Prognostic implications of Wilms’ tumor gene (WT1) expression in patients with de novo acute myeloid leukemia

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Background and Objectives. The Wilms’ tumor (WT1) gene is overexpressed in patients with most forms of acute leukemia. Several studies have reported the usefulness of quantitative assessment of WT1 expression as a molecular marker of minimal residual disease. However, the biological significance and the prognostic impact of WT1 overexpression in acute myeloid leukemia (AML) is still uncertain.

Design and Methods. We analyzed the prognostic relevance of WT1 expression in a cohort of 77 adult patients with AML, using a real-time quantitative reverse-transcription polymerase chain reaction approach.

Results. WT1 expression was significantly higher in AML patients than in normal controls (p = 0.0001). The normalized levels of WT1 with respect to the control gene for β-glucuronidase (GUS) in AML samples showed a median WT1/GUS ratio of 0.93 (range 0–25). We classified the patients into two groups according to this ratio. Forty patients (52%) showed a WT1/GUS ratio ≤ 1 and 37 (48%) had a ratio > 1. A ratio > 1, although significantly associated with FLT3 mutations, was the strongest independent prognostic factor for disease-free survival (p = 0.004), relapse risk (p = 0.005) and cumulative incidence risk (p = 0.01). This adverse prognostic value was more evident in patients aged 60 years and younger.

Interpretation and Conclusions. The WT1/GUS ratio is an independent prognostic factor for predicting relapse in patients with AML and it could be included as part of the initial evaluation to establish more defined risk groups.

Key words: WT1 expression, real-time reverse transcription PCR, leukemia, prognostic value.

The WT1 gene is a tumor suppressor originally associated with Wilms’ tumor and other related syndromes, such as WAGR (Wilms’ tumor, aniridia, genitourinary anomalies and mental retardation) and Denys-Drash syndrome.1-3 The WT1 locus is located at chromosome band 11p13 and encodes a transcription factor.4-6 In contrast with the wild type expression of other tumor suppressor genes, such as p53 or RB1, normal expression of WT1 is restricted in adults to a limited number of tissues, mainly the genitourinary system.6 In normal bone marrow (BM), WT1 is expressed at a very low level by normal primitive progenitor cells.7 However, several studies have demonstrated that WT1 is consistently overexpressed in most forms of acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS) and blast crisis of chronic myeloid leukemia (CML).8-10 This expression of WT1 could thus represent a universal molecular marker of malignant hematopoiensis, and several recent studies claim the usefulness of quantitative assessment of WT1 expression as a molecular marker for minimal residual disease (MRD).1,11,12 Although the biological significance of WT1 overexpression in patients with leukemia is far from being clarified, it has been suggested that WT1 could be involved in the pathogenesis of human leukemia though a role during growth arrest and cellular differentiation as well as by means of its function as a transcriptional repressor.13-15 Moreover, some preliminary studies indicate that prognosis in leukemia patients could be inversely correlated with the levels of WT1 expression.9-10 Here we analyzed WT1 expression in a cohort of 77 adult patients with AML using...
real-time quantitative reverse transcription polymerase chain reaction (RQ–PCR), and evaluated the utility of this biological marker for predicting relapse.

Design and methods

Patients and controls

A total of 77 adult non-promyelocytic patients, diagnosed with de novo AML in four Spanish institutions between March 1998 and March 2003, were included. Diagnoses were made according to morphologic and cytochemical criteria of the French–American–British (FAB) classification. The only criterion for inclusion was the availability of RNA. The main characteristics of the patients are shown in Table 1. In addition, 9 peripheral blood (PB) and 4 BM samples were collected from healthy donors as normal controls and the K562 cell line was examined as a positive control.

Treatment

Sixty-six of the 77 patients were enrolled into intensive chemotherapy trials in which induction chemotherapy consisted of standard combinations of anthracycline plus cytarabine, with or without etoposide. As post-remission therapy 26 patients followed a standard chemotherapy program; 17 patients received an autologous hematopoietic stem cell transplant (HSCT) and 11 received an allogeneic HSCT.

Cytogenetic analysis

Karyotype analysis was performed using unstimulated short-term cultures according to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN, 1995). Whenever possible, at least 20 metaphases were evaluated. Cytogenetic risk groups were defined as follows: high risk, -5/del(5q), -7/del(7q), abn 3q, complex aberrations (≥3 independent aberrations), t(9;22) and t(6;9); low risk, t(8;21) and inv(16); intermediate risk, all other karyotypic aberrations or a normal karyotype.

Molecular analysis: detection of ITD and D835 mutations of FLT3

FLT3 internal tandem duplications (ITD) and asparagine 835 (D835) mutations were studied in cDNA samples following the method described by Nakao et al. for ITD and by Moreno et al. for D835 mutations. In selected cases, the presence of D835 mutations was confirmed by sequencing the PCR products.

RQ–PCR for WT1

Blood BM samples or PB, in three cases in which BM was not available but the PB contained more than 80% blasts, were collected into EDTA K3 tubes; erythrocytes were lysed using lysis buffer (0.155M NH₄Cl, 10mM KHCO₃, 0.1 mM Na.EDTA, pH 7.4) and white cells separated by centrifugation at 1,500g. The collected cells were resuspended in guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, containing 5% N-lauroylsarcosine and 0.1 M 2-mercaptoethanol), and stored at −80°C. RNA was extracted following the guanidinium–thiocyanate, phenol-chloroform procedure of Chomczynski and Sacchi. For cDNA synthesis 0.5 µg aliquots of RNA were reverse-transcribed in a 25-µL reaction volume using random hexamer primers with the TaqMan Gold RT–PCR Kit (PE Applied Biosystems, Foster City, CA USA) following the manufacturer’s protocol. The reaction was preincubated for 10 min at 25°C to allow annealing of random hexamers followed by reverse transcription at 48°C for 30 min and denaturation at 95°C for 5 min to inactivate the reverse transcriptase.

In order to generate standards, the WT1 rearrangement and the control gene for β-glucuronidase (GUS)
and cDNA. The was >30 cycles and the control gene valor DNA polymer ratios were >1.0. Detection was performed using a inserts were measured by spectrofluorimetry to assess the copy number. Standard curves were then prepared using ten-fold serial dilutions, ranging from 1.3×10⁶ to 1.3×10⁹ copies of plasmid pCR II-TOPO⁶⁶¹ and pCR II-TOPO⁶⁶⁵. These dilutions were prepared with DNA from salmon testes at stock concentrations of 1 µg in 25 µL water and stored at -20°C until use to prevent degradation.

RO–PCR for WT1 detection was performed using a LightCycler (Roche Mannheim, Germany). The primers WT1A and WT1B and TaqMan probe WT1M were designed and synthesized by TIB MOLBIOL (Berlin, Germany) (WT1A, 5’–AGC TGT CGG TGG CCA AGT TGT C–3’; WT1B 5’–TGC CTG GGA CAC TGA ACG GTC–3’ and WT1M, 6FAM–ACC CCT CAA AGC GCC AGC TGG AGT–XT p) (Figure 1). The PCR was performed in a 10 µL final volume, using 1 µL FastStart LightCycler DNA Master Hybridization Probes (Roche Molecular Biochemicals, IN, USA, Cat. No. 3003248). This mixture contains buffer, dNTPs, 1 mM MgCl₂, and inactive Taq DNA polymerase. The primers were used at a final concentration of 0.3 µM. The mix contained TaqMan probe at 0.2 µM, MgCl₂ to a final concentration of 3 mM and 2 µL of sample cDNA.

As a reference, GUS was quantified in all samples using ENF1102 and ENR1162 primers and the ENPr1142 probe described by Beillard et al., and synthesized by TIB MOLBIOL. The PCR mixtures were the same as for WT1 but the primers were added at a final concentration of 0.5 µM.

Each PCR program started with an incubation at 94°C for 10 min to activate the Taq DNA polymerase, followed by 45 cycles of amplification, each involving an annealing–extension step at 60°C for 30 s and denaturation at 94°C for 3 s. Fluorescence was measured at the end of the annealing using the F1 channel (530 nm). Results were calculated using LightCycler 3.0 software, which fits an empirical straight line to the points of the standard curve, based on the established relationship between the crossing point (Cp) and the logarithm of the initial number of target copies (N) of the sample. This allows estimation of N for each sample on the basis of its Cp, for both WT1 and the control gene GUS.

All samples were analyzed in duplicate. The results were expressed as the WT1 or GUS copies per microliter of cDNA. Normalized levels were calculated as the WT1/GUS ratio. The expression of GUS control gene in 100 leukemic samples showed a median Cp of 24.91 and limits (3rd and 97th percentiles) of 20.5–30.1 (data not shown). Samples for which the Cp of GUS was >30 cycles were considered invalid and therefore excluded.

Definitions

Complete remission (CR) and hematologic relapse were defined according to the National Cancer Institute criteria. The patients were classified into two groups according to whether their WT1/GUS ratios were >1.0 and ≤1.0.

Statistics

All descriptive statistics and tests (Mann–Whitney non-parametric U test, χ² and Fisher’s exact test) were calculated using the statistical package SPSS 8.0. A p < 0.05 was considered statistically significant. Unadjusted time-to-event analyses were performed using the Kaplan–Meier method and log-rank tests for comparisons. The probability of relapse was also estimated by the cumulative incidence method. Disease-free survival (DFS), relapse risk (RR) and cumulative incidence of relapse (CIR) were calculated from the date of CR. In the analysis of DFS, relapse and death in CR were considered uncensored events, whichever occurred first. For RR, relapse in CR was considered an uncensored event and for CIR analysis, death in CR was considered as a competing cause of failure. The follow-up of the patients was updated on September 30, 2003. All p values reported are two-sided. Multivariate analysis was performed using the Cox proportional hazards model. Except for the cumulative incidence method, computations were performed using the 4F, 1L and 2L programs from the BMDP statistical library (BMDP Statistical Software Inc, Los Angeles, CA, USA).

Results

Standard curves, sensitivity and reliability of the assay

The regression coefficients obtained for WT1 and GUS standard curves were all greater than 0.99. For WT1, the estimated mean ± standard deviation (SD) for the slope was −3.68 ± 0.08, with a mean ± SD for the intercept...
WT1 ratio: a new prognostic factor

For the GUS standard curves, the slope obtained was $-3.47 \pm 0.06$, and the intercept was $39.79 \pm 0.67$.

When the sensitivity of the procedure was assessed using serial ten-fold dilutions (from $10^{-4}$ to $10^{-9}$) of plasmid pCR II-TOPO WT1, the method could detect at least 13 copies. When cDNA dilutions from the K562 cell line were used, the method could amplify the WT1 transcript from a dilution of $10^{-4}$ (Figure 3).

The intra-assay reproducibility was assessed repeating the analysis of the K562 $10^{-2}$ dilution 10 times in the same assay. The mean $C_v \pm SD$ for WT1 was $29.10 \pm 0.08$ cycles, which represented a coefficient of variance (CV) of 0.27%. These results corresponded to mean $\pm$ SD of 15245$\pm$721.5 copies/$\mu$L cDNA and an estimated within-assay CV of 4.7%. For GUS, the mean $C_v \pm SD$ was 26.89$\pm$0.07 cycles, with a CV of 0.26%, or 2336$\pm$117 copies/$\mu$L cDNA; a CV of 5.0%.

The inter-assay reproducibility was assessed by repeating the analysis of the K562 $10^{-2}$ dilution in eight consecutive assays. The mean $C_v \pm SD$ for WT1 was 28.95$\pm$0.19 cycles, a CV of 0.8%. These results correspond to a mean$\pm$SD of 25230$\pm$2568 copies/$\mu$L cDNA and an estimated within-assay CV of 10%. For GUS, the mean $C_v \pm SD$ was 27.28$\pm$0.25 cycles, with a CV of 0.9%, or 2105$\pm$183 copies/$\mu$L cDNA; a CV of 8.6%.
**WT1 expression in controls and AML at presentation**

WT1 expression was very low or undetectable in normal controls. All nine PB and two BM samples were negative. The two remaining BM samples were positive but they expressed WT1 at very low levels, with WT1/GUS ratios of 0.00069 and 0.00025.

In the 77 BM and PB samples from patients at diagnosis the WT1/GUS level was significantly higher than in normal controls ($p = 0.0001$: Figure 4). Quantification showed a WT1/GUS median ratio of 0.93 (range 0–25). Forty patients (52%) had a WT1/GUS ratio ≤ 1 with a median ratio of 0.36 (range 0–1.0) and 37 patients (48%) had a WT1/GUS ratio > 1 and a median ratio of 2.76 (range 1.1–25.0).

**WT1 ratio and FLT3 mutations**

Mutations in the FLT3 gene were detected in 20 out of 76 (26%) patients with FLT3 expression. These alterations included 13 patients with ITD, six with D835 mutations and one patient with combined mutations. FLT3 mutations were found in 15 of 36 (42%) patients with WT1/GUS > 1 whereas only six patients out of 40 (15%) with WT1/GUS ≤ 1 had FLT3 mutations ($p = 0.009$: Table 1).

**WT1 ratio: clinical characteristics, response to therapy and clinical outcome**

No significant association was found between the WT1 ratio and age, gender, leukocytes, FAB or cytogenetic risk groups (Table 1). Moreover, the WT1 ratio had no influence on the patients’ responses to induction therapy: 29 (80.6%) of patients with a WT1/GUS ratio of ≤ 1 and 25 (83.3%) of patients with a WT1/GUS ratio > 1 achieved CR after one or two cycles of treatment (Table 2).

**Disease-free survival (DFS)**

The estimated probability of DFS at four years for the whole series was 36 ± 7%. Univariate analysis showed that this was significantly influenced by age over 60 years ($p = 0.0005$) and WT1 ratio ($p = 0.004$). The four-year probability of DFS was 13±8% for patients with WT1/GUS > 1 whereas for patients with WT1/GUS ≤ 1 it was 57 ± 10% (Figure 5). For patients 60 years and younger, a shorter DFS was found for patients with high-risk karyotypes ($p = 0.03$), FLT3 mutations ($p = 0.04$) and a WT1/GUS ratio of > 1 ($p = 0.0003$). The four-year probability of DFS in patients with a WT1/GUS ratio of ≤ 1 was 73±11% whereas for those with a WT1/GUS ratio > 1 it was 9±8%.

**Relapse risk**

The actuarial probability of RR at four years in the total group was 53 ± 9%. Univariate analysis showed...
statistical significance for age over 60 years ($p = 0.0006$) and WT1 ratio ($p = 0.005$). Thus, the probability of relapse was significantly increased in patients with a WT1/GUS ratio of $>1$ ($78\pm11\%$ vs $32\pm10\%$) (Figure 6). For patients 60 years and younger, the RR was significantly influenced by karyotype ($p = 0.02$), FLT3 mutations ($p = 0.009$) and WT1 ratio ($p = 0.00001$). The probability of relapse in patients with WT1/GUS $> 1$ was $84\pm13\%$, while for patients with WT1/GUS $\leq 1$ it was $15\pm10\%$.

**Cumulative incidence of relapse**

For patients with WT1/GUS $> 1$ the CIR at four years was $70\%$ whereas for patients with WT1/GUS $\leq 1$ it was $28\%$ ($p = 0.01$) (Figure 7). Analyzing only patients 60 years or younger, the CIR at four years was $88\%$ for patients with WT1/GUS $> 1$ while it was $22\%$ for patients with WT1/GUS $\leq 1$ ($p = 0.0001$).

**Multivariate analysis**

Multivariate modeling including age, gender, number of courses of treatment to achieve CR, FAB subtype, leukocytosis, cytogenetic risk group, FLT3 mutations and WT1/GUS ratio showed that WT1/GUS and age over 60 years were both independent prognostic factors for DFS (WT1/GUS, $p = 0.003$; age, $p = 0.0001$) and RR (WT1/GUS, $p = 0.001$; age, $p = 0.003$) (Table 3). For patients 60 years and younger, multivariate analysis showed that the WT1/GUS ratio was the sole independent adverse prognostic factor for DFS ($p = 0.001$) and RR ($p = 0.0001$) (Table 3).

**Discussion**

This study shows that quantification of WT1 expression by RQ-PCR is a useful method for predicting relapse in patients with AML. This method allows us to classify AML patients into two groups according to the WT1/GUS ratio at presentation (WT1/GUS $> 1$ and WT1/GUS $\leq 1$). The adverse prognosis for patients with WT1/GUS $> 1$ was particularly significant in patients aged 60 years and younger.

Reported data on the prognostic significance of WT1 expression are controversial, mainly because of the limited number of patients and the diversity of methods used. Two studies claimed that WT1 expression does not have prognostic significance in patients with...
AML, but the qualitative methods used appear unsuitable to assess expression variations. Other studies using (semi) quantitative RT–PCR methods to assess WT1 expression have suggested that WT1 levels could be useful for predicting prognosis in such patients. However, these methods were complex, time-consuming and poorly reproducible. Trka et al. reported preliminary results using a real-time quantitative PCR that suggest a prognostic relevance of WT1 expression in pediatric patients with AML. To our knowledge, the present study reports the first adult series in which WT1 expression, tested with a reliable and reproducible real-time PCR method, shows a prognostic impact in patients with AML.

Contrary to other reports, we did not find any association between WT1/GUS ratio and FAB subtype, cytogenetic risk group or other pre-treatment characteristics. However, a strong association was found between this ratio and FLT3 mutations. FLT3 often associates with other acquired mutations and this may reveal a co-operating model leading to the transformed phenotype. The WT1 protein is necessary for cell proliferation and differentiation and inhibits apoptosis by interacting with p53 and bcl2. FLT3 mutations are also associated with proliferation of leukemic cells and inhibition of apoptosis. Thus, both alterations might contribute to leukemogenesis resulting in proliferating leukemic cells incapable of differentiation and with their programmed cell death inhibited.

In conclusion, this study shows that the WT1/GUS ratio is associated with FLT3 mutations at presentation and that it is an independent prognostic factor for predicting relapse in patients with AML. If these results are confirmed in prospective studies involving large number of patients, WT1 quantification could be included as part of an initial evaluation to establish more defined risk groups.

EB designed the analysis, interpreted the data and wrote the paper; JC performed cytogenetic analyses and contributed to the interpreting of the data and writing of the paper; PB supervised the experimental work and contributed to revision; SB performed molecular studies; GM contributed to statistical analysis and collecting data; PE and MJS contributed by including patients and collecting data; MAS contributed to the writing of the paper and was responsible for final approval of the version submitted. The authors reported no potential conflicts of interest.

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References

al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'realtime' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) — a Europe Against Cancer program. Leukemia 2003;17:2474-86.


