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Research paper

Role of 3′ repressor sequences of p53 in anti-cancer drug sensitivity of human lung tumor cells

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ABSTRACT

Introduction: The C-terminus of p53 and non-coding mRNAs play critical roles in negative regulation. However, their impact on anti-cancer drug sensitivity remains unclear.
Methods: In this study, we investigated the effects of p53 deleting these sequences on anti-cancer drug sensitivity by drug sensitivity test, flow cytometry, Agilent mRNA expression microarray detection, transplantation tumor in nude mice.
Results: The results showed that the cell line with p53 deleted the C-terminal sequences (p53(del)) was more sensitive to navelbine (NVB) compared to the cell line that carried the full length p53 (p53(wt)). The p53(del) cells was more sensitive to cisplatin (PDD) and 5-fluorouracil (5FU) than p53(wt) cells but there was not significant difference. NVB treatment led to significant G2 arrest and apoptosis in p53(del) cells but not in p53(wt) cells. mRNA expression profile of p53(del) cells indicated that approximately 11% of the 41,000 genes in genome showed differential expression after NVB treatment, among which 2064 genes were up-regulated and 2784 were down-regulated with fold change >2 ($P < 0.01$). Tumor transplantation assay in nude mice showed that the p53 truncation significantly increased tumor sensitivity to NVB compared to the full-length p53, with 99.46% tumor inhibition.
Conclusions: In summary, deletion of 37 amino acid residues (356–393) and 3′ non-coding mRNAs at the C-terminus of p53 selectively increased tumor sensitivity to the mitotic inhibitor NVB.

1. Introduction

The p53 tumor suppressor is a nuclear phosphoprotein that acts as a DNA damage-inducible sequence specific transcription factor targeting multiple downstream genes, and regulates critical life processes, including DNA repair, cell cycle arrest, apoptosis, angiogenesis and aging. It plays an important role in the suppression of tumorigenesis and progression (Levine and Oren, 2009; Sullivan et al., 1825).

In normal cells, mouse double minute 2 homolog (MDM2) also known as E3 ubiquitin-protein ligase Mdm2 blocks p53 transcription *via* binding to its transcriptional activation domain. MDM2 promotes p53 degradation, thus preventing it from regulating the downstream targets (Freedman et al., 1999; Stommel and Wahl, 2005). In addition, p53 abnormality and loss of function was found in approximately 50–60% human cancers (Olivier and Hussain, 2004). The C-terminal amino acid residues (291–393) in the p53 protein serve as the negative regulatory domain (Iorio and Croce, 2009).

The microRNAs (miRNA) (Hamard et al., 2012) and long non-coding mRNAs (lncmRNA) (Lim et al., 2005) are critical negative regulators that target the 3′ UTR of p53 mRNA to inhibit p53 transcription and translation as well as its function (Loewer et al., 2010). Following drug treatment, lncmRNAs inhibited p53-mediated cell cycle arrest and apoptosis *via* binding to its 5′ and 3′ UTRs (Zhang et al., 2009).

Cancer prevention and treatment requires removal of p53 repression and reactivation and restoration of p53 functions. Tremendous effort and progress has been made in the field (Zhang et al., 2013; Selivanova et al., 1997; Waning et al., 2010; Wu, 2010; Chen and Kastan, 2010; Suzuki and Matsubara, 2011). We proposed to study

Abbreviations: NVB, navelbine; PDD, cisplatin; 5FU, 5-fluorouracil; MDM2, mouse double minute 2 homolog; miRNA, microRNAs; lncmRNA, long non-coding mRNAs; p53(wt), full-length p53; p53(del), the deleted-C-terminal p53; ANOVA, analysis of variance; Ad, adenovirus; MOI, multiplicity of infection; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; WAF1, wild-type p53 activated fragment-1; CIP1, cyclin-dependent kinase inhibitor 1; RAS, small G-protein; RAF, Ras-associated factor −1; PKC, protein kinase C; PKA, cAMP dependent protein kinase; Bcl2, B cell leukemia/lymphoma 2; TNF, tumor necrosis factor; DAXX, death-domain associated protein; BRCA1, breast cancer 1; Bax, BCL2-associated X protein.

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whether the 37 amino acid residues at the C-terminus of p53 (356–393) and 3′ non-coding RNAs served as negative regulators of p53 and their role in tumor sensitivity to anti-cancer drugs. We investigated whether the truncated p53 that lacked C-terminal sequences increased drug sensitivity in the tumor cells compared to the full length p53. We created two p53 transgenic cell lines and recombinant adenoviruses (Wang et al., 2003). We also established *in vivo* and *in vitro* assays to evaluate anti-cancer drug sensitivity and the results were as expected (Wang et al., 2010).

We previously reported the differences in growth and proliferation *in vitro* between the two p53 transgenic cell lines and confirmed that the cell line carrying truncated p53 showed more significant suppression of lung tumor growth and proliferation (Wang et al., 2003). In this study, we compared the anti-cancer drug sensitivity of these two p53 transgenic human lung tumor cell lines both *in vivo* and *in vitro* and confirmed that 3′ truncated p53 selectively increased the sensitivity to NVB in human lung tumor cells. We also explored the regulatory mechanisms, key genes and pathways involved in signal transduction using cell cycle assays and mRNA expression array chips.

2. Materials and methods

2.1. 801D cell line and p53 gene status

801D cell (kindly provided by the People's Liberation Army General Hospital) was a human pulmonary giant cell carcinoma cell line and cultured in 1640 medium supplemented with 10% fetal bovine serum. The p53 gene in these cells exhibits loss of heterozygosity in the 248th codon and a CGG → CTT transversion.

2.2. BALB/c nude mice and chemotherapeutic drugs

All animal experiments and maintenance conformed to the guidelines of both the Animal Care and Use Committee and the American Association of Laboratory Animal Care. Female BALB/c nude mice (Vital River, Beijing, China) aged 4–6 weeks (average weight 20 g) were used in this study. These mice were raised in a pathogen-free environment, at a temperature of 21 ± 2 °C and relative humidity of 30–70%. Specialized persons were responsible for their feeding.

Cisplatin (PDD) was purchased from Qilu Pharmaceutical Co., Ltd. (Shangdong, China). Navelbine (NVB) was obtained from Haoseng Pharmaceutical Co., Ltd. (Jiangsu, China). 5-fluorouracil (5FU) was supplied by Xudong Haipu Pharmaceutical Co Ltd. (Shanghai, China). Three drugs were dissolved in saline to a concentration of 0.3 mg/ml.

2.3. Construction of recombinant p53 adenovirus

Recombinant p53 adenoviral plasmids were constructed using the Ad-Track-Easy transgenic system as described previously (He et al., 1998). Two deficient adenoviruses carrying either full-length p53 (p53(wt)) or the deleted-C-terminal p53 (p53(del)) were prepared. The two recombinants, plus the empty vector, were then infected into HEK293T packaging cells, together with the defective adenovirus, to generate the control adenovirus (Ad), Ad-p53(del) and Ad-p53(wt) (Wang et al., 2010). Virus concentrations were determined by multiplicity of infection (MOI) (He et al., 1998).

2.4. Studies in vitro

2.4.1. Drug sensitivity assays in human lung tumor cells

801D-p53(del), 801D-p53(wt) and 801D-p (empty vector) cell lines were established by transfection of plasmids expressing the truncated (deletion of 37 amino acid residues and 3′ non-coding mRNA), full-length p53 and the empty vector into the 801D cell line, respectively (Wang et al., 2003), which were then used as models for *in vitro* drug sensitivity assays.

Cells were seeded into 96-well plates. PDD, NVB and 5FU were added to the cells at various concentrations. After 24 h incubation at 37 °C in the CO_2 incubator, drug sensitivity was tested using MTT method in the parental 801D cell line as well as the transfected cell lines. IC50 was calculated according to the plot. The experiment was conducted in triplicate, and *t*-test was used to compare significant differences in IC50s between groups.

2.4.2. Cell cycle assays by FACS

The parental 801D cell line and the transfected cell lines were treated with NVB (the concentration was 20 ng/ml) for 24 h. The cells were collected at 0, 24, 48 and 72 h after removing NVB. Cell cycles were examined with the non-treated cells as control.

2.4.3. Cell apoptotic analysis

Cells were inoculated in 96-well plate and grew to 75% confluence. The cells were treated with NVB (the concentration was 20 ng/ml) for 24 h. The cells were detected at 0, 24, 48 and 72 h after removing NVB. After washing with RPMI-1640 Medium without serum, cells were incubated in 100 μl RPMI-1640 Medium without serum [50 mg/ml propidium iodide (PI, Sigma-Aldrich), 5 μg/ml hoechest33342 (Thermo)]. Cells were incubated for 10 m at 37 °C and the percentage of apoptotic cells were determined by Thermo Scientific ArrayScan VTI HCS (Thermo, the USA). Cellquest software (Becton Dickinson) was used for analysis. This experiment was repeated three times.

2.4.4. Differential gene expression profiling

Four samples including 801D-p53(del), 801D-p53(wt), 801D-p53(del) + NVB and 801D-p53(wt) + NVB were collected. The 801D-p53(del) and 801D-p53(wt) were cells at logarithmic growth phase collected after 72-hour incubation; 801D-p53(del) + NVB and 801D-p53(wt) + NVB were cells collected after 48–72 hour treatment with 20 ng/ml NVB. Total RNA of the four samples was extracted by TRIzol (Invitrogen) and RNeasy (Qiagen) kits and labeled by Agilent Quick Amp labeling kit. The 44 K Agilent whole genome array containing 41,000 human genes was used to identify the differential gene expression in the samples. The samples were hybridized on the Agilent whole genome array chip and scanned using mRNA expression profiling, gene clustering and statistical analysis.

2.5. Studies in vivo

2.5.1. Viral infection of 801D cells

801D cells were co-cultured with the three viruses at a concentration of 25, 50 and 100 times the number of cells. Colony assays were performed. Number of colonies was counted and colony formation rate and inhibition rate were calculated.

2.5.2. Drug sensitivity assays in nude mice

Nude mice were randomly divided into four groups. There were six mice in each group. These groups were the Ad control group (drug untreated group) and the Ad, Ad-p53(del), Ad-p53(wt) groups (drug treatment groups). The three viruses, Ad, Ad-p53(del) and Ad-p53(wt), infected 801D cells at a MOI of 50. After 24 h, cells were harvested and washed twice with saline. Cells were resuspended at a density of 1.0×10^7 cells/ml. 0.2 ml (2.0×10^6 cells) suspension (equal to 1.0×10^8 virus) was injected subcutaneously into the flank of BALBc (nu/nu) mice. When tumors became palpable, PDD, 5FU and NVB were injected into abdominal cavity at 0.17 μg/g, 6.55 μg/g and 6.55 μg/g body weight respectively. Control groups did not received drug treatment. After 7 days, three drugs were injected again at the same dose respectively. All mice were sacrificed after 45 days and the number of tumors,