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

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A B S T R A C T

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.

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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.[4,5] 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.[7,8] However, several studies have demonstrated that *WT1* is consistently overexpressed in most forms of acute myelobastic leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS) and blast crisis of chronic myeloid leukemia (CML).[7,9,10] This expression of *WT1* could thus represent a universal molecular marker of malignant hematopoiesis, and several recent studies claim the usefulness of quantitative assessment of *WT1* expression as a molecular marker for minimal residual disease (MRD).[7,11,12]

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.[9,16]

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.[17]

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).[18] 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 aspartate 835 (D835) mutations were studied in cDNA samples following the method described by Nakao *et al.*[19] for ITD and by Moreno *et al.*[20] 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 NH_4Cl, 10mM $KHCO_3$, 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.[21]

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*)

Table 1. Clinical characteristics of patients with *de novo* AML divided according to *WT1/GUS* ratio.

	WT1/GUS ≤ 1		*WT1/GUS >1*		
Characteristics	*n*	*(%)*	*n*	*(%)*	*P*
Patients	40	(52)	37	(48)	
Age (years)					
≤ 60	26	(65)	19	(51)	n.s.
> 60	14	(35)	18	(49)	
Gender					
Male	20	(50)	17	(46)	n.s.
Female	20	(50)	20	(54)	
WBC ($\times 10^9$/L)					
≤ 50	33	(83)	29	(78)	n.s.
> 50	7	(18)	8	(22)	
FAB subtype					
M0	3	(8)	3	(8)	n.s.
M1	9	(23)	14	(38)	
M2	13	(33)	12	(33)	
M4	8	(20)	4	(11)	
M5	5	(13)	3	(8)	
M6	2	(5)	1	(3)	
Cytogenetic risk group*					
Low	2	(6)	2	(6)	
Intermediate	24	(73)	21	(64)	n.s.
High	7	(21	10	(30)	
FLT3**					
ITD+D835 pos	6	(15)	15	(42)	0.009
ITD+D835neg	34	(85)	21	(58)	

**66 patients with evaluable metaphases. **One patient was excluded because no expression of FLT3 was detected. n.s. Not significant.*

Figure 1. Schematic representation of *WT1* cDNA. The numbers indicate the exons, the arrows indicate localization of primers and the bar the TaqMan probe. The sites of alternative splicing are represented by diagonal lines.

from patients' samples were amplified using the primers and conditions described below. The PCR products were cloned into the pCR® II-TOPO vector, following the protocol provided with the TOPO™ Cloning® kit, to generate the plasmids pCR II-TOPO^{WT1} and pCR II-TOPO^{GUS}.

The *WT1* and *GUS* inserts were measured by spectrofluorimetry to assess the copy number. Standard curves were then prepared using ten-fold serial dilutions, ranging from 1.3×10^6 to 1.3×10^2 copies of plasmid pCR II-TOPO^{WT1} and 2×10^4 to 2×10^2 of plasmid pCR II-TOPO^{GUS}. 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.

RQ–PCR for *WT1* detection was performed using a LightCycler (Roche Mannheim, Germany). The primers WT1A and WT1B and TaqMan probe WT1TM 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 WT1TM, 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 $MgCl_2$, 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, $MgCl_2$ 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.*,[22] 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 (C_p) and the logarithm of the initial number of target copies (N) of the sample.[23] This allows estimation of N for each sample on the basis of its C_p, 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 C_p of 24.91 and limits (3rd and 97th percentiles) of 20.5-30.1 (*data not shown*). Samples for which the C_p 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.[24] 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, χ^2 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[25] and log-rank tests for comparisons.[26] The probability of relapse was also estimated by the cumulative incidence method.[27] 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.[28] 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

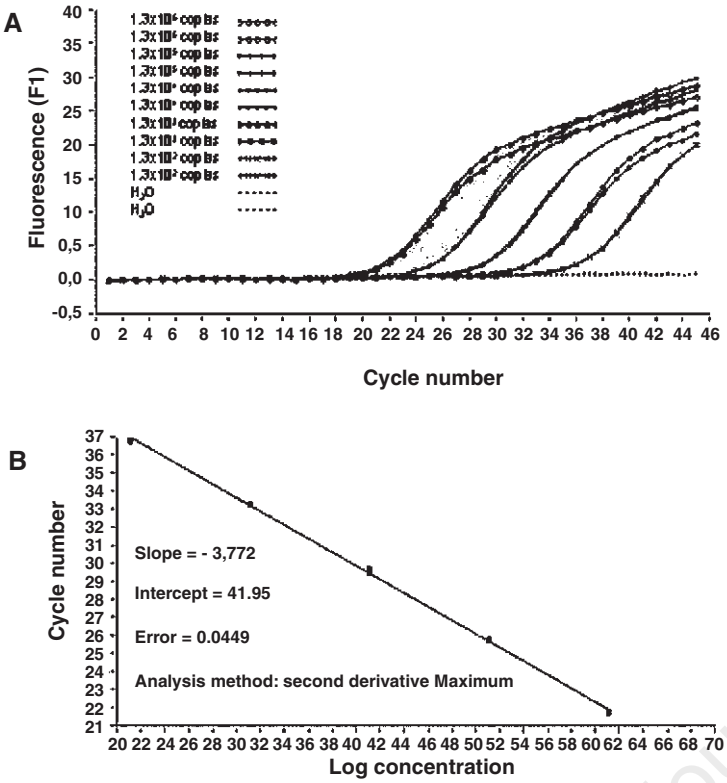

Figure 2. Standard curve and straight line for *WT1* gene expression. (A) Standard curve for the detection of *WT1* obtained by 10-fold serial dilutions ranging from 10^{-4} to 10^{-8} of stock solution of plasmid pCR II-TOPO^{WT1}. (B) Straight line obtained from the crossing point (Cp) and concentration at each point of the standard curve.

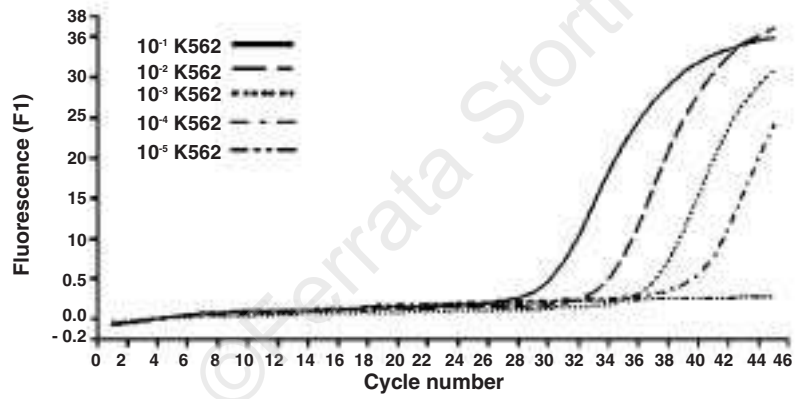

Figure 3. Sensitivity analysis. Real-time PCR detects the *WT1* transcript from the K562 cell line down to a cDNA dilution of 10^{-4}.

of 41.09 ± 0.79 (Figure 2). For the *GUS* standard curves, the slope obtained was – 3.47 ± 0.06, and the intercept was 39.79 ± 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_p±SD for *WT1* was 29.10±0.08 cycles, which represented a coefficient of variance (CV) of 0.27%. These results corresponded to mean ± SD of 15245±721.5 copies/µL cDNA and an estimated within-assay CV of 4.7%. For *GUS*, the mean C_p ± SD was 26.89±0.07 cycles, with a CV of 0.26%, or 2336±117 copies/µL 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_p±SD for *WT1* was 28.95 ± 0.19 cycles, a CV of 0.8%. These results correspond to a mean±SD of 25230±2568 copies/µL cDNA and an estimated within-assay CV of 10%. For *GUS*, the mean C_p±SD was 27.28±0.25 cycles, with a CV of 0.9%, or 2105±183 copies/µL cDNA: a CV of 8.6%.

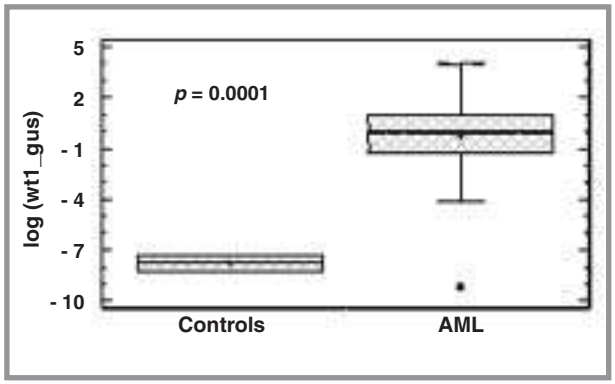

Figure 4. *WT1/GUS* ratio in healthy controls and AML patients, presented as box-and-whisker plots. Boxes represent values between the 25th and 75th percentiles with the median and mean, whiskers represents 10th and 90th percentiles and outlying values are represented by squares. The *WT1/GUS* ratios were significantly higher in patients with AML than in normal controls ($p = 0.0001$).

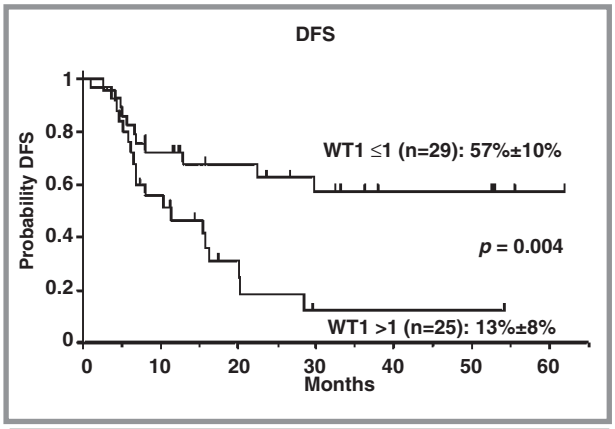

Figure 5. Kaplan–Meier product-limit estimate of disease-free survival according to *WT1/GUS* ratio.

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).

Table 2. Impact of *WT1/GUS* ratio on clinical outcome and treatment.

	WT1/GUS ≤ 1		*WT1/GUS >1*		
Characteristics	*n*	*(%)*	*n*	*(%)*	*P*
Induction response					
CR	29	(81)	25	(83)	
Failure					
Resistance	4	(11)	3	(10)	n.s.
Death	3	(8)	2	(7)	
Post-induction therapy					
Chemotherapy only	13	(45)	13	(52)	
Autologous HSCT	8	(28)	9	(36)	n.s.
Allogeneic HSCT	8	(28)	3	(12)	

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

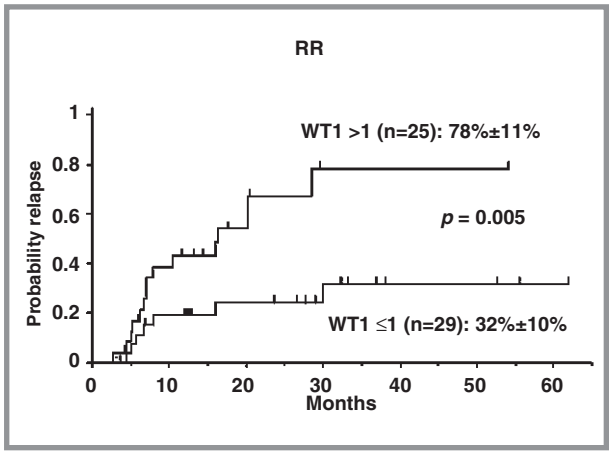

Figure 6. Kaplan–Meier product-limit estimate of risk of relapse according to *WT1/GUS* ratio.

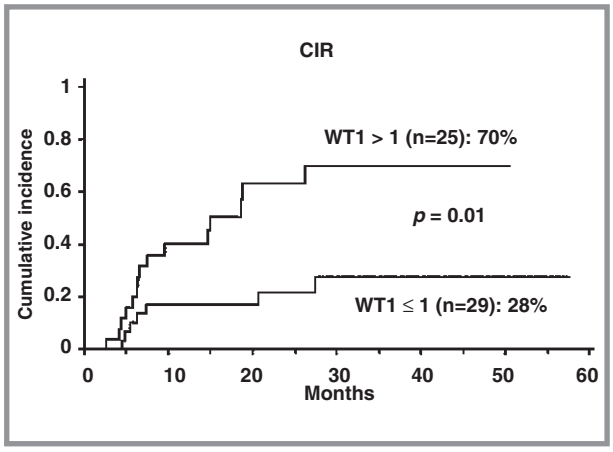

Figure 7. Cumulative incidence of relapse from the time of complete remission according to the *WT1/GUS 1* ratio.

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±11% vs 32±10%) (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±13%, while for patients with *WT1/GUS* ≤ 1 it was 15±10%.

Cumulative incidence of relapse

For patients with *WT1/GUS* > 1 the CIR at four years was 70% whereas for patients with *WT1/GUS* ≤ 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* ≤ 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* ≤ 1). The adverse prognosis for patients with *WT1/GUS* ratio > 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.[16,29-33] Two studies claimed that *WT1* expression does not have prognostic significance in patients with

Table 3. Multivariate analysis for disease-free survival (DFS) and relapse risk (RR).

	All patients				*Patients 60 years or younger*			
	DFS		*RR*		*DFS*		*RR*	
	P	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*
Age (≤ 60 vs > 60)	0.0001	2.4 (1.1–5.4)	0.003	2.7 (1–6.8)	–	–	–	–
WT1 (≤ 1 vs > 1)	0.003	2.5 (1.1–5.7)	0.001	2.6 (1–6.9)	0.001	5.5 (1.9–16.2)	0.0001	12.8 (2.7–59.9)

AML,[29,30] but the qualitative methods used appear unsuitable to assess expression variations. Other studies,[16,31,32] 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.*[33] 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,[9,31,33] 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.[34] The WT1 protein is necessary for cell proliferation and differentiation and inhibits apoptosis by interacting with p53 and bcl2.[35,36] *FLT3* mutations are also associated with proliferation of leukemic cells and inhibition of apoptosis.[37,38] 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; PF,RC 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

1. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 1990;60:509-20.
2. Brown KW, Watson JE, Poirier V, Mott MG, Berry PJ, Maitland NJ. Inactivation of the remaining allele of the WT1 gene in a Wilms' tumour from a WAGR patient. Oncogene 1992;7:763-8.
3. Akasaka Y, Kikuchi H, Nagai T, Hiraoka N, Kato S, Hata J. A point mutation in a sporadic Wilms' tumor without genitourinary abnormalities is identical with the most frequent mutation in Denys-Drash syndrome. FEBS Lett 1993;317:39-43.
4. Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III. Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science 1991;253:1550-3.
5. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms' tumor gene wt-1. Proc Natl Acad Sci USA 1991;88:9618-22.
6. Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, et al. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 1990;346:194-7.
7. Cilloni D, Gottardi E, de Micheli D, Serra A, Volpe G, Messa F, et al. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. Leukemia 2002;16:2115-21.
8. Kreuzer KA, Saborowski A, Lupberger J, Appelt C, Na IK, Le Coutre P, et al. Fluorescent 5'-exonuclease assay for the absolute quantification of Wilms' tumor gene (WT1) mRNA: implications for monitoring human leukemias. Br J Haematol 2001;114:313-8.
9. Inoue K, Sugiyama H, Ogawa H, Yamagami T, Miea H, Kita K, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994; 84: 3071-9.
10. Brieger J, Weidmann E, Maurer U, Hoelzer D, Mitrou PS, Bergmann L. The Wilms' tumor gene is frequently expressed in acute myeloblastic leukemias and may provide a marker for residual blast cells detectable by PCR. Ann Oncol 1995; 8: 811-6.
11. Cilloni D, Saglio G. Usefulness of quantitative assessment of Wilms tumor supresor gene expresión in chronic myeloid leukemia patients undergoing imatinib therapy. Semin Hematol 2003;40:37-41.
12. Ogawa H, Tamaki H, Ikegame K, Soma T, Kawakami M, Tsuboi A, et al. The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogenic stem cell transplantation in acute type leukemia. Blood 2003;10:1698-702.
13. Phelan SA, Lindberg C, Call KM. Wilms' tumor gene WT1, mRNA is down-regulated during induction of erytroid and megakaryocytic differentiation of K562 cells. Cell Growth Differentiation 1993; 5:677-86.
14. Yamagami T, Sugiyama H, Inoue K, Ogawa H, Takekawa T, Hirata M, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. Blood 1996;87:2878-84.
15. Hirose M. The role of Wilms' tumor genes. J Med Invest 1999;46:130-40.
16. Bergmann L, Miething C, Maurer U, Brieger J, Karakas T, Weidmann E, et al. High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood 1997;90:1217-25.
17. Benett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative group. Br J Haematol 1976; 33:451-8.
18. Mitelman F, editor. ISCN 1995: An International System for Human Cytogenetic Nomenclature 1995. Basel, Switzerland: S Karger. 1995.
19. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplications of the FLT3 gene found in acute myeloid leukemia. Leukemia 1996;10:1911-8.
20. Moreno I, Martín G, Bolufer P, Barragán E, Rueda E, Roman J, et al. Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukaemia. Haematologica 2003; 88:19-24.
21. Chonczynsky P, Sacchi N. Single-step method of RNA isolation by acid guanidinium-thiocyanate phenol-chloroform extraction. Anal Biochem 1987;162:156-9.
22. Beillard E, Pallisgaard N, Bi W, van der Velden VHJ, Dee R, van der Schoot CE, et

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.
23. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986-94.
24. Cheson BD, Cassileth PA, Head Dr, Schiffer CA, Bennett JM, Bloomfield CD, et al. Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 1990;8:813-9.
25. Kaplan EL, Meier P. Nonparametric estimations from incomplete observations. J Am Stat Assoc 1958;53:457-81.
26. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163-70.
27. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat 1988; 16: 1141-54.
28. Cox DR. Regression models and life tables (with discussion). J R Stat Soc B 1972; 34:187-220.
29. Schmid D, Heinze G, Linnerth B, Tisljar K, Kuseck R, Geissler K, et al. Prognostic significance of WT1 gene expression at diagnosis in adult de novo acute myeloid leukemia. Leukemia 1997;11:639-43.
30. Gaiger A, Schmind D, Heinze G, Linnerth B, Greinix H, Kalhs P, et al. Detection of the WT1 transcript by RT-PCR in complete remission has no prognostic relevance in de novo acute myeloid leukemia. Leukemia 1998;12:1886-94.
31. Garg M, Moore H, Tobal K, Liu Yin J. Prognostic significance of quantitative analysis of WT1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukaemia. Br J Haematol 2003;123:49-59.
32. Karakas T, Miething CC, Maurer U, Weidmann E, Ackermann H, Hoelzer D, et al. The coexpression of the apoptosis-related genes bcl-2 and wt1 in predicting survival in adult acute myeloid leukaemia. Leukemia 2002;16:846-54.
33. Trka J, Kalinova M, Hrusak O, Zuna J, Krejci O, Madzo J, et al. Real-time quantitative PCR detection of wt1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry. Leukemia 2002;16:1381-9.
34. Kottaridis PD, Gale RE, Linch DC. Flt3 mutations and leukaemia. Br J Haematol 2003;122:523-38.
35. Maheswaran S, Englert C, Bennett P, Heinrich G, Haber DA. The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. Genes Dev 1995; 9: 2143-65.
36. Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, et al. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. EMBO J 1999;18:3990-4003.
37. Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia 1996;10:588-99.
38. Lisovsky M, Estrov Z, Zhang X, Consoli U, Sancgez-Williams G, Snell V, et al. FLT3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukaemia cells: regulation of Bcl-2 and Bax. Blood 1996;88:3987-97.