The expression of ΔNTP73, TATP73 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 TATP73 and ΔNTP73 expression in acute myeloid leukaemia (AML) samples and normal CD34+ progenitors. Both forms were more highly expressed in leukaemic cells. Amongst AML blasts, TATP73 was more expressed in AML harbouring the recurrent genetic abnormalities (RGA): PML-RARA, RUNX1-RUNX1T1 and CBFB-MYH11, whereas higher ΔNTP73 expression was detected in non-RGA cases. TP53 expression did not vary according to ΔNTP73/TATP73 expression ratio. Leukaemic cells with higher ΔNTP73/TATP73 ratios were significantly more resistant to cytarabine-induced apoptosis.

Keywords: acute myeloid leukaemia, TATP73, ΔNTP73, 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 NH2-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 ΔNTP73 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 ΔNTP73 in acute promyelocytic leukaemia (APL) (Rizzo et al, 2004). As the preferential upregulation of ΔNTP73 may bestow oncogenic activity on TP73, we compared the expression of the TATP73 and ΔNTP73 in AML blasts and normal CD34+.
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: TAP73 forward primer: GGGACGCAA-GAAAGAAC, TAP73 reverse primer: GTGAGCTGGGCGCATCTG, TAP73 probe: FAM–TGCTGCTCTGACG–NFQ; ΔNTP73 forward primer: CGCTTACCATGCTG-TAGCT, ΔNTP73 reverse primer: GGTGGCCTCCCTTGTCAT, ΔNTP73 probe: FAM–ACCTGCGCCAGGC–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 (ΔCt) were derived by subtracting the Ct value for the median of internal reference (GAPDH and ABL1) from the Ct values of the evaluated genes. The relative fold value was obtained by the formula 2−ΔΔCt using the median ΔCt 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 iodide (#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**

**TATP73 and ΔNTP73 gene expression in leukaemic and normal haematopoietic progenitors**

The relative expression of TATP73 and ΔNTP73 was higher in leukaemic blasts compared with normal CD34+ progenitors (TATP73: 3.69 ± 0.7 vs. 0.02 ± 0.01; P = 0.03; ΔNTP73: 25.1 ± 4.41 vs. 0.46 ± 0.16; P = 0.02; Fig 1A and B). Amongst AML samples, a significant higher expression of TATP73 was detected in the RGA group (5±1 ± 1.17 vs. 1.73 ± 0.3; P = 0.01), whereas the non-RGA samples presented higher ΔNTP73 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 TATP73 and ΔNTP73 (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 maturation stages of the blasts, we compared the expression of ΔNTP73 and TATP73 according to CD34 expression. No significant difference was detected between CD34+ (n = 67) and CD34− (n = 80) AML cases (TATP73: 2.01 ± 0.7 vs. 4.41 ± 0.96, P = 0.06; ΔNTP73: 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 ΔNTP73/TATP73 ratio in AML samples**

Figure 1E shows the relationship between TP53 and ΔNTP73 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 ΔNTP73/TATP73...
expression ratio, no significant difference was detected (Fig 1F).

In vitro cytotoxicity assay

In order to test whether the ΔNTP73/TATP73 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(ΔNTP73/TATP73 ratio) and the Log(fold change of apoptotic cells) ($r^2 = -0.607$, $P = 0.005$; Fig 2).

Discussion

In the present study, we detected a higher expression of the TATP73 and ΔNTP73 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 ΔNTP73/TATP73 ratio compared with non-RGA blasts. Rizzo et al (2004) also...
reported that APL blasts presented lower $\Delta$NTP73 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 $\Delta$NTP73 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 ($P = 0.06$). The differences in $\Delta$NTP73 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 $\Delta$NTP73 and TATP73 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 $\Delta$Np73 is a transcriptional target of PML-RARα and that treatment with retinoic acid restored the levels of expression of $\Delta$Np73.

The present study is the first to demonstrate that leukaemic cells with higher $\Delta$NTP73/TATP73 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 $\Delta$NTP73/TATP73 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 $\Delta$NTP73 and TATP73 may be involved in leukaemogenesis and in determining the response to chemotherapy.

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References


