JCP Online First, published on July 25, 2017 as 10.1136/jclinpath-2017-204499

TP73 DNA methylation and upregulation of ΔNp73 are associated with an adverse prognosis in breast cancer

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2017-204499).

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Received 6 April 2017
Revised 12 May 2017
Accepted 12 May 2017

ABSTRACT

Aim Accumulated evidence suggests that aberrant methylation of the *TP73* gene and increased levels of ΔNp73 in primary tumours correlate with poor prognosis. However, little is known regarding the transcriptional and functional regulation of the *TP73* gene in breast cancer. The aim of the present study was to determine the expression of the ΔNp73 isoform, its relationship with DNA methylation of *TP73* and their clinical prognostic significance in breast cancer patients.

Methods *TP73* gene methylation was studied in TCGA datasets and in 70 invasive ductal breast carcinomas (IDCs). The expression of p73 isoforms was evaluated by immunohistochemistry (IHC) and Western blot and correlated with clinicopathological variables and clinical outcome.

Results We observed that the methylation of diverse CpG islands of *TP73* differed significantly between molecular subtypes. An inverse correlation was found between p73 protein expression and the methylation status of the *TP73* gene. The expression of exon 3′ of p73 (only expressed in ΔNp73) was significantly higher in patients with wild-type p53. Immunohistochemical analysis revealed that all p73 isoforms were localised in both the nuclear and cytoplasmic compartments. We confirmed a positive association between the expression of ΔNp73 and high histological grade.

Conclusions Our findings suggest that high expression of ΔNp73 could be used to determine the aggressiveness of IDCs and could be incorporated in the pathologist's report.

INTRODUCTION

Breast cancer (BC) is the most common female cancer worldwide, accounting for one-third of newly diagnosed malignancies. Invasive ductal carcinoma (IDC) is the most frequent type of BC (75%–85%) and exhibits distinct biological behaviour as compared with invasive lobular carcinoma (ILC), the second most common type (10%–15%).[1 2] Accumulating evidence suggests that BC is a heterogeneous disease with distinct histopathological and biological features that require different clinical management strategies.[3] The status of lymph nodes, primary tumour size and tumour histological grade are among the most important prognostic factors for patients with early-stage BC.[4] The Nottingham combined histological grade is well-established and takes into account three morphological features of the tumour cells: degree of differentiation, nuclear pleomorphism and mitotic count. Thus, using this system, primary tumours can be classified into low, intermediate and high histological grade.[5 6]

During the last few decades, the classification of BC patients according to traditional characteristics has been modified to include gene expression profiles to further classify breast tumours into molecular subtypes: normal breast-like, luminal A, luminal B, HER2 and basal-like.[3 7] As we and others have demonstrated, breast carcinogenesis is a multistep process that involves a combination of genetic and epigenetic alterations.[8–10] One of the most extensively studied and clinically relevant epigenetic alterations in BC is the aberrant DNA methylation of gene promoter CpG islands.[11] Several studies have shown that the BC molecular subtypes are associated with characteristic methylation patterns.[12–16] Furthermore, Yan *et al*[17] have suggested that increased CpG island hypermethylation is associated with high-grade tumours. In this context, we have previously reported a CpG site located at +258 bpfrom the transcription start site (TSS) of *TP73* which was more frequently methylated in high histological grade and high proliferating rate IDCs.[8] Additional reports have shown that hypermethylation of the *TP73* gene can also be detected in other solid tumours such as squamous cell lung cancer,[18] gastric carcinoma[19] and cervical cancer.[20]

The *TP73* gene belongs to the *TP53* family, which includes *TP53* itself and the *TP63* gene.[21] All three genes are structurally and functionally similar and are involved in controlling cellular proliferation, differentiation and cell death. *TP73* is critical for normal cell homeostasis because it partially compensates for the loss of p53 function.[22] Unlike *TP53*, which is mutated in more than 50% of all human tumours, *TP73* is rarely mutated (less than 1%) but rather overexpressed as compared with normal tissue. The *TP73* gene has at least 24 isoforms which are the products of two different promoters (P1 and P2) and alternative splicing. The alternative activation of the P1 and P2 promoters generates p73 protein isoforms containing (TA) or

To cite: Gomez LC, Sottile ML, Guerrero-Gimenez ME, *et al*. *J Clin Pathol* Published Online First: [*please include* Day Month Year]. doi:10.1136/jclinpath-2017-204499

lacking (ΔN) the transactivation domain. While TAp73 is considered a pro-apoptotic tumour suppressor protein, ΔNp73 acts as an anti-apoptotic oncoprotein since it has a dominant negative effect on TAp73 and *TP53*.[23 24] TAp73 and ΔNp73 are frequently overexpressed in a number of solid tumours, including lung, ovarian, hepatocellular, breast and colon cancers, and their expression levels are associated with prognosis.[25–29] Several studies have found that over-expression of ΔNp73 inhibits apoptosis in many human cancers[30–33] and correlates with poor prognosis.[28 34 35] More recently, a high ΔNp73/TAp73 ratio has been associated with lower overall survival (OS) and lower disease-free survival (DFS) in patients with acute promyelocytic leukaemia.[34] Even though some studies have reported the prognostic value of TAp73 and ΔNp73 expression levels individually, the ratio between them has not been studied in BC.[28 36] Here we aimed to investigate the expression of ΔNp73/TAp73 isoforms, their relationship with *TP73* DNA methylation and their clinical significance in BC patients.

MATERIALS AND METHODS

Patients and tissue samples

From March 2007 to December 2010, a total of 103 BC patients who did not receive neoadjuvant treatment were enrolled. Ethics approval was obtained from the Ethics Committee of the Medical School, National University of Cuyo, Mendoza, Argentina. The tumours of 70 patients were identified as IDCs. The clinical features of patients with IDCs are given in the online supplementary table. After surgery each sample was divided into two portions: one was sent for routine H&E staining and pathological analysis, while the other was dissected to enrich tumour cell content and cryopreserved at −80°C. In these patients we had previously studied *TP73* gene methylation in a CpG site located within a very strong CpG island (at +258 bp from the TSS) by MS-MLPA.[8] Six samples of normal breast tissue and three fibroadenomas were included as controls. The methylation percentage results of CpG sites were dichotomized into methylated and unmethylated status, based on a 8% cut-off criterion as previously reported by us.[37]

Western blotting

Of the 70 frozen samples of IDC tissue only 42 yielded enough protein of sufficient quality for Western blot analysis. Three samples of normal breast tissues and one fibroadenoma were included. Proteins were analysed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and incubated with mouse monoclonal antibody (mAb) anti-p73, which recognises all p73 isoforms (1:500 Abcam, Cambridge, MA), mouse mAb anti-p73 Delta N (1:500 Abcam) and mouse mAb anti-α-tubulin (1:1000 Sigma, St. Louis, MO, USA). The expression of ΔNp73 and TAp73 was relativized to the loading control α-tubulin. The ΔNp73 expression levels were dichotomized according to the ΔNp73/TAp73 ratio into two categories: low ΔNp73 (<1) and high ΔNp73 (≥1).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 5 μm-thick sections. Tissue sections were then incubated with either the rabbit mAb anti-p73 (1:100 Abcam) or the mouse mAb anti-p73 Delta N (1:500 Abcam). We used biotin-conjugated anti-rabbit and anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) as secondary antibodies. Diaminobenzidine (0.5 mg/mL)/hydrogen peroxide (0.01%) was used as chromogen substrate. p73 expression was evaluated in tumour cells as well as in the morphologically normal tissue of the surgical margins. The samples were evaluated regarding intensity and the proportion of immunostained cells using a scoring system reported previously.[38]

TCGA data analysis

TCGA breast invasive carcinoma CNV (GISTIC2 method), RNA-seq (Illumina HiseqV2) and DNA methylation (Illumina Infinium Human Methylation 450 BeadChip) datasets were extracted from the UCSC Cancer Browser (https://genome-cancer.ucsc.edu/), along with the clinico-pathological phenotypes and *TP53* mutational status. The expression levels of 50 genes included in the PAM 50 signature were used to carry out classification into BC intrinsic subtypes.[39] The methylation levels of each subtype relative to normal breast tissue were compared using the R bioconductor package bumphunter.[40] To evaluate differentially expressed exons, the EdgeR algorithm was used. Log_2 fold change values associated with p values were obtained and corrected using false discovery rate (FDR) values.

Statistical analysis

The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to assess the normality of the data distribution. Associations between *TP73* methylation, ΔNp73 expression and clinico-pathological characteristics were examined using the χ^2 test or Fisher's exact test. The strength of associations was assessed by the φ coefficient for dichotomous variables and Cramer's V coefficient for polytomous variables. The correlations were assessed by Spearman's ρ coefficient. Analyses of DFS and OS were performed by the Kaplan–Meier method. The difference between curves was evaluated with the log-rank test. For all tests, two-sided $p<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS software version 16.0.

RESULTS

Differential DNA methylation of *TP73* among BC molecular subtypes

We evaluated the association between methylation of *TP73* in the +258 CpG site and the BC molecular subtype. In the 70 specimens studied using MS-MLPA, we observed that the frequency of tumours with methylated *TP73* differed among BC subtypes. Association analysis revealed that most luminal A tumours exhibited unmethylated *TP73* ($p=0.004$). In contrast, all triple negative tumours exhibited methylated *TP73* ($p<0.001$) (table 1).

To study the methylation status of *TP73* in the entire promoter region in BC molecular subtypes, we analysed the

Table 1 Statistical relationships between molecular subtype and *TP73* methylation status

	TP73 methylation				
Variable molecular subtype	No.	%	Methylated *TP73* %	Unmethylated *TP73* %	p Value
Total patients	70	100			
Luminal A	35	50	40	60	0.004*
Luminal B	19	27.1	47.4	52.6	NS
Triple negative	14	20	100	0	<0.001
HER2	2	2.9	100	0	NS

*p Value from the χ^2 test.
NS, not significant.

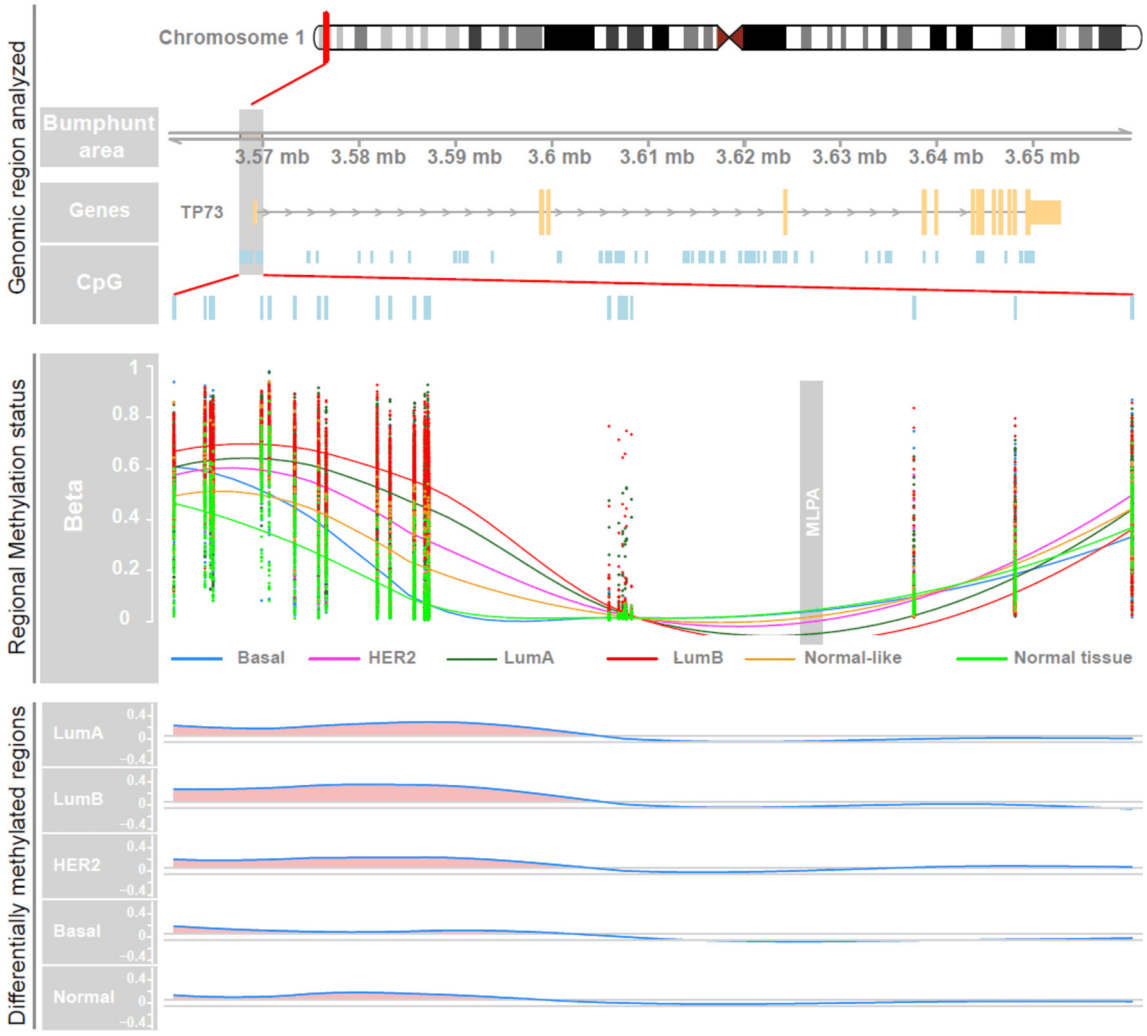

Figure 1 Subtype-specific methylation of *TP73* in breast tumours. Methylation values (beta values) are shown along the y-axis, while each column of dots represents a given CpG site located in relation to their chromosomal region (shown in the bottom tracks). Each dot represents the methylation value for a single sample in a given CpG site. A smoothed regression curve was added to the scatter plot to show the methylation differences between molecular subtypes and normal tissue. The lanes below show the 'bumps' for each tumour subtype, representing the methylation levels across each region as a whole. Note the greater height of the bumps in the luminal B subtype indicating higher methylation levels in this subtype.

methylation levels of all CpG islands in or close to promoters using TCGA Human Methylation 450 Array data. These results are expressed as beta values, which are continuous variables between 0 and 1. The level of *TP73* methylation in the CpG island located in the region +258 was similar to that obtained in our patients. We observed that *TP73* was less methylated in luminal A tumours in this region. Moreover, in the CpG islands located in the promoter region, we also observed that the methylation pattern of *TP73* was significantly different among the subtypes ($p<0.001$) (figure 1). However, in these CpG islands, the luminal A and luminal B tumours showed a higher methylation level in the *TP73* gene promoter compared with normal breast tissues and basal tumours.

TP73 methylation is inversely correlated with p73 expression

The expression of p73 was assessed by Western blot in 42 IDCs in which the methylation status of *TP73* had been previously studied by MS-MLPA.[8] We used an antibody against the C-terminus domain of p73 which recognises all isoforms and another antibody specifically against ΔNp73 (see the online supplementary figure). We observed methylation of the *TP73* +258 CpG site in 23/42 IDCs. We detected an inverse correlation ($\rho=-0.42$; $p=0.039$) between *TP73* methylation and p73 expression. Among the 23 samples, we noted that although the gene was methylated in 16/23 (69.6%), the protein was still expressed. Subsequently, we studied the relationship between *TP73* methylation and the expression levels of the TAp73 isoform and the ΔNp73 isoform. For this, we dichotomized the tumour specimens into two groups according to ΔNp73/TAp73 ratio (low ΔNp73 expression <1; high ΔNp73 expression ≥1). We observed a non-statistically significant increased expression of the ΔNp73 isoform (high ΔNp73/TAp73) when the *TP73* gene was methylated (figure 2).

The ΔNp73 isoform is frequently expressed in invasive ductal carcinomas

Expression of p73 protein (TAp73, ΔNp73 or both isoforms) was found in 69% of cases (29/42), 65.5% of which (19/29) showed high ΔNp73 expression (high ΔNp73/TAp73 ratio). The 66.7% of tumours exhibiting high expression of ΔNp73 variants also displayed negative staining of p53 (wild type p53) by inmunohistochemistry compared with 33.3% of tumours with low levels of

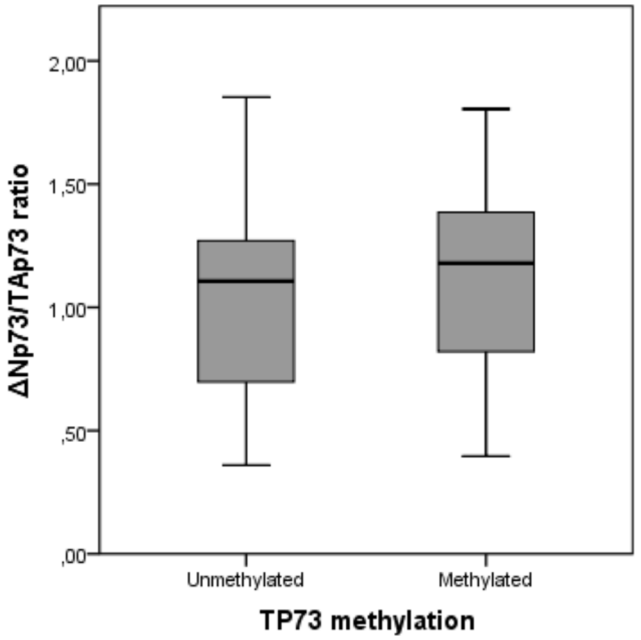

Figure 2 Distribution of the ΔNp73/TAp73 ratio within *TP73* DNA methylation status. The y-axis represents the value of the ΔNp73/TAp73 ratio and the x-axis shows *TP73* promoter methylation status. As can be observed, although not statistically significant (p=0.260), a tendency towards an enhanced ΔNp73/TAp73 ratio is detected among tumours with methylated +258 CpG.

ΔNp73; however, this association did not reach statistical significance (table 2).

We also analysed the differential exon expression of p73 with respect to the ratio of mutated *TP53*/wild type *TP53* in 399 samples using the TCGA database. We observed that the expression of exon 3' of p73, which is only expressed in the ΔNp73 isoform, was significantly higher in patients with wild type p53 ($p<0.05$) (figure 3).

Twenty-three tumour tissue samples were evaluated for total p73 expression and localization by IHC. Seventeen samples (73%) showed high p73 expression. IHC analysis also revealed that all p73 isoforms were localised in both nuclear and cytoplasmic compartments in invasive and in situ tumour cells, while normal tissues showed weak staining. The ΔNp73 isoform was mainly expressed in cytoplasm (figure 4).

ΔNp73 isoform expression is associated with higher histological grade

Pathological and molecular characteristics were similar between patients with a low and high ΔNp73/TAp73 ratio, except for patients with higher histological grade, which showed a high ΔNp73/TAp73 ratio. Fisher's test and Cramer's V coefficient

Table 2 Statistical relationships between ΔNp73 expression and p53 protein status

			ΔNp73 expression		
Variable p53 status	No.	%	Low ΔNp73 %	High ΔNp73 %	p Value
Total patients	42	100			
Mutated p53	14	33.3	41.2	51.8	0.126
Wild type p53	28	66.6	33.3	66.7	

The p value is from Fisher's exact test.

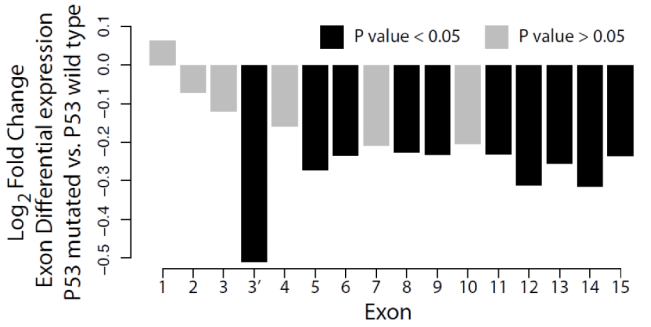

Figure 3 *TP73* exon differential expression of mutated p53 versus wild type p53 tumours. Samples were grouped according to *TP53* mutational status into a non-silent mutated *TP53* group and a wild type *TP53* group. Log_2 fold change values associated with p values and false discovery rate (FDR) values were obtained using the Benjamini and Hochberg method. To assess differential exon usage, the log_2 fold change of each exon corresponding to *TP73* was compared with the log_2 fold change of the entire gene. The expression of exon 3′ of p73 (only expressed in the ΔNp73 isoform) was significantly higher in patients with wild type p53 ($p<0.05$).

confirmed the association between high expression of ΔNp73 (high ΔNp73/TAp73 ratio) and high histological grade: ΔNp73 overexpression was found in 86.7% (13/15) of high-grade, 77.8% (14/18) of intermediate grade, and in only 33.3% (3/9) of low-grade tumours (figure 5). Our analysis of ΔNp73 expression in high-grade versus low-grade tumours revealed a significantly moderate association ($\varphi=0.509$, $p=0.015$). In addition, comparison between ΔNp73 expression in intermediate grade versus low-grade tumours showed a significantly moderate association ($\varphi=0.447$, $p=0.04$).

Interestingly, with a mean follow-up of 120 months, we observed that 18.2% of patients died, 15.1% experienced disease progression, and 66.7% became disease free. Patients were divided into two groups for analysis according to low or high ΔNp73 expression levels. The results showed a non-significant correlation between high ΔNp73 expression with DFS (log rank $\chi^2=0.511$, $p=0.475$) and OS (log rank $\chi^2=0.444$, $p=0.555$) (figure 6).

DISCUSSION

The critical role of the tumour suppressor p73 has been extensively studied. The *TP73* gene gives rise to several protein isoforms with antagonistic functions. The two different promoters (P1 and P2) of p73 protein have several CpG islands, most of which are located in promoter P1 with fewer in promoter P2. [36]

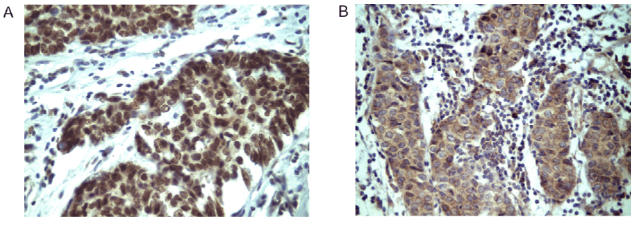

Figure 4 Immunohistochemical detection of p73 isoforms in breast cancer. (A) Tumour breast sample with high expression of all p73 isoforms (TAp73, ΔNp73 or both) localised in both the nuclear and cytoplasmic compartments (×400). (B) Breast tumour sample with high expression of the ΔNp73 isoform localised mainly in the cytoplasmic compartment (×400).

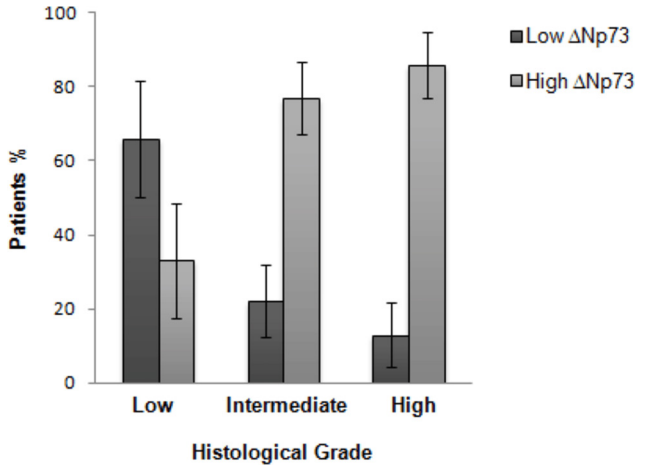

Figure 5 Expression level of the ΔNp73 isoform and histological grade. Distribution of high ΔNp73 expression (grey bars) versus low ΔNp73 expression (black bars) and low, intermediate and high histological grade.

To investigate the relationship between *TP73* promoter methylation and molecular subtype in breast carcinomas, we performed in silico and in vivo analyses. Our results indicate that *TP73* in CpG islands located in or close to promoter P1 has a different methylation status in different BC subtypes. The *TP73* methylation status in the +258 CpG site was different in all subtypes. Luminal A tumours showed a significantly low frequency of *TP73* methylation. A similar level of *TP73* methylation in this region was observed in luminal A tumours when we performed the in silico analysis. We also confirmed our previous results regarding the methylation status of *TP73* in triple negative tumours.[9]

Moreover, the in silico analysis of human breast tumours revealed that the CpG islands in the promoter region are highly methylated in luminal A and luminal B tumours, and hypomethylated in normal breast epithelium and basal-like tumours. Holm *et al*[14] also reported that the BC subtypes, especially luminal A, luminal B and basal-like, exhibit different methylation profiles. The different methylation status of *TP73* between the two regions could be related to the regulatory function of each region. These results indicate that the methylation status of *TP73* can contribute to differentiate between the luminal and basal-like subtypes of BC.

We also studied the relationship between *TP73* DNA methylation and expression of the protein in 42 BC patients. We observed elevated expression of the p73 protein in tumours even though they showed *TP73* DNA methylation. In general, increased ΔNp73 isoform expression was observed in breast tumours in which *TP73* was found methylated close to promoter P1. In accordance with the present study, Lai *et al*[36] reported that under a promoter hypermethylation state, ΔNp73 is expressed at high levels while TAp73 expression remains low. Given that P1 contains more CpG islands while P2 contains fewer CpG islands, methylation affects the transcription and expression of TAp73.

Immunoblot analysis revealed that p73 was expressed in 69.7% of cancer samples, and in 65% of these cases increased ΔNp73 isoform levels were found. This latter observation is in accordance with other authors who reported significant ΔNp73 isoform overexpression in a number of solid tumours.[28 41–45] The balance between TAp73 and ΔNp73 isoforms may play a role in the regulation of cell proliferation and cell death.[46]

An association between wild-type p53 status and upregulation of the ΔNp73 isoform has been shown in breast tumour.[28] Although our patients do not show significant correlations between p53 and ΔNp73 expression, we have noted a link between wild type p53 and ΔNp73 isoform expression in 66.7% of cases. This finding is in accordance with the in silico analysis where we observed a correlation between wild type p53 and ΔNp73 expression. This led us to assume that transactivation of the ΔNp73 promoter by p53, as postulated by Dominguez *et al*,[28] could possibly explain this behaviour. However, the relationship between p53 status and ΔNp73 expression is controversial. Bozetti *et al*[47] reported a significant correlation between mutated p53 and ΔNp73 expression in BC, suggesting that both alterations could confer an additional advantage on tumour cells. More studies are needed to elucidate whether these alterations in *TP53*-family genes may or not be mutually exclusive.

Immunohistochemical analysis of BC tissues revealed the presence of p73 in the nuclear and cytoplasmic compartments, while the ΔNp73 isoform was predominantly expressed in the cytoplasm of tumour cells. Inoue *et al*[48] recognised nuclear localization and export signals in p73, suggesting that the localization of the protein can be controlled by both nuclear import and export, thus affecting the cellular distribution of p73 by the balance between these two processes. In contrast to our work, Di Vinci *et al*[49] reported nuclear localization of ΔNp73 and cytoplasmic expression of TAp73 in almost all non-small cell

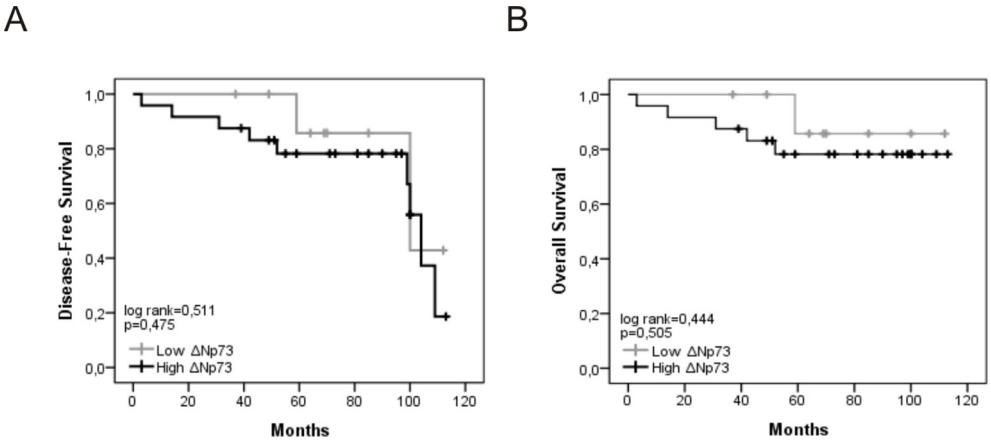

Figure 6 Survival of breast cancer patients according to high and low ΔNp73 expression. Probability of (A) disease-free survival and (B) overall survival.

lung cancer cases analysed. In accordance with our observations, several reports indicated that ΔNp73 may be confined mainly to the cytoplasm of tumour cells.[26 36 47 50] For example, Bozetti *et al* observed that ΔNp73 is predominantly cytoplasmic while TAp73 localization may be confined to the nucleus and to the cytoplasm in BC.[47] It is important to note that the latter work used a TAp73 specific antibody which did not cross-react with ΔNp73. Although our p73 antibody recognises all p73 isoforms, the findings were similar. Most previous studies focused on p73 isoforms have analysed their expression levels using real time PCR.[51] The poor availability of specific antibodies which recognise each p73 isoform with no cross-reaction has been a limiting factor for these studies. Therefore, there is no extensive information about subcellular localization of p73 isoforms for all type of tumours or about the functional significance of cytoplasmic ΔNp73.

The most remarkable association found in our results was the correlation between high expression of the ΔNp73 isoform in patients and elevated histological grade. In the assessment of BC, a substantial proportion (30%–60%) of breast tumours are classified as intermediate histological grade, which is not very informative for making a treatment decision.[52] Thus, in these cases, the chosen treatment is generally based on other prognostic factors. Comparisons between the expression of ΔNp73 in high-grade tumours and low-grade tumours revealed a positive correlation with higher-grade cancers. Our results suggest that breast tumours with higher ΔNp73 expression have a poor prognosis. Therefore, the detection of ΔNp73 expression in intermediate histological grade samples could be useful to determine the aggressiveness of tumours and thereby contribute to treatment planning. Our findings are consistent with previous studies which also associated ΔNp73 overexpression (alone or in association with TAp73, ΔN/TA ratio) with poor prognosis in acute promyelocytic leukaemia, BC, colon cancer and lung cancer.[26 28 34]

In conclusion, our results suggest that hypermethylation of *TP73* and high expression of ΔNp73 could be used to predict the aggressiveness of breast tumours. Concomitant upregulation of ΔNp73 expression and hypermethylation of *TP73* could be an effective indicator of poor prognosis and therefore could be determined to evaluate the aggressiveness of IDC, specially in intermediate histological grade cancers. Further studies are required to reach a definitive conclusion.

Take Home Messages

- *TP73* DNA methylation differs among breast cancer molecular subtypes.
- *TP73* DNA methylation in the +258 CpG site is inversely correlated with p73 expression.
- ΔNp73 isoform expression is associated with higher histological grade.

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Handling editor Runjan Chetty.

Acknowledgements We want to thank Dr F Darío Cuello-Carrión for his technical assistance.

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Funding This work was supported by the National Cancer Institute, Ministry of Health of Argentina (LMV-R), CONICET, Argentina Grant: PIP 27913 (LMV-R), and National University of Cuyo, Argentina Grant: 06-J463 (LMV-R); the Associates for Breast and Prostate Cancer Studies (ABCs) (Grant ID: 887377001-40000) award (DM and JIO) and the Fashion Footwear Association of New York (FFANY) (Grant ID: 88737700160000) foundation (DMM).

Competing interests None declared.

Ethics approval Ethics Committee of the Medical School, National University of Cuyo, Mendoza, Argentina.

Provenance and peer review Not commissioned; externally peer reviewed.

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J Clin Pathol published online July 25, 2017