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Monitoring Minimal Residual Disease in Pediatric Acute Lymphoblastic Leukemia Using WT1 Expression Level in Relation to Immunopheno Typing

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Abstract

Objective: Continuous Wilms Tumor gene (WT1) expression is a typical feature of leukemic blasts. Conversely, WT1 is only transiently expressed in normal hematopoiesis. Therefore detection of the submicroscopic levels of leukemic cells (MRD) in peripheral blood samples from children with A WT1 level in children with ALL by a quantitative method based on the Real-time quantitative RT-PCR (RQ-PCR) and assessed its relation to the immunophenotypic ALL subtypes.

Materials and Methods: Twelve newly diagnosed ALL children with different immunophenotypic features were included in this study. The peripheral blood samples were collected before induction, at the second week of induction, at the start of consolidation and beginning of the maintenance phase of chemotherapy. The last blood sample was collected from each patient 2 to 6 months after the maintenance phase started. For RQ-PCR we have used Light Cycler device and SYBR Green I dye. K562 cell line was considered as the control sample and the expression level of WT1 in K562 was defined as 1.0E+4.

Results: At diagnosis the WT1expression level in patients with T-cell immunophenotype was as the same level of K562 cell line (p=0.185). This level was significantly higher than the level in Early pre-B cell ALL patients (p=0.012). There was no significant expression changes in pre-B cell ALL patients in all stages of therapy. In patients with T-cell ALL the expression showed accordance with the clinical course of patients. WT1 was undetectable in the fifth samples of patients with pre-B cell ALL.

Conclusion: These preliminary results indicated that WT1 is better to be employed to monitor MRD in the leukemic patients with T-cell ALL during chemotherapy.

Keywords: Leukemia; Genes; Residual; Real-Time polymerase chain reaction; WT1

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Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common malignancy of childhood, representing nearly 97% of all childhood leukemia's. Approximately 80% of childhood ALL cases are B-cell ALL, have lympho blasts with surface expression of two or more B lineage-associated antigens. T-cell ALL is identified by the expression of T-cell-associated surface antigens, of which cytoplasmic CD3 is specific. Historically, the prognosis of patients with T-cell ALL has been worse than that of patients with B-lineage ALL [1].

The Wilms Tumor gene (WT1) gene was originally defined as a tumor-suppressor gene. However, it is proposed that the wild-type WT1 gene performs an oncogenic rather than a tumor-suppressor function in leukemia [2,3]. WT1 encodes a zinc finger transcription factor with a complex pattern of alternative splicing with different binding specificities. It locates at chromosome 11 p13. WT1 mRNA is highly expressed in the Bone Marrow (BM) of patients with different types of leukemia, Compared with normal BM cells. Indeed, it has been established that the WT1 mRNA levels are

correlated with the disease status. So, the value of WT1 detection in monitoring leukemia treatment efficacy is of high significance [4].

The WT1 is expressed in blasts of patients with acute leukemia, irrespective of lineage, and WT1 nuclear protein is detectable in the majority of such blasts [5]. The expression of WT1 gene in blast leukemia may cause resistance of blast to apoptosis and cause poor prognosis [6]. Also, the WT1 assay can be very useful for the prediction and management of relapse following allogeneic stem cell transplantation regardless of the presence of chimeric gene markers [7].

The detection of residual leukemic cells at a submicroscopic level, Minimal Residual Disease (MRD) is a powerful tool for monitoring the disease response after treatments [8,9]. Therefore, detection of MRD in peripheral blood samples from children with ALL using WT1 as a marker should have clinical importance.

Recently, WT1 over expression has been suggested, as a promising MRD marker. Also, WT1 is highly over expressed in many hematopoietic tumors reaching positivity up to 80% to 90% in AML [10].

Despite extensive investigation of WT1 expression in leukemic patients from different ethnic populations, little is known about the profile of its expression in Iranian patients. In this study we aimed to monitor WT1 level in children with ALL by a quantitative method based on the Real-time quantitative RT-PCR (RQ-PCR) and assess its relation to the immunophenotypic subtypes.

Materials and Methods

Patients

The main criterion for inclusion in this study was that all patients were new cases and not having taken any chemotherapeutic regimens at the time of first sampling. Diagnosis of ALL was based on cytomorphological findings (FAB criteria) and immunophenotypic characteristics of BM leukemic cells. A total of 49 samples including preservative free heparinized Peripheral Blood (PB) samples were obtained from 12 newly diagnosed ALL patients. The peripheral blood samples were collected before induction, at the second week of induction, at the start of consolidation and beginning of the maintenance phase of chemotherapy. The last blood sample was collected from each patient 2 to 6 months after the maintenance phase started.

The patients were selected from those attending the Hematology and Oncology Clinics of Dr. Sheikh Pediatric Hospital, affiliated to Mashhad University of Medical Sciences. This study was approved by Mashhad University of Medical Sciences and informed consents were obtained from patients or their parents.

Isolation of mononuclear cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from peripheral blood using Ficol (Cinnagen, Iran) density gradient centrifugation. Mononuclear cells were washed once in normal saline and cells were transferred to eppendorf tubes for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from PBMCs using Trizol (Cinnagen, Iran), according to the manufacturer's instructions. First strand cDNA was synthesized using 2 µg of total RNA in 20 µl reaction mixture consisting of 4 µl of 5 × RT PCR buffer, 2 µl 20 mM dNTPs (Roche, Germany), 1 µl oligo dT (Roche, Germany), 2 µl DEPC treated water and 1 µl M-Mulv reverse transcriptase (200 u/µl) (Fermentase, Russia). Thereafter the mixture was incubated at 42°C for 60 min, followed by 70°C for 5 min.

WT1 and β-actin RQ-PCR

Amplification was performed by WT1 specific primers GATAACCACACAACGCCCATC as sense and CACACGTCGCACATCCTGAAT as antisense and β–actin specific primers GTGGGGCGCCCCAGGCACCA as sense and GTCCTTAATGTCACGCACGATTTC as antisense. The amplicon sizes of WT1 and β-actin PCR products were 90 bp and 538 bp, respectively.

PCR reaction mixture was prepared according to the manufacturer's instructions (Light Cycler - Fast Start DNA Master SYBR Green). Each cycle of WT1 amplification consisted of 95°C for 15 sec, 60°C for 6 sec, 72°C for 6 sec. Each cycle of β-actin amplification consisted of 95°C for 15 sec, 60°C for 10 sec, 72°C for 20 sec.

The WT1 gene expression level of K562 leukemia cells, which strongly express WT1, was designated 1.0E+4, and the levels for the experimental samples were calculated relative to this value. Because relative WT1 expression levels in most normal tissues are $<10^{-2}$ (if the expression level of WT1 in K562 is defined as 1.0E+4.), levels of $>10^{-1}$ were considered positive (cut-off level) [11].

Statistical analysis

The analysis was performed using the REST-RG software. For comparing quantitative differences of WT1 expression in patients and normal subjects, the permutation test in the REST-RG software (Pair-wise Randomization Reallocation test) was used.

Results

The current study was carried out on 12 patients with childhood ALL. Based on FAB criteria and immunophenotyping results 50% of patients were classified in B-cell ALL group and 50% as T-cell ALL. The mean of age was 5.25 years (SD, 3.5). There were 4 male and 8 female in this study. Major clinical and paraclinical features of patients are shown in Table 1.

WT1 monitoring in B-cell ALL group

WT1 expression was positive in 3 patients (50%) in B cell ALL patients at diagnosis time. In last blood sample collected from each patient 2 to 6 months after the beginning of the maintenance phase, WT1 was positive in 33% of samples. There was no significant expression changes in Early Pre-B cell patients and B cell ALL group (patients with Pre-B cell and or early Pre-B cell ALL) in all stages of therapy ($p>0.05$). In Pre-B cell ALL patients the expression level decreases significantly in the second, fourth and fifth samples ($p<0.01$). Only in the fifth samples of patients with pre-B cell ALL the WT1 level decreased lower than the cut-off level.

WT1 monitoring in T-cell ALL group

WT1 expression was positive in all 6 T cell ALL patients (100%) at diagnosis time. In last blood sample collected from each patient 2 to 6 months after the beginning of the maintenance phase, WT1 was positive in 0% of samples and all of them became negative at the end of the assessment. The WT1 expression level in patients with T-cell immunophenotype was as the same level of K562 cell line ($p=0.185$). This level was significantly higher than early pre-B cell ALL level ($p=0.012$). In patients with T-cell ALL the expression showed accordance with the clinical course of patients. At the second

Table 1: The Clinical and Para-clinical Findings in Patients.

NAME	Sex	AGE(Y)	WBC (10^9/L)	BLAST%	HB	Leukemia type	PLT (10^9/L)	CNS involvement	LDH
Pt1	M	10	20.2	85	8.9	Early pre B cell	56	Yes	7726
Pt2	M	3.5	1.6	20	4.9	Early pre B cell	12	No	334
Pt3	F	3.5	1.9	0	5.2	Early pre B cell	37	No	436
Pt4	F	3	6.6	2	7.3	Early pre B cell	77	No	834
Pt5	F	2	6.2	2	5.9	Pre B cell	152	No	884
Pt6	F	2.5	6	53	7.5	Pre B cell	38	No	798
Pt7	M	12	72	84	6	T-cell	64	Yes	3303
Pt8	F	2.5	87	40	10	T-cell	58	No	365
Pt9	F	9	136	98	7.5	T-cell	23	No	799
Pt10	F	5	117	77	6.5	T-cell	44	No	1411
Pt11	M	8	206	95	6.1	T-cell	37	No	5122
Pt12	F	2	51	78	3.2	T-cell	10	No	1307

week of induction the expression decreased slightly but it was not significant (p=0.240). At the start of consolidation phase there was a significant decrement compared to the first sample (p=0.009) but the level is still more than the cut-off level. In this group the expression level decreased lower than the cut-off level in the maintenance phase.

Discussion

Molecular biology and tumor immunology advances have resulted in the identification of a number of tumor associated antigens [12]. In the majority (70% to 90%) of acute leukemia's, WT1 is over expressed and has been identified as a convenient MRD leukemic marker [5,7,13].

Many studies have shown that 70% to 80% of leukemia patients over express the WT1 gene, and it can be a universal leukemia marker. Although the pathogenesis of WT1 in leukemia has not been completely revealed, the phenomenon that low expression of WT1 and high expression of WT1 are associated with clinical remission and relapse respectively shows that WT1 can be a potential prognostic factor, MRD marker and therapeutic target in acute leukemia [4].

In normal BM, progenitor cells expression of WT1 gene is very low; however, numerous studies suggest that increased expression of WT1 can be seen in AML and ALL. Hence, The WT1 gene expression can be used as a molecular marker for leukemia [14,15]. Many studies have demonstrated that the prognosis in leukemia patients is inversely associated with the expression of WT1 [4,16]. This over expression of WT1 in acute leukemia could provide a useful molecular marker of malignant hematopoiesis and is suggested as the importance of quantitative assessment of WT1 expression as a marker for MRD [17].

Indeed, because the WT1 gene is expressed at low levels even in normal hematopoietic stem cells, most researches using analyses of WT1 transcripts have produced positive results with regard to the prediction of relapse. So, early recognition of relapse at the molecular level helps to therapeutic intervention while the burden of disease is still relatively low [15].

However, WT1 as a molecular marker for MRD and prognostic factor remains controversial. Some researchers regard the WT1 transcript level as a reliable marker for MRD monitoring in childhood acute leukemia, others argued that the WT1 transcript level was not suitable for MRD monitoring [4,18]. The biological functions of WT1 over expression in patients with acute leukemia are not clearly understood, but it has been suggested that WT1 affects the pathogenesis of human leukemia during cellular differentiation and growth arrest. The main purpose of this study was to monitor WT1 level in children with ALL and assess its relation to immunophenotypic features.

In our patients WT1 expression level was more prominent in T-cell ALL patients than B-cell precursor ALL subtypes. Boublikova L et al. [13] showed that contrary to B-cell ALL, most of the childhood T-ALL patients showed WT1 over expression, with the median being significantly higher than in B-cell ALL patients. In that study, older children (> or =10 years) had higher WT1 levels than children under 10 years of age, while there was no association between WT1 expression and peripheral blood leukocyte count (WBC). In other previous studies on ALL patients, a higher frequency of WT1 expression was found in B-ALL in one study and in T-ALL by another [19,20]. Studies on the role of WT1 mutations in T-ALL are limited. In pediatric and adult T-ALL, the WT1 mutations were not predictive of poor clinical outcome [21]. But, one research described that within the standard risk group of thymic T-ALL, the small group of WT1-mutant patients had inferior relapse-free survival [22]. Also in one study found that increased WT1 gene expression level at +60 and + 90 days after allo-HSCT significantly associated with worse prognosis [23]. In this study we have found WT1 expression in 92% of samples before induction. That was comparable to reports demonstrating WT1 expression in 44% to 86% of ALL [15]. Busse A et al. [24] also found WT1 expression in 92% of ALL samples which was similar to our findings. In a study on 19 children with ALL, researchers found that WT1 was over expressed in 89% of pediatric ALL patients. Also, WT1 expression increased after induction chemotherapy in the 3 pediatric patients who had relapse [25]. In a study on 50 children with ALL, WT1 gene was expressed in 52% of the patients while it was only expressed in 2% of the controls. It was found that there were highly statistically significant differences between patient and control groups regarding WT1 genes expression [15]. Also they found that WT1 genes expression was significantly associated with better Relapse Free Survival (RFS).

Asgarian et al. assessed the WT1 expression in Iranian ALL patients and founded that leukemic cells from 51.6% (32/62) of the newly diagnosed patients and 57.9% (11/19) of the relapsed patients were WT1 positive and all of the patients in remission (35/35)

were WT1 negative. WT1expression in newly diagnosed and relapsed ALL patients were significantly higher than that of the ALL patients at remission and normal subjects. They demonstrated that WT1 could be employed as a reliable marker to monitor minimal residual disease in Iranian patients with ALL [26].

Conclusion

In conclusion our results on pediatric ALL showed that WT1 is expressed at high levels in Iranian patients with T-cell ALL and its expression level was more compatible with other clinical and paraclinical findings. These preliminary results indicated that WT1 is better to be employed as a marker to monitor MRD in the ALL patients with T-cell ALL during chemotherapy. However more researches on larger number of patients are needed for better understanding the roles of WT1 genes in the process of leukemogenesis, their significance as a prognostic factor, MRD marker and being a possible target for immunotherapy in acute leukemia.

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