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Research paper

Prognostic significance of The Wilms' Tumor-1 (*WT1*) rs16754 polymorphism in acute myeloid leukemia

Jessica Petiti[a], Valentina Rosso[a], Marco Lo Iacono[a], Chiara Calabrese[a], Elisabetta Signorino[a], Valentina Gaidano[a], Massimo Berger[b], Giuseppe Saglio[a], Daniela Cilloni[a,*]

[a] Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
[b] Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Divisions, Regina Margherita Children Hospital, Turin, Italy

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ABSTRACT

Acute myeloid leukemia is a genetically heterogeneous disease characterized by the accumulation of mutations in hematopoietic progenitor cells. For its heterogeneity, prognostic markers are very useful for therapeutic choice. The most important prognostic markers are age, white blood cell count, chromosomal alterations and gene mutations. Recent works have studied the prognostic significance of *WT1* polymorphisms and mutations, highlighting the role of SNP rs16754 as a positive prognostic factor in AML patients. Nevertheless, the data are still unclear. To investigate the role of *WT1* rs16754 polymorphism in AML, we designed a new tool for the detection using PNA directed PCR Clamping technology. Our data were able to establish a correlation between SNP rs16754 and the clinical outcome. Our results support the hypothesis that rs16754 polymorphism is an independent positive prognostic molecular marker that could be useful for therapeutic choice. In view of this, we described a novel assay faster, more sensitive and cheaper than DNA sequencing. The assay allows evaluating *WT1* rs16754 polymorphism in diagnostic routine to improve prognostic information faster and without overcosting for diagnostic laboratories.

1. Introduction

Acute myeloid leukemia is a genetically heterogeneous disease characterized by the accumulation of mutations in hematopoietic progenitor cells [1]. AML is the most common acute leukemia affecting adults and its incidence increases with age [2]. AML is divided in to several cytogenetic risk groups and treatment and prognosis vary among this subtypes [3]. For a therapy decision, it is very important to determine the individual patient's risk and chances of success of a treatment. Prognostic factors are associated with outcome parameters, they can be incorporated into scoring systems and influence therapeutic choice [4]. Therefore, the identification of prognostic factors would be the most useful step to improve our ability to forecast the outcome [5].

Among the prognostic factors, the most important are age, white blood count (WBC), karyotypic alterations and gene mutations [6]. The most common karyotypic alterations are t(15;17)(q22;q12) or *PML-RARα*; t(8;21)(q22;q22) or *AML1-ETO* and inv(16)(p13.1q22) or *CBFB-MYH11*. These cytogenetic abnormalities are unique and identify AML with favorable prognosis [7]. Many mutations are involved in AML pathogenesis and for many of them the prognostic significance has been clearly established [8]. Among the mutations with positive prognostic significance, *NPM1* is the most frequent and confers to AML a superior rate of chemosensitivity, but only if not associated to *FLT3 ITD* alteration [9]. Another mutation with positive prognostic significance is the biallelic mutation of *CEBPa*. By contrast *FLT3 ITD*, mutations of *RUNX1, DNMT3* and *TP53* have a clear adverse impact on prognosis. Additional mutations in genes such as *ASXL1, IDH1, IDH2, EZH2* and *WT1* have been reported but the prognostic significance is still controversial in the majority of the cases [10].

Despite the presence of mutations in the *WT1* gene, which are quite rare and whose prognostic significant is not so defined, *WT1* has been demonstrated to be a very sensitive and universal marker of the leukemic clone [11]. *WT1* is highly expressed in 90% of AML but it was shown that the absolute value of expression at diagnosis is not correlated with the outcome of the patient [12,13]. This gene, located on chromosome 11p13, encodes a transcription factor that contains zinc-finger motifs and plays an important role in cellular development and cell survival [14,15].

* Corresponding author at: Dept of Clinical and Biological Sciences of the University of Turin, San Luigi Hospital, Gonzole 10, 10043 Orbassano-Torino, Italy.
E-mail addresses: jessica.petiti@unito.it (J. Petiti), valentina.rosso@unito.it (V. Rosso), marco.loiacono@unito.it (M. Lo Iacono), chiara.calabrese@unito.it (C. Calabrese), elisabetta.signorino@unito.it (E. Signorino), valentina.gaidano@unito.it (V. Gaidano), massimo.berger@unito.it (M. Berger), giuseppe.saglio@unito.it (G. Saglio), daniela.cilloni@unito.it (D. Cilloni).

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Recent works have studied the prognostic significance of *WT1* polymorphisms (SNPs) and mutations in different malignant disease [16–18]. Because synonymous SNPs do not cause a change in the protein sequence, for a long time it was thought they did not have a functional role. Conversely, recent papers have shown how synonymous SNPs can directly affected gene function and phenotype by various mechanisms [19]. Among all the *WT1* polymorphisms studied, data have highlighted the role of SNP rs16754 (A > G), that seems to represent an independent positive prognostic factor in AML patients [20–22]. Nevertheless, the data on its prognostic significance are still confused, in particular for the difficulty to reproduce the results in different cohorts [23–26]. In our study, we tried to solve this question using a faster, more sensitive, and cheaper tool based on a PNA directed PCR Clamping.

Peptide nucleic acid (PNA) is a synthesized polymer similar to DNA and RNA, with a backbone composed of repeating *N*-(2-aminoethyl)-glycine units linked by peptide bonds [27]. PNA/DNA binding is stronger and more specific than DNA/DNA binding, so that a single base mismatch appears destabilizing for the duplex [28]. Furthermore, PNA oligomers cannot be used as substrates for polymerase, suppressing DNA amplification [29].

Our data were able to establish a correlation between SNP rs16754 and the clinical outcome.

2. Material and methods

2.1. Cells culture conditions

K562 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, USA). K562 were grown in RPMI 1640 medium supplemented with 200 nM Glutamine (EuroClone, Milan, Italy), 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and 0.1% penicillin/streptomycin and maintained at 37 °C with 5% CO_2.

2.2. Patients

The study was approved by the local ethic committee of San Luigi Hospital, Orbassano, Turin (number of approval 201/2014). After informed consent, human bone marrow (BM) or peripheral blood (PB) mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Sentinel Diagnostic, Milan, Italy) from 87 AML patients. Characteristics of patients are listed in Table 1.

Total RNA was extracted using TRIzol Reagent (Ambion, Thermo Fisher Scientific, Massachusetts, USA), following the manufacturer's instructions, and 1 μg was reverse transcribed using random hexamers as primers in a final volume of 25 μL.

All cases were characterized at the cytogenetic level by conventional karyotyping and screened by RT-PCR for the presence of the most frequent fusion transcripts, as previously described [30]. *NPM1* mutations [31], *FLT3 ITD* or *D835* and *DNMT3* [32] mutations were screened. *WT1* quantitative assessment is available for all samples included in the study [13].

Patients younger than 60 years were treated following standard protocols established by the GIMEMA Cooperative Group for the treatment of adult patients with acute myeloid leukemia which included: induction treatment with a 3-drug regimen: daunorubicine (DNR) 50 mg/sqm/day on days 1, 3 and 5; cytosine-arabinoside (ARA-C) 100 mg/sqm/day on days 1–10; etoposide 100 mg/sqm/day on days 1–5; to be repeated in case of partial remission (PR). Consolidation therapy with DNR 50 mg/sqm/day on days 4–6 and intermediate-doses ARA-C (500 mg/sqm/12 h on days 1–6) for patients achieving complete remission (CR) after either the first or the second induction cycle. Additional consolidation treatments with high dose ARA-C were used followed, in high risk patients by allogeneic stem cell transplantation. Secondary AML were treated following the Mito-FLAG scheme (fludarabine 30 mg/sqm day 1–5, ARA-C 2000 mg/sqm day 1–5, mitoxantrone 7 mg/sqm day 1,3,5 and G-CSF 5 μg/kg from day -1) and consolidated as described above. Elderly and unfit patients were treated monthly with azacitidine 75 mg/m^2 for 7 days or decitabine 20 mg/m^2 for 5 days.

Table 1
Characteristics of patients with and without *WT1* rs16754 polymorphism.

Characteristics	*WT1* rs16754 AA (n = 59		*WT1* rs16754 GA/GG (n = 28)		*P*
	No	%	No	%	
Sex					
Male	33	55,9	12	42,3	0,36
Female	26	44,1	16	57,1	
Age (years)					
Median	66		57		0,21
Range	34–87		14–79		
0–20	0	0	2	7,1	
21–40	5	8,5	4	14,3	
41–60	18	30,5	8	28,6	
60–90	36	61,0	12	42,8	
n.d.	0	0	2	7,1	
Diagnosis					
De novo	50	84,7	25	89,3	0,74
Secondary	9	15,3	3	10,7	
Therapy					
Chemotherapy	27	45,7	14	50	
Chemotherapy + HSCT	8	13,6	8	28,6	
Demethylating therapy	11	18,6	4	14,3	
BSC	2	3,3	0	0	
n.d.	11	18,6	2	7	
Cytogenetic					
Normal karyotype	47	79,7	25	89,3	0,37
t (15;17)	4	6,8	1	3,6	
t (8;21)	2	3,4	0	0,0	
inv. 16	2	3,4	1	3,6	
MLL fusion	2	1,4	0	0,0	
Complex karyotype	2	3,4	1	3,6	
NPM1 status					
Mutant (type A or B)	16	27,1	9	32,1	0,62
w.t.	43	72,9	19	67,9	
FLT3 status					
Mutant (cod. 835 or ITD)	12	20,3	11	39,3	0,07
w.t.	47	79,7	17	60,7	
*DNMT3*a status					
Mutant (R882H)	4	6,8	3	10,7	0,68
w.t.	55	93,2	25	89,3	
WBC count × 10^9/L					
Median	23,2		26,5		0,98
Range	4–353		2–237		
% of Blasts					
Median	60		70		0,87
Range	5–99		5–100		
WT1/10^4 *ABL* copies					
Median	3707		3401		0,92
Range	10–33011		4–30215		

Abbreviations: *WT1*, Wilms' tumor; w.t., wild-type; HSCT, hematopoietic stem cells transplant; BSC, best supportive care; ITD, internal tandem duplication; WBC, white blood cells.

2.3. Cloning PCR controls with pGEM®-T Easy Vector

WT1 rs16754 AA, *WT1* rs16754 GA/GG and *GUSB* were amplified from K562 cell line, purified by QIAquick Gel Extraction Kit (Qiagen, Hildem, Germany) and cloned in pGEM-T Easy Vector (Promega, Milan, Italy). Primers sequences were as follow: *WT1* Fwd 5′-ACAGCCGGAG CCTGTCGCTA-3′, *WT1* Rev 5′-TCAAAGCGCCAGCTGGAGTTTG-3′, *GUSB* Fwd 5′-GATCCACCTCTGATGTTCACTG-3′ and *GUSB* Rev 5′-TCAAGTAAACAGGCTGTTTTCCA-3′. Sequences were verified by capillary Sanger sequence method. All reactions were performed following the manufacturer's instructions. Plasmids were used as positive controls in PCR steps.