Prognostic impact of ∆TAp73 isoform levels and their target genes in colon cancer patients

Running title: ∆TAp73 impact on patient survival

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Key words: ∆TAp73 isoforms, survival, colon cancer patients, ABCB1, HMGB1

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STATEMENT OF TRANSLATIONAL RELEVANCE

The tumor suppressor and/or oncogenic functions of TP73 isoforms have been intensely debated recently. The 2010 publication of an article on the specific role of ΔTAp73 in knock-out mice strongly supports their role as oncogenes in the tumorigenic process. Thus, there is currently increasing interest in unraveling the mechanisms that underlie the oncogenic potential of ΔTAp73 isoforms. Our report is the first that shows the prognosis value of ΔTAp73 variants and their target genes involved in drug resistance in colon cancer patients. The positive correlation found in our colon cancer series between ΔTAp73 variants and HMGB1 and ABCB1 expression supports them as TP73 targets in vivo. The upregulation of these two genes after ectopic expression of ΔNp73 in colon tumor cells also sustains this statement. The fact that ΔTAp73 isoforms are associated with shortened overall survival and with increase in proliferation and drug resistance confirms their oncogenic role and plausible value as prognostic markers. ABCB1 and HMGB1 strongly predict overall survival in an independent manner, making clear the importance of studying downstream TP73 targets that could predict the outcome of colon cancer patients better than ΔTAp73 themselves do.
ABSTRACT

Purpose: Cumulative data support the role of ∆TAp73 variants in tumorigenic processes such as drug resistance. We evaluate the impact of TP73 isoforms and their putative target genes ABBC1, HMGB1 and CASP1 on the survival of colon cancer patients and the correlation between their expressions.

Experimental Design: We determined in 77 colon cancer patients the expression of ∆Ex2p73, ∆Ex2/3p73, ∆Np73, TAp73, ABBC1, HMGB1 and CASP1 by quantitative real-time RT-PCR. Tumor characteristics, disease-free survival (DFS) and overall survival (OS) were examined in each patient. Functional experiments were performed to check whether ectopic expression of ∆Np73 modifies the proliferation, drug resistance, migration and invasion properties of colon tumor cells and the expression of ABBC1, HMGB1 and CASP1.

Results: Positive correlations were observed between the expression levels of ∆TAp73 variants and HMGB1. A trend was also observed for ABBC1. Overexpression of ∆Ex2/3p73 and ∆Np73 isoforms significantly associated with advanced stages (p = 0.04 and p = 0.03, respectively) and predicted shortened OS (p = 0.04 and p = 0.05, respectively). High levels of ABBC1 and HMGB1 associated with shorter OS (p = 0.04 and p = 0.05, respectively). Multivariate analysis showed that, in addition to the tumor stage, ABBC1 and HMGB1 had independent relationships with OS (p = 0.008). Ectopic expression of ∆Np73 associated with an increase in proliferation and drug resistance.

Conclusions: The positive correlation between ∆TAp73 variants and HMGB1 and ABBC1 expression supports them as TP73 targets. The fact that upregulation of ∆TAp73 isoforms associated with shortened OS, increase in proliferation and drug resistance confirms their oncogenic role and plausible value as prognostic markers. ABBC1 and HMGB1, putative ∆TAp73 target genes, strongly predict OS in an independent manner.
making clear the importance of studying downstream TP73 targets that could predict the outcome of colon cancer patients better than ∆TAp73 themselves do.

**INTRODUCTION**

TP73, a member of the p53 family, is expressed in multiple variants. TAp73 isoforms (full-length) have tumor suppressor potential (1), while ∆TAp73 variants (∆Ex2p73, ∆Ex2/3p73, ∆Np73 and ∆N´p73), lacking the transactivation domain, show oncogenic properties (2-7). TP53 and TP73 share significant structural and functional homology (8), although some evidence shows that their roles differ in human tumorigenesis. The two genes are activated through different pathways after DNA damage, and are capable of inducing cell cycle arrest and cell death. Unlike TP53, inactivating mutations of TP73 are extremely rare in human tumors (9). Moreover, although TP73 can activate some TP53-responsive genes to varying degrees, such as those induced after DNA damage (10, 11), recent analyses demonstrated that p73 has its own set of target genes (11, 12), indicating unique and overlapping functions for this family. Further complexity is revealed by the fact that the members of the TP53 family can transactivate common target genes but through the recognition of distinct binding elements (11). In addition, several reports have indicated that ∆Np73 acts downstream from TP53 and TAp73 as a transcriptional negative regulator that competes for binding motifs in target promoters (4, 13). The latest is supported by the evidence that loss of ∆Np73 makes some target promoters more accessible to TP53 and TAp73 (14).

TP53 knockout mouse develops normally, but shows high predisposition to spontaneous tumors (15). In contrast, TP73-null mice (all TP73 isoforms absent) have developmental neurological and immunological defects with early deaths, but no tumor predisposition (16). Specifically, mice lacking TAp73 show neurological defects and
develop spontaneous malignancies (17), while ΔTAp73-null mice display signs of neurodegeneration and impaired tumorigenic capability (14).

Several studies have reported the expression status of TP73 isoforms in human tumors (18-22), but few showed the involvement of ΔTAp73 isoforms in the prognosis of patients (23-31). The failure or lack of tumor response is mainly due to drug resistance mechanisms. In clinical oncology, multidrug resistance remains an unresolved problem. TP53 and TAp73 isoforms have functions in the apoptotic response to drug-induced DNA damage (32-34). ΔNp73 acts as a dominant-negative inhibitor of TAp73 and wild-type p53 (4), inhibiting drug-induced apoptosis (25, 35). In addition, TP73 regulates the expression of genes associated with drug resistance. Specifically, upregulation of the activity of ABCB1, HMGB1 and CASP1 promoters by p73 variants have been described previously (35-38). MDR1 is a P-glycoprotein that acts as an energy-dependent drug efflux pump and is often overexpressed in multidrug-resistant tumor cells (39). HMGB1 is involved in several biological processes, including invasiveness, transcription, DNA repair and drug resistance mechanisms (40, 41). Lastly, CASP-1 plays an important role in several apoptosis pathways (38) and has been described as being downregulated in several tumor types (42, 43). Additionally, overexpression of ABCB1 and HMGB1 has been associated with poor prognosis of cancer patients (44-46).

This is the first report that shows the prognosis value of ΔTAp73 variants and their target genes involved in drug resistance in patients diagnosed with colon cancer. The positive correlation found between ΔTAp73 variants and HMGB1 and ABCB1 expression supports them as TP73 targets. The fact that ΔTAp73 isoforms are associated with shortened OS confirms their oncogenic role and plausible value as prognostic markers. ABCB1 and HMGB1 strongly predict OS in an independent manner, making
clear the importance of studying downstream TP73 targets that could predict the outcome of colon cancer patients better than ΔTAp73 themselves do.

MATERIALS AND METHODS

Patients, tumor samples and nucleic acid extraction

The present study, approved by the research ethics board of the Puerta de Hierro Majadahonda University Hospital (Madrid, Spain), was based on a consecutive series of 77 patients undergoing surgery for colorectal cancer, between January 2001 and January 2003. All colon cancer patients were considered sporadic cases because no clinical antecedents of FAP were reported and those with clinical criteria of hereditary non-polyposis colorectal cancer (Amsterdam criteria) were excluded. Both normal and tumor tissues were obtained sequentially, immediately after surgery, snap-frozen in liquid nitrogen and stored at -80ºC until processing.

All tumors were histologically examined by a pathologist to confirm the diagnosis of colon cancer, verify the presence of tumor, select those samples with at least 75% tumor tissue and establish the pathological stage.

RNA was extracted from approximately 30mg of colon tumor and normal samples using the RNeasy Mini Kit (Quiagen Inc, Hilden, Germany). After extraction, RNA was quantified spectrophotometrically.

Real-Time Polymerase Chain Reaction

mRNA levels were detected in the normal and tumor counterpart samples by a relative quantification approach in which the amount of the targets is expressed in relation to the geometric average of the three reference housekeeping genes, as described in detail elsewhere (20). The relative concentrations of the target and the reference genes were calculated by interpolation, using a standard curve of each gene plotted from a serial dilution of a cDNA prepared from the RNA of an individual...
expressing the specific analyzed gene. The expression level of the target gene in a patient was calculated as a ratio: target in tumor tissue to target in normal tissue (T:N). For the synthesis of the first-strand cDNA, 400ng of RNA was reverse-transcribed, using the Gold RNA PCR Core Kit (Applied Biosystems) according to the manufacturer’s instructions. Random hexamers were used as primers for cDNA synthesis.

Real-time polymerase chain reaction (qPCR) was performed in a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Each reaction was performed in a final volume of 20 µl containing 2 µl of the cDNA product sample, 0.5 µM of each primer and 1x reaction mix including FastStar DNA polymerase, reaction buffer, deoxyribonucleotide triphosphates (dNTPs) and SYBR green.

Thermal cycling for all genes was initiated with a denaturing step at 95°C for 10 min and then subjected to 40 cycles of PCR (denaturing at 94°C 10 s, annealing at a different temperature for each gene -- 67° 5s for ABCB1, 58° 5s for HMGB1 and 62° 4s for CASP1 -- and elongation at 72°C for 5 s, in which fluorescence was acquired). At the end of the PCR cycles, melting curve analyses were performed, followed by sequencing, in order to validate the generation of the specific PCR product expected.

Primer sets for ΔEx2p73, ΔEx2/3p73, ΔNp73 and TA73 and the conditions for each reaction have been described elsewhere (20). Primer pairs for ABCB1, HMGB1 and CASP1 were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA). The following primers were used: forward, 5’CTATGCATCTTATGCTCTGGCC3’; reverse, 5’CCTGTCCAACACTAAAAGCCC3’; forward, 5’ACCCAGATGCTTCAGTCAACTTC3’; reverse,
5’TGCCATATCTTCAAATTTTCCTTTC3’ for HMGB1; and forward, 5’AGTTACCTGGCAGGGACGCT3’; reverse, 5’TGGAAAGGAAGAAAGTACTCCTTGA 3’ for CASP1.

Proliferation, migration, invasion and drug resistance experiments

The colon cancer cells HCT116 were obtained from the American Type Cell Collection and maintained in DMEM (Lonza group Ltd, Switzerland). Cells were seeded in triplicate and transiently transfected with a pcDNA plasmid encoding ΔNp73 or the empty vector (kindly provided by Dr Marín, Instituto de Biomedicina, Universidad de León, Spain), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 24, 48, 72 and 96 hr post-transfections, different fractions were kept to preserve cells, isolate RNA and/or protein. RNA samples were submitted to a DNAase treatment for evaluation of the levels of ΔNp73, HMGB1, ABCB1 and CASP1.

Proliferation was evaluated by three different approaches: First, cell density with a cell counter apparatus (Digital Bio, Seoul, Korea). Second, 2x10^4 colon cells were seeded in quadruplicates in 96 well E-plates to perform: a) MTT cell proliferation and survival assay kit (Cayman Chemical Company, Ann Arbor, MI). At 24, 48 and 72h post-transfections, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazole (MTT) reagent was added and absorbance was measured on a microplate reader at 570nm (Multiskan Ex, Thermo Scientific). b) analysis by RT-CES™ microelectronic cell sensor System (ACEA, San Diego, CA). Cells were placed on the reader in the incubator for continuous recording of impedance (every 10 minutes for 96 hours) as reflected by cell index (Abassi et al., 2009). Cells were transfected when attached (15 hours after seeded), and impedance changes are shown 12 hours after transfection (after 27 hours of the beginning of the process).
For drug resistance experiments, HCT116 cells were treated with 100 μM oxaliplatin for 36 hours. Subsequently, floating and adherent cells were trypsinized and checked for viability by flow citometry using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, California). Specifically, cells were resuspended in 1x binding buffer at a concentration of 1x10^6 cells/ml. Two hundred microliters of the cell suspension were transferred to a 5-ml polypropylene tube, and 5 μl each of PI (50μg/ml stock) and Annexin V-FITC were added simultaneously. Cells were mixed and incubated at room temperature for 15 minutes in the dark. Cells were analyzed within 30 minutes.

HCT116 cells were cultured on 8.0 μm pore Transwells (Corning Incorporated) previously covered with either 0.5% gelatin for migration assays or with Matrigel matrix (125 μg/ml, BD Biosciences) for invasion assays. Previously to culture, cells were labeled with Cell Tracker Green (CMFDA C2925, Invitrogen, Oregon, USA). After several time-points, from 8 to 72h, cells getting the lower surface of the filter were recovered by trypsinization and counted by fluorescence with WALLAC plate reader (Ex: 485nm; Em: 535nm) by interpolation using a standard curve.

**TP53 analysis**

TP53 immunophenotypic analysis in the colon samples was performed according to standard procedures, with overnight incubation in the presence of the cl1801 mouse monoclonal antibody (Oncogene Sciences, Manhasset, NY). Immunodetection was performed with peroxidase-labeled streptavidin biotin (LSA; DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as substrate. All immunostaining was performed using the TechMate 500 (DAKO) automatic immunostaining device. cl1801 mouse monoclonal antibody was used because of its ability to detect up to 89% of TP53 point mutations (48). Tissue samples exhibiting definitive nuclear (or nuclear and
cytoplasmic) staining in > 10% of the epithelial cells were considered positive for TP53. Cases displaying no nuclear staining were considered negative.

**Clinico-pathological parameters**

The following parameters were obtained from the medical records of the 77 patients: age, tumor size, tumor location, lymph node metastases, pathological stage, histological grade and vascular invasion (VI). Pathological stage was assessed by the tumor-node-metastases (TNM) classification. Presence of lymph node metastases was evaluated by optical microscopy. No other immunohistochemical or molecular techniques were used. No patient received chemotherapeutic treatment prior surgery.

**Patients’ follow-up**

Clinical follow-up after diagnosis and surgery was based on periodic visits (every 3 months during the first year, every 6 months during the second year, and then yearly until relapse in our Medical Oncology Department, complemented by other periodic controls in Health Centers of our Hospital), on clinical and biochemical tests and on CT scans. In addition, an ultrasonic study was performed when liver function was impaired. Overall and Disease-Free Survival (OS and DFS) were the study endpoints. OS was defined as the period from time of diagnosis until death. Disease-free survival was defined as the interval between diagnosis and first recurrence.

**Statistical analysis**

As the values of gene expression (T:N ratio) displayed non-normal distribution (Kolmogorov-Smirnov test, Lilliefors correction), the data were normalized by log\(_{10}\) transformation. For the same reason, we used the geometric rather than the arithmetic average of the T:N ratio to describe the gene expression data.

Expression of \textit{TP73} isoforms, \textit{ABCB1}, \textit{CASP1} and \textit{HMGB1} were divided into bicentiles and tertiles. The DFS analysis did not include the patients with pathological
stage IV. Overall survival distribution was estimated by Kaplan-Meier method (49), and differences between groups were tested using the log-rank test (50). A Cox proportional hazard univariate and multivariate analysis was also performed, including relative risk and confidence intervals (95%CI). Finally, the Cox proportional risk regression model was fitted to data to estimate the independent prognostic importance of OS and DFS and confuser variables were analyzed (51). The model’s basic assumptions were evaluated (proportional hazards).

For statistical study of quantitative variables in the proliferation assays, the mean and standard deviation were calculated. Student t-test were performed to compare mean values of mock and ΔNp73 cells.

All p values were two-sided, and values <0.05 were considered to indicate statistical significance. Analyses were performed using SPSS v.14 (SPSS, Chicago, IL, USA).

RESULTS

Association between TP73 isoform levels and tumor stage

Pathological stage is the prognostic factor that has the most clearly demonstrated practical use in colorectal cancer. In a previous report of a series of 113 colorectal cancer patients, we found an association between tumor stage and expression levels of ΔEx2/3p73 and ΔNp73 isoforms (20). Our current series of 77 patients is included in the aforementioned one and we posited whether this association was maintained. ΔEx2/3p73 expression was significantly higher in stage IV (p = 0.04), with geometric averages of 0.24 for stage I, 0.25 for stage II, 0.16 for stage III and 7 for stage IV. ΔNp73 levels increased in parallel with stage (p = 0.03). The geometric average expressions were 0.009, 0.27, 0.36 and 5.33 in stages I, II, III and IV, respectively.

Correlation between expression of TP73 variants and prognosis
The follow-up period of our series was the interval between surgery and the time of last medical appointment or death. As of October 2009, the series had been followed for a median of 70 months (range of follow-up: 3 – 104 months). During this period 19 recurrences (24.3%) were recorded and 21 patients (27%) died. Description of the number of recurrentes and exitus in the different categories for each variable is shown in Table 1.

**Disease-free survival (DFS)**

Kaplan-Meier and univariate analyses were performed to determine the influence of stage and TP73 isoform levels on DFS. No statistical associations were observed between TP73 variant levels and DFS. As expected, tumor stage correlated in both statistical approaches with DFS ($p = 0.002$ and $p = 0.02$ for Kaplan-Meier and univariate analysis, respectively). Patients at stage III had a 5-year DFS rate of 59.6% (95% CI, 36.3%-82.9%); patients at stage II, 76.4% (95% CI, 57.6%-95.2%); and those at stage I, 100%. In the multivariate analysis, the pathological stage was seen as a statistically supported factor in DFS prediction ($p = 0.015$).

**Overall survival (OS)**

In the final analysis, the 5-year OS for patients was 57% (95% CI, 43.5%-70.5%).

Tumor stage correlated in Kaplan-Meier and univariate analysis with OS ($p < 0.0001$ and $p < 0.0001$, respectively). Patients at stage IV had a 5-year OS rate of 20% (95% CI, 0%-55.1%); patients at stage III, 32.4% (95% CI, 10.3%-54.5%); patients at stage II, 76.7% (95% CI, 62.4%-91%); and those at stage I, 87.7% (95% CI, 64.6-100%). The Kaplan-Meier survival analysis revealed an association between OS and $\Delta Ex2/3p73$ expression when its levels were divided into bicentiles ($p = 0.038$) (Figure
1A). Patients with low $\Delta Ex2/3p73$ expression had a 5-year OS rate of 66.8% (95% CI, 47.2%-86.4%), whereas patients with high levels had a rate of 48.2% (95% CI, 31.1%-65.3%) (Figure 1B). A trend was observed in OS for the expression of $\Delta Np73$ ($p = 0.06$). Patients with low expression had a 5-year OS rate of 72.4% (95% CI, 56.9%-87.9%), whereas patients with high levels had a rate of 39.6% (95% CI, 17.3%-61.9%).

**Correlation between TP73 isoform expression and mRNA levels of drug-resistant related genes**

Direct correlations were found between the levels of $\Delta Ex2p73$, $\Delta Ex2/3p73$ and $\Delta Np73$ and $HMGB1$ expression (Table 2). Similarly, a significant statistical trend was observed between $\Delta Ex2p73$, $\Delta Ex2/3p73$ and $\Delta Np73$ expression and $ABCB1$ levels (Table 2). No other correlations were identified.

**Correlation between levels of drug-resistant related genes and prognosis**

**Disease-free survival**

Kaplan-Meier and univariate analyses were performed to determine the influence of $ABCB1$, $CASP1$ and $HMGB1$ levels on DFS. No statistical associations were observed.

**Overall survival**

Patients were divided into bicentiles based on $ABCB1$, $CASP1$ and $HMGB1$ levels. Since no differences between low and high levels were observed for OS, we decided to divide patients into tertiles. Thus, patients presented low, median or high levels of expression. No association was observed for the expression of $ABCB1$ ($p = 0.1$). Patients with low expression had a 5-year OS rate of 74.9% (95% CI, 55.7%-94.1%); patients with median levels, a rate of 69.6% (95% CI, 50.8%-88.4%); and those with the highest levels, a rate of 35.5% (95% CI, 12.6%-58.4%) (Figure 2A). The
Kaplan-Meier graph revealed similar behavior of median- and low-level tertiles (Figure 2A). Thus, these patients were grouped as above, and ABCB1 expression was analyzed further with only two categories: low and high expression levels of ABCB1. When OS was analyzed in these two groups a significant difference was observed, since patients with low ABCB1 expression had a 5-year OS rate of 71.8% (95% CI, 58.3%-85.3%) and patients with high expression, a rate of 35.5% (95% CI, 12.6%-58.4%) (p = 0.03) (Figure 2B).

No correlation was observed, either, for HMGB1 expression (p = 0.1). Patients with low expression had a 5-year OS rate of 69.7% (95% CI, 44.2%-95.2%); patients with median levels, a rate of 45.2% (95% CI, 24%-66.4%); and those with the highest levels, a rate of 58.3% (95% CI, 38.5%-78.1%) (Figure 3A). The Kaplan-Meier graph revealed similar behavior of median- and high-level tertiles (Figure 3A). Thus, these patients were grouped as above, and HMGB1 expression was analyzed further with only two categories: low and high expression levels of HMGB1. When OS was analyzed in these two groups a significant difference was observed, since patients with low HMGB1 expression showed a 5-year OS rate of 69.7% (95% CI, 44.2%-95.2%) and patients with high expression, a rate of 51% (95% CI, 36.1%-65.9%) (p = 0.04) (Figure 3B).

Similar results were found in the univariate analysis, in which expression levels of ABCB1 and HMGB1 were seen as a statistically supported factor in OS prediction (p = 0.04 and p = 0.05, respectively).

Multivariate analysis showed that tumor stage, ABCB1 and HMGB1 had independent relationships with OS. When TP73 isoform expression data were included in the multivariate analysis, tumor stage, HMGB1 and ABCB1 levels again showed independent relationships with OS (Table 3).
Correlation between expression of TP73 variants and prognosis depending on TP53 status

Positive TP53 immunostaining (nuclear), suggesting TP53 mutations, was observed in 53 out of 77 colon patients (70%)

Disease-free survival

Kaplan-Meier and univariate analyses were performed to determine the influence of TP73 isoforms on DFS depending on TP53 status. Patients were divided into bicentiles based on ∆Ex2p73, ∆E2/3p73, ∆Np73 and TAp73 levels. In those cases showing a positive immunostaining for TP53 (suggestive of mutation) a significant difference was observed regarding ∆E2/3p73 since patients with low ∆E2/3p73 expression had a 5-year SLE rate of 71.2% (95% CI, 51.6%-90.8%) and patients with high expression, a rate of 88% (95% CI, 64%-98%) (p = 0.035). The univariate and multivariate analyses revealed no differences.

Overall survival

The Kaplan-Meier analysis revealed a trend when TAp73 levels were divided in quartiles since patients in the first lower quartiles had a 5-year OS rate of 70% (95% CI, 59%-88%) and patients in the 4th quartile, showing the higher TAp73 expression, a rate of 81% (95% CI, 65%-89%) (p = 0.1). The univariate and multivariate analyses revealed no differences.

Ectopic expression of ∆Np73 increases proliferation, drug resistance and modify the levels of HMGB1, ABCB1 and CASP1

We transiently transfected HCT116 colon cancer cells with an expression vector containing ∆Np73 or the empty vector. After 72 hours of transfection a statistical significance increase in the cell number was observed in those cells ectopically
expressing ΔNp73 (Figure 4A). The MMT cell proliferation assay also confirmed this fact (Figure 4B). Additionally, a significant difference in the initiation and rate of proliferation measured by the cell index and the slope of the curves in the RT-CES™ System was observed between both cells (Figure 4C). The ectopic expression of ΔNp73 does not compromise the viability of the cells, being in both cell types, those overexpressing the isoform and the control one >95-98%.

Cells expressing the ΔNp73 vector showed 30% higher viability after oxaliplatin exposure than those transfected with the mock vector (Figure 5). Oxaliplatin did not modify the endogenous levels of ΔNp73.

No modification in migration and invasion was detected.

Ectopic expression of ΔNp73 led to a 6-20 fold increase in its mRNA levels compared with the mock vector. This increase was accompanied by an upregulation in the mRNA levels of ABCB1 and HMGB1 of 2-8 folds. No modifications in CASP1 levels were detected.

**DISCUSSION**

Although several studies have linked the upregulation of specific TP73 isoforms with poor tumor prognosis parameters (20), little information is available on the impact of the altered expression of TP73 variants on patient survival. ΔNp73 overexpression is associated with shorter survival in patients with neuroblastoma (27), medulloblastoma (28) and lung (26), hepatocellular (30) and cervical squamous cell carcinoma (29). In addition, ΔEx2/3p73 variant upregulation associates with low-grade glioma patients’ survival (31). In our colon cancer patient series, we observed that overexpression of ΔEx2/3p73 and ΔNp73 forms predict OS, although only the pathological stage remains
as an independent predictor in the multivariate analysis. Two reports have described the upregulation of TP73 as an independent marker of colorectal cancer patient survival (23, 52). These publications analyzed the general levels of TP73 without taking into consideration the different variants that could really be involved in the shortening of survival. Although the general levels of TP73 could be used in the clinical setting as a survival predictor, there has recently been increasing focus on unravelling which specific TP73 isoforms really support the oncogenic role in human cancer processes. Our results point to ∆Ex2/3p73 and ∆Np73 as the variants which may contain these oncogenic properties. Intriguingly, we have observed that those cases with concomitant overexpression of specific TP73 isoforms and inactive TP53 showed a better outcome. It may be possible that the inactivation of TP53 through mutation could trigger specific tumor suppressor pathways that might partially compensate the oncogenic environment generated by the overexpression of the ∆TAp73 variants. It is mandatory to unravel the complex mechanisms involved in the simultaneous regulation of target genes by TP53, TAp73 and ∆Np73 isoforms and the putative feedback among them in order to get solid conclusions from the cancer patient studies. Lastly, it is interesting to note that both TAp73 and ∆TAp73 forms were found to be upregulated in a significant number of our colon tumors. It is possible that the presence of ∆TAp73 variants, even at low levels, completely suppresses the transactivation activity of TAp73, with the consequent elimination of essential TAp73 antitumorigenic function. Furthermore, at the protein level, ∆TAp73 isoforms have been described to be more stable than those of TAp73, what can contribute to promote a cellular oncogenic context (2, 13). The use of compounds that can increase the stability of TAp73 variants, as netrin-1 (53), could diminish this tumorigenic environment.
The association of the overexpression of $\Delta TAp73$ isoforms with shorter survival could be due to some putative TP73 target genes being involved in drug resistance, invasiveness and other stages of the tumorigenesis process. $ABCB1$, $HMGB1$ and $CASP1$, among others, have been described as TP73 targets (35-38). These previous data are supported by the fact that the ectopic expression of $\Delta Np73$ in our cellular system induces the upregulation of $ABCB1$ and $HMGB1$. In our study, direct statistical correlation was found between expression of $\Delta Ex2p73$, $\Delta Ex2/3p73$ and $\Delta Np73$ and $HMGB1$ levels. Furthermore, a direct trend was observed between the same variants and $ABCB1$ expression levels. This supports the possible positive regulation of $HMGB1$ and $ABCB1$ by the $\Delta TAp73$ forms in vivo in colorectal carcinomas. In a larger colon cancer patient series, the correlation between $ABCB1$ and $\Delta TAp73$ variants might reach statistical significance. Although $TP73$ has been described as regulating $CASP1$ expression, no such a direct correlation between $CASP1$ levels and $TAp73$ expression was found in our set of patients (38).

Additionally, $ABCB1$ and $HMGB1$ overexpression was associated with shorter OS of patients. In the multivariate analysis including clinico-pathological parameters of the tumors and the levels of $TP73$ variants, $ABCB1$, $HMGB1$ and $CASP1$, we observed that, in addition to tumor stage, $ABCB1$ and $HMGB1$ expression were also strong independent predictors of OS. These data underline the importance of identifying the specific targets downstream of $\Delta TAp73$ isoforms, which might have an oncogenic function and could be stronger than TP73 variants themselves in predicting patient outcome. As such, they could be used as prognosis markers in the clinical setting. Little is known about the relevance of $ABCB1$ and $HMGB1$ to the outcome of cancer patients, although the fact that upregulation of $ABCB1$ and $HMGB1$ has been associated in a few reports with poor prognosis of cancer patients sustains our hypothesis (44-46).
Remarkably, the finding that the ectopic expression of ΔNp73 increases the proliferation rate and confers resistance to oxaliplatin to colon cancer cells strengthens the oncogenic potential of this specific isoform and its involvement in specific tumorigenesis processes. As previously reported by other groups (54, 55), the exposure of the cells to oxaliplatin did not modify the endogenous levels of TP73 variants. It is possible that those tumours already expressing high levels of ΔNp73 can show resistance to the treatment, additionally, the oxaliplatin action could select gradually the cells overexpressing this putative oncogenic p73 variant, ending up with a resistant tumour.

As cumulative data support the oncogenic role of ΔTAp73 isoforms (14, 56), the mechanisms and targets underlying these functions are currently of great interest. Here, we present original data regarding the impact of specific TP73 variants in the outcome of colon cancer patients. Additionally, we found that putative ΔTAp73 isoform targets are independent prognosis markers of OS. Specifically, upregulation of ABCB1 and HMGB1 predicts in a strong independent manner the OS of patients diagnosed with colon cancer. Further experiments are needed to identify specific targets of ΔTAp73 isoforms that perform an oncogenic role and could be used as clinical markers of relapse.

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**REFERENCES**


Table 1. Number of recurrences and exitus in the different categories for each variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Recurrences (n = 19/77)</th>
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<td>7/42</td>
<td>16,6</td>
<td>5/42</td>
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<tr>
<td></td>
<td>III</td>
<td>9/20</td>
<td>45</td>
<td>9/20</td>
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<td>IV</td>
<td>3/6</td>
<td>50</td>
<td>6/6</td>
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<td>14,6</td>
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<td>41,4</td>
<td>14/29</td>
<td>48,3</td>
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<td>8/50</td>
<td>16</td>
<td>11/50</td>
<td>22</td>
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<td></td>
<td>Moderate</td>
<td>10/22</td>
<td>45</td>
<td>9/22</td>
<td>41</td>
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<tr>
<td></td>
<td>Poor</td>
<td>1/5</td>
<td>20</td>
<td>1/5</td>
<td>20</td>
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<tr>
<td>LNM</td>
<td>No</td>
<td>8/53</td>
<td>15</td>
<td>7/53</td>
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<td>Yes</td>
<td>11/24</td>
<td>46</td>
<td>14/24</td>
<td>58</td>
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<td>Bicentiles △Ex2p73 expression</td>
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<td>26</td>
<td>9/38</td>
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<tr>
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<td>23</td>
<td>12/39</td>
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<td>Bicentiles △Ex3p73 expression</td>
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<td>High</td>
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<td>13/39</td>
<td>33</td>
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<tr>
<td>Bicentiles △Np73 expression</td>
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<td>29</td>
<td>8/38</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>8/39</td>
<td>21</td>
<td>13/39</td>
<td>33</td>
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<tr>
<td>Bicentiles TAp73 expression</td>
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<td>29</td>
<td>12/38</td>
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<td>High</td>
<td>8/39</td>
<td>21</td>
<td>9/39</td>
<td>23</td>
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<td>Bicentiles ABCB1 expression</td>
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<td>22</td>
<td>9/51</td>
<td>18</td>
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<td>High</td>
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<td>31</td>
<td>12/26</td>
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<td>Bicentiles HMGB1 expression</td>
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<td>11</td>
<td>4/26</td>
<td>15</td>
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<tr>
<td></td>
<td>High</td>
<td>16/51</td>
<td>31</td>
<td>17/51</td>
<td>33</td>
</tr>
<tr>
<td>Bicentiles CASP1 expression</td>
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<td>26</td>
<td>12/38</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>9/39</td>
<td>23</td>
<td>9/39</td>
<td>23</td>
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</tbody>
</table>
Table 2. Correlations between expression levels of p73 isoforms and HMGB1, ABCB1 and CASP1 for colon human cancer patients

<table>
<thead>
<tr>
<th></th>
<th>ΔEx2p73</th>
<th>ΔEx2/3p73</th>
<th>ΔNp73</th>
<th>TAp73</th>
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</thead>
<tbody>
<tr>
<td>HMGB1</td>
<td>p &gt; 0.0001, r = 0.4</td>
<td>p = 0.012, r = 0.28</td>
<td>p = 0.04, r = 0.23</td>
<td>ns</td>
</tr>
<tr>
<td>ABCB1</td>
<td>p = 0.06, r = 0.28</td>
<td>p = 0.08, r = 0.25</td>
<td>p = 0.08, r = 0.25</td>
<td>ns</td>
</tr>
<tr>
<td>CASP1</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

*p* is calculated by analysis of variance; *r* is the Pearson coefficient. *ns*, no significant statistical correlation.
**Table 3.** Univariate and multivariate analysis of the association between $p73$ isoforms and MDR1, HMG1 and Caspase-1 expression and clinicopathological characteristics and overall survival of colon cancer patients. The blank cells correspond to variables which showed no independent relationship to OS in the multivariate analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Univariate analysis</th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>$p$ Value</td>
<td>HR (95% CI)</td>
<td>$p$ Value</td>
</tr>
<tr>
<td>Stage</td>
<td>I vs II</td>
<td>1.6 (0.19-12.86)</td>
<td>0.66</td>
<td>0.57 (0.06-5.5)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>I vs III</td>
<td>8.8 (1.13-68.5)</td>
<td>0.037</td>
<td>7.18 (0.86-60.1)</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>I vs IV</td>
<td>75.6 (7.47-764.8)</td>
<td>&lt;0.0001</td>
<td>26.5 (2.42-289.9)</td>
<td>0.007</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>Yes vs No</td>
<td>5.13 (2.23-11.81)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Differentiation</td>
<td>Well vs Poor</td>
<td>2.29 (0.83-6.3)</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate vs Poor</td>
<td>1.69 (0.21-13.64)</td>
<td>0.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LNM</td>
<td>Yes vs No</td>
<td>7.23 (3.19-16.37)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicentiles $\Delta Ex2p73$ expression</td>
<td>Low vs high</td>
<td>1.53 (0.7-3.35)</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicentiles $\Delta Ex2/3p73$ expression</td>
<td>Low vs high</td>
<td>2.28 (1.02-5.1)</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicentiles $\Delta Np73$ expression</td>
<td>Low vs high</td>
<td>2.1 (0.94-4.68)</td>
<td>0.07</td>
<td></td>
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<tr>
<td>Bicentiles $\Delta Tap73$ expression</td>
<td>Low vs high</td>
<td>0.73 (0.34-1.56)</td>
<td>0.4</td>
<td></td>
<td></td>
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<tr>
<td>Bicentiles $ABCB1$ expression</td>
<td>Low vs high</td>
<td>2.28 (1.04-4.99)</td>
<td>0.04</td>
<td>4.5 (1.48-13.92)</td>
<td>0.008</td>
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<tr>
<td>Bicentiles $HMG1$ expression</td>
<td>High vs Low</td>
<td>2.61 (0.99-6.9)</td>
<td>0.05</td>
<td>6.25 (1.61-24.19)</td>
<td>0.008</td>
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<tr>
<td>Bicentiles $CASP1$ expression</td>
<td>Low vs high</td>
<td>0.70 (0.31-1.57)</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LEGENDS TO FIGURES

Figure 1: Influence of colon carcinoma levels of $\Delta Ex2/3p73$ (A) and $\Delta Np73$ (B) variants on overall survival (OS), Kaplan-Meier curves and $p$ values. Expression was distributed in low and high levels by the median.

Figure 2: Influence of colon carcinoma levels of $ABCB1$ on overall survival (OS), Kaplan-Meier curves and $p$ values. A) $ABCB1$ expression in colon cancer patients distributed by tertiles in low, median and high levels. B) $ABCB1$ expression in colon cancer patients distributed in two groups: low (new variable combining low and median levels) and high expression.

Figure 3: Influence of colon carcinoma levels of $HMGB1$ on overall survival (OS), Kaplan-Meier curves and $p$ values. A) $HMGB1$ expression in colon cancer patients distributed by tertiles in low, median and high levels. B) $HMGB1$ expression in colon cancer patients distributed in two groups: high (new variable combining median and high levels) and low expression.

Figure 4: Ectopic expression of $\Delta Np73$ increases the proliferation of HCT116 colon cancer cells 72 hours after transfection. A) Statistical significant increase in the number of cells. Experiments were done in quadruplicates and counted in a cell counter apparatus (Digital Bio, Seoul, Korea). B) MTT assay shows that ectopic expression of $\Delta Np73$ lead to an increase in the cell proliferation rate compared to the mock HCT116 cells (*$p<0.0001$). C) Significant difference in the initiation and rate of proliferation measured by the cell index and the scope of the curves in the RT-CES™ System
(ACEA, San Diego, CA) (**p<0.0001; p value was calculated taken the different cell index measurements in the exponential cellular growth phase).

**Figure 5:** Ectopic expression of ΔNp73 induces resistance to oxaliplatin to colon cancer cells. HCT116 cells were exposed to 100 μM oxaliplatin for 36 hours and check for viability by flow citometry. Those cells containing the ΔNp73 expressing vector showed an increase of 30% of cell viability. Representative of 3 independent experiments.
- Figure 1 -

A

ΔEx2/3p73

Low

High

Cumulative survival

Overall survival (months)

p = 0.038

B

ΔNp73

Low

High

Cumulative survival

Overall survival (months)

p = 0.06
- Figure 2 -
- Figure 3 -
Clinical Cancer Research

Prognostic impact of ΔTAp73 isoform levels and their target genes in colon cancer patients

Beatriz Soldevilla, Raquel Díaz, Javier Silva, et al.

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