bjh short report

The expression of ΔN*TP73*, TA*TP73* and *TP53* genes in acute myeloid leukaemia is associated with recurrent cytogenetic abnormalities and *in vitro* susceptibility to cytarabine cytotoxicity

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Summary

TP73 encodes for two proteins: full-length TAp73 and ΔNp73, which have little transcriptional activity and exert dominant-negative function towards TP53 and TAp73. We compared TA*TP73* and ΔN*TP73* expression in acute myeloid leukaemia (AML) samples and normal $CD34^+$ progenitors. Both forms were more highly expressed in leukaemic cells. Amongst AML blasts, TA*TP73* was more expressed in AML harbouring the recurrent genetic abnormalities (RGA): *PML-RARA*, *RUNX1-RUNX1T1* and *CBFB-MYH11*, whereas higher ΔN*TP73* expression was detected in non-RGA cases. *TP53* expression did not vary according to ΔN*TP73*/TA*TP73* expression ratio. Leukaemic cells with higher ΔN*TP73*/TA*TP73* ratios were significantly more resistant to cytarabine-induced apoptosis.

Keywords: acute myeloid leukaemia, TA*TP73*, ΔN*TP73*, *TP53*, apoptosis.

TP73 is a homologue of the tumour suppressor gene *TP53* and shares substantial structural and functional homology with p53 protein (TP53) (Kaghad *et al*, 1997). Nevertheless, *TP73* is not a classic tumour suppressor gene and inactivating mutations in cancer are rare. Unlike *TP53* knockout mice ($p53^{-/-}$), $TP73^{-/-}$ mice do not develop tumours (Moll & Slade, 2004). This gene has two distinct promoters that allow the formation of two isoforms: full-length TAp73 and ΔNp73, which lacks the NH_2-terminal transactivating domain and is thought to act in dominant-negative manner on TP53 and TAp73 functions (Yang *et al*, 2000).

Higher *TP73* mRNA and protein levels have been detected in tumoural tissues (Stiewe & Putzer, 2002). However, the differential expression of *TP73* forms in the distinct malignancies is unclear. Zaika *et al* (2002) reported the first evidence that ΔN*TP73* is frequently upregulated in a variety of solid tumours. *TP73* is commonly expressed and shows a very low frequency of mutations and hypermethylation in acute myeloid leukaemia (AML) (Sahu *et al*, 2005). Furthermore, this gene has a distinct expression pattern in AML subtypes, presenting lower levels of ΔN*TP73* in acute promyelocytic leukaemia (APL) (Rizzo *et al*, 2004).

As the preferential upregulation of ΔN*TP73* may bestow oncogenic activity on *TP73*, we compared the expression of the TA*TP73* and ΔN*TP73* in AML blasts and normal $CD34^+$

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progenitors and correlate their relative expression with susceptibility to apoptosis.

Patients and methods

Patient samples

Bone marrow (BM) cells from 147 non consecutive patients with *de novo* AML were obtained at diagnosis after informed consent. The diagnosis and classification of the disease had considered morphological and immunophenotypic features and the detection of recurrent genetic abnormalities (RGA) *PML-RARA*, *RUNX1-RUNX1T1* or *CBFB-MYH11*, which were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) (van Dongen *et al*, 1999). These rearrangements were detected in 79 cases (RGA group): *PML-RARA* in 32, *RUNX1-RUNX1T1* in 17 and *CBFB-MYH11* in 30 samples. The non-RGA group ($n = 68$) constituted AML subtypes M0 ($n = 4$), M1 ($n = 9$), M2 ($n = 32$), M4 ($n = 14$), M5 ($n = 7$) and M6 ($n = 2$) according to the French–American–British classification. The presence of the *MLL-AFF1* rearrangement was investigated by RT-PCR (van Dongen *et al*, 1999), but no positive case was detected.

In addition, 22 BM samples were obtained from healthy adult donors for BM transplantation, and $CD34^+$ haematopoietic progenitors were isolated using immunomagnetic beads (#130-046-702; Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation, samples contained more than 80% of $CD34^+$ cells. The study was approved by the local Ethics Committee (12474/2004).

Quantitative real-time PCR (RQ-PCR)

TaqMan-based RQ-PCR was performed using the following primers and probes: TA*TP73* forward primer: GGGACGCAGAAGAAACC, TA*TP73* reverse primer: GGTGGACTGGGCCATCTTC, TA*TP73* probe: FAM–CAGCTCGCTCTGCAGCC–NFQ; ΔN*TP73* forward primer: CGCCTACCATGCTGTACGT, ΔN*TP73* reverse primer GGCTGCTCATCTGGTCCAT, ΔN*TP73* probe: FAM–ACCTCGCCACGGCCCA–NFQ. For *TP53* analyses, the primes and probe were developed by *Assay on Demand* (Hs01034253_m1; Applied BioSystems, Foster City, CA, USA). All experiments were carried out in duplicate. The differences of threshold cycles (ΔC_t) were derived by subtracting the C_t value for the median of internal reference (*GAPDH* and *ABL1*) from the C_t values of the evaluated genes. The relative fold value was obtained by the formula $2^{-\Delta\Delta C_t}$ using the median ΔC_t value of k562 cells as a reference.

In vitro cytotoxicity assay

Samples from 20 AML patients (*PML-RARA* = 6; *CBFB-MYH11* = 1; *RUNX1-RUNX1T1* = 2; non-RGA = 11) with cell viability >90% and time of collection under 24 h were selected. Cells were incubated with vehicle or cytarabine (Ara-C) 100 μg/ml for 24 h, and apoptosis was assessed by Annexin-V and propidium idodide (#556547; BD Biosciences Pharmingen, San Jose, CA, USA) and analysed by flow cytometry FACScalibur (Becton Dickinson Immunocytometry Systems, San Diego, CA, USA) using the CELL QUEST software (BD BioSciences, San Diego, CA, USA).

Statistical analysis

Data were normally distributed and presented as mean ± SD. Parametric Student's *t*-test and Pearson correlation were performed using statistical package for the social sciences (SPSS) 11.0 software. *P*-values <0·05 were considered significant.

Results

*TA*TP73 *and* ΔNTP73 *gene expression in leukaemic and normal haematopoietic progenitors*

The relative expression of TA*TP73* and ΔN*TP73* was higher in leukaemic blasts compared with normal $CD34^+$ progenitors (TA*TP73*: 3·69 ± 0·7 vs. 0·02 ± 0·01, $P = 0·03$; ΔN*TP73*: 25·1 ± 4·41 vs. 0·46 ± 0·16, $P = 0·02$; Fig 1A and B). Amongst AML samples, a significant higher expression of TA*TP73* was detected in the RGA group (5·17 ± 1·17 vs. 1·73 ± 0·3, $P = 0·01$), whereas the non-RGA samples presented higher ΔN*TP73* expression (10·62 ± 1·55 vs. 44·23 ± 9·48, $P < 0·0001$). Samples harbouring *PML-RARA*, *RUNX1-RUNX1T1* and *CBFB-MYH11* rearrangements did not differ in the expression of TA*TP73* and ΔN*TP73* (Fig 1C and D).

RGA and non-RGA groups did not differ regarding age, sex and leucocyte counts, but the frequency of $CD34^+$ cases (presence of >20% of $CD34^+$ blasts) was higher in the non-RGA group (58·3% vs. 34·2%, $P = 0·003$). To test whether the differences in gene expression were due to distinct maturational stages of the blasts, we compared the expression of ΔN*TP73* and TA*TP73* according to CD34 expression. No significant difference was detected between $CD34^+$ ($n = 67$) and $CD34^-$ ($n = 80$) AML cases (TA*TP73*: 2·01 ± 0·7 vs. 4·41 ± 0·96, $P = 0·06$; ΔN*TP73*: 26·92 ± 7·43 vs. 20·17 ± 3·69, $P = 0·39$, in $CD34^+$ and $CD34^-$ group respectively). In comparison with normal $CD34^+$ progenitors, the expression of TAp73 and ΔNp73 was significantly higher in $CD34^+$ and $CD34^-$ AML blasts.

Correlation between TP53 *gene expression and* Δ*N*TP73/*TA*TP73 *ratio in AML samples*

Figure 1E shows the relationship between *TP53* and ΔN*TP73* expression in *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11* and non-RGA cells. Similar levels of the expression of both genes were detected. *TP53* expression was similar in $CD34^+$ and $CD34^-$ AML (data not shown). Moreover, when *TP53* expression was analysed according to ΔN*TP73*/TA*TP73*

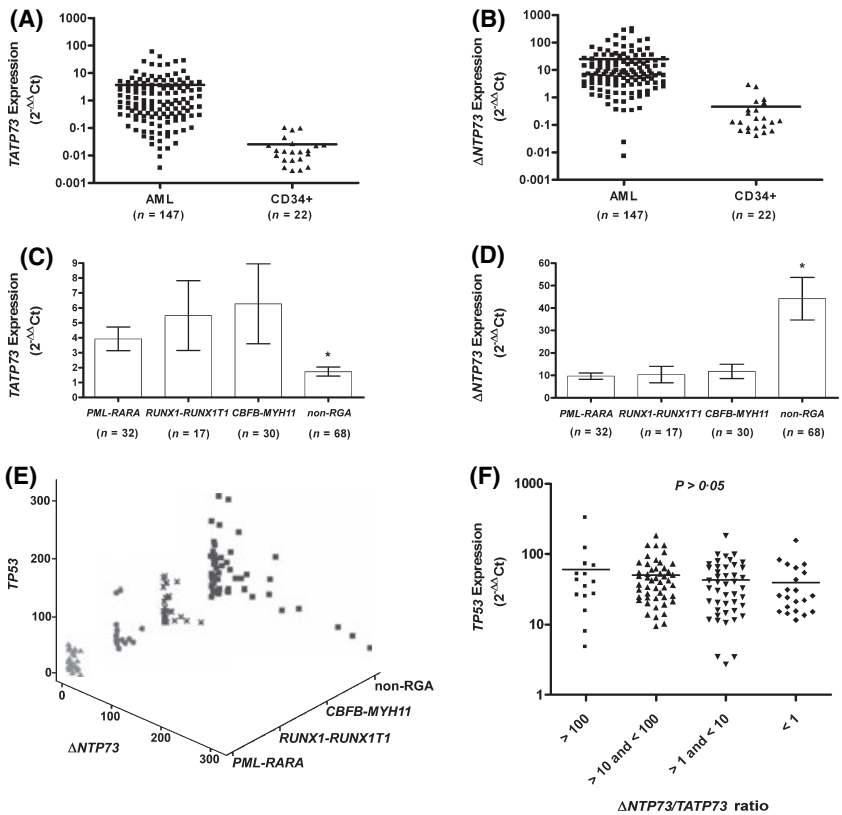

Fig 1. Quantitative analysis of TA*TP73*, ΔN*TP73* and *TP53* gene expression. Leukaemic cells from 147 patients with acute myeloid leukaemia (AML) at diagnosis and $CD34^+$ haematopoietic progenitors from the bone marrow of 22 healthy adults were obtained by aspiration and TA*TP73* (A) and ΔN*TP73* (B) expression was quantified by quantitative real-time PCR (RQ-PCR). The horizontal bars represent the mean of relative gene expression calculated using the formula $2^{-\Delta\Delta C_t}$, in which the geometric mean of *GAPDH* and *ABL1* expression (housekeeping genes) was used for normalization and the expression in the k562 cell line as internal control. AML samples were subdivided according to the presence or not of the recurrent genetic abnormalities (RGA): *PML-RARA*, *AML1-ETO* and *CBFB-MYH11*. TA*TP73* (C) and ΔN*TP73* (D) gene expression was quantified by RQ-PCR as above. Asterisks indicate significant difference in comparison with the other groups. The correlation between the level of expression of *TP53* and ΔN*TP73* genes in the AML samples was analysed according to the RGA subtype. (E) Distribution of *TP53* gene expression values according to ΔN*TP73*/TA*TP73* expression ratio in AML samples (F).

expression ratio, no significant difference was detected (Fig 1F).

In vitro cytotoxicity assay

In order to test whether the ΔN*TP73*/TA*TP73* expression ratio was correlated with resistance to apoptosis in AML blasts, we performed an *in vitro* cytotoxicity assay using Ara-C as stimulus. There was a significant negative association between Log(ΔN*TP73*/TA*TP73* ratio) and the Log(fold change of apoptotic cells) ($r^2 = -0{\cdot}607$, $P = 0{\cdot}005$; Fig 2).

Discussion

In the present study, we detected a higher expression of the TA*TP73* and ΔN*TP73* forms in AML blasts compared with normal $CD34^+$ progenitors. Moreover, the comparative analysis of 147 AML samples demonstrated that blasts with RGA presented significantly lower ΔN*TP73*/TA*TP73* ratio compared with non-RGA blasts. Rizzo *et al* (2004) also

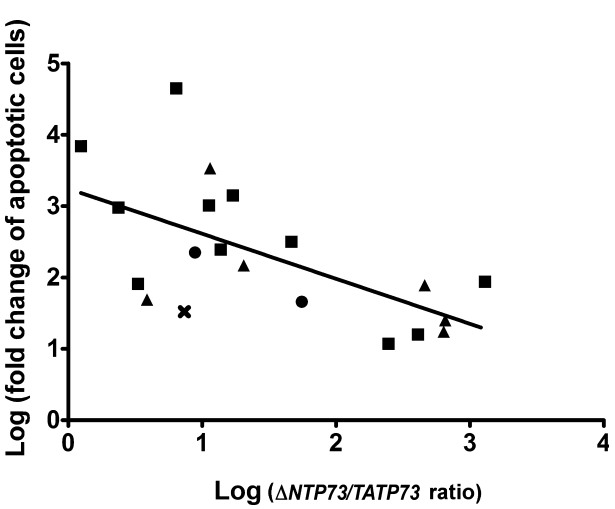

Fig 2. Correlation between *in vitro* cytotoxicity induced by Ara-C (100 μg/ml) and ΔN*TP73*/TA*TP73* expression ratio in acute myeloid leukaemia blasts harbouring or not the recurrent genetic abnormalities (non-RGA, ■): *PML-RARA* (▲), *RUNX1-RUNX1T1* (●) and *CBFB-MYH11* (×).

reported that APL blasts presented lower ΔN*TP73* expression (Rizzo *et al*, 2004). However, these authors did not specifically analyse *RUNX1-RUNX1T1* and *CBFB-MYH11* leukaemic cells. Therefore, our results suggested that lower ΔN*TP73* expression may be associated with AML with good prognosis and not only with APL. We should point out that there was a relative excess of *CBFB-MYH11* cases in the present analysis, which may be explained by the fact that samples were referred to our centre for molecular diagnosis because of morphological features associated with RGA. Apart from the high proportion of *CBFB-MYH11*, the distribution of AML subtypes was similar to that described elsewhere in Brazil (Onsten *et al*, 2006).

TAp73 expression was higher in $CD34^-$ compared with $CD34^+$ AML, but this difference was not significant ($P = 0{\cdot}06$). The differences in ΔN*TP73* and *TP53* expression in these two AML subgroups were lower than those detected for TAp73 and also not significant. During normal haematopoiesis, a narrow range of variation of *TP73* expression among normal $CD34^+$ progenitors, peripheral blood leucocytes and lymphocytes was found during normal haematopoiesis (Peters *et al*, 1999). In contrast, TAP53 was not detected in the normal $CD34^+$ progenitors, and low but detectable levels were observed in the mature lymphoid, granulocytic and monocytic cell populations (Kastan *et al*, 1991).

The quantification of both forms of *TP73* is relevant because the balance between ΔN*TP73* and TA*TP73* is the key to determine the oncogenic activity of the gene (Zaika *et al*, 2002). However, the mechanisms underlying the regulation of *TP73* forms by AML-associated oncoproteins are unknown. Bernassola *et al* (2004) demonstrated that the COOH-terminal region of PML physically interacted with TAp73. PML overexpression resulted in the inhibition of TAp73 ubiquitination and degradation (Bernassola *et al*, 2004). As the PML-RARα oncoprotein retains the COOH-terminal region of PML, it may interfere with TAp73 expression, post-transcriptional modification and stability. In fact, Mainardi *et al* (2007) demonstrated that ΔNp73 is a transcriptional target of PML-RARα and that treatment with retinoic acid restored the levels of expression of ΔNp73.

The present study is the first to demonstrate that leukaemic cells with higher ΔN*TP73*/TA*TP73* ratios were significantly more resistant to apoptosis induced by Ara-C, and thus may be associated with the resistant phenotype. No correlation was detected between ΔN*TP73*/TA*TP73* ratio and *TP53* expression, suggesting that resistance was not dependent on TP53. One may argue that determining whether *TP53* and *TP73* genes are mutated in resistant cells would be more relevant than the gene expression analysis, but considering the rarity of these mutations in AML this possibility seems unlikely.

Taken together, our results demonstrated that both forms of *TP73* were overexpressed in AML, with a distinct pattern of expression associated with the presence of RGA, suggesting that the deregulation of the balance between ΔN*TP73* and TA*TP73* may be involved in leukaemogenesis and in determining the response to chemotherapy.

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