arrest, genomic stability, and apoptosis, providing a protective effect against tumorigenesis. However, p53 is mutated in more than 50% human cancer types including FaDu cells [1,20].

To investigate the effect of DNA damage agents on cells expressing mutant p53, we treated FaDu cells with doxorubicin (Dox) and measured the cellular response by MTT assay. We observed that doxorubicin-induced FaDu cell death is dose-dependent (Fig. 1A). Flow cytometry analysis revealed that percentage of cells in sub-G1 is increased by doxorubicin treatment in a dose-dependent manner (Fig. 1B), suggesting that DNA damage agent, doxorubicin, induces apoptosis independent of wild type p53 in FaDu cells.

It has been reported that ΔNp63α can be down-regulated by treatment of DNA damage agents such as ultraviolet radiation [23,24]. To understand the influence of chemotherapeutic agents on the expression of ΔNp63α, a predominant isoform of p63 protein expressed in FaDu and HaCat cells, we treated FaDu cells with doxorubicin at different time duration under different concentrations. We found that the protein level of ΔNp63α, but not the mutant p53, was down-regulated by doxorubicin in a dose- (Fig. 1C) and time- (Fig. 1D) dependent manner. In concomitant with the down-regulation of ΔNp63α, PARP1 cleavage, which is a molecular marker of apoptosis, was increased with the treatment of doxorubicin in a dose- and time- dependent manner (Fig. 1C and D). The treatment with another genotoxic stress drug, cisplatin (CDDP), can also induce the down-regulation of ΔNp63α (Fig. 1E and F) as the fashion treated with doxorubicin (Fig. 1C and D). The down-regulation of ΔNp63α and the increase of PARP1 cleavage by the treatment of Dox or CDDP can also be observed in HaCat