

Tumor-derived exosomes are enriched in Δ Np73, which promotes oncogenic potential in acceptor cells and correlates with patient survival

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Tumor-derived exosomes are emerging as local and systemic cell-to-cell mediators of oncogenic information through the horizontal transfer of mRNAs, microRNAs and proteins during tumorigenesis. The exosomal content has been described as biologically active when taken up by the recipient cell. Identifying the specific molecular cargo of exosomes will help to determine their function in specific steps of the tumorigenic process. Here we evaluate whether Δ Np73 is selectively packaged in tumor-derived exosomes, its function in the acceptor cells *in vitro* and *in vivo* and its prognosis potential in cancer. Δ Np73 messenger is enriched in tumor-derived exosomes, suggesting its active sorting in these microvesicles. We observed the transmission of this exosome cargo to different cell types and how it confers proliferation potential and chemoresistance to the acceptor cells *in vitro* and in animal models. Finally, our data support the potential prognostic value of exosomal Δ Np73 in colon cancer patients.

INTRODUCTION

Exosomes are intraluminal microvesicles not exceeding 100 nm in size and formed inside late endosomes, which release them by fusing with the plasma membrane (1,2). They are developed in a variety of normal and pathological cells, including tumor cells. Their different cell origins may condition their cargo and membrane lipid and protein composition. Exosome content reprograms the recipient cells epigenetically and participates in the evasion of host immune response against tumors, among other functions. Their role in communication between cells is a phenomenon that is being increasingly studied (3,4). In this context, tumor-derived exosomes are emerging as local and systemic intercellular mediators of oncogenic information through

the horizontal transfer of mRNAs, microRNAs and proteins during tumorigenesis (5,6). Recent reports indicate that these molecules included in exosomes are biologically active when taken up by the acceptor cell and modulate its function or even direct its fate (5–8). In consequence, tumor-released exosomes may contribute to cancer progression in key stages of the process by participating in cell-to-cell communication. Certainly, deciphering the specific molecular cargo of exosomes will help us to determine their function. The genetic content of exosomes is not simply a reflection of the genetic content of the cell of origin: specific populations of RNAs are selectively packaged into exosomes (9–11), which indicates the existence of an unknown mechanism controlling the sorting of a specific cargo. Researchers are now starting to understand the specific

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content and function of tumor-derived exosomes (1,5–7,9–14). Also relevant is the assessment of the putative use of either the quantity of exosomes or their specific molecular content as diagnosis and/or prognosis markers. As here we have the extraordinary advantage of getting patient samples through a non-invasive procedure, this approach should be easily transferable to clinical practice.

The recent description of the involvement of Δ Np73 in different stages of the carcinogenesis process (15) points to it as a good candidate for selective inclusion by the tumor in released exosomes. Δ Np73 is a TP73 gene-derived isoform that lacks the amino terminal transactivation domain (15). The TP73 gene also gives rise to variants that contain the transactivation domain and show tumor-suppressor properties similar to those of TP53. Δ Np73 exerts its oncogenic potential in different ways. Initially, Δ Np73 was described as acting as a direct competitor for the DNA binding sites and/or, by heteroduplex formation with TP53 and the transactivation-competent TAp73 variants, inhibiting their tumor-suppressor properties (16–18). In addition, Δ Np73 may induce the expression of a set of genes responsible for their oncogenic potential (19).

Our study shows the presence of Δ Np73 mRNA in tumor-released exosomes. Remarkably, the Δ Np73 messenger levels contained in tumor-derived exosomes are higher than those in tumor cells, which suggests its active sorting in exosomes. In addition, we observed the transmission of this exosome cargo to epithelial, fibroblast and endothelial cells and its significant effect in proliferation and drug resistance in the acceptor cells. Finally, our data support the potential prognostic value of exosomal Δ Np73 in colon cancer patients.

RESULTS

Δ Np73 β mRNA is incorporated in exosomes released from tumor cells

The colon cancer cell line HCT116 was transiently transfected with a vector containing Δ Np73 β . The ectopic expression of Δ Np73 β led to a 700-fold increase in its mRNA levels 72 h after transfection, when compared with the mock vector. Seventy-two hours posttransfection, exosomes were isolated from the culture media and analyzed, and their identity was confirmed by size, using electron microscopy (Fig. 1A), and the presence of CD63 and CD9, known specific exosome markers (Fig. 1B). Released exosomes and size distribution were performed by LM10 nanoparticle characterization system (NanoSight) (Fig. 1C). Although no differences in their number were observed (Fig. 1C), those exosomes coming from cells transiently expressing Δ Np73 β contained higher levels of its mRNA than those expressing the empty vector (Fig. 1D). We confirmed the absence of the plasmid containing Δ Np73 β in the released exosomes (Supplementary Material, Fig. S1).

Δ Np73 β mRNA is transferred via exosomes in a cell type-specific way

Particular populations of RNAs are selectively packaged in exosomes and their cargo transferred in a cell type-specific fashion (9–11). To further confirm this way of transferring Δ Np73 β mRNA, we incubated HCT116 and SW480-ADH colon cancer

cells for 72 h with exosomes coming from HCT116 cells expressing Δ Np73 β . The recipient HCT116 cells showed a 52-fold increase in Δ Np73 β mRNA levels over the same cells incubated with exosomes from control mock cells ($P = 0.0001$; Fig. 1E). No such a difference was observed in the cancer colon cell line SW480-ADH (Fig. 1E). In addition, we evaluated the transfer of Δ Np73 β mRNA via exosomes to cell types related to tumor microenvironment. The BJ-5ta fibroblast and HUVEC endothelial cells showed 22- and 98-fold increases, respectively, of Δ Np73 β after 72 h of incubation ($P = 0.0001$), whereas no differences were observed in pericytes (Fig. 1E). We also evaluated this phenomenon using other cells as donors. Thus, exosomes from HCT116 stably transfected with Δ Np73 β and SW480-ADH and SW1417 transiently transfected cells were used as donors. The same cell lines were used as acceptors. A 3.1- to 3.4-fold increase in Δ Np73 β mRNA levels compared with control mock cells was observed for HCT116 and SW480-ADH ($P = 0.004$ and 0.003 , respectively) (Fig. 1E). No differences were observed for SW1417. These data support the view that Δ Np73 β is transferred via exosomes in a cell type-specific manner. In parallel, we confirm the exosome traffic by labeling exosomal membrane with PKH67 (Fig. 1F).

Exosomal Δ Np73 β modulates proliferation and confers drug resistance on the recipient cells

We described elsewhere how the ectopic expression of Δ Np73 β increases the proliferation rate and confers resistance to oxaliplatin on colon cancer cells (15). Thus, to test the functionality of the exosomal Δ Np73 β mRNA in the acceptor cell, we carried out proliferation and drug-resistance assays after the intake of the exosome cargo. The methylthiazolotetrazolium (MTT) cell proliferation assay showed that HCT116 colon cancer cells and BJ-5ta fibroblasts incubated with exosomes from Δ Np73 β overexpressing cells increased their proliferation rate over cells incubated with exosomes from mock cells (Fig. 2A). Furthermore, pretreatment of HCT116 cells for 72 h with exosomes from Δ Np73 β overexpressing cells led to a significant increase in their survival rate after oxaliplatin exposure (Fig. 2B and C). No differences in angiogenesis were observed in the HUVEC cells pretreated with the exosomes from Δ Np73 β overexpressing cells when compared with controls (Supplementary Material, Fig. S2).

Transfer of exosomal Δ Np73 β to xenograft tumors regulates their proliferation 'in vivo'

HCT116 cells were subcutaneously implanted in 6-week nude mice. Xenograft tumors from cells ectopically overexpressing Δ Np73 β had higher levels of Δ Np73 in their exosomes than in tumors ($P = 0.062$) (Fig. 3A).

To evaluate whether Δ Np73 β constrained in exosomes can confer oncogenic potential on more indolent xenograft tumors, immediately after HCT116 mock cell inoculation, 20 μ g of exosomes from Δ Np73 β overexpressing cells or mock cells were injected 3 days/week into the tail vein. Throughout the treatment and at the end point, we observed that tumor size in those mice injected with exosomes from Δ Np73 β overexpressing cells were significantly higher than in controls ($P = 0.09$ and 0.05 , respectively; Fig. 3B). Accordingly, these tumors showed a

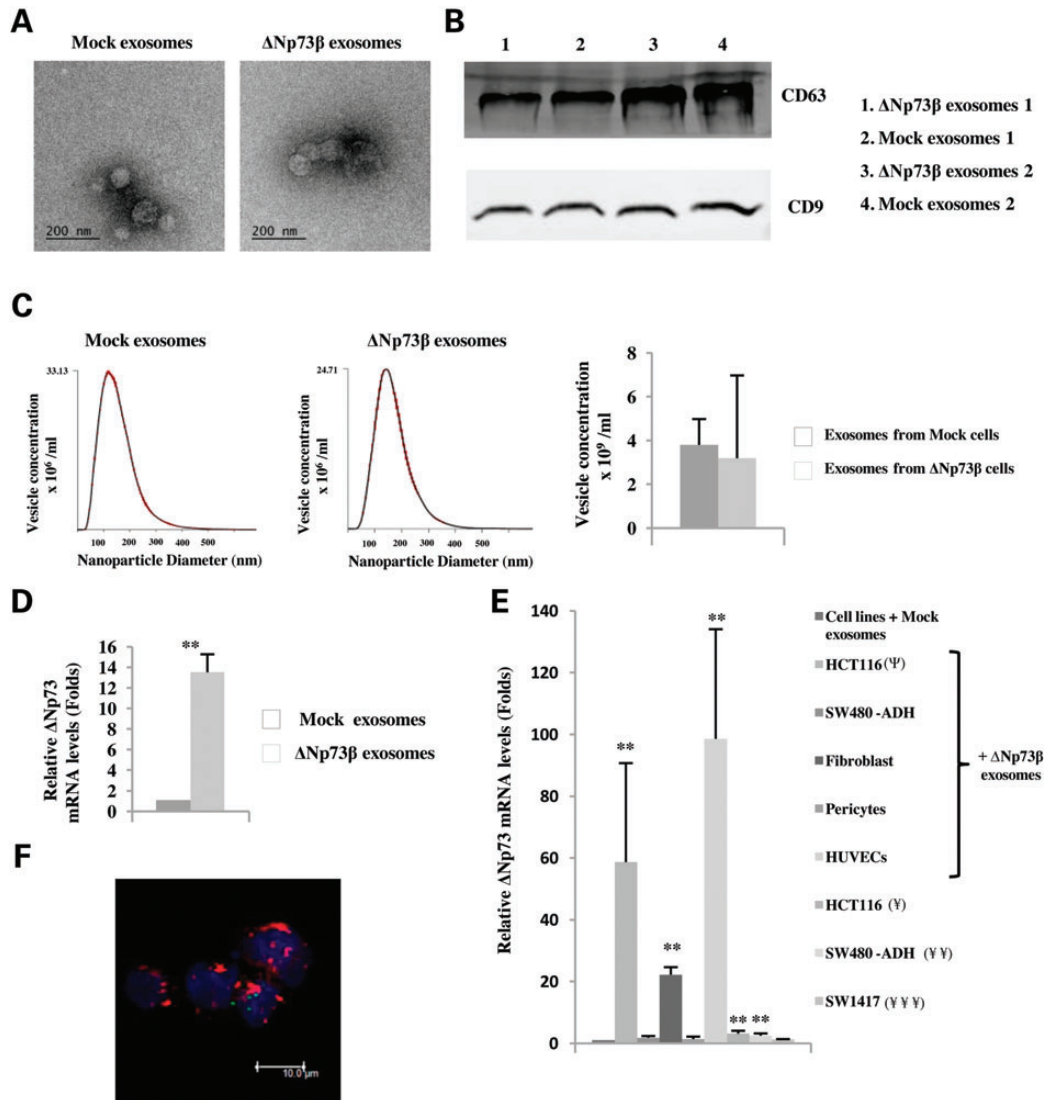


Figure 1. Analysis of exosome identity and transference capacity. (A) Representative electron microscopy image of exosomes and (B) western blot of CD63 and CD9 in exosomes isolated from the HCT116 cell line transfected with ΔNp73β or mock vector (duplicate experiments). (C) Number of exosomes released by ΔNp73 over-expressing HCT116 cells, quantified by NanoSight system. Mock cells were used as control. (D) Quantitative real-time PCR analysis of ΔNp73 mRNA expression in exosomes derived from transfected cell lines. Expression levels were normalized with cDNA concentration (ng/μl). (E) Quantitative real-time PCR analysis of ΔNp73 levels in cell lines incubated with exosomes from HCT116 cells transient (Ψ) and stably (Y) transfected with ΔNp73β, and exosomes from SW480-ADH (YY) and SW1417 (YYY) transiently transfected respect their mock control. Representative of four independent experiments (** $P < 0.0001$). (F) Uptake of PKH67-labeled exosomes by HCT116 cells. In the confocal microscopy image, Topro-3 was used to detect nuclei of cells (blue), E-cadherine was used to label cell membrane (red) and PKH67 was used to label the exosomes (green).

significantly higher number of cells that were positive for the proliferative marker Ki67 than control mice tumors (injected with exosomes from mock cells; Fig. 3C; $P = 0.04$). Moreover, mice injected with exosomes coming from ΔNp73β over-expressing cells had significantly higher levels of ΔNp73β in the xenograft tumor and in their own exosomes than mice injected with exosomes from mock cells (Fig. 3D, $P = 0.01$ and 0.029 , respectively).

To evaluate in an *in vivo* model whether the aggressiveness of a tumor modifies the behavior and progression of a more indolent contralateral tumor via exosomes, we injected HCT116 cells stably transfected with ΔNp73β in the left flank of nude mice and HCT116 mock cells in the opposite flank. A group of mice

injected only with HCT116 mock cells in one flank were used as control. At days 13 and 15 after cell inoculation, tumors from mock cells in mice bearing contralateral ΔNp73 tumors showed significantly bigger tumors than those from control mice (Fig. 3E). As, 20 days after inoculation, no differences in tumor size were observed, a first cycle of oxaliplatin treatment was initiated. After the first cycle (5 days of drug administration) (20), we observed that these tumors did not respond to the chemotherapeutic approach, while a decrease in size was observed in tumors from control mice (Fig. 3E). Although both tumors grew between the two drug cycles, the significant differences in size were maintained (Fig. 3E). Additionally, mice with ΔNp73β HCT116 tumors in the right flank and a mock tumor

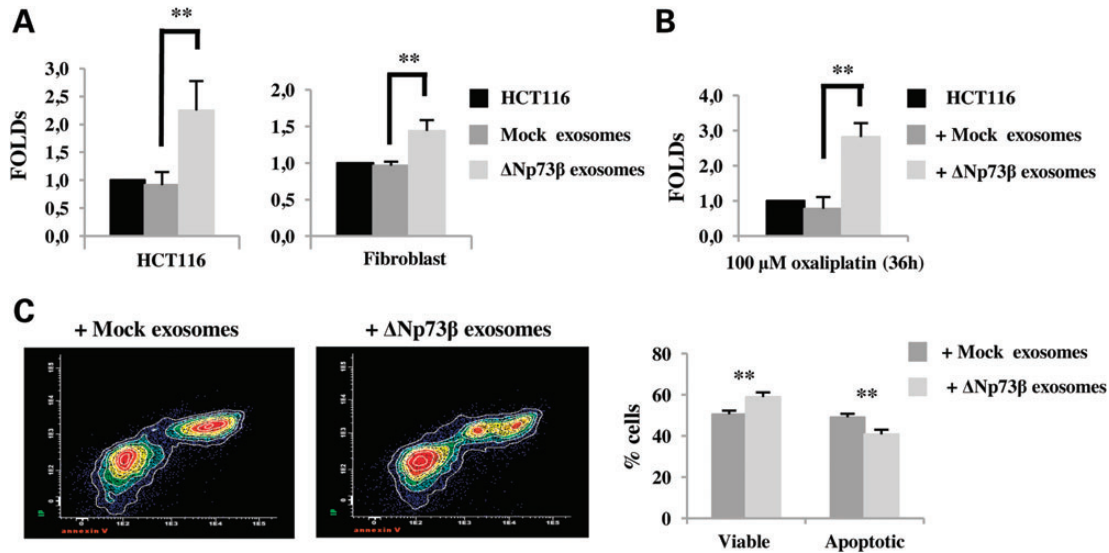


Figure 2. Transfer of exosomal Δ Np73 increases proliferation and drug resistance in recipient cells. (A) Proliferation and (B) viability for drug-resistance analyzed by MTT assay were performed after exosome addition. Treatment with mock exosomes was used as control. (C) Representative of four independent experiments for the drug-resistance experiments by flow cytometry (left) and its quantification (right) (** $P < 0.0001$).

in the opposite flank had higher levels of Δ Np73 in their exosomes than control mice did (Fig. 3E).

Exosomal Δ Np73 is detected in colon cancer patients

The mRNA levels of Δ Np73 were examined in colon cancer and normal counterpart tissues of 69 patients. Description of the patient clinicopathological characteristics is shown in Table 1. High levels of Δ Np73 were found in 30% of tumors, which confirmed our previously reported results (15). In addition, those patients harboring higher levels of Δ Np73 tended to show advanced stage of the disease and shorter disease-free survival (DFS; Fig. 4).

Exosomes in plasma from colon cancer patients and healthy controls were identified by electron microscopy (Fig. 5A), Nano-Sight technology (Fig. 5B) and evaluation of flotillin and CD9 protein levels (21). We found that levels of Δ Np73 in exosomes from plasma of cancer patients are statistically higher than those in healthy controls ($P = 0.0001$; mean rank in cancer patients = 65.96 versus mean rank in healthy controls = 29.40; Kruskal–Wallis test; Fig. 5C). In addition, levels of Δ Np73 in exosomes from patients are higher than in tissue samples ($P < 0.0001$, Fig. 5D). Remarkably, no correlation was found between Δ Np73 levels in tumor samples and in the counterpart exosomes (Supplementary Material, Fig. S3).

We also quantified exosomes by analyzing the expression of Ber-EP-4 (specific protein of the epithelial cell membrane), acetylcholinesterase activity and the concentration of total protein in exosomes (21). Interestingly, negative significant correlations were observed between the expression levels of Δ Np73 in tumor tissue samples and levels of Ber-EP-4 ($P = 0.03$, $r = -0.3$), total protein ($P = 0.007$, $r = -0.35$) and acetylcholinesterase activity ($P = 0.04$, $r = -0.3$, Fig. 5E).

To test whether exosomes of colon cancer patients can transfer Δ Np73, we incubated HCT116 cells with exosomes from colon cancer patients harboring Δ Np73 at detectable levels, either low,

moderate or high. We observed that recipient HCT116 cells increased 4.1- and 5.1-fold their Δ Np73 mRNA levels after incubation with exosomes showing low, moderate and high exosomal Δ Np73 when compared with HCT116 cells incubated with exosomes from healthy donors. In the latter, Δ Np73 levels were very low or undetectable ($P = 0.0001$ and 0.001 , respectively; Fig. 5F).

Prognostic value of exosomal Δ Np73

As with the results observed in tumors, those patients at an advanced stage of the disease tended to show higher levels of exosomal Δ Np73 (Supplementary Material, Fig. S4). Interestingly, we observed that those patients with higher levels of exosomal Δ Np73 had a significantly higher expression of carcinoembryonic antigen (CEA) in serum samples ($P = 0.008$, Fig. 6A). To further evaluate the prognostic value of exosomal Δ Np73 in colorectal cancer patients, we analyzed its possible association with patient survival.

Disease-free survival

Kaplan–Meier and univariate analyses were performed to determine the influence of Δ Np73 levels on DFS. Patients were divided into tertiles based on Δ Np73 expression. Thus, patients had low, median or high levels of expression. No association was observed for the expression of Δ Np73 ($P = 0.3$), but the Kaplan–Meier graph revealed the similar behavior of median- and high-level tertiles (Fig. 6B). Thus, these patients were grouped as above, and Δ Np73 expression was analyzed further with only two categories: low and high expression levels of Δ Np73. When DFS was analyzed in these two groups a trend was observed, since patients with low Δ Np73 expression had a 5-year DFS rate of 57% (95% CI, 51–60%) and patients with high expression had a rate of 49% (95% CI, 41–58%; $P = 0.07$; Fig. 6B).

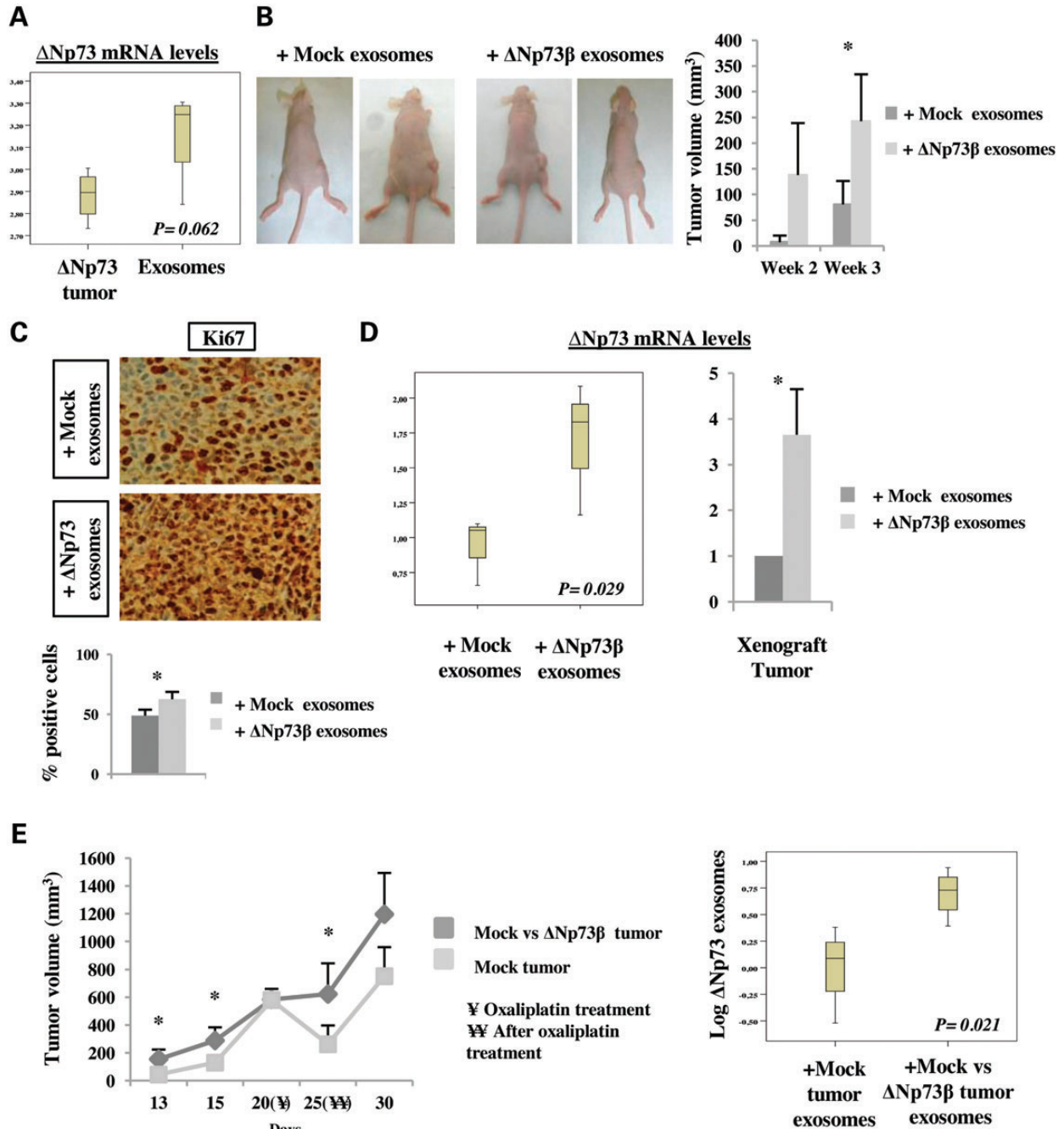


Figure 3. Exosomal Δ Np73 β confers oncogenic potential on xenograft tumors. (A) mRNA expression levels of Δ Np73 were evaluated in the xenograft tumor and in the mice exosomes after exosome treatment. (B) Analysis of xenograft tumor growth treated with Δ Np73 β or mock exosomes (left) and size quantification at Weeks 2 and 3 after exosome treatment. (C) Ki67 immunohistochemistry in xenograft tumors after exosome treatment. (D) mRNA expression levels of Δ Np73 in exosomes and xenograft tumor ($n = 4$ mice per group, Student's t -test, $*P = 0.01$). (E) Analysis of xenograft tumor growth with a contralateral Δ Np73 overexpressing tumor. Mice with mock xenograft tumors in only one flank were used as control.

Overall survival

Kaplan–Meier and univariate analyses were also performed to determine the influence of exosomal Δ Np73 levels on overall survival (OS). Again, patients were divided into tertiles based on Δ Np73 expression. No association was found for the expression of Δ Np73 ($P = 0.3$), but the Kaplan–Meier graph revealed similar behavior of median- and high-level tertiles (Fig. 6C).

Thus, these patients were grouped as above, and Δ Np73 expression was analyzed further with only two categories: low and high expression levels of Δ Np73. When OS was analyzed in these two groups a trend was observed, since patients with low Δ Np73 expression had a 5-year DFS rate of 59% (95% CI, 53–65%) and patients with high expression had a rate of 55% (95% CI, 50–60%; $P = 0.1$; Fig. 6D).

Table 1. Levels of ΔNp73 in Exosomes from Colorectal Cancer Patients

Clinicopathological parameters	Cases (%)	ΔNp73 exosomal levels (geometric average ± SD)	P
Sex			
Male	58.6	1.99 (± 0.20)	NS
Female	41.4	1.94 (± 0.17)	
Localization			
Rectum	32.1	2.12 (± 0.23)	0.009
Left	25.6	1.39 (± 0.23)	
Right	42.3	2.34 (± 0.18)	
Vascular invasion			
Yes	34.2	2.16 (± 0.27)	NS
No	65.8	1.88 (± 0.16)	
Tumor size			
≤4 cm	39.7	2.19 (± 0.19)	NS
>4 cm	60.3	1.86 (± 0.17)	
CEA			
≤3 mg/ml	30.6	1.64 (± 0.24)	0.008
>3 mg/ml	69.4	2.12 (± 0.17)	
LMN			
Yes	46.8	2.08 (± 0.21)	NS
No	53.2	1.87 (± 0.19)	
Stage			
I	7.6	1.9 (± 0.37)	NS
II	43.0	2 (± 0.17)	
III	40.5	2.4 (± 0.14)	
IV	8.9	1.9 (± 0.48)	
Stage			
I + II	50.6	1.95 (± 0.17)	NS
III + IV	49.4	2.34 (± 0.15)	(0.1)

Correlations with clinicopathological parameters.
LNM, lymph node metastases; NS, no significant.

No associations for either DFS or OS were found for exosome protein concentration.

DISCUSSION

Tumor-derived exosomes are emerging as local and systemic cell-to-cell mediators of oncogenic information through the horizontal transfer of mRNAs, microRNAs and proteins during tumorigenesis (5,6). The exosomal content has been described as biologically active when taken up by the acceptor cell and as altering the latter’s function and fate (5–8). Identifying the specific molecular cargo of exosomes will help us to determine their function at specific steps of the tumorigenic process.

It has been suggested that the genetic exosomal cargo is not simply a reflection of the cell content of origin, but that specific populations of RNAs are selectively packaged into exosomes (2,11–13), which indicates the existence of an unknown mechanism controlling the sorting of a specific cargo. Our results support this. In our *in vitro* experiments, we observed that, although the quantity of released exosomes did not differ between cells overexpressing ΔNp73β and the mock cells, the ΔNp73β levels did, being higher in exosomes coming from cells upregulating ΔNp73β. Likewise, in our series, exosomes from healthy donors contained lower levels of ΔNp73 than those from colon cancer patients. Both approaches suggest that those cells expressing higher levels of ΔNp73 could package more ΔNp73 in exosomes. Nevertheless, ΔNp73 exosome content is not a faithful reflection of its levels in the tumor

tissues, since relative levels of ΔNp73 were significantly lower in tissue samples than in their counterpart exosomes, which supports a possible ΔNp73 enrichment mechanism in the released exosomes. This is also sustained by the lack of correlation between ΔNp73 levels in patient tumor tissues and their counterpart exosomes. Thus, although it seems that the higher the ΔNp73 levels in cells or tissues, the greater the quantity in exosomes, it is clear that a ΔNp73 mRNA sorting mechanism may exist between our data point and its enrichment in the exosomes, regardless of its expression in the cell or tissue of origin. Such a sorting process for ΔNp73 and other reported mRNAs and miRNAs still remains elusive.

Unexpectedly, the amount of released exosomes in patients (measured by Ber-EP4 expression, acetylcholinesterase activity and exosomal protein concentration) correlated inversely with its tissue ΔNp73 content. Further experiments are needed to evaluate whether ΔNp73 is involved in the mechanism of exosome release *in vivo*.

Why are tumor cells interested in shipping ΔNp73 to the environment? Although the oncogenic role of ΔNp73 became clearer after the release of the phenotype of the specific knockout mice (22), unfortunately not many data exist on the particular tumorigenesis steps it might be involved in. Our data support the view that exosomes-containing ΔNp73 can be taken up selectively by fibroblasts, epithelial and endothelial cells. Once incorporated in the acceptor cell, ΔNp73 seems functionally active, as there is a significant increase in its proliferation rate and resistance to apoptosis, properties that we recently described for ΔNp73 overexpression (15). Our experiments in mice also support the functionality of ΔNp73 in the recipient cells and its possible release through exosomes by the acceptors. Our approach also supports that ΔNp73 confined in exosomes may be mediators of a systemic instigation similar to that described by Weinberg (23). In the case presented here, an aggressive tumor instigated a more indolent one through exosomes instead of through cytokines. Whether exosomes-containing ΔNp73 could alter the bone marrow, as Peinado *et al.* described (5), or affect directly the tumor is still to be clarified. There is a clear advantage for tumor progression if proliferative and survival input information is transferred to clones, when no such phenotypes had been acquired during the malignant evolution. Similarly, the acquisition of information by tumor-associated fibroblasts (TAF), which ensure their survival, could directly benefit tumor proliferation, invasion and metastasis (24). Furthermore, the achievement by fibroblasts of higher proliferative potential could trigger signaling pathways involved in the gaining of TAF properties (25). Undeniably, the description by the scientific community of new, specific oncogenic roles for ΔNp73 will help us to clarify its function when encapsulated and released to the environment by the tumor.

The scenario in colon cancer patients is promising, since ΔNp73 was not only detected in patient exosomes but also predicted survival better than tumor ΔNp73 did. In this sense, it should be noted that levels of serum CEA, a classic marker of colon cancer prognosis, are significantly associated with exosomal ΔNp73. Our results are preliminary given the small size of our patient series, but the trends observed for DFS and OS and exosome ΔNp73 content (*P* = 0.07 and 0.1, respectively) already highlight the potential of using exosomal ΔNp73 levels as a prognosis marker. Even if tumor and exosomal ΔNp73

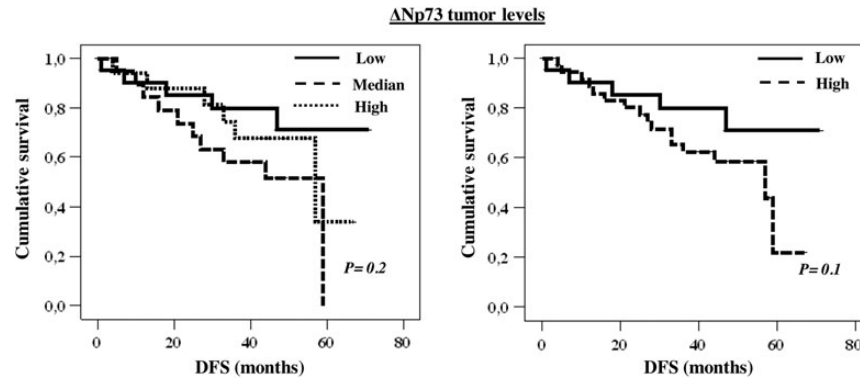


Figure 4. Influence of colon carcinoma levels of Δ Np73 on DFS, Kaplan–Meier curves and *P*-values. Δ Np73 expression in colon cancer patients distributed by tertiles, low, median and high levels (left) and in two groups of low (new variable combining low and median levels) and high expression (right).

could predict with similar statistical power patient survival, obtaining the sample through a non-invasive method, i.e. a routine blood extraction, would greatly facilitate the transfer of such a biomarker to clinical practice. Although Peinado *et al.* (5) observed that exosome protein concentration predicted patient outcome, our data do not support these findings. This association is probably tumor dependent. Further results in independent and larger cohorts may validate our results.

Intercellular communication via exosomes may be essential for tumor progression and spread (5,6). We put forward here that Δ Np73 is preferentially constrained in exosomes by colon tumor cells and can confer proliferation potential and chemoresistance to the acceptor cells *in vitro* and *in vivo*. Importantly, exosomal levels of Δ Np73 in colon cancer patients could be used in the clinical setting as prognosis markers, with the significant advantage of obtaining the sample through a non-invasive method.

MATERIALS AND METHODS

Cell culture

Cells were obtained from American Type Culture Collection and grown at 37°C in a humidified atmosphere of 95% and 5% CO₂. Human colon cancer cells (HCT116, SW480-ADH and SW1417) and foreskin fibroblasts (BJ-5ta) were cultured in Dulbecco's modified Eagle medium (DMEM; Lonza Group Ltd.), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen, Paisley, UK), penicillin (100 units/ml), streptomycin (100 ng/ml) and fungizone (0.25 µg/ml). SW480-ADH are a subpopulation from SW480 (26). HBVP cells (human brain vascular pericytes) were grown in Pericyte Medium (PM; ScienCell Research Laboratories) and 2% of FBS. HUVEC cells (human umbilical vein endothelial cells) were cultured in complete endothelial cell growth medium-2 (EGMTM-2MV BulletKitTM, Lonza) and serum-starved prior to experiments. HCT116 cells were transiently and stably transfected with pcDNA or pEGFP-1 plasmid-encoding Δ Np73 β or mock (kindly provided by Dr. C. Marín, University of León, Spain), using Lipofectamine 2000 (Invitrogen). Seventy-two hours posttransient transfection, different fractions were

kept to preserve cells and isolate RNA and protein. One week posttransfection, pEGFP-1 expressing cells were selected by sorter and expanded in DEMEN supplemented with 20% FBS.

Exosome isolation, quantification and identification

HCT116 cells transiently transfected with Δ Np73 β or the empty vector were cultured in media supplemented with 10% exosome-depleted FBS. FBS was depleted of bovine exosomes by filtration through a 0.22-µm PVDF filter (Millipore, Carrigtwohill, Ireland) and ultracentrifugation (OptimaTM MAX-XP, Beckman Coulter, Fullerton, CA) at 120 000g for 70 min. Seventy-two hours after transfection, supernatant fractions collected (40 ml from 24 × 10⁶ cells) were pelleted by centrifugation at 500g for 10 min to eliminate cells. The supernatant was centrifuged at 17 000g for 20 min, followed by filtration through a 0.22-µm PVDF filter. Exosomes were then harvested by ultracentrifugation at 120 000g for 70 min. The exosome pellet was resuspended in 400 µl of phosphate-buffered saline (PBS) or DMEM with 1% exosome-depleted FBS. Peripheral blood obtained from humans and mice was centrifuged at 500g for 10 min at 4°C and circulating exosomes were isolated from plasma, as described above.

For identification by transmission electron microscopy (model JEOL Jem1010, 100 kV), exosomes were fixed in 2% PFA (w/v) in 200 mM phosphate buffer (pH 7.4). Fixed exosomes were dropped onto a formvar-carbon-coated grid and left to dry at room temperature for 20 min. After washing in PBS, the exosomes were fixed in 1% glutaraldehyde for 5 min, washed in water and stained with saturated aqueous uranyl oxalate for 5 min. Samples were then embedded in 0.4% (w/v) uranyl acetate and 1.8% (w/v) methylcellulose and incubated on ice for 10 min. The excess liquid was then removed. The grid was dried at room temperature for 10 min and viewed at 80 000 and 120 000 magnification. The LM10 nanoparticle characterization system (NanoSight) was used for real-time characterization and quantification of the vesicles.

For quantification in patients samples, acetylcholinesterase activity (27), Ber-EP4 intensity and total exosomal protein concentration were assessed (21). For flow cytometry quantification, samples were incubated with monoclonal antibody mouse

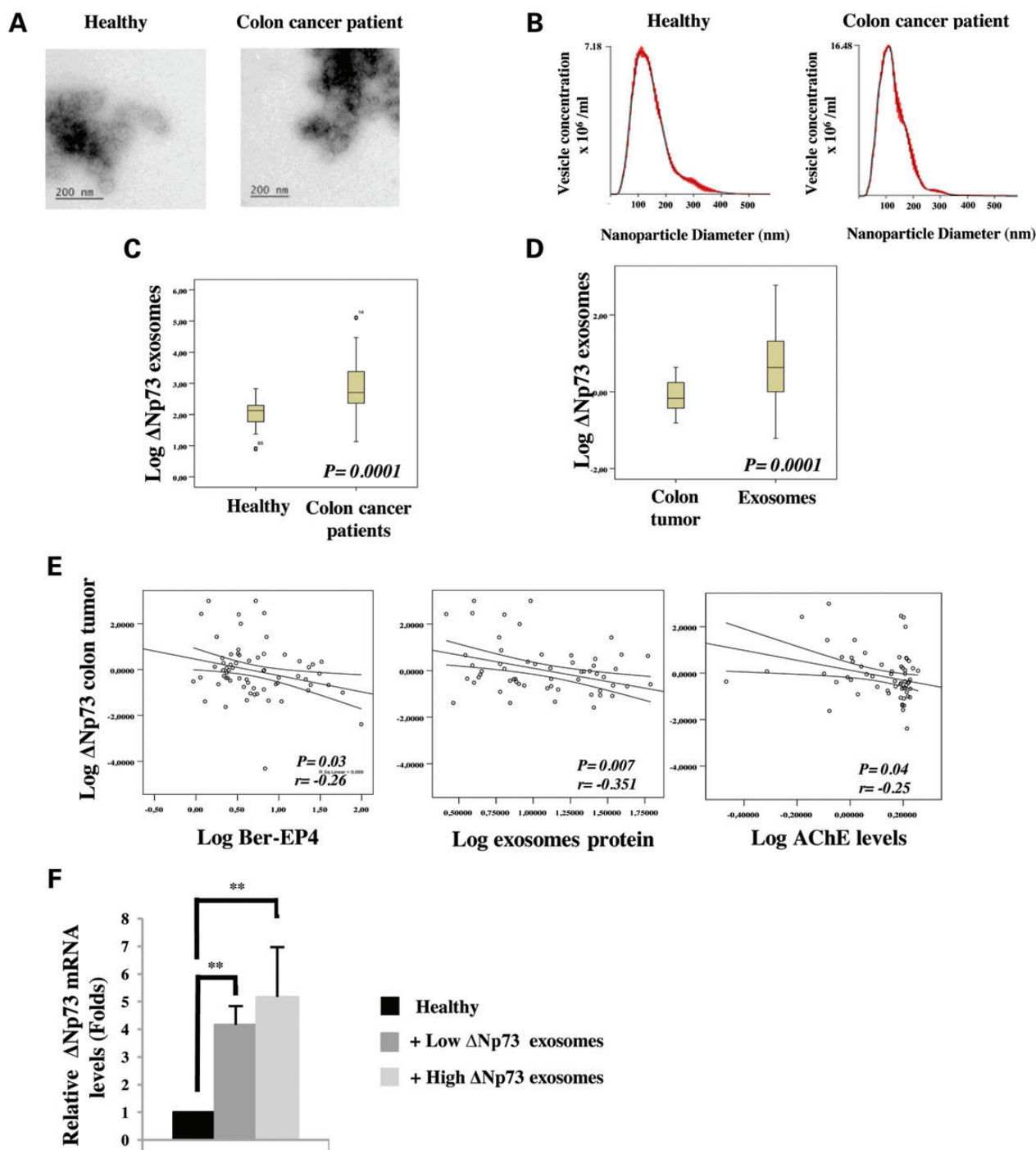


Figure 5. Analysis of Δ Np73 exosomes from colon cancer patients. (A) Identity of exosomes by electron microscopy and (B) NanoSight system. (C) Statistical analysis of Δ Np73 mRNA levels in circulating exosomes relative to cDNA concentration from plasma of colon cancer patients and healthy controls. (D) mRNA expression levels of Δ Np73 were evaluated in the primary tumor and in exosomes. $^{**}P < 0.0001$ using Mann–Whitney *U*-test. (E) Correlations between Δ Np73 mRNA expression levels in primary tumor and exosome quantity measured by Ber-EP4 and total protein levels and acetylcholinesterase activity (*P* is calculated by analysis of variance; *r* is the Pearson coefficient). (F) Quantitative real-time PCR analysis of Δ Np73 expression in HCT116 cell line incubated with exosomes from healthy and colon cancer patients bearing low or high levels of Δ Np73 (Student's *t*-test, $^{**}P < 0.0001$).

anti-human epithelial cell adhesion molecule (EpCAM antigen) clone Ber-EP4 (DAKO Cytomation, Glostrup, Denmark) conjugated with fluorescein isothiocyanate (FITC). A mouse IgG1-FITC irrelevant antibody (Abcam, Cambridge, UK) was used as a negative control of staining.

Exosome labeling

For the exosome-tracking experiments, purified exosomes from HCT116 cells transiently transfected with Δ Np73 β or the empty vector were fluorescently labeled using PKH67 membrane dye

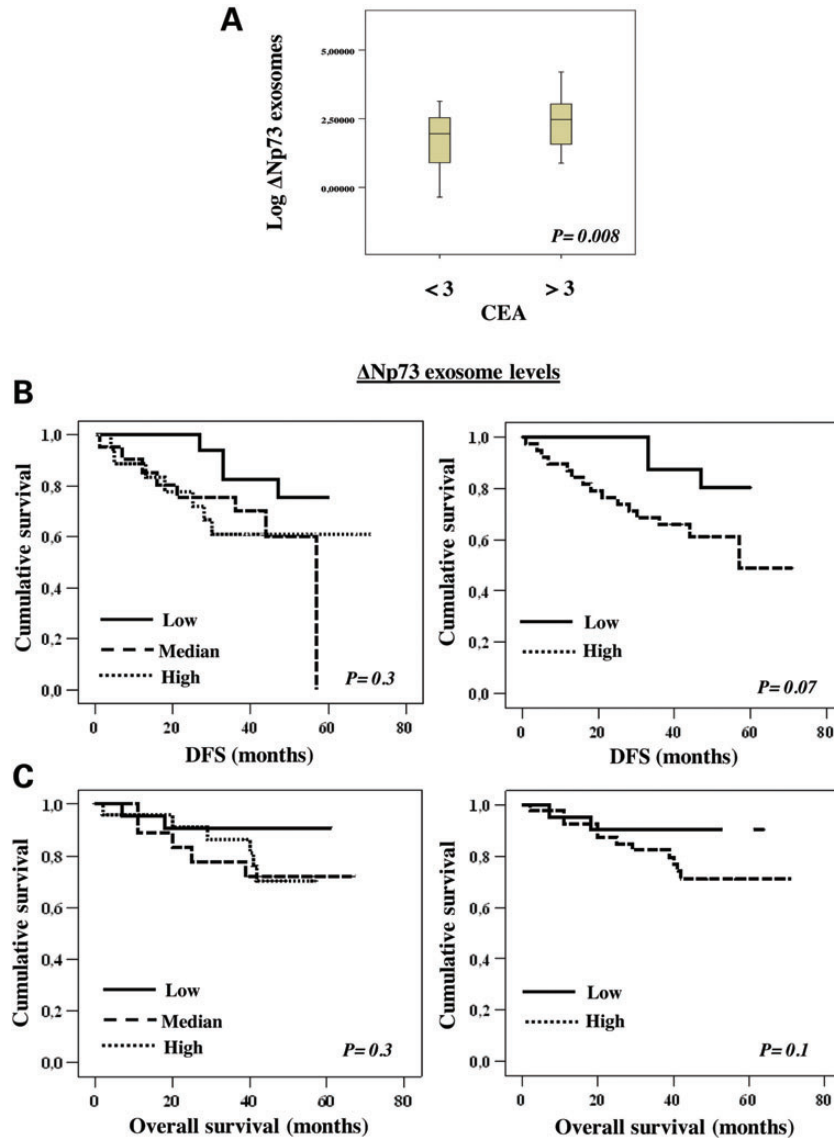


Figure 6. Δ Np73 levels and prognosis in colon cancer patients. (A) Correlation between Δ Np73 mRNA expression levels in exosomes and CEA (P is calculated by analysis of variance; r is the Pearson coefficient). Influence of exosome levels of Δ Np73 on DFS and (B) OS. (C) Kaplan–Meier curves and P -values. Exosome Δ Np73 expression levels from colon cancer patients were distributed by tertiles (left) and in two groups of low (new variable combining low and median levels) and high expression (right).

(Sigma-Aldrich) according to the manufacturer's protocol. Briefly, labeled exosomes were washed in 9 ml of PBS, collected by ultracentrifugation as described above and resuspended in DMEM supplemented with 10% exosome-depleted FBS. HCT116 cells were seeded in duplicate in a chamber slides of four-well (Thermo Scientific) and incubated for 6 h at 37°C with 100 μ l of Δ Np73 β or mock PKH67-labeled exosomes. After that, the cells were subsequently fixed with 4% paraformaldehyde and washed twice with PBS. Then, the samples were incubated with E-Cadherine 1/20 (BD Biosciences) overnight at 4°C, washed with PBS and incubated with Alexa Fluor 546 anti-mouse (Invitrogen Life Technologies, 1/1000) during 45 min at room temperature. Nuclei were stained with Topro-3 (Invitrogen Life Technologies 1/1000) for 15 min and the sections were mounted with PBS glycerol.

Images were collected with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with 40 \times HCX PL APO (1.25–1.52 numerical aperture) oil-immersion optics. Images were captured with a scanning speed of 400 Hz and image resolution of 1024 \times 1024 pixels and then analyzed by Leica Application Suite 2.02.

Incubation experiments with exosomes

HCT116, SW480-ADH, SW1417, BJ-5ta fibroblast, HBVP and HUVEC cells were seeded in quadruplicate in 24-well E-plates and after 24 h incubated with 5 μ g of Δ Np73 β or mock exosomes from HCT116 cells. For experiments with exosomes from human subjects, HCT116 cells were incubated with 5 μ g of exosomes from a pool of five healthy donors, 10 colon

cancer patients showing low levels of $\Delta Np73$ and 10 harboring high expression. mRNA of acceptor cells was obtained 72 h postincubation and $\Delta Np73$ levels were evaluated by real-time PCR.

Protein extraction and western blot analysis

Exosomes were lysed with RIPA buffer (Sigma, St. Louis, MO, USA) containing a protease and phosphatase inhibitor cocktail (Thermo, Rockford, IL, USA). Lysates were cleared by centrifugation and supernatant used for Western blot. Protein extracts were denatured in $2\times$ SDS buffer at 95°C , separated in 10% SDS–polyacrylamide gel electrophoresis and transferred by the iBlot System (Invitrogen, Paisley, UK). Membranes were blocked and incubated with mouse monoclonal antibody for CD63 (ab59479; abcam, Cambridge, UK) or with rabbit polyclonal antibody for CD9 (ab92726; abcam, Cambridge, UK) in a 1:500 dilution. Anti-mouse IRDye 800 CW infrared polyclonal secondary antibody was used at a dilution of 1:10 000 (Rocklan, Gilbertsville, PA, USA). The band intensities were quantified by densitometry, using Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK). The Bio-Rad Protein Assay was used for protein quantification.

RNA extraction, reverse transcription and real-time RT–PCR

RNA from tissues was extracted by the trizol and *mirVana*TM miRNA Isolation Kit (Ambion Inc., TX, USA) from cells and exosomes and quantified spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). For cDNA synthesis, random hexamers were used as primers and RNA retro-transcribed with the Transcription First-Strand cDNA Synthesis Kit (Roche). Real-time PCR of $\Delta Np73$ and the reference housekeeping genes was performed and quantified as described previously (15). The difference between $\Delta Np73\beta$ expression was quantified by the normalized relative ratio (NRR) = $E_T^{\text{CpT(C)}-\text{CpT(S)}} \times E_R^{\text{CpR(S)}-\text{CpR(C)}}$; *E*: efficiency, T: target gene, R: reference gene, C: calibrator and S: sample. For exosomes, mRNA levels were normalized by cDNA concentration (ng/ μl).

MTT assay

Cells were seeded in quadruplicate in 96-well E-plates and incubated at 0, 24 and 48 h with 100 μl of $\Delta Np73\beta$ or mock exosomes. Forty-eight hours postincubation, HCT116 cells were treated with 100 $\mu\text{mol/l}$ oxaliplatin for 36 h (15). After 48 and 72 h incubation (HCT116 and BJ-5ta fibroblasts, respectively) or oxaliplatin treatment, MTT Reagent was added (Cayman Chemical Company) and absorbance measured on a microplate reader at 570 nm (Multiskan Ex; Thermo Scientific).

Flow cytometry for drug-resistance studies

HCT116 cells were incubated with 100 μl of $\Delta Np73\beta$ or mock exosomes for 0, 24 and 48 h. At 48 h, postincubation cells were treated with 100 $\mu\text{mol/l}$ oxaliplatin for 36 h. Floating and adherent cells were trypsinized and checked for viability by the Annexin V–FITC Apoptosis Detection Kit (BD Pharmingen). Data were acquired in a FACS-Aria II sorter (BD

Biosciences, San Jose, CA, USA), using the FACSDiva software program (BD Biosciences). The Infinicyt program (Cytognos SL, Salamanca, Spain) was used for data analysis. Experiments were done in quadruplicate and results reported as percentage of positive and negative cells for both markers.

Immunohistochemistry analysis

Two to 4 μm -thick paraffin-embedded tissue sections were cut onto Dako slides (DAKO Cytomation, Glostrup, Denmark) and subsequently dewaxed, rehydrated, subjected to antigen retrieval by means of Dako's Solution with 10 mM of sodium citrate, at pH 9, and heated at 95°C . The slides were cooled and treated with peroxidase-blocking solution (DAKO Cytomation, Glostrup, Denmark) for 5 min. Sections were then immunostained with mAb by the two-stage peroxidase-based EnVision technique (DAKO Cytomation, Glostrup, Denmark), counterstained with hematoxylin and mounted. Tissues were then stained with anti-Ki67 (rabbit polyclonal; DAKO, Carpinteria, CA, USA), diluted 1:200 in 5% goat serum. Cases displaying no nuclear staining were considered negative. Ki67 was performed by two independent observers (B.S and M.R.), with differences resolved by use of a conference microscope. The PI was defined as the percentage of tumor nuclei showing Ki67 staining per total of 1000 neoplastic cells counted in five fields of 200 tumor cells. Tumor cells were considered positive for Ki67 whenever diffuse or punctuate brown nuclear staining could be identified.

Angiogenesis assay

HUVEC cells were seeded in quadruplicate in 96-well E-plates and preincubated with 100 μl of $\Delta Np73\beta$ or mock exosomes. After 24 h, cells were trypsinized and seeded in a matrigel matrix (10.6 mg/ml, MatrigelTM Basement Membrane Matrix, BD Biosciences) with new addition of exosomes in a 96-well plate. The matrix was allowed to solidify at 37°C and 5% CO_2 for 1 h. After 16 h incubation, the tubular structures were analyzed and photographed under inverted microscopy with a digital camera and flexible cold light. The vascular structures were quantified by number of knots and vessels and length of each.

Patient samples, clinicopathological parameters and follow-up

The study, approved by the Research Ethics Board of the Hospital Universitario Puerta de Hierro Majadahonda, was based on a consecutive series of 69 patients who had surgery for colon cancer between February 2003 and January 2009. Informed written consent was obtained from all participants. All patients were considered sporadic cases. Those that met the clinical criteria for hereditary non-polyposis colon cancer (Amsterdam criteria) were excluded. Samples of tumor and normal tissues were obtained after surgery, snap frozen and stored at -80°C . All tumors were histologically examined. Adjuvant treatment based on oxaliplatin (FOLFOX6, leucovorin 400 mg/m^2 IV on day 1 as a 2-h infusion, followed by 2400 mg/m^2 IV 46-h infusion and oxaliplatin 100 mg/m^2 IV as a 2-h infusion on Day 1) was administered to all Stage III patients without medical contraindications who gave their written

informed consent. Blood samples (20 ml) were taken before intervention. Blood samples from 12 healthy blood donors were obtained at the Hematology Department.

Levels of CEA were obtained from the medical records of the patients. Clinical follow-up after diagnosis and surgery was based on periodic visits and clinical, biochemical and imaging techniques. An ultrasonic study was performed when liver function was impaired.

Animals and tumor xenografts

We subcutaneously injected 1×10^6 HCT116-pEGFP-1- Δ Np73 β or HCT116-pEGFP-1 cells suspended in 20% matrigel into recipient mice (Hsd:athymic Nude-Foxn1nu mice, female, 6 weeks old, Harlan Laboratories). Tumor weight diameter was measured with calipers; volume was calculated as $\text{width}^2 \times \text{length} \times 0.52$. Mice were distributed into four groups (5 mice per group): mock tumor and mock versus Δ Np73 β contralateral tumor and with addition of Δ Np73 β and mock exosomes. All experiments were performed in accord with regulations of the research animal welfare board of Hospital Universitario Puerta de Hierro Majadahonda. For exosome treatment 1 day after cell inoculation, 20 μ g of total Δ Np73 β and mock exosome protein (in a total volume of 200 μ l of PBS) were injected by tail vein three times a week for 3 weeks. At the end of experiments, mice were anesthetized via isoflurane inhalation and peripheral blood was obtained by intracardiac puncture of sterile PBS through the left ventricle. Then, mice were euthanized and their tumors were excised for subsequent analysis.

Statistical analysis

CEA was contrasted with levels of exosomes in plasma and Δ Np73 β expression in tumor tissues by the Kruskal–Wallis and Pearson's χ^2 -tests. Box-plot variables were modified with \log_{10} transformation. OS was defined as the period from time of diagnosis until death. DFS was defined as the interval between diagnosis and first recurrence. The DFS evaluation did not include patients with pathological stage D. The median follow-up was 19 months (range of patient follow-up: 1–72 months). During this period, 24% of patients progressed and 16% died. At 36 months follow-up, the OS of patients was 72% (95% CI, 55–88%) and the DFS was 66% (95% CI, 52–81%). The relationship between the cumulative probability of OS and DFS, as well as analyzed predictors, was calculated by the Kaplan–Meier method, while significant differences between curves were evaluated with Mantel's log-rank test. Results obtained from experiments requiring cultured cell lines and mice were expressed as mean \pm standard deviation of at least three separate experiments. Levene's test was performed to evaluate the equality of variances. The paired Student's *t*-test was used for statistical analysis. Two-tailed *P*-values of ≤ 0.05 were considered statistically significant. Statistical analysis used the SPSS, version 14.0.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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