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Imaging, Diagnosis, Prognosis

Prognostic Impact of ΔTAp73 Isoform Levels and Their Target Genes in Colon Cancer Patients

Beatriz Soldevilla[1], Raquel Díaz[1], Javier Silva[1], Yolanda Campos-Martín[3], Concepción Muñoz[2], Vanesa García[1], José M García[1], Cristina Peña[1], Mercedes Herrera[1], Marta Rodriguez[1], Irene Gómez[1], Nagat Mohamed[1], Margarita M. Marques[4], Félix Bonilla[1], and Gemma Domínguez[1]

Abstract

Purpose: Cumulative data support the role of ΔTAp73 variants in tumorigenic processes such as drug resistance. We evaluate the impact of *TP73* isoforms and their putative target genes *ABCB1*, *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 Δ*Ex2p73*, Δ*Ex2/3p73*, Δ*Np73*, *TAp73*, *ABCB1*, *HMGB1*, and *CASP1* by quantitative real-time reverse transcriptase-PCR. Tumor characteristics, disease-free survival, and overall survival (OS) were examined in each patient. Functional experiments were carried out to check whether ectopic expression of ΔNp73 modifies the proliferation, drug resistance, migration, and invasion properties of colon tumor cells and the expression of *ABCB1*, *HMGB1*, and *CASP1*.

Results: Positive correlations were observed between the expression levels of Δ*TAp73* variants and *HMGB1*. Furthermore, a trend was observed for *ABCB1*. Overexpression of Δ*Ex2/3p73* and Δ*Np73* isoforms was 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 *ABCB1* and *HMGB1* were associated with shorter OS ($P = 0.04$ and $P = 0.05$, respectively). Multivariate analysis showed that, in addition to the tumor stage, *ABCB1* and *HMGB1* had independent relationships with OS ($P = 0.008$). Ectopic expression of ΔNp73 was associated with an increase in proliferation and drug resistance.

Conclusions: The positive correlation between Δ*TAp73* variants and *HMGB1* and *ABCB1* expression supports them as TP73 targets. The fact that upregulation of Δ*TAp73* isoforms was associated with shortened OS, increase in proliferation, and drug resistance confirms their oncogenic role and plausible value as prognostic markers. *ABCB1* and *HMGB1*, putative Δ*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 ΔTAp73 variants themselves do. *Clin Cancer Res; 17(18); 6029–39. ©2011 AACR.*

Introduction

TP73, a member of the *p53* family, is expressed in multiple variants. TAp73 isoforms (full-length) have tumor-suppressor potential (1), whereas ΔTAp73 variants (ΔEx2p73, ΔEx2/3p73, ΔNp73, and ΔNÇp73), 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 2 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 showed 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 ΔNp73 acts downstream from TP53 and TAp73 as a transcriptional negative regulator that

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Translational Relevance

The tumor-suppressor and/or oncogenic functions of TP73 isoforms have been intensely debated recently. The publication of an article in 2010 on the specific role of Δ*TAp73* in knockout 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 prognostic value of Δ*TAp73* variants and their target genes involved in drug resistance in colon cancer patients. The positive correlation found in our colon cancer series between Δ*TAp73* variants and *HMGB1* and *ABCB1* expression supports them as TP73 targets *in vivo*. The upregulation of these 2 genes after ectopic expression of ΔNp73 in colon tumor cells also sustains this statement. The fact that Δ*TAp73* isoforms are associated with shortened overall survival (OS) and with increase in proliferation and drug resistance 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.

competes for binding motifs in target promoters (4, 13). The latest finding is supported by the evidence that loss of ΔNp73 makes some target promoters more accessible to TP53 and TAp73 (14).

TP53 knockout (KO) mice develop normally, but show high predisposition to the development of spontaneous tumors (15). In contrast, *TP73*-null mice (all *TP73* isoforms absent) have developmental neurologic and immunologic defects with early deaths, but no tumor predisposition (16). Specifically, mice lacking *TAp73* show neurologic defects and develop spontaneous malignancies (17), whereas Δ*TAp73*-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 only a few showed the involvement of Δ*TAp73* 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 has 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 biologic 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). In addition, overexpression of *ABCB1* and *HMGB1* has been associated with poor prognosis of cancer patients (44–46).

This is the first report that shows the prognostic value of Δ*TAp73* variants and their target genes involved in drug resistance in patients diagnosed with colon cancer. The positive correlation found between Δ*TAp73* variants and *HMGB1* and *ABCB1* expression supports them as TP73 targets. The fact that Δ*TAp73* isoforms are associated with shortened overall survival (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 familial adenomatous polyposis (FAP) were reported and those with clinical criteria of hereditary nonpolyposis colorectal cancer (Amsterdam criteria) were excluded. Both normal and tumor tissue samples were obtained sequentially, immediately after surgery, snap-frozen in liquid nitrogen, and stored at −80°C until further 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 pathologic stage.

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

Real-time PCR

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 3 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, 400 ng of RNA was reverse-transcribed, using

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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 PCR (quantitative PCR) was carried out in a Light Cycler apparatus (Roche Diagnostics) using the Light-Cycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Each reaction was carried out in a final volume of 20 μL containing 2 μL of the cDNA product sample, 0.5 μmol/L of each primer, and 1× reaction mix including FastStar DNA polymerase, reaction buffer, deoxyribonucleotide triphosphates, and SYBR green.

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

Primer sets for Δ*Ex2p73*, Δ*Ex2/3p73*, Δ*Np73*, and *TAp73* 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). The following primers were used: forward, 5′CTATGCATCTTATGCTCTGGCC3′; reverse, 5′CCTGTCCAACACTAAAAGCCC3′ for *ABCB1*; 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 Culture Collection and maintained in Dulbecco's Modified Eagle Medium (DMEM; Lonza Group Ltd). 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 hours posttransfection, different fractions were kept to preserve cells and 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 3 different approaches: First, cell density was assessed with a cell-counter apparatus (Digital Bio). Second, 2×10^4 colon cells were seeded in quadruplicates in 96-well E-plates to carry out an MTT cell proliferation assay (Cayman Chemical Company). At 24, 48, and 72 hours posttransfection, MTT reagent was added and absorbance was measured on a microplate reader at 570 nm (Multiskan Ex; Thermo Scientific). The RT-CES microelectronic cell sensor system (ACEA) was used for analysis. 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 (47). Cells were transfected when attached (15 hours after seeding), 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 μmol/L oxaliplatin for 36 hours. Subsequently, floating and adherent cells were trypsinized and checked for viability by flow cytometry using the Annexin V–FITC Apoptosis Detection Kit (BD Pharmingen). Specifically, cells were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Two hundred microliters of the cell suspension was transferred to a 5-mL polypropylene tube, and 5 μL each of propidium iodide (PI; 50 μg/mL stock) and Annexin V–fluorescein isothiocyanate (FITC) was 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 Inc.) previously covered with either 0.5% gelatin for migration assays or with Matrigel matrix (125 μg/mL; BD Biosciences) for invasion assays. Before culture, cells were labeled with Cell Tracker Green (CMFDA C2925; Invitrogen). After several time points, from 8 to 72 hours, cells adhering to the lower surface of the filter were recovered by trypsinization and counted by fluorescence with the Wallac Plate Reader (Ex: 485 nm; Em: 535 nm; Perkin Elmer Life Science) by interpolation using a standard curve.

TP53 analysis

TP53 immunophenotypic analysis in the colon tissue samples was conducted according to standard procedures, with overnight incubation in the presence of the cl1801 mouse monoclonal antibody (Oncogene Sciences). Immunodetection was carried out with peroxidase-labeled streptavidin biotin (LSA; DAKO) using diaminobenzidine chromogen as substrate. All immunostaining was done using the TechMate 500 (DAKO) automatic immunostaining device. The 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 more than 10% of the epithelial cells were considered positive for TP53. Cases displaying no nuclear staining were considered negative.

Clinicopathologic parameters

The following parameters were obtained from the medical records of the 77 patients: age, tumor size, tumor location, lymph node metastases, pathologic stage, histologic grade, and vascular invasion (VI). Pathologic stage was assessed by the tumor-node-metastasis (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 before undergoing surgery.

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Patient 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), clinical and biochemical tests, and computed tomography scans. In addition, an ultrasonic study was done when liver function was impaired. OS and disease-free survival (DFS) were the study endpoints. OS was defined as the period from time of diagnosis until death. DFS was defined as the interval between diagnosis and first recurrence.

Statistical analysis

As the values of gene expression (T:N ratio) displayed nonnormal 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 *TP73* isoforms, *ABCB1*, *CASP1*, and *HMGB1* was divided into bicentiles and tertiles. The DFS analysis did not include the patients with pathologic stage IV disease. OS distribution was estimated by the Kaplan–Meier method (49), and differences between groups were tested using the log-rank test (50). Cox proportional hazard univariate and multivariate analyses were also conducted, including relative risk and 95% confidence intervals (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 basic assumptions of the model were evaluated (proportional hazards).

For statistical study of quantitative variables in the proliferation assays, the mean and SD were calculated. Student *t*-test was conducted to compare mean values of mock and ΔNp73 cells.

All *P* values were 2-sided, and values less than 0.05 were considered to indicate statistical significance. Analyses were conducted using the Statistical Package for Social Sciences version 14 (SPSS v.14).

Results

Association between *TP73* isoform levels and tumor stage

Pathologic stage is the prognostic factor that has most clearly shown 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 Δ*Ex2/3p73* and Δ*Np73* isoforms (20). Our current series of 77 patients is included in the aforementioned report, and we posited whether this association was maintained. Δ*Ex2/3p73* 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. Δ*Np73* 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 recurrences and deaths in the different categories for each variable is shown in Table 1.

Disease-free survival

The Kaplan–Meier and univariate analyses were conducted 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 analyses, 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 pathologic stage was seen as a statistically supported factor in DFS prediction ($P = 0.015$).

Overall survival

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

The tumor stage correlated in the Kaplan–Meier and univariate analyses 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 Δ*Ex2/3p73* expression when its levels were divided into bicentiles ($P = 0.038$; Fig. 1A). Patients with low Δ*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; Fig. 1B). A trend was observed in OS for the expression of Δ*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 resistance related genes

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

Correlation between levels of drug resistance related genes and prognosis

Disease-free survival. Kaplan-Meier and univariate analyses were conducted to determine the influence of

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

Variable	Category	Recurrences (n = 19/77)	%	Deaths (n = 21/77)	%
Stage	I	0/9	0	1/9	11
	II	7/42	16.6	5/42	12
	III	9/20	45	9/20	45
	IV	3/6	50	6/6	100
Vascular invasion	No	7/48	14.6	7/48	14.6
	Yes	12/29	41.4	14/29	48.3
Tumor differentiation	Well	8/50	16	11/50	22
	Moderate	10/22	45	9/22	41
	Poor	1/5	20	1/5	20
LNM	No	8/53	15	7/53	13
	Yes	11/24	46	14/24	58
Bicentiles Δ*Ex2p73* expression	Low	10/38	26	9/38	24
	High	9/39	23	12/39	31
Bicentiles Δ*Ex2/3p73* expression	Low	11/38	29	8/38	21
	High	8/39	21	13/39	33
Bicentiles Δ*Np73* expression	Low	11/38	29	8/38	21
	High	8/39	21	13/39	33
Bicentiles *TAp73* expression	Low	11/38	29	12/38	32
	High	8/39	21	9/39	23
Bicentiles *ABCB1* expression	Low	11/51	22	9/51	18
	High	8/26	31	12/26	46
Bicentiles *HMGB1*expression	Low	3/26	11	4/26	15
	High	16/51	31	17/51	33
Bicentiles *CASP1* expression	Low	10/38	26	12/38	32
	High	9/39	23	9/39	23

Abbreviation: LNM, lymph node metastasis.

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. Because 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

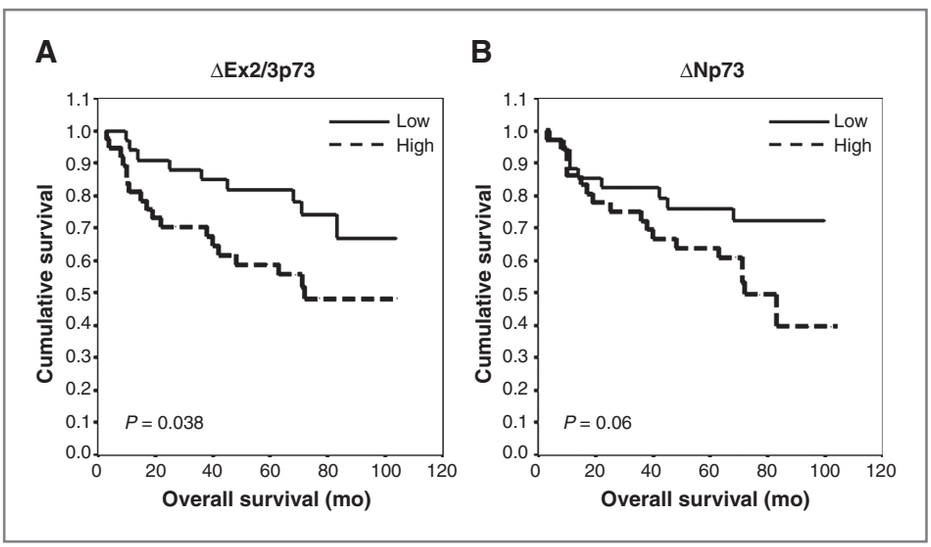

Figure 1. Influence of colon carcinoma levels of Δ*Ex2/3p73* (A) and Δ*Np73* (B) variants on OS, Kaplan–Meier curves, and *P* values. Expression was distributed in low and high levels by the median.

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Table 2. Correlations between expression levels of *p73* isoforms and *HMGB1*, *ABCB1*, and *CASP1* for human colon cancer patients

	ΔEx2p73	*ΔEx2/3p73*	*ΔNp73*	*TAp73*
HMGB1	$P > 0.0001$; $r = 0.4$	$P = 0.012$; $r = 0.28$	$P = 0.04$; $r = 0.23$	NS
ABCB1	$P = 0.06$; $r = 0.28$	$P = 0.08$; $r = 0.25$	$P = 0.08$; $r = 0.25$	NS
CASP1	NS	NS	NS	NS

NOTE: *P* is calculated by analysis of variance; *r* is the Pearson coefficient.
Abbreviation: NS, no statistically significant correlation.

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; Fig. 2A). The Kaplan–Meier graph revealed similar behavior of median- and low-level tertiles (Fig. 2A). Thus, these patients were grouped as described above, and *ABCB1* expression was analyzed further with only 2 categories: low and high expression levels of *ABCB1*. When OS was analyzed in these 2 groups, a significant difference was observed, because 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; $P = 0.03$; Fig. 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; Fig. 3A). The Kaplan–Meier graph revealed similar behavior of median- and high-level tertiles (Fig. 3A). Thus, these patients were grouped as above, and *HMGB1* expression was analyzed further with only 2 categories: low and high expression levels of *HMGB1*. When OS was analyzed in these 2 groups, a significant difference was observed, because 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; $P = 0.04$; Fig. 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 conducted to determine the influence of *TP73* isoforms on DFS depending on TP53 status. Patients were divided into bicentiles based on *ΔEx2p73*, *ΔE2/3p73*, *ΔNp73*, and *TAp73* levels. In those cases showing a positive immunostaining for TP53 (suggestive of mutation) a significant difference was observed with regard to *ΔE2/3p73*

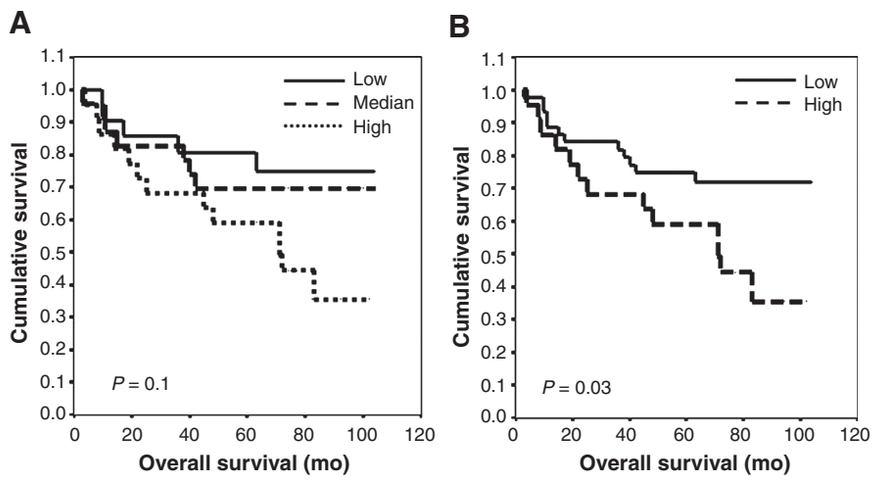

Figure 2. Influence of colon carcinoma levels of *ABCB1* on OS, Kaplan–Meier curves, and *P* values. A, *ABCB1* expression in colon cancer patients distributed by tertiles in low, median, and high levels. B, *ABCB1* expression in colon cancer patients distributed in 2 groups: low (new variable combining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

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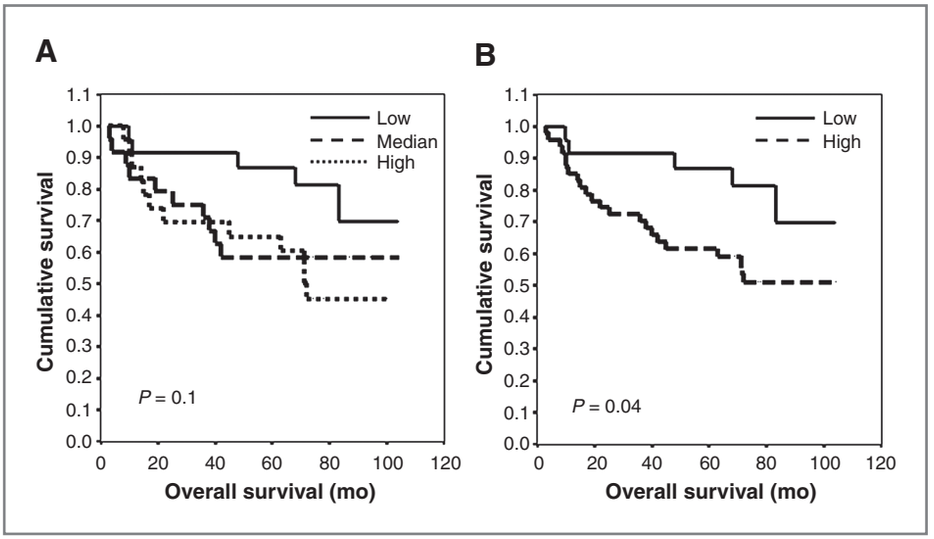

Figure 3. Influence of colon carcinoma levels of *HMGB1* on OS, Kaplan–Meier curves, and *P* values. A, *HMGB1* expression in colon cancer patients distributed by tertiles in low, median, and high levels. B, *HMGB1* expression in colon cancer patients distributed in 2 groups: high (new variable combining median and high levels) and low expression.

because patients with low Δ*E2/3p73* expression had a 5-year DFS 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 because 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 Δ*Np73* increases proliferation and drug resistance and modifies the levels of *HMGB1*, *ABCB1*, and *CASP1*

We transiently transfected HCT116 colon cancer cells with an expression vector containing Δ*Np73* or the empty vector. After 72 hours of transfection, a statistically significant increase in the cell number was observed in those cells ectopically expressing Δ*Np73* (Fig. 4A). The MTT cell proliferation assay also confirmed this fact (Fig. 4B). In addition, 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

Table 3. Univariate and multivariate analyses of the association between *p73* isoforms and *MDR1*, *HMG1*, and *caspase-1* expression and clinicopathologic characteristics and OS of colon cancer patients

		Univariate analysis			Multivariate analysis		
Variable	**Category**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Stage	I vs. II	1.6	0.19–12.86	0.66	0.57	0.06–5.5	0.63
	I vs. III	8.8	1.13–68.5	0.037	7.18	0.86–60.1	0.069
	I vs. IV	75.6	7.47–764.8	<0.0001	26.5	2.42–289.9	0.007
Vascular invasion	Yes vs. no	5.13	2.23–11.81	<0.0001			
Tumor differentiation	Well vs. poor	2.29	0.83–6.3	0.11			
	Moderate vs. poor	1.69	0.21–13.64	0.62			
LNM	Yes vs. no	7.23	3.19–16.37	<0.0001			
Bicentiles Δ*Ex2p73* expression	Low vs. high	1.53	0.7–3.35	0.28			
Bicentiles Δ*Ex2/3p73* expression	Low vs. high	2.28	1.02–5.1	0.044			
Bicentiles Δ*Np73* expression	Low vs. high	2.1	0.94–4.68	0.07			
Bicentiles *TAp73* expression	Low vs. high	0.73	0.34–1.56	0.4			
Bicentiles *ABCB1* expression	Low vs. high	2.28	1.04–4.99	0.04	4.5	1.48–13.92	0.008
Bicentiles *HMGB1*expression	High vs. low	2.61	0.99–6.9	0.05	6.25	1.61–24.19	0.008
Bicentiles *CASP1* expression	Low vs. high	0.70	0.31–1.57	0.39			

NOTE: The blank cells correspond to variables that showed no independent relationship to OS in the multivariate analysis.
Abbreviation: LNM, lymph node metastasis.

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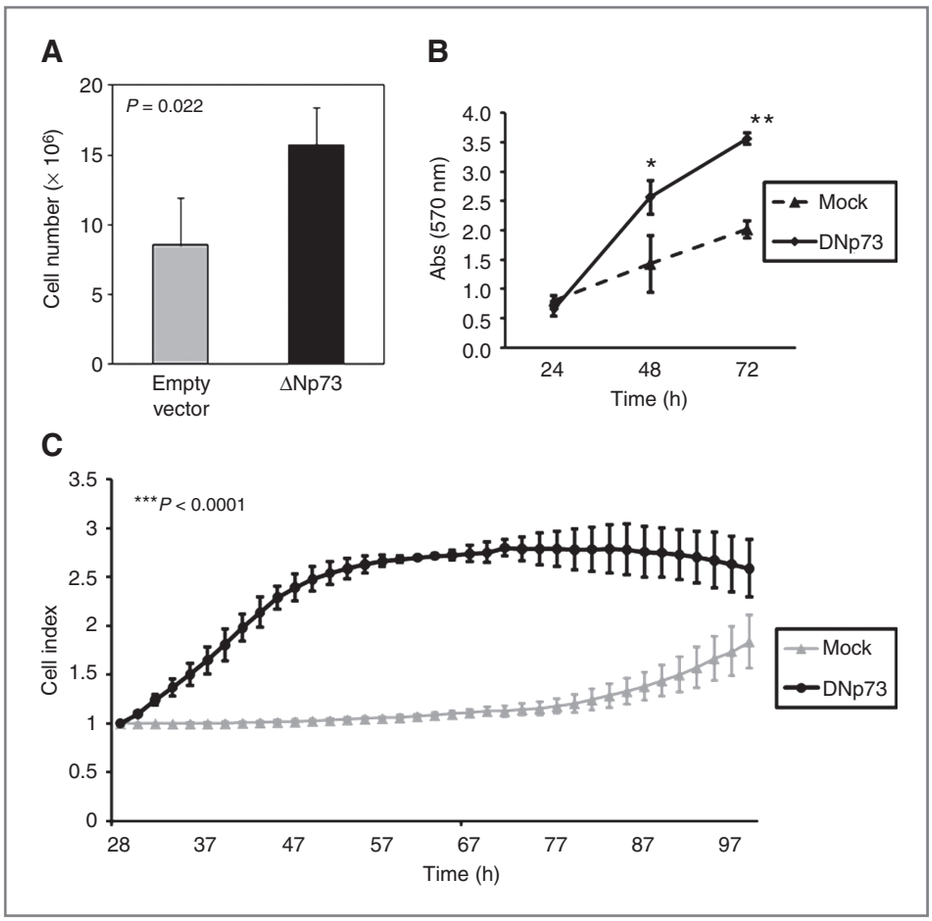

Figure 4. Ectopic expression of Δ*Np73* increases the proliferation of HCT116 colon cancer cells 72 hours after transfection. A, statistically significant increase in the number of cells. Experiments were done in quadruplicates and counted in a cell counter apparatus (Digital Bio). B, MTT assay shows that ectopic expression of Δ*Np73* leads to an increase in the cell-proliferation rate compared with the mock HCT116 cells (*, $P < 0.001$; **, $P < 0.0001$). 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 (***, $P < 0.0001$. P value was calculated taking the different cell-index measurements in the exponential cellular growth phase).

both cells (Fig. 4C). The ectopic expression of ΔNp73 does not compromise the viability of the cells, being in both cell types, with cells overexpressing the isoform and the control variant in the range of 95% to 98%.

Cells expressing the Δ*Np73* vector showed 30% higher viability after oxaliplatin exposure than those transfected with the mock vector (Fig. 5). Oxaliplatin did not modify the endogenous levels of ΔNp73.

No modification in migration and invasion was detected.

Ectopic expression of Δ*Np73* led to a 6- to 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- to 8-fold. 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. Δ*Np73* overexpression is associated with shorter survival in patients with neuroblastoma (27), meduloblastoma (28), and lung (26), hepatocellular (30), and cervical squamous cell carcinomas (29). In addition, Δ*Ex2/3p73* variant upregulation is associated with survival in patients with low-grade glioma (31). In our colon cancer patient series, we observed that overexpression of Δ*Ex2/3p73* and Δ*Np73* forms predict OS, although only the pathologic stage remains 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 unraveling which specific TP73 isoforms really support the oncogenic role in human cancer processes. Our results point to ΔEx2/3p73 and ΔNp73 as the variants that 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 for the oncogenic environment generated by the overexpression of the ΔTAp73 variants. It is mandatory to unravel the complex mechan-

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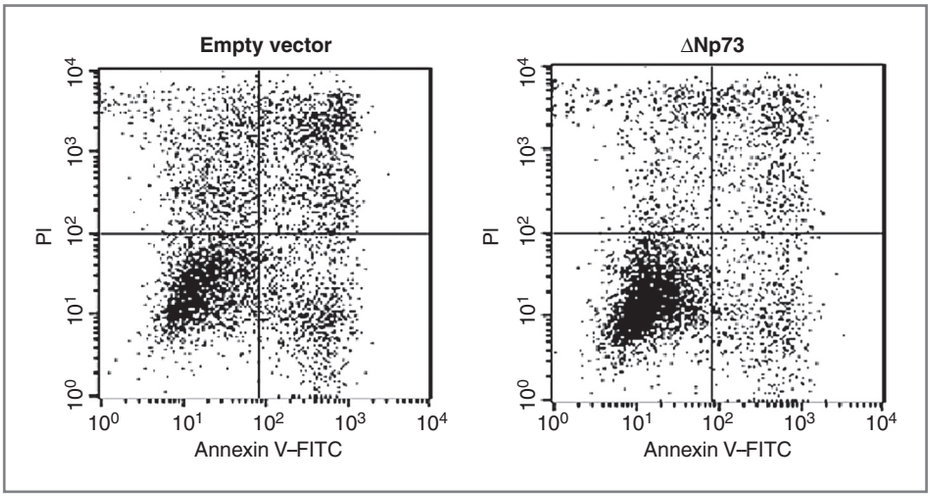

Figure 5. Ectopic expression of Δ*Np73* induces resistance to oxaliplatin in colon cancer cells. HCT116 cells were exposed to 100 μmol/L oxaliplatin for 36 hours and checked for viability by flow cytometry. Cells containing the ΔNp73-expressing vector showed an increase in 30% of cell viability. Results are representative of 3 independent experiments.

isms involved in the simultaneous regulation of target genes by TP53, TAp73, and ΔNp73 isoforms and the putative feedback among them to obtain solid conclusions from the cancer patient studies. Lastly, it is interesting to note that both TAp73 and ΔTAp73 forms were found to be upregulated in a significant number of our colon tumors. It is possible that the presence of ΔTAp73 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, ΔTAp73 isoforms have been described to be more stable than those of TAp73, in terms of what can contribute to promote a cellular oncogenic context (2, 13). The use of compounds that can increase the stability of TAp73 variants, such as netrin-1 (53), could diminish this tumorigenic environment.

The association of the overexpression of Δ*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 Δ*Np73* in our cellular system induces the upregulation of *ABCB1* and *HMGB1*. In our study, direct statistical correlation was found between expression of Δ*Ex2p73*, Δ*Ex2/3p73*, and Δ*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 Δ*TAp73* forms *in vivo* in colorectal carcinomas. In a larger colon cancer patient series, the correlation between *ABCB1* and Δ*TAp73* variants might reach statistical significance. Although *TP73* has been described as regulating *CASP1* expression, no such direct correlation between *CASP1* levels and *TAp73* expression was found in our set of patients (38).

In addition, *ABCB1* and *HMGB1* overexpression was associated with shorter OS of patients. In the multivariate analysis including clinicopathologic 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 ΔTAp73 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 prognostic 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 Δ*Np73* 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 tumors already expressing high levels of ΔNp73 can show resistance to the treatment; in addition, the oxaliplatin action could gradually select the cells overexpressing this putative oncogenic p73 variant, resulting in a resistant tumor.

As cumulative data support the oncogenic role of ΔTAp73 isoforms (14, 56), the mechanisms and targets underlying these functions are currently of great interest. In this article, we present original data with regard to the impact of specific *TP73* variants in the outcome of colon cancer patients. In addition, we found that putative ΔTAp73 isoform targets are independent prognostic 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 carry out an oncogenic role and could be used as clinical markers of relapse.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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