WT1 GENE EXPRESSION IN THE PERIPHERAL BLOOD OF PATIENTS SUFFERING FROM HEMATOLOGICAL MALIGNANCIES

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Abstract

The WT1 gene, localized on the 11p13 region of the human genome has an important role in the development of the urogenital system, but also in the regulation of cell proliferation and differentiation. WT1 gene is overexpressed in 70-90% of leukemic cells, irrespective of the type of leukemia. WT1 gene expression in peripheral blood may detect minimal residual disease in all types of leukemias. We measured the WT1 gene expression level with qRT-PCR in the peripheral blood of 34 patients with malignancies, mainly leukemias (2 AML, 2 CML, 2 NHL, 1 HD and 27 ALL) under cytostatic treatment, 13 oncological patients who completed their chemotherapy and were in remission and 21 patients suffering from non-malignant diseases. We found WT1 gene expressions in 19 PB samples out of 47 oncological patients: 17 WT1 expressions appeared in ALL patients (15 cases under treatment and 2 post treatment) and 1-1 in a CML and NHL patient. We found a significant inverse relationship between WT1 gene positivity and time elapsed between diagnosis and blood sample examination. We did not find any relationship between WT1 gene expression and age, gender, WBC at diagnosis, FAB type of lymphoblasts, immunophenotype, risk groups, survival rate and relapse. Repeated measurements of WT1 gene expression may become a useful tool in MRD monitoring, especially in leukemias which lack specific DNA markers.

Keywords: WT1 gene, leukemia, children, MRD

Introduction

A zinc finger polypeptide gene (WT1 gene), at the human chromosome 11p13, plays an important role in normal kidney development. (Call et al.,1990). Expression of WT1 gene was found in bone marrow

samples of acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic myelocytic leukemia (CML) patients (Miwa, Beran & Saunders,1992), raising the possibility of its involvement in leukemia hematopoiesis. Inoue et al. (1994, 1996) suggested that quantitative WT1 gene expression can be used as a panleukemic marker for minimal residual disease (MRD) detection in acute leukemias. WT1 gene expression may be used for monitoring disease behavior (MRD, relapse) in leukemias, regardless of type and the presence or absence of DNA markers. (Keiholz et al., 2005; Kletzel, Olzewski, Huang, & Chou, 2002). Exponential increase of WT1 gene expression precedes clinical relapse with several months, both in patients treated with chemotherapy and allogeneic bone marrow transplantation. (Ogawa et al., 2003). Expression of WT1 gene has been associated with chemoresistance in leukemias (Hirose, 1999).

The WT1 gene product is a regulatory molecule of cell growth and differentiation. In intrauterine life, WT1 is expressed primarily in the urogenital system. In adult life, WT1 gene expression is found in the urogenital system, central nervous system (SNC), bone marrow and lymph nodes. (Hirose, 1999). The WT1 gene located at chromosome 11p13 has 10 exons, through two splicing events generates 4 variants with different functions. (Yang, Han, Saurez Saiz, & Minden, 2007). The N-terminus domain is involved in ribonucleic acid (RNA) and protein interactions. The C-terminus domain consists of 4 cysteine 2- histidine 2 zinc fingers, which bind to target DNA sequences and regulates many growth and differentiating factors (bcl-2, c-myc, IGF-II, IGF-I receptor, CSF-1, PAX-1, RAR-α). (Rosenfeld, Cheever, & Gaiger, 2003; Yang et al., 2007).

WT1 was first described as a tumor-suppressor gene in Wilms tumor. Inactivation of both alleles of WT1 gene resulted in Wilms tumor. Its function, however, is more complex and depends on the environment and tissue specificity. Wild-type WT1 gene is overexpressed in haematological cancers (AML, ALL, CML) and other cancers (lung, prostate, CNS, breast cancer, neuroblastoma, ovarian, pancreatic, desmoid tumor, etc.). (Hohenstein, & Hastie, 2006). WT1 gene is expressed in low levels in CD34+ primitive cells, probably induces a controlled self-renewal of cells. (Yang et al., 2007). In leukemias, it acts more likely as an oncogene. The precise mechanism of WT1 gene in leukemogenesis is not fully understood, however, a link between hematologic malignancies and WT1 gene expression level clearly exists.

WT1 protein-containing vaccines are currently under phase I/II/III evaluation; WT1 protein is immunogenic, it elicits a humoral and cytotoxic T-cell mediated immune response. (Maslak et al., 2010; Keiholz et al., 2009; Hashii et al., 2011; Gaiger et al., 1999). “WT1 is a promising tumor antigen for the development of a novel class of universal vaccines” (Van Driessche,

Berneman, & Van Tendeloo, 2012) and the National Cancer Institute named it as the most important antigen among 75, worth to investigate. (Brayer & Pinilla-Ibarz, 2013).

Aim of the paper

Monitoring childhood acute leukemias involve flow cytometry or PCR methods of bone marrow cells. PCR assessment is more sensitive but applicable only in a small percent of leukemias (20-30% in acute lymphoblastic leukemia), where specific fusion genes have been detected at the time of diagnosis. (Raso, Varga, Timar, & Magyarosy, 2000). In AML, more than 50% of the patients lack clonality markers suitable for MRD monitoring. (Saglio et al., 2005). As WT1 gene is not expressed in normal peripheral blood cells (PB) (Saglio et al., 2005), we investigated the potential role of WT1 gene expression measurement from peripheral blood during the monitoring of childhood malignancies, mainly leukemias.

Material and methods

WT1 gene expression was measured with quantitative real-time PCR analysis from the peripheral blood of 34 children treated for leukemias and lymphomas, 13 children with leukemias who have already finished their cytotoxic regimens and 21 children suffering from other non-malignant diseases. Two ml peripheral blood in EDTA tube was sent to the Molecular Biology Laboratory of our university for WT1 gene expression measurement with RT-PCR technique. RNA wasextracted from 1 ml blood with Qiamp RNA Blood Mini Kit (Qiagen Group), according to the manufacturer's instructions. The RNA was diluted in 40 μL ultra pure water, and the RNA concentration was determined by NanoDropND–100 Spectrophotometer (Thermo Scientific, USA). Total RNA from each sample was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, USA) with Gene Amp PCR System 9700 (Applied Biosystems, USA). A final concentration of 1 μg/100 μL was obtained. TaqMan real-time quantitative PCR amplification reactions were carried out in an AB 7500 Real–Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) – 40 ng cDNA and 10 μL 2 × TaqMan Buffer with standard conditions (initial setup 2 minutes – 500C, 10 minutes – 950C, denaturation 15 seconds – 950C, annealing 1 minutes – 600C) and 50 cycles. ABL was used as reference gene with the following forward (f) and reverse (r) primers: (f),5'-TGGAGATAACACTCTAAGCATAACTAAAGG -3'; (r), 5'-GATGTAGTTGCTTGGGACCCA-3'; and the hybridization probe: F 5'-CCATTTTTGGTTTGGGCTTCACACCATT-3 T' (Applied Biosystems). WT1 expression was studied using the following forward (f) and reverse (r)

primers: (f),5'-CAGGCTGCAATAAGAGATATTTTAAGCT-3'; (r), 5'-GAAGTCACACTGGTATGGTTTCTCA -3'; and the hybridization probe: F 5'-CTTACAGATGCACAGCAGGAAGCACACTG-3' T (Applied Biosystems). All reactions were performed in a 20 μL volume with 6 pmol forward and reverse primer and 4 pmol TaqMan probe. All reactions were executed in triplicate. In the case of negative control, cDNA was not added. The relative quantification of WT1 gene expression was performed by calculating the concentration difference (Y) against the reference gene: $Y = 2^{-\Delta Ct}$ where $\Delta Ct = Ct(WT1) - Ct(ABL)$; Ct – threshold cycle; 3. 32 cycle difference meaning 10-fold concentration difference. Non-parametric Mann-Whitney and chi-square tests were applied for data processing.

Results

The 3 groups in which WT1 gene expression level was measured from the peripheral blood of the patients were:

Group I consisted of 34 patients with malignancies who performed their cytostatic regimens. Group II included 13 patients with malignancies after completion of their chemotherapy whom all were in complete remission. Group III included 21 patients admitted to the hospital with other non-malignant diseases. The follow-up time-span after WT1 gene assessment in all groups was 30 months. The cancer patients suffered from ALL (n=40), AML (n=2), non-Hodgkin lymphoma (n=2), Hodgkin disease (n=1) and CML (n=2), with a slight predominance of males (59, 6%). Six out of 40 patients with ALL belonged to high risk group (15%), bone marrow lymphoblasts were of L1 morphology in 43, 9% and L2 type in 56, 1% of the cases, whereas immunology of lymphoblasts showed the precursor B type in 18 cases (48, 6%) and other, less favorable types including T-cell, CD10 negative and biphenotypic cases, in 19 cases (51, 4%). The average age at diagnosis was 6,52 years (median 4,54 years), with a median number of leucocytes at presentation 17, 120/mm^3 (range between 1200-424000/mm^3).

We found 17 positive WT1 gene expression measurements in Group I, 2 positive cases in Group II and other 2 positive results in Group III. In six patients, the WT1 gene expression was assessed at diagnosis, before any specific treatment, all results were positive, with higher quantitative expressions in high risk patients and T-cell ALL. After 1 month of antileukemic treatment, only 1 patient out of 6 had positive WT1 gene expression. Altogether we have found WT1 gene expressions in 19 PB samples of 47 oncological patients: 17 WT1 expressions appeared in ALL patients (15 cases under treatment and 2 post treatment) and 1-1 in a CML and NHL patient. We found a significant inverse relationship between WT1 gene positivity and time elapsed between diagnosis and sample examination. The interval between diagnosis, and WT1 measurement varied between 0

and 180 months (mean 34, 25 months, median 26, 00 months; confidence interval 22, 12-46, 38 months). The mean time elapsed between diagnosis and WT1 gene expression measurement was 39, 18 months in WT1 negative cases (min 1, max. 180 months) and 23, 89 months in WT1 positive cases (min 0, max. 144 months), a statistically significant difference, non-parametric Mann-Whitney test (p=0, 059).

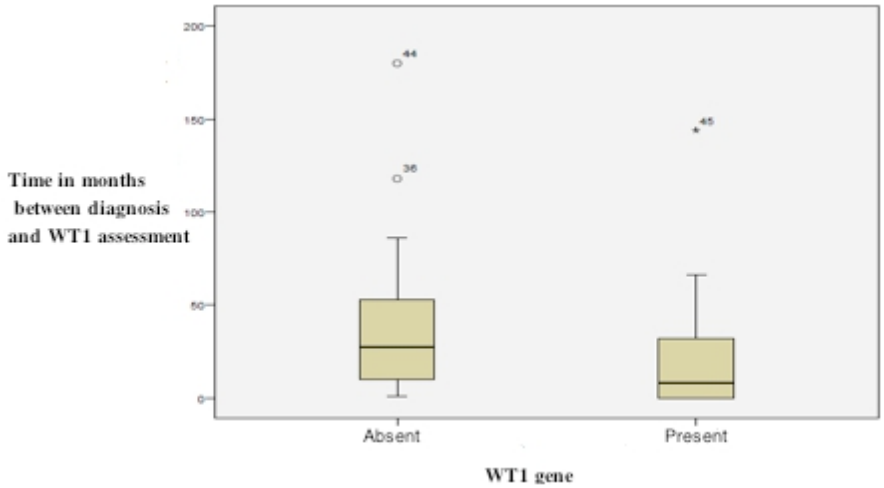

Figure 1. Correlation between WT1 gene expression in PB and time elapsed from diagnosis till WT1 measurement

We did not find any relationship between WT1 gene expression and age, gender, WBC at diagnosis, FAB type of lymphoblasts, immunophenotype, risk groups, survival rate and relapses. Patients who expressed WT1 gene had a mean age at diagnosis of 6, 225 years, and those without WT1 expression of 6, 576 years. In WT1 positive cases the WBC at diagnosis was higher (mean 66.103, 33/mm^3), versus WT1 negative cases (mean WBC 56.441, 92/mm^3), however, the difference is not statistically significant. More boys had WT1 gene expression in their PB samples than girls (50% versus 26, 3%), though the difference is not statistically significant (p=0, 104 Pearson chi-square test). In patients with absolute lymphoblast count (ALyC) above 1000/mm^3 on day 8, fifty percent expressed WT1 gene in their PB, whereas this value was 40% in patients with ALyC < 1000/mm^3. Correlated with FAB types of BM lymphoblasts, WT1 positivity had been found in 33, 3% of patients with L1 and 47, 8% of those with L2 type lymphoblasts. According to blast immunology, patients were assigned into 2 groups: one group with precursor B lymphoblasts (n=18) where the ratio of WT1 positivity was 38, 9% and the second group (n=19) with less favorable immunology (T-cell, CD10 negative precursor B cell lymphoblast, precursor B cell with myeloid markers) where WT1 positivity reached higher ratio, 47, 4%. However, this difference is

statistically insignificant (Pearson chi-square test p=0,271). The 2 patients from Group II (leukemias in remission, after completion of therapy) who expressed WT1 gene in their PB (0, 051% and 0, 01%), were in hematological remission after 30 months of follow-up. In Group III we found 2 positive WT1 results: 1 patient with immune thrombocytopenic purpura (WT1 0, 041%) and other with adenopathy-hepatomegaly-splenomegaly of viral origin (WT1 0, 001%), but none of them got sick of cancer during the following 30 months. One patient in whom MRD had been evidenced with PCR test, expressed WT1 gene in his peripheral blood sample in 0, 025% and continued specific treatment for additional 1 year, after which he became MRD-free. In 30 months time of follow-up, this patient is in remission. The 17- year old adolescent girl with high-risk ALL who had a negative WT1 expression result 12 months after diagnosis, relapsed at the end of maintenance therapy, underwent allogeneic bone marrow transplantation but died in refractory disease. Among the two patients treated with CML, WT1 gene expression correlated well with the level of BCR-ABL transcripts from peripheral blood. The patient who achieved major molecular response with BCR-ABL assessment by qRt-PCR was also negative for WT1 gene expression and the other patient who was positive for BCR-ABL rearrangement, had an elevated WT1 gene expression level (0, 190%) in his peripheral blood. This 12- year old boy with CML with positive WT1 result 7 months after diagnosis, who received imatinib treatment but with episodes of non-compliance, died from disease progression. The WT1 positive patients and some of their disease features are presented in table 1.

Table 1. Clinical and laboratory data of patients with WT1 expression in peripheral blood

Idnr	WT1 % PB	Age at Dg yr	Diagnosis	WBC at Dg /µl	AbsLyCd 8	FAB BM	MO_imm	Dg-WT1int months	Relapse
30	44,840	8	ALL-T	211690	< 1000/µl	L2	2	0	No
21	41,870	13	ALL-B	21440	> 1000/µl	L2	2	0	No
32	05,140	16	ALL-T	68700	< 1000/µl	L2	2	0	No
16	00,850	11	ALL-T	239460	> 1000/µl	L2	2	0	No
12	00,400	2.02	ALL-B	18540	< 1000/µl	L1	1	8	No
5	00,250	4.08	ALL-B	1930	< 1000/µl	L2	2	28	No
22	00,190	12	CML	365770			2	7	Yes
28	00,122	5	NHL					0	No
24	00,092	12	ALL-B	2070	< 1000/µl	L2	1	1	No
33	00,066	7	ALL-B	2200	< 1000/µl	L2	1	0	No
8	00,051	4.05	ALL-B	8500	< 1000/µl	L1	1	66	No
18	00,046	2.6	ALL-B	12200	< 1000/µl	L1	2	35	No
1	00,025	5.10	ALL-B	9800	< 1000/µl	L2	2	38	Yes

Idnr	WT1 % PB	Age at Dg yr	Diagnosis	WBC at Dg /µl	AbsLyCd 8	FAB BM	MO_imm	Dg-WT1int months	Relapse
11	00,010	6	ALL-B	6300	< 1000/µl	L1	1	144	No
7	00,007	2.02	ALL-B	8140	< 1000/µl	L2	2	26	No
8	00,007	2.04	ALL-B	14800	< 1000/µl	L1		25	No
13	00,001	5.04	ALL-B	159840	< 1000/µl	L2	1	6	No
34	00,001	4	ALL-B	8200	> 1000/µl	L1	1	41	No
17	00,001	4	ALL-B	30280	< 1000/µl	L2	2	29	No

Legend: AbsLyCd8= absolute lymphoblast count in peripheral blood on day 8; MO immunology 1= precursor B, 2= group consisted of T-cell, CD10 negative, aberrant myeloid markers; Dg-WTint months= time in months elapsed from diagnosis till WT1 gene expression measurement.

Discussions

Monitoring residual leukemic cells in bone marrow is important in assessment of treatment efficacy and prognosis. (Rak, 2001). Cytomorphological techniques offer detection of 1-5 malignant cells out of 100 cells, immunophenotyping can detect 1 leukemic cell among 10^4 normal cells. (Braham et al., 2010). Revers-transcriptase polymerase chain reaction (PCR) based on demonstration of specific immunoglobulin heavy chain or TCR receptor gene rearrangements has slightly higher sensitivity. (Coustan-Smith et al., 2002; Cavé,1999). However, the PCR technique necessitates the presence of fusion genes, like TEL-AML1, BCR-ABL, MLL-AF4 and others. These DNA markers are not found in all leukemias.

To found a “panleukemic” marker, which reliably shows MRD and foresee an imminent relapse, which can easily be carried out from peripheral blood samples, has been tempting ever since the nineties, shortly after the discovery of the WT1 gene. It has been shown that WT1 gene expression appears in 70-90% of newly diagnosed leukemias, regardless of their type and the presence or absence of DNA markers. Its feature to decrease to very low levels or even disappear during remission, and reaching high levels several months before clinical relapse, qualifies WT1 gene as a sensitive tool in leukemia monitoring. In simultaneous measurements, WT1 levels mirrored the other molecular markers (where fusion gene transcripts were available for MRD assessment with qRt-PCR) where parallel assessments have been carried out. (Cilloni et al., 2002; Saglio et al., 2005, Kerst et al., 2008).

WT1 gene expression has been found in all 6 ALL patients, who were assessed at diagnosis, before the introduction of chemotherapy. Other authors in larger patient groups found WT1 gene expression in 70-90% of acute leukemias.

After 1 month of chemotherapy, only 1 patient out of 6 expressed WT1 gene in 0, 092%. We found an inverse positive correlation between WT1 gene expression and time elapsed from diagnosis (p=0, 059) (figure 1).

Eleven patients were assessed after 8 months of chemotherapy, and 4 of them expressed WT1 gene. Among the 20 patients assessed during the 9-41 months of treatment, 7 cases were WT1 positive. Only 1 CML patient of these died from disease progression. The other patients have not experienced relapse during the 30 months follow-up. This may suggest that transitional WT1 gene expression may appear during maintenance therapy of ALL, without prognostic significance and the need of repeated WT1 measurements for MRD monitoring in leukemias.

We measured increased quantitative WT1 gene expressions in patients with high WBC at diagnosis and T cell ALL, though the statistical results have not reached significant difference. Some authors have suggested that high WT1 expression at diagnosis is an unfavorable prognostic factor (Spanaki et al., 2007; Rosenfeld et al. 2003), while others have not found this association if intensive chemotherapy was applied. (Gaiger et al., 1999). In AML patients, pretreatment WT1 expression level was not prognostically predictive, but the kinetics of WT1 transcript reduction during treatment provided a more accurate prognostic factor. (Cilloni et al., 2009, Lapillonne et al., 2006).

In one of the 2 NHL patients, WT1 gene was expressed at diagnosis (0, 122%), though larger studies confirmed that in NHL WT1 gene expression is lower or absent because the malignant clone arises from more differentiated lymphoid cell. (Inoue et al., 1994)

We found no significant correlation between WT1 gene expression and age at diagnosis, gender, WBC count at presentation, FAB type, immunophenotype, risk groups, survival and relapses. Similar results were reported by other authors (Gaiger et al., 1999; Elmaagacli, Beelen, Trenschel & Schaefer, 2000)

In the 2 CML patients, quantitative WT1 gene expression levels were concordant with the parallel BCR-ABL level measurements from PB.

It has been shown that WT1 overexpression found in leukemic samples is related to the presence of leukemic cells and is not due to the immaturity of the cell population. (Saglio et al., 2005).

Most observations related to WT1 gene expression have been made in adults. In pediatric leukemias, it has been shown that WT1 gene expression is higher in AML than ALL patients and FABL2 type compared to FABL1 type, in T-cell leukemia compared to B-cell ALL, in relapse versus diagnosis and in M1 FAB type versus M5 FAB. (Rosenfeld et al., 2003; Rodrigues et al., 2007).

Conclusion

WT1 gene was expressed in the PB in all 6 patients tested at diagnosis, before the introduction of chemotherapy. Chemotherapy as short as one month has led to undetectable WT1 gene levels in PB in the majority of patients. We found a positive inverse correlation between WT1 gene expression, and time elapsed between diagnosis and WT1 assessment. In CML patients, WT1 level showed good correlation with BCR-ABL levels in peripheral blood. WT1 gene expression may become a useful tool in MRD monitoring, especially in leukemias which lack specific DNA markers, but repeated measurements are mandatory to fulfill this aim.

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