Early prediction of treatment outcome in acute myeloid leukemia by measurement of *WT1* transcript levels in peripheral blood samples collected after chemotherapy

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ABSTRACT

The Wilms' tumor gene *WT1* is a reliable marker for minimal residual disease assessment in acute leukemia patients. The study was designed to demonstrate the potential use of *WT1* to establish quality of remission in acute leukemia patients for early identification of patients at high risk of relapse. A prospective study based on a quantitative Real–Time PCR (TaqMan) assay in 562 peripheral blood samples collected from 82 acute leukemia patients at diagnosis and during follow-up was established. The evaluation of *WT1* in peripheral blood samples after induction chemotherapy can distinguish the continuous complete remission patients from those who obtain only an "apparent" complete remission and who could relapse within a few months. *WT1* helps identify patients at high risk of relapse soon after induction chemotherapy allowing post-induction therapy in high risk patients to be intensified.

Key words: *WT1*, minimal residual disease, acute leukemia, RQ-PCR

Citation: Cilloni D, Messa F, Arruga F, Defilippi I, Gottardi E, Fava M, Carturan S, Catalano R, Bracco E, Messa E, Nicoli P, Diverio D, Sanz MA, Martinelli G, Lo-Coco F, and Saglio G. Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral blood samples collected after chemotherapy. Haematologica 2008 June; 93(6):921-924. doi: 10.3324/haematol.12165

Introduction

Evaluation of minimal residual disease (MRD) in acute myeloid leukemia (AML) after initial chemotherapy is important to predict prognosis and may improve selection of the type and intensity of post-remission treatment.[1] The mainstays of MRD studies in this setting include molecular tests, such as RT-PCR amplification of chromosome translocations[2,3] and multidimensional flow cytometry detection of aberrant phenotypes.[4-6] The major obstacle in MRD detection by RT-PCR or quantitative PCR (RQ-PCR) is represented by the limited percentage of AML patients presenting with detectable genetic aberrations.[7] This prompted several investigators to validate the use of alternative markers for MRD detection suitable in the vast majority of AML patients, and in particular to test *WT1* expression as a universal marker of leukemic cells.[8-12] The Wilms's tumor gene (WT1) codes for a transcription factor that has been shown to be highly expressed in several hematopoietic tumors including AML.[8-12] Given the existence of background levels determined by *WT1* expression in normal bone marrow, studies using qualitative RT-PCR have provided conflicting results on the clinical value of this marker,[13,14] whereas most recent investigations by quantitative real-time RT-PCR (RQ-PCR) clearly distinguished *WT1* transcript amounts related to AML cells, normal hemopoietic cells and post-chemotherapy regenerating normal bone marrow cells.[15,16] Therefore, longitudinal RQ-PCR analysis of *WT1* transcript amount may prove clinically relevant for AML monitoring. Furthermore, since *WT1* expression in normal peripheral blood (PB) is about 1 log lower than in normal BM with the majority of normal PB samples scoring negative, we hypothesized that sequential RQ-PCR study of *WT1* expression in PB might further improve the sensitivity of MRD evaluation in AML and might also favor compliance and sample availability. Finally, one of the main goals of MRD assessment is represented by the possibility of identifying, as soon as possible after induction chemotherapy the subset of

Funding: this work has been supported by grants from AIRC (Associazione Italiana per la Ricerca sul Cancro), CNR (Progetto Finalizzato Oncologia), MURST-COFIN 2003, AIL (Associazione Italiana contro le Leucemie), CRT, and by Regione Piemonte. EM is a fellow of the Gigi Ghirotti Foundation.
Manuscript received August 28, 2007. Revised version arrived on January 2, 2008. Manuscript accepted January 29, 2008.
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The online version of this article contains a supplemental appendix.

patients who, although in CR, present a high risk of relapse. This means these patients can be treated with intensified chemotherapy protocols. To this purpose, in the present study, we analyzed *WT1* expression levels at diagnosis and during follow-up in 82 AML patients treated with standard chemotherapy protocols. Our data indicate that this approach provides important prognostic information in AML by identifying patients at higher risk of relapse early after induction chemotherapy.

Design and Methods

After obtaining informed consent, 562 peripheral blood (PB) samples were collected from 82 AML patients. PB sampling was performed at diagnosis, after each cycle of chemotherapy and at sequential time intervals during follow-up and at relapse. All cases were classified according to FAB criteria, characterized at the cytogenetic level, and screened by RT-PCR for the presence of the most frequent fusion transcripts as previously described.[17] The main clinical and biological characteristics of the cohort of analyzed patients are reported in Table1 (*Online Appendix 1*). Patients under 60 years of age were treated following standard protocols established by the GIMEMA Cooperative Group. (treatment details in *Online Appendix 2*) After each cycle of chemotherapy, a BM aspirate and biopsy were performed in order to assess the response to therapy. Complete remission was defined according to standard criteria.[18] PB samples were collected at diagnosis, after induction and consolidation chemotherapy. PB samples were collected 1-5 days after the achievement of a neutrophil count 0.5×10^9/L. A mean of 6.8 samples per patient were available for RQ-PCR analysis (range 3-16). The median follow-up was 17 months (mean 20.7, range 5-66). Finally, as previously described,[10] 70 PB samples and 22 BM samples from healthy volunteers were used as control in order to define the normal range of *WT1* expression in healthy subjects.

Cytogenetic and molecular analysis

Cytogenetic and molecular analysis was performed in all patients included in the study following standard procedures.[17,3] *WT1* RQ-PCR reactions and fluorescence values were measured as previously described.[10,19]

Results

As previously reported in other papers[10,11] BM and PB samples obtained from healthy volunteers express very low levels of WT1. In our study, the majority of normal PB are negative for *WT1* expression and the mean value in positive samples is 4±3 *WT1* copies/10^4 ABL copies (median 0, range 0-20). BM samples of normal donors express a mean value of *WT1* copies of 32±19 (median 28, range 0-90). A median value of 3,725 *WT1* copies/10^4 ABL copies (mean value 7,222±12,496, range 68-95,549) was detected in the 82 PB samples collected at diagnosis and evaluated in the present study, and a median value of 30,212 (mean 57,830±22,980, range 416-122,714) in BM samples. Seventy-one out of 82 patients achieved complete remission (CR) and 11 patients were resistant to induction chemotherapy. Twenty-seven out of 71 patients attaining CR after chemotherapy persisted in remission with a median of 28 months of follow-up (mean 30.3 months, range 12-60)(Figure 1) and 44 relapsed after a median of ten months (range 5-66) during the follow-up period. (Figures 2 and 3). No significant differences were observed in *WT1* transcript amount at diagnosis between the patients who persisted in CR and those who relapsed, either in PB (p=0.13) or in BM (p=0.27). No difference in the *WT1* amount at diagnosis was detected in patients resistant to chemotherapy when compared with responders (p=0.05). Regression analysis demonstrates the absence of correlation between *WT1* expression and WBC count at diagnosis (r=0.0008). There was no significant difference in *WT1* transcript within the FAB and cytogenetic risk subgroups and in patients carrying ITD or point mutation of FLT3 when compared with the wild type *FLT3* group (p=0.29). *WT1* quantitative assessment was performed in PB samples obtained soon after recovery from induction chemotherapy induced aplasia. As shown in Figure 2, 23 patients out of 71, although in CR, displayed *WT1* values above the normal range with a median value of 112 *WT1* copies (mean 292±638, range 23-2840). By contrast, in 48 out of 71 patients who entered CR, the amount of *WT1* transcript measured after induction treatment fell within the range detected in healthy controls with a median value of *WT1* of 6 copies/10^4 ABL (mean=7.1±5, range 0.5-19). Interestingly, all patients showing *WT1* values above the normal upper limit after induction chemotherapy relapsed after a median of seven months (range 4-16). As shown in Figure 3, 21 of the 48 patients with normal *WT1* values after chemotherapy relapsed after a median of 12 months from diagnosis (range 6-44 months) and 27 patients persisted in CR after a median of 28 months of follow-up (range 12-60 months) (Figure 1). No significant differences were observed between *WT1* transcript levels detected at CR after induction chemotherapy in the cohort of 27 out of 48 patients who subsequently persisted in remission when compared with the 21 who relapsed during follow-up (p=0.33). In all patients who reached a normal *WT1* value after induction chemotherapy and later relapsed during follow-up, at least one

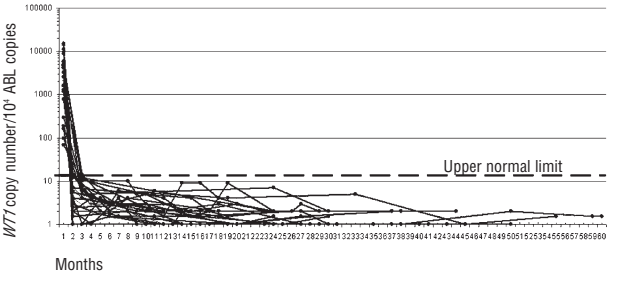

Figure 1. *WT1* expression at diagnosis and during follow-up of 27 patients in CR characterized by *WT1* values within the normal range after induction chemotherapy. All these patients persisted in CR during follow-up. *WT1* never increased above the normal range during follow-up. The broken line represents the upper normal limit.

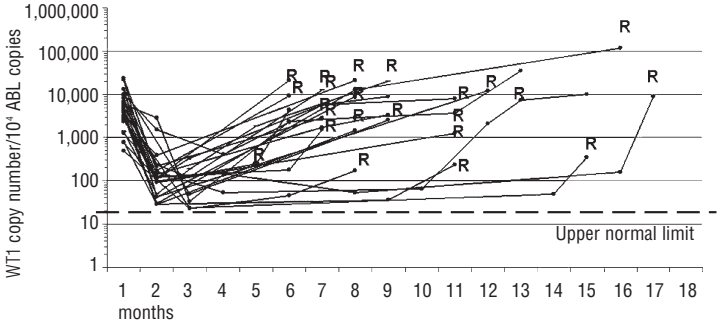

Figure 2. ***WT1*** **expression at diagnosis and during follow-up of 23 patients in CR characterized by** ***WT1*** **values above the normal upper limit in PB after induction chemotherapy. All these patients relapsed after a median of seven months from diagnosis. R: relapse. The broken line represents the upper normal limit.**

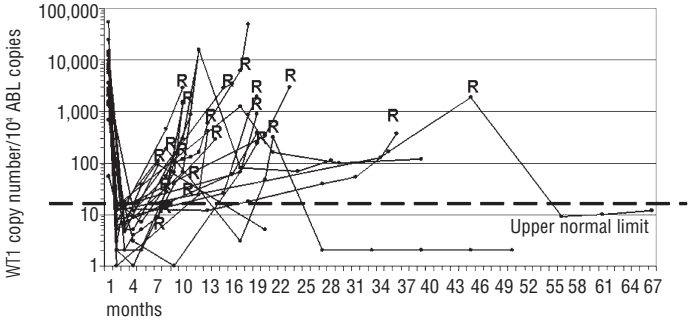

Figure 3. ***WT1*** **expression at diagnosis and during follow-up of 21 patients in CR characterized by** ***WT1*** **values within the normal range after induction chemotherapy. All these patients relapsed after a median of 13 months from diagnosis. The broken line represents the upper normal limit.**

abnormal value was detected before relapse. The detection of an abnormal *WT1* value preceded by 1-6 months (mean 2.4 months) the hematologic relapse. In none of the 27 patients who persisted in CR were abnormal *WT1* values detected during follow-up. Compared with the 21 patients who relapsed after achieving normal *WT1* levels after induction therapy, the patients who showed abnormal *WT1* levels after chemotherapy underwent disease relapse after a significantly shorter time interval from diagnosis to relapse (median of 7 *vs.* 12 months; mean 8.3±3.3 *vs.* 14.8±9.4; p=0.0033). In the 11 patients resistant to chemotherapy, the *WT1* transcript amount persisted at very high levels after chemotherapy with a median value of 5,647 (mean 13,886±27,548) *WT1* copies/10^4 *ABL* copies at diagnosis and a median of 3,180 (mean 10,232±19,103) after induction chemotherapy. This study shows that longitudinal quantitative analysis of *WT1* expression in the PB of AML patients may provide relevant prognostic information by early identification of patients at highest risk of relapse. Doubts about the use of *WT1* expression as a marker for MRD monitoring in leukemia have mainly been based on the existing background expression derived from normal hematopoietic cells.[20] The recent advent of RQ-PCR technique for more precise and standardized quantification has allowed this problem to be partially overcome by identifying threshold values distinguishing the *WT1* transcript amounts expressed in normal subjects from those of leukemic cells.[10-16] We found that such discrimination, which is extremely relevant for the purpose of clinical studies of MRD, is best accomplished by using PB instead of BM, due to considerably lower *WT1* expression levels and smaller individual variability in normal PB compared with BM. Whether *WT1* amount at diagnosis in leukemia patients has any prognostic significance is still a subject of debate.[21-23] In our study, the *WT1* transcript level at diagnosis seems not to be correlated with patient outcome. The most important information derived from our study comes from *WT1* expression analysis after induction chemotherapy, when the standard criteria define the patient as being in complete remission, and morphological criteria and flow cytometry on PB did not reveal the presence of circulationg blast cells. Since *WT1* is overexpressed in the large majority of AML cases, it could represent the ideal marker for MRD evaluation. Recently, Lapillonne *et al.*[24] demonstrated that *WT1* quantitative assessment after a first course of induction treatment in BM samples represents the ideal tool to identify pediatric acute leukemia patients at high risk of relapse. Our data demonstrate that the sensitivity of *WT1* analysis in PB is equal if not better than BM. Furthermore, it identifies a precise time point, in particular soon after the first cycle of chemotherapy for the evaluation of *WT1* transcript with the intent of identifying patients at high risk of relapse. At this time point, *WT1* copy number allows a better assessment of the quality of remission. In particular, the observation that abnormal *WT1* values in PB after the first course of therapy in CR patients strictly correlate with relapse represents an important achievement as it would allow clinicians to intensify post-induction therapy and use more aggressive consolidation cycle to prevent relapse. From our study, however, we have seen that approximately half of the patients who reach normal *WT1* values after induction chemotherapy relapse, although the reappearance of disease occurs later compared with those who do not reach the normalization of *WT1*. Therefore, only the abnormal *WT1* values after induction treatment are

unfailingly predictive of relapse. For the remaining patients, a stringent molecular follow-up post-remission is recommended since this may allow relapse to be predicted some months before its occurrence, when the conventional methods used are still unable to identify the reappearance of leukemic cells. The ongoing efforts to standardize real time methods of *WT1* assessment and the introduction of rigorous, internationally accepted controls will enable RQ-PCR to become a robust and routine basis for diagnostic and prognostic procedures.

Authorship and Disclosures

DC designed the study and wrote the manuscript; FM, FA and IDF collected the biological samples; EG, MF and DD performed RQ-PCR; SC, VR and RC performed RNA collection and sample storage; EM and PN collected clinical data; MS and FL-C revised the manuscript and GS provided the final approval. The authors reported no potential conflicts of interest.

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