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Effects of ΔNp73β on Cisplatin Treatment in Colon Cancer Cells

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Abbreviated title: p73 in colon cancer cells

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ABSTRACT

p73 can activate transcription of p53-responsive genes, thereby inhibiting cell growth. An alternative promoter in the TP73 gene gives rise to an N-terminally truncated isoform of p73, ΔNp73, which lacks the transactivation domain of the full length TAp73 protein. TAp73 is considered pro-apoptotic, and ΔNp73 anti-apoptotic. In this study, we overexpressed ΔNp73β in p53 wild type and p53 mutant colon cancer cell lines and further exposed the cells to cancer therapeutic drug cisplatin. The results showed that cisplatin decreased the protein expression levels of ΔNp73β in a dose-dependent manner, and both TAp73 and p53 were upregulated after cisplatin treatment. Further, clonogenic potential and cell viability were decreased, and apoptotic cells increased, in p53 mutant and in p53 wild type cells. Cellular viability was significantly higher in ΔNp73β-cells than mock-transfected cells. However, ΔNp73β overexpression did not affect the cellular susceptibility to cisplatin. In conclusion, the overexpression of ΔNp73β increases viability in p53 wild type and p53 mutant colon cancer cells, and cisplatin induces the degradation of ΔNp73β in a dose-dependent manner.
INTRODUCTION

p73 protein is encoded by the TP73 gene located to chromosome 1p36, a region frequently deleted in various cancers [1]. The structure and function of the p73 protein are homologous to p53, and it activates the transcription of p53-responsive genes and inhibits cell growth in a p53-like manner by inducing apoptosis [2]. It has been speculated that p73, like p53, is a tumor suppressor. However, initial genetic studies showed that p73-deficient mice do not develop spontaneous tumors [3]. Inactivation of p53 has been shown in the majority of human malignancies [4], while p73 mutations are rare in primary tumors [5]. Overexpression of p73 has been found in various tumor types, such as colorectal, breast, ovarian and lung cancers [6-9], suggesting that p73 plays an oncogenic role in tumorigenesis. Further, overexpression of the p73 protein has been associated with a poor prognosis in several types of cancers [6,10].

TP73 gene gives rise to several different mRNAs, and thus a number of different isoforms of the protein differing both N- and C- terminally. There are two N-terminal isoforms of p73, the full length proapoptotic variant of the protein, TAp73, and the N-terminally truncated form, ΔNp73. The ΔNp73 isoform arises through the use of an alternative promoter located in intron 3 [3,11]. There are also several C-terminal splice variants of the protein termed α, β, etc [1,12-14].

The TAp73 isoform is homologous to p53; harboring the N-terminal transactivating (TA) domain responsible for gene activation [2], while the Δ-isoforms lack the TA domain [3]. ΔNp73 shares the DNA-binding and oligomerisation domains with TAp73 and inhibits p53 and TAp73, either by competing for DNA-binding sites or by oligomerizing with the full length proteins [14-15]. Interestingly, the alternative
behind various cisplatin apoptosis response to DNA cycle arrest and apoptosis. It is well known that p53 plays an important role in cell cycle arrest and apoptosis. It is well-known that p53 plays an important role in response to DNA-damaging agents, with p53−/− cells being resistant to drug-induced apoptosis [23]. Also, p73 is activated by DNA damaging drugs, including cisplatin [24-25], and silencing of p73 with siRNA results in drug resistance in various cancer cells [24,26]. In this study, we attempted to clarify the mechanisms behind the effects of ΔNp73β on cisplatin treatment in colon cancer cells.

Cisplatin (cis-diammine-dichloro-platinum) is widely used as DNA-damaging drug in cancer therapy. It interacts with DNA, resulting in the formation of DNA adducts, primarily intrastrand crosslinks [22]. Subsequently, it induces DNA damage recognition proteins to signal to downstream effectors such as p53, resulting in cell cycle arrest and apoptosis. It is well-known that p53 plays an important role in response to DNA-damaging agents, with p53−/− cells being resistant to drug-induced apoptosis [23]. Also, p73 is activated by DNA damaging drugs, including cisplatin [24-25], and silencing of p73 with siRNA results in drug resistance in various cancer cells [24,26]. In this study, we attempted to clarify the mechanisms behind the effects of ΔNp73β on cisplatin treatment in colon cancer cells.
MATERIALS AND METHODS

Cell Culture and Transfection

Human colon carcinoma cell lines, HCT116 with wild type p53 (HCT116\(^{p53+/-}\)) and truncated p53 missing 40 amino acid residues (HCT116\(^{p53-/-}\)) [27], as well as HT29 were cultivated in McCoy’s 5A medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (GIBCO, Invitrogen, Carlsbad, CA), 1.5 mM L-glutamine (GIBCO) and 1X PEST (GIBCO) at 37°C in a 5% CO\(_2\) incubator. The HCT116\(^{p53-/-}\) cells are considered functionally p53 negative. The cells (1.5×10\(^4\) cells/cm\(^2\)) were seeded in 6-, 12-, or 96- well plates under standard cell culture conditions as described above for 24 h. HCT116 cells were then transfected with a pCMV6-XL5 vector (OriGene, Rockville, MD) containing transfection-ready cDNA for ΔNp73β (NM_001126241.1, OriGene) using transfection reagent FuGENE® 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer’s instructions. HT29 cells were transfected using the calcium precipitation method. Briefly, plasmid DNA was mixed with HEPES buffer and calcium chloride, and added with equal volume of 2 times HeBs buffer in a dropwise fashion. The mixture was incubated for 20 min at room temperature and added to the culture medium. The pCMV6-XL5 vector lacking the cDNA insert was used as a negative control. The cells were then treated with various concentrations of cisplatin (Sigma-Aldrich) 24 h after transfection.

Colony Forming Assay

HCT116 cells and HT29 were transfected in six-well plates as described above. After 24 h cells were trypsinized with TrypLE (Invitrogen, Carlsbad, CA) and
1×10³ cells were seeded in six-well plates with or without cisplatin (0.6 μM). The cells were grown under standard cell culture condition for seven days, fixed in 4% buffered formaldehyde and stained with 5% Giemsa. The cell colonies were counted and a survival fraction between cisplatin treated and control cells was calculated.

**XTT Viability Assay**

Cellular viability was determined at 48 and 72 h after the transfection using TACST™ XTT assay in 96-well plates according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The assay is based on the cleavage of the yellow tetrazolium salt XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-amilide) into a soluble orange formazan dye. This reaction is attributed mainly to the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells. The absorbance measured at 450 nm is proportional to the number of viable cells.

**Western Blot**

The cells were lysed in RIPA buffer (150 mM NaCl, 2% Triton, 0.1% SDS, 50 mM Tris pH 8.0) containing 1% Protease Inhibitor Cocktail (Sigma) at 48 and 72 h after the transfection. Protein concentrations were determined by BCA Protein Assay (Pierce, Woburn, MA). The samples (20 μg protein) were subjected to electrophoresis (35 min, 200V) on precast Criterion Tris-HCl gels, 4-15% (Bio-Rad, Hercules, CA) and electrotransferred on to polyvinylidene difluoride membranes (Amersham Biosciences/GE healthcare, Piscataway, NJ). The membranes were incubated with mouse monoclonal antibodies against ΔNp73
(1:500), TAp73 (1:500) and p53 (1:1000) (Abcam, Cambridge, MA) at 4°C over night, then with a secondary HRP-conjugated goat-anti-mouse antibody (1:1000, DAKO Cytomation, Glostrup, Denmark) at room temperature for 1 h. The proteins were detected using the Amersham ECL Plus Western Blot detection system (Amersham biosciences/GE Healthcare) and visualized in a Fujifilm LAS-1000 CCD camera (Fujifilm, Tokyo, Japan). Equal loading of protein samples was verified using a primary polyclonal rabbit anti-β-actin antibody (1:1000, Cell Signalling Technology, Danvers, MA) and a secondary polyclonal goat anti-rabbit antibody (1:2000, DAKO).

**M30-Apoptosense ELISA and DAPI Assay**

Apoptosis was quantitatively detected by using M30-Apoptosense® ELISA kit (Peviva, Bromma, Sweden) at 72 h after the transfection according to the manufacturer’s instructions. Absorbance was measured at 450 nm and normalized against total protein concentration in each sample.

The plates with cells were centrifuged to spin down apoptotic cells. Cells were trypsinized, resuspended in PBS and centrifuged on to glass slides using a Shandon Cytospin® 2 Cytocentrifuge (Thermo Scientific, Waltham, MA), then fixed with 4% buffered formaldehyde and mounted with VECTASHIELD® HardSet™ Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA). The cells were visualized in a UV-light fluorescence microscope, and apoptotic cells were examined.
Statistical Analysis

All experiments were performed in triplicates on three different occasions. Student’s t-test was performed to evaluate statistical significance. Data are represented as means ± standard error of mean (SEM). Two-sided p-values below 0.05 were considered as statistically significant.
RESULTS

Colony Forming Assay

Transfected cells were treated with 0.6 µM cisplatin for 7 d, and cell colonies were counted (Figure 1). Cisplatin markedly reduced the number of colonies, and a quota between cisplatin treated samples and controls, the survival fraction, was calculated. ∆Np73β-transfected HCT116^p53+/+ cells had a survival fraction of 0.53±0.08 and mock-transfected cells had a fraction of 0.44±0.1. The survival fractions were not significantly different (p=0.13). The survival fraction for ∆Np73β-transfected HCT116^p53−/− cells was 0.49±0.07 and for mock-transfected cells 0.43±0.06. The survival fractions did not significantly differ (p=0.07). HT29 cells were highly resistant to cisplatin, as shown by results both from our experiments and other reports [28-29]. The survival fraction for ∆Np73β-transfected HT29 cells was 0.95±0.20 and for mock-transfected cells 0.91±0.22. There was no significant difference (p=0.80).

Cellular Viability

Cellular viability was examined using the XTT assay after the treatment with increasing concentrations of cisplatin (0-60 µM). The HCT116 cells were found to respond to 10-20 µM of cisplatin, therefore these concentrations were chosen for subsequent experiments.

Cellular viability was determined at 48 and 72 h after transfection. ∆Np73β-transfected HCT116^p53+/+ cells were significantly more viable than mock-transfected cells at 48 h after the transfection (p=0.005, Figure 2A). The cellular viability of HCT116^p53+/+ cells was significantly decreased after treatment with 20
Fig 1. Colonies of HCT116<sup>p53+/+</sup> colon cancer cells fixed and stained with Giemsa 7 days after seeding the cells without (A) or with 0.6 µM cisplatin (B). Colonies of HCT116<sup>p53-/-</sup> cells appeared similar under the same conditions. Colonies of HT29 cells were treated without (C) or with 0.6 µM cisplatin (D).

µM cisplatin compared to treatment with 0 and 10 µM cisplatin, both in ∆Np73β-transfected cells (p=0.026 and 0.034 for 0 and 10 µM, respectively) and mock-transfected cells (p=0.038 and 0.023 for 0 and 10 µM, respectively, Figure 2A). There was no difference between HCT116<sup>p53+/+</sup> cells treated with 0 and 10 µM cisplatin, neither in ∆Np73β-transfected cells (p=0.40) nor in mock-transfected cells (p=0.86). Cellular viability was decreased after treatment with 20 µM cisplatin compared to treatment with 0 and 10 µM, both in ∆Np73β-transfected cells at 72 h after the transfection (p=0.008 and 0.0007 for 0 and 10 µM, respectively) and in mock-transfected cells (p=0.015 and 0.024 for 0 and 10 µM, respectively, Figure 2B). Further, cellular viability of ∆Np73β-transfected
HCT116<sup>p53+/+</sup> cells were significantly higher than mock-transfected cells treated with 20 µM cisplatin (p=0.006, Figure 2B).

**Fig 2.** Cellular viability measured using XTT assay in A) HCT116<sup>p53+/+</sup> cells at 48 h after the transfection B) HCT116<sup>p53+/+</sup> cells at 72 h after the transfection C) HCT116<sup>p53/-</sup> cells at 48 h after the transfection D) HCT116<sup>p53/-</sup> cells at 72 h after the transfection E) HT29 cells at 48 h after the transfection and F) HT29 cells at 72 h after the transfection. All cells were transfected with either ΔNp73β- or mock-vector (control), treated or not treated with cisplatin. Untreated mock-transfected cells are set as 100% viable. The p-values from two-sided t-tests are in A) *<sup>1</sup>=0.023, *<sup>2</sup>=0.038, *<sup>3</sup>=0.005, *<sup>4</sup>=0.026 and *<sup>5</sup>=0.034 B) *<sup>1</sup>=0.024, *<sup>2</sup>=0.015, *<sup>3</sup>=0.006, *<sup>4</sup>=0.008 and *<sup>5</sup>=0.007 C) *<sup>1</sup>=0.005, *<sup>2</sup>=0.02, *<sup>3</sup>=0.01 and *<sup>4</sup>=0.048 D) *=0.028 E) *=0.001 F) *<sup>1</sup>=0.001, *<sup>2</sup>=0.045 and *<sup>3</sup>=0.002.
ΔNp73β-transfected HCT116p53-/ cells were significantly more viable than mock-transfected cells at 48 h after transfection (p=0.01, Figure 2C). The viability of ΔNp73β-transfected cells treated with 10 µM cisplatin was also higher than untreated mock-transfected cells (p=0.02, Figure 2C). The treatment with 20 µM cisplatin decreased cellular viability in ΔNp73β-transfected HCT116p53-/ compared to no cisplatin treatment (p=0.048, Figure 2C). In mock-transfected HCT116p53-/ cellular viability was lower after the treatment with 20 µM cisplatin compared to treatment with 10 µM cisplatin (p=0.005, Figure 2C). ΔNp73β-transfected HCT116p53-/ cells were more viable than mock-transfected cells both with 0 and 10 µM cisplatin at 72 h after transfection, however, the difference was not significant (p=0.076 and 0.34 for 0 and 10 µM, respectively, Figure 2D). In mock-transfected cells viability was decreased after the treatment with 20 µM cisplatin as compared to the control (p=0.028, Figure 2D). A similar trend was found in the ΔNp73β-transfected cells (p=0.09, Figure 2D).

HT29 cells were treated with the same concentrations of cisplatin. At 48 h after transfection, there was no difference between 0 and 10 µM cisplatin, neither in ΔNp73β-transfected cells (p=0.35) nor in mock-transfected cells (p=0.76). Cellular viability remained similar with treatment of 20 µM cisplatin compared to treatment with 0 or 10 µM, both in ΔNp73β-transfected cells (p=0.10 and 0.84 for 0 and 10 µM, respectively) and in mock-transfected cells (p=0.47 and 0.85 for 0 and 10 µM, respectively, Figure 2E). ΔNp73β-transfected HT29 cells were more viable than mock-transfected cells at the concentration of 20 µM (p=0.001). A similar trend was seen at 10µM (p=0.055). At 72 h after transfection, cellular viability was decreased after treatment with 20 µM cisplatin compared to no treatment, both in ΔNp73β-
transfected cells (p=0.001) and mock-transfected cells (p=0.045 Figure 2F). There was a significant decrease in viability with 20 μM cisplatin compared to 10 μM in ΔNp73β-transfected cells (p=0.002) but not in mock-transfected cells (p=0.13). Again, there was a trend that ΔNp73β- transfected cells were more viable than the mock-transfected cells at the concentration of 10 μM (p=0.074) but not at 20 μM (p=0.28).

**Protein Expression**

Expression of ΔNp73, TAp73 and p53 proteins in HCT116p53+/+ and HCT116p53−/− cells was determined by Western blot at 48 and 72 h after the transfection (Figure 3). The expression of ΔNp73 was markedly increased after ΔNp73β-transfection. Levels of the ΔNp73β protein in both HCT116p53+/+ and HCT116p53−/− cells were decreased in a dose-dependent manner after cisplatin treatment. TAp73 expression was increased in both ΔNp73β- and mock–transfected HCT116p53+/+ and HCT116p53−/− cells after the treatment with cisplatin. In HCT116p53+/+ p53 expression was increased in response to cisplatin. p53 expression was also increased in ΔNp73β overexpressing cells, without the cisplatin treatment. The HT29 cells were transfected using the calcium phosphate transfection protocol as other transfection reagents were ineffective. An increase in the ΔNp73 protein levels could be observed after transfection (supplementary data).
Fig 3. Protein expression examined by Western blot of ΔNp73, TA p73 and p53 in cell lines HCT116p53+/− and HCT116p53−/− at 72 h after the transfection with ΔNp73β- or mock- vector, treated with increasing concentrations of cisplatin (0-20 µM).

Apoptosis

Apoptosis was evaluated in HCT116 cells at 72 h after transfection using the M30-Apoptosense® ELISA kit (Peviva). The amount of apoptotic cells in ΔNp73β-transfected HCT116p53+/− cells was increased 1.8× at 10 µM (p=0.062) and significantly increased 3.9× at 20 µM (p=0.049), after the treatment with cisplatin compared to the controls. Corresponding figures for mock-transfected HCT116p53+/− cells were 1.5× at 10 µM (p=0.33) and 3.9× at 20 µM (p=0.004). There was no difference between ΔNp73β-transfected and mock-transfected HCT116p53+/− cells, either with or without treatment of cisplatin (p=0.68, 0.10 and 0.81 for 0, 10 and 20 µM cisplatin, respectively) (Figure 4).

Apoptotic HCT116p53−/− cells increased after the cisplatin treatment, although not significantly, by 1.6× at 10 µM (p=0.16) and 2.0× at 20 µM (p=0.059) in ΔNp73β-transfected cells, and by 1.5× at 10 µM (p=0.042) and 1.8× at 20 µM (p=0.056) in mock-transfected cells. No difference between ΔNp73β- and mock transfected cells
was found, neither at 0, 10 or 20 µM cisplatin (p=0.79, 0.38 and 0.92, respectively) (Figure 4).

**Fig 4.** Apoptosis at 72 h after the transfection with ΔNp73β- or mock-vector in HCT116p53+/+ and HCT116p53−/− cells measured by M30-Apoptosense® ELISA kit (Peviva), with and without cisplatin treatment. Values are normalized against total protein content of the samples, and presented as units (U) of M30 per mg of protein. The p-values from two-sided t-tests are *1=0.004, *2=0.048, *3=0.049 and *4=0.042.
DAPI staining was used to validate the results of the M30 assay. Apoptotic cells were distinguished from non-apoptotic cells by morphological hallmarks such as nuclear condensation and fragmentation. Representative pictures of DAPI stained cells are shown in Figure 5.

**Fig 5.** HCT116<sup>p53<sup>+/-</sup></sup> and HCT116<sup>p53<sup>-/-</sup></sup> cells undergo apoptosis after treatment with 0, 10 or 20 µM cisplatin for 48 h. The cells are stained with DAPI, apoptotic cells were observed (arrows).
DISCUSSION

In this study, we investigated the effects of $\Delta Np73\beta$ on colon cancer cells with respect of cytotoxicity of cisplatin. HCT116$^{p53+/+}$, HCT116$^{p53-/-}$ and HT29 cells, were transfected with a $\Delta Np73\beta$ expression vector and further treated with cisplatin, then colony formation potential, cellular viability, apoptosis, and protein expression were examined. We found that overexpression of $\Delta Np73\beta$ significantly increased cellular viability in all the three cell lines. There were no significant differences between $\Delta Np73\beta$- and mock-transfected cells regarding apoptosis in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cell lines, indicating that the differences in cellular viability do not seem to be primarily due to increased apoptosis. A reduction in cell cycle arrest could possibly explain the difference were due to decreased cell cycle arrest rather than apoptosis.

Since $\Delta Np73\beta$ was degraded in a dose dependent manner in response to the cisplatin, any resistance to cisplatin treatment conferred by $\Delta Np73\beta$ should be minor at the concentrations used in the cellular viability and apoptosis assays. A relatively low concentration of cisplatin was used in the colony forming assay, and the survival fractions slightly increased in $\Delta Np73\beta$-transfected cells compared to mock-transfected cells, indicating resistance to cisplatin treatment.

It has been shown in sarcoma cell line SAOS-2 that $\Delta Np73$ is rapidly degraded in a proteasome dependent manner in response to DNA-damage [30]. $\Delta Np73$ expression is induced by p53 and TAp73 [11], constituting a regulatory feedback mechanism. The degradation of $\Delta Np73$ may be a safety mechanism, allowing cell cycle arrest and apoptosis to occur properly in damaged cells without inhibition from the anti-apoptotic $\Delta Np73$. In contrast, cisplatin up-regulates $\Delta Np73$ in SH-
SY5Y neuroblastoma cells [17,31]. The signaling pathways leading to DNA damage-induced degradation of ΔNp73 could be disturbed in some tumor types, providing a mechanism for resistance to various DNA-damaging treatments.

p53 contributes to cisplatin treatment by inducing cell cycle arrest and apoptosis [22], and we found the protein levels of p53 to be increased after the cisplatin treatment. Further, HCT116p53−/− cells were less sensitive to the cisplatin treatment than HCT116p53+/+ cells, confirming the involvement of p53 in cisplatin treatment. We also found an increase in p53 protein in cells overexpressing ΔNp73β, even without the cisplatin treatment. This could be due to blocking of the mdm2 promoter; ΔNp73β has been previously shown to reduce the transcriptional activation of mdm2 [32]. Mdm2 promotes the ubiquitin-mediated degradation of p53 [33], and high levels of ΔNp73β may, therefore, result in reduced degradation of p53.

In addition, we found that expression of TAp73 protein was increased after the cisplatin treatment. Inhibition of TAp73 has been shown to increase the resistance to the cisplatin treatment [24], suggesting that TAp73 is involved in cisplatin-induced cell cycle arrest and apoptosis. On the contrary, it has been reported that p73 is not induced in response to cisplatin in the HCT116 cell line as a result of deficient mismatch repair [25]. Cisplatin increases the half-life of p73 by activating the tyrosine kinase c-abl in mismatch-repair-proficient cells [25]. However, p73 has been stabilized by the transcription factor c-Jun in response to cisplatin, in cells defective in c-abl binding and phosphorylation [34], suggesting alternative pathways for p73 induction independent of c-abl and mismatch-repair signaling pathway.
Upregulation of ΔNp73 in tumors may disturb apoptotic signaling pathway by interfering with p53 and TAp73, and high expression of ΔNp73 has been correlated with a worse outcome of patients [35-36] and resistance to DNA-damaging drugs [37-38]. Recent studies have shown that overexpression of ΔNp73 protects cells from apoptosis, also when treated with cisplatin, by inhibiting p53 target genes such as p21 [30], while downregulation of ΔNp73 increases apoptosis [39]. However, the effect of the ΔNp73β isoform remains indistinct. Unlike ΔNp73α, ΔNp73β possessed the capability of activating some p53-responsive genes, thereby inducing both cell cycle arrest and apoptosis [20]. Resulting from the use of alternative intron 3 promoter, the ΔNp73 isoforms contain 13 unique residues at the N-terminus shown to contain an activation domain [20]. The ΔNp73α and β isoforms are identical apart from the C-terminal where the β-isoform lacks exon 13 [1], suggesting that the C-terminal of α-isoform represses transcription. Both ΔNp73α and β have been shown to inhibit the transcription of p53 responsive genes, although the α-isoform is much more potent than the β-isoform which shows some intrinsic transactivation capacity [16]. Also in TAp73, the α-isoform has lower transactivation ability than the β-isoform [40].

In conclusion, we demonstrate that cisplatin decreased clonogenic potential and cellular viability while increasing apoptosis. These effects were more pronounced in HCT116p53+/+ than HCT116p53−/−, confirming the involvement of p53 in the cisplatin treatment. Both p53 and TAp73 were upregulated in cisplatin treated HCT116 cells. Overexpression of ΔNp73β increased cellular viability in HT29 cells as well as both HCT116p53+/+ and HCT116p53−/−. Resistance to cisplatin treatment increased slightly in ΔNp73β overexpressing HCT116p53+/+ and
HCT116<sup>p53<sup>-/-</sup></sup> cells, indicating that both p53 and TAp73 were involved in cisplatin treatment.
REFERENCES


Supplementary. Western blot of ΔNp73 in HT29 colon cancer cells after the transfection with ΔNp73β- or mock-vector.