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To cite this article: Varvara Petrova, Mara Mancini, Massimiliano Agostini, Richard A Knight, Margherita Annicchiarico-Petruzzelli, Nikolai A Barlev, Gerry Melino & Ivano Amelio (2015) TAp73 transcriptionally represses BNIP3 expression, Cell Cycle, 14:15, 2484-2493, DOI: 10.1080/15384101.2015.1044178

To link to this article: https://doi.org/10.1080/15384101.2015.1044178
TAp73 transcriptionally represses BNIP3 expression

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Keywords: autophagy, HIF, lung cancer, p73, p53

Introduction

p73 is a transcriptional factor, belonging to p53 family. The presence of 2 promoters in the TP73 gene gives rise to 2 sets of isoforms: transactivational (TA) domain-containing isoforms, TAp73, regulated by the first promoter (P1), and the N-truncated isoforms, lacking TA domain (from promoter P2), ΔNp73. Alternative splicing can also take place at 3’-end, leading to 7 isoforms varying in activity and specificity α, β, γ, δ, ε, ζ, η.1-5 p53 family is one of the most powerful families of genes6,7; it plays fundamental roles in protection of genome integrity8-13 and in germline and somatic cells impacting fertility14-21 and cancer.22-36 In cancer cells p73 is rarely mutated, but its expression is often deregulated. There is increasing evidence, that TAp73/ ΔNp73 expression ratio affects tumor development and progression.37-39 TAp73 is considered a bona fide tumor suppressor, largely mimicking p53 function. It controls cell cycle arrest, apoptosis as well as DNA damage repair.40 Tumor suppressor function of TAp73 has also been recently associated to repression of tumor angiogenesis, through regulation of hypoxia inducible factor (HIF) signaling. TAp73 indeed directly binds HIF-1α protein, promoting its oxygen-independent degradation.41,42 Conversely, ΔNp73 antagonizes TAp73 and it is considered an oncogenic protein.43-46 It can form inactive complexes with TAp73, and also bind common promoters with p53 and TAp73, thus inhibiting their transcriptional activity.47-50 Besides its cancer related function, TAp73 also plays a role in neurogenesis, and its dysregulation is linked with developmental defect and neurodegenerative diseases. In fact, TAp73 is necessary for neuronal differentiation and maintenance of neuronal stem cells.51-54

Hypoxia inducible factors (HIFs) mediate the physiological response to hypoxia55 regulating processes, such as angiogenesis,56-58 proliferation59-68 and metabolism.64,69-73 The wide transcriptional reprogramming operated by HIF-1, includes the direct transcriptional induction of the Bcl-2 Nineteen kilodalton Interacting Protein (BNIP3).74,75 BNIP3 is a Bcl2-family BH3-only protein, which contributes to cellular processes, such as apoptosis, autophagy, mitophagy and mitochondrial metabolism.76 BNIP3-deficient mice do not display significant physical abnormalities and altered lifespan, however they show decreased postischemic myocardial apoptosis,77-82 suggesting an involvement in hypoxia-dependent cell death. BNIP3 was first shown to localize in mitochondria, although later in glial cells was also observed a nuclear localization.84,85 BNIP3 activation causes mitochondrial dysfunction through mitochondrial apoptosis, reduced oxidative phosphorylation and induction of autophagy and mitophagy.86-89

Here, we show a direct regulation by TAp73 on BNIP3 transcription, and we report a possible clinical relevance of this axis...
for lung cancer patients. Consistently with reduced TAp73 activity, high BNIP3 expression correlates with bad prognosis in patients with lung cancer.

Results

TAp73 represses HIF1α and its target BNIP3

To investigate the influence of TAp73 on BNIP3 expression we used SaOS-2 cells with Tet-On system. SaOs-2 is a p53/p63/p73 deficient human osteosarcoma cell line. Expression of TAp73 in these cells can be induced by doxycycline treatment. As shown in Fig. 1A, B and Fig. S1A, 2 μg/ml of doxycycline induced TAp73 expression in a time-dependent manner. Along with TAp73 accumulation we detected decrease in BNIP3 protein levels and as previously described reduced HIF1α (Fig. 1A).41,43 p21 was used as positive control of p73 transcriptional activation (Fig. 1A). To evaluate whether BNIP3 downregulation was associated to altered transcription of the BNIP3 gene, we performed real-time qPCR in SaOs-2 Tet-On cell line. qPCR also highlighted decrease in BNIP3 mRNA level (Fig. 1B). Taken together these data indicate that consistently with TAp73-dependent downregulation of HIF1α BNIP3 is downregulated.

Next we employed H1299, p53-null human non-small cell lung carcinoma (NSCLC) cell line, expressing endogenous TAp73. First, we overexpressed HA-tagged TAp73 for 24 h and exposed the cells to hypoxia during the last 8h of transfection (1% O2) (Fig. 2A, B). Protein and RNA levels of TAp73 and its transcriptional target p21 confirmed TAp73 transcriptional activation (Fig. 2B, Fig. S1B, C). Increased levels of BNIP3 mRNA and protein were observed in cells, under hypoxia, as BNIP3 is a hypoxia response gene. TAp73 overexpression in normoxia and hypoxia confirmed the BNIP3 repression observed in SaOs-2 Tet-On mRNA (Fig. 2A, B).

Then we performed knockdown experiment in H1299 cells by transfecting selective siRNA, for TAp73 isoforms. mRNA levels of BNIP3 resulted upregulated after TAp73 silencing, stronger effect was seen after 48h (Fig. 2C). Similarly western blot (WB) analysis showed BNIP3 protein accumulation after TAp73 depletion (Fig. 2D). Together with the data reported in Figure 1, these data proved a TAp73-dependent inhibition of BNIP3 expression in an oxygen-independent manner.

TAp73 binds BNIP3 promoter

The ability of TAp73 to inhibit the expression of BNIP3 in an oxygen-independent manner indicated the possibility of an additional HIF-independent regulation of BNIP3 by TAp73. We therefore investigated the hypothesis that TAp73 acts also as a transcriptional factor directly regulating BNIP3 promoter. In support of this hypothesis BNIP3 has been shown as a direct p53 transcriptional target.90 We therefore assessed whether the previous validated p53RE in BNIP3 promoter could also be regulated by TAp73 (Fig. 3A). The p53RE is located between −987 and
−1021 bp upstream of the transcription start site (TSS) and comprises 2 closely located p53 binding sites. To experimentally validate our hypothesis we used a reporter gene vector, containing the region of BNIP3 promoter showed in Fig. 3A (between −1638 and +186 bp from the TSS) upstream of the luciferase reporter gene. We co-transfected H1299 cells with this construct, HA-TAp73-expressing plasmid and control Renilla vector for 20 h. Transfection efficiency was confirmed by WB (Fig. 3C). Consistently with our hypothesis luciferase assay showed significant decrease in luciferase activity of approximately 40% after HATAp73 transfection (Fig. 3B).

Next, we performed chromatin immunoprecipitation (ChIP) assay for the indicated p53RE in the BNIP3 promoter, in TAp73 SaOs-2 Tet-On cells, after 16 h of doxycycline induction. The specific amplification in anti-HA immunoprecipitated chromatin confirmed a direct binding of TAp73 on BNIP3 human promoter (Fig. 3D). Data obtained from luciferase gene reporter assay and ChIP demonstrated that TAp73 suppresses BNIP3 gene expression directly binding its promoter.

The BNIP3 regulation is of clinical importance for lung cancer patients

Trp73−/− and TAp73−/− mice spontaneously develop lung carcinomas, and altered ratio TAp73/ΔNp73 is frequently reported in human lung cancer.91,92 TAp73 is therefore considered a bona fide tumor suppressor, particular relevant in lung tumorigenesis. We wanted therefore to verify whether down-stream to TAp73 alteration, BNIP3 upregulation might play a role in lung carcinoma. We employed a bioinformatic approach to assess BNIP3 expression in human lung cancer patient specimens. We used publicly available Hou Lung patient data set to analyze BNIP3 expression in 156 patient samples. Dataset includes 4 groups of patient samples: derived from normal lung, large cell lung carcinoma, lung adenocarcinoma or squamous cell lung carcinoma. Median BNIP3 expression was significantly higher in all lung carcinomas compared to normal lung tissue (Fig. 4A–C). These data suggest that failure of TAp73/BNIP3 axis in lungs may lead to BNIP3 upregulation and may contribute to tumorigenicity.
We next used publicly available data set to assess BNIP3 expression impact on patients’ survival. Survival rate appeared significantly higher in patients with low BNIP3 expression (Fig. 4D). Our data suggest that BNIP3 may have a role in tumorigenesis and progression of lung cancers.

Discussion

We identified BNIP3 as a novel TAp73 target gene. BNIP3 expression can be regulated by TAp73 via 2 mechanisms (Fig. 5). Here we show that BNIP3 expression is inhibited by TAp73, through its direct binding on BNIP3 promoter. We demonstrated that the p53-like responsive element in the BNIP3 promoter, previously experimentally validated for p53 can also be recognized by TAp73. As BNIP3 is a HIF1α target gene,90 and HIF1α is repressed by TAp73,41 relationship between TAp73 and BNIP3 can also depend on an indirect regulation via HIF1.

TAp73 has tumor-suppressor function, we therefore also investigated a possible involvement of BNIP3 in tumourigenesis. BNIP3 contributes to several processes in cell, which potentially can affect tumor development. The ability to activate apoptosis would indicate a tumor suppressor function for BNIP3, however its pro-necrotic role may lead to pro-tumorigenic effects, as necrosis can promote tumor growth and associates with poor prognosis for patients.93 BNIP3 is also known to lead to autophagy, which may promote both tumor suppression and tumor growth, and the implication of autophagy in cancer progression can be different.94-99 BNIP3 has also been shown to act as a transcriptional factor: if it translocates to nucleus, it suppresses Apoptosis-Inducing Factor expression, preventing cell death, thus showing tumorigenic function.85 Our bioinformatics analysis would suggest an oncogenic function for BNIP3. BNIP3 expression is upregulated in lung carcinomas, and correlates with bad prognosis for patients with lung cancer. Therefore our current data, although still preliminary, might indicate TAp73/BNIP3 negative axis as a novel pathway for TAp73 tumor suppressor function.
function. Consistently, TAp73 loss results in mitochondrial dys-
function.\textsuperscript{100,101} BNIP3 upregulation as a consequence of TAp73 loss might therefore contribute to TAp73–dependent mitochondria-
ral phenotype and be associated to the complex involvement of p73\textsuperscript{102-104} and the other family members in regulation of mito-
ochondrial activity,\textsuperscript{105-108} cell metabolism\textsuperscript{109-114} and redox 
homeostasis.\textsuperscript{115-118} However, currently it is still unclear 
whether the complex integration of all the p53 family mem-
biers, in particular the truncated isoform of p73, DNp73, 
and the cancer-associated mutants of p53, impacts and affects the 
TAp73-dependent antagonism of BNIP3 expression and more generally of hypoxia response. Future studies are demanded to address these aspects.

Overall, we described a novel transcriptional target of TAp73, 
also involved in hypoxia response, confirming the antagonistic 
role of TAp73 on HIF signaling and tumourigenesis.

Materials and Methods

Cell cultures

H1299 and SaOS2-Tet-On cell lines were used. Cells were 
grown in humidified incubator, at 37°C, in atmosphere of 5% 
CO\textsubscript{2} in air. Cells were cultivated in RPMI medium, containing 
L-glutamine, 4.5 g/L of D-glucose, 2,383 g/L of HEPES Buffer, 
1.5 g/L of Sodium Bicarbonate, 110 mg/L of Sodium Pyruvate
RNA was extracted from cells by means of RNEasy Mini Kit (Qiagen), according to the Qiagen company protocol. The RNA obtained was quantified by spectrophotometric analysis, and 1 μg of total RNA was used to prepare cDNA with RevertAid H minus First Strand cDNA Synthesis kit (ThermoScientific), using Random primers and protocol from the kit. qPCR was carried out with 1/10 of prepared cDNA and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was analyzed in accordance to 7500 Software version 2.0.6 of Applied Biosystems, normalized to housekeeping gene TBP.

Figure 5. TAp73 regulates the BNIP3 expression via 2 mechanisms. TAp73 can directly bind the BNIP3 promoter and inhibit its expression. BNIP3 is upregulated by HIF1 in hypoxia. TAp73 drives HIF1α degradation and, subsequently, can prevent BNIP3 upregulation. No expression of TAp73 enables expression of BNIP3 (upper panel). TAp73 expression leads to lower BNIP3 level impacting different processes, including autophagy, mitophagy, mitochondrial metabolism and necrotic cell death.

Western blot analysis

For the protein extraction cells were lysed in RIPA buffer with protease inhibitor cocktail tablets Complete, EDTA-free (Roche) and phosphatase inhibitor cocktail tablets PhosSTOP (Roche). Lysate was measured for protein concentration by using Bio-RAD Protein assay (Bio-RAD), then mixed with Laemmly loading buffer, and 100 μg of proteins were loaded on 10% SDS-PAGE, and then transferred to polyvinylidene difluoride blotting membranes (Amersham, GE Healthcare). Membranes were blocked for 1 hour in 5% (m/vol) dry milk dissolved in PBS with 1% (vol/vol) Tween-20 (PBST); incubated with primary antibodies overnight and with secondary ones, conjugated with
horseradish peroxidase, for 1 hour. Antibodies were diluted in 5% dry milk in PBST: anti-HIF1α 1:250 (Novus Biologicals), anti-HA 1:1000 (Covance), anti-GAPDH 1:40000 (Sigma), anti-p21 1:1000 (Santa Cruz Biotechnology), anti-BNIP3 1:600 (Abcam), anti-β-tubulin 1:3000 (Santa Cruz Biotechnology), anti-β73 1:2000 (Bethyl). SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) was used to detect signal on membranes.

Cell transfection
For TAp73 overexpression in H1299 cell line 1.2 E6 cells were seeded per 10 cm dish 24 h before transfection. Transfection was performed with 10 ug DNA (pcDNA empty or pcDNA with HA-TAp73) per 10 cm dish using Lipofectamine 2000 Reagent (Invitrogen). Cells were collected 24 h after transfection.

For TAp73 knockdown in H1299 cell line 1.2 E6 cells were seeded per 10 cm dish 24 h before transfection. Transfection was performed using 50 nM siRNA (control siRNA (Ambion) or siTAp73 (Ambion)) and Lipofectamine RNAiMAX (Invitrogen). Each dish was split in two 24 h after transfection; cells were collected 48 h and 72 h after transfection.

For luciferase assay H1299 cells were seeded 20 h before transfection in 12-well plates, 1.5 E5 cells per well. Transfection was carried out by means of Lipofectamine 2000 (Invitrogen). Cells were cotransfected with 0.05 ng/well pcDNA with HA-TAp73 plasmid or empty pcDNA plasmid, 1 ug/well pRL-cytomegalovirus vector and 800 ng/well BNIP3 promoter luciferase reporter vector or p21 promoter luciferase reporter vector.

Luciferase assay
Cells were lysed 20 h after transfection, and Firefly luciferase activity was measured, normalized to Renilla luciferase activity with Dual-Glo Luciferase Assay System (Promega), in accordance with Dual-Glo Luciferase Assay System protocol. Light emission over 1s was measured with luminoimeter.

Chromatin immunoprecipitation assay
SaOS2-Tet-On cell line was used for ChIP assay. TAp73 overexpression for 24 h was achieved by doxycycline treatment. Then cells were collected, fixed in 37% formaldehyde, and subjected to sonication for DNA shearing. Chromatin was immunoprecipitated with anti-HA antibodies (Covance) or unspecific immunoglobulin G (IgG) antibodies (Invitrogen) with a ChIP assay Kit (Invitrogen), and the promoter region, containing potential p73 response element, was amplified using the designed BNIP3 promoter primers. For positive control p21 promoter primers were used. The sequences of BNIP3-ChIP primers are following: 5′-AGCGTTTCTGCGCGACCTTG- 3′ and 5′-GGGACTGGGAGGCACTTTTCAGAGGA- 3′

Bioinformatic analyses
By using Oncomine® database and Oncomine® Research Edition (available via Internet https://www.oncomine.org/resource/main.html) we gained access to Hou Lung dataset, analyzed it for BNIP3 expression and compared BNIP3 expression in normal lung with expression in large cell carcinoma, squamous cell lung carcinoma or lung adenocarcinoma.

Gene expression data set GSE4573 was downloaded. Patients were divided in 2 cohorts, in accordance to level of the BNIP3 expression. Kaplan-Meier curves, demonstrating survival, were built up for both cohorts. P-value is measured by Students t-test.11,120

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.

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