Original Article

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Quantitative Assessment of WT1 Expression by Real Time Quantitative PCR in Pediatric Patients with Acute Myeloblastic Leukemia

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Abstract

Background: Acute myeloid leukemia (AML) is one of myeloid malignancies which the risk increases with age increment. It is categorized based on genetic aberrations. Some of these genetic disorders can determine minimal residual diseases (MRD) and prognosis of AML patients. Wilms tumor (WT1) over expression is found in AML patients. The aim of this study was to determine the frequency of WT1 over expression in AML pediatric cases in North -East of Iran.

Materials and Methods: This retrospective Study was done in Mashhad, Iran during 2016 in 100 pediatric AML cases. WT1 expression was evaluated by quantitative reverses transcription PCR (qRT-PCR) and cloning method. Both WT1 and ABL genes were cloned to create a standard curve and then copy number of WT1 gene in patients was evaluated.

Results: One hundred children under 15 with mean age of 6.50± 4.22 were evaluated in this study. There were no significant differences between age and sex and WT1 expression (P>0.05). Mean expression of cited gene was 200.52±210.62 copies of WT1/ABL 104 in studied samples. WT1 gene over expression was observed in 82% of all patients.

Conclusion: WT1 assessment can be applied as a prognostic and diagnostic marker in AML patients under 15.

Keywords: Acute myeloid leukemia pediatrics, quantitative reverses transcription PCR Wilms tumor

Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia among adults, which the risk increases in association with age (1) .It happens spontaneously, and is not related to compatible factors in most cases; although some genetic disorders have been introduced which are impressive in AML progression (2). Heredity factors, radiation, Occupational exposure and some medicines were mentioned as the AML causes (3). World health organization (WHO) categorized AMLs based on genetic disorders and their probable prognosis. Modern techniques revealed that more than 70-80% of AML cases had clonal cytogenetic disorders (4) , which are diagnostic and applicable for prognosis determination (5-7). Moreover, AML molecular abnormalities can be used as suitable targets for treatment (8). Chromosomal findings are considered as independent index to determine prognosis

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in normal cytogenetic cases. Patients with translocations such as t (15;17), t (8;21) and inv (16) or with no cytogenetic disorders are associated with favorable prognosis; while cases with FLT3 ITD mutation, inv (3), del (7q) and t (10;11) are accompanied with poor prognosis ; the last three abnormalities are candidate to receive allogeneic bone marrow (BM) transplantation(3, 9). Wilms tumor 1 (WT1) gene which is located on the long arm of chromosome 11 at position 11q13 is a tumor suppressive gene. WT1 expression is limited to myeloid blasts in bone marrow. Oncogenic role of WT1 is reported in AML, acute lymphoid leukemia (ALL), chronic lymphocytic leukemia (CML) and myelo-dysplastic syndrome (MDS) (10). Some studies explained WT1 role in cell proliferation, differentiation and apoptosis (11). It's over expression is reported in 90% of AML patients (12). It can be used as a valuable index when different diagnoses are suspected. Relative WT1 expression is linked in with age (8).

A few studies have been done in field of WT1 gene over expression in pediatrics cases; they assessed this gene in small group of patients. Indeed previous researches did not perform cytogenetic and molecular tests in their patients, completely. This study was done to assess WT1 expression in pediatric AML patients and the relation with molecular findings.

Materials and Methods

Sample preparation:

This retrospective study was done in Molecular pathology research center of Mashhad University of medical sciences (MUMS), under supervision of MUMS ethics committee (ethics code: 910531). 100 children under 15 years old were evaluated. Twenty four samples of Peripheral blood (PB) and 76 samples of BM aspiration of AML cases and 12 samples from healthy controls were assessed. Archived extracted RNAs were used for this study. Slides from patients were stained by Gimsa and myeloperoxidase. After preparing slides, the rest of samples of PB and BM were also collected in EDTA tube for molecular tests. 1 milliliter of BM and PB sample collected in heparin container tubes and cultured for cytogenetic study. AML was defined based on criteria which are written as below and definitive diagnosis was done by an expert hematologist. It is worth mentioning that Samples without following mentioned criteria were excluded from this study.

1- Presence of 20%≤ myeloid blasts in BM or PB specimen.

2- Positive myeloperoxidase, specific and nonspecific esterase, Soudan black and Periodic-Acid Schiff staining of patient's slide.

3- Positive markers such as: CD13, CD117, CD64, CD14, CD117 in peripheral samples.

4- Discovering AML related genetic disorders such as: t (8.21), t (15.17), and inv (16).

Cytogenetic culture

Cultured BMs grew within 24 and 48 hours according to Ayatollahi et al. (2015) article protocol (13). BM culture results were used to diagnose recurrent and novel chromosomal aberrations.

Preparation of RNA and cDNA synthesis

Mononuclear cells, including leukemic blasts were separated from both BM and PB samples by Ficoll–Hystopaque 1077 (SigmaAldrich Company, Saint Louis, MO, USA) density gradient. Total RNA was extracted using RNeasy mini kit, following the manufacturer's protocol (Qiagen, Hilden, Germany). The RT (reverse transcription) steps were adapted from the BIOMED 1 protocol (14). Samples were incubated for 10 min at 20 ◦C, 45 min at 42 ◦C and 3min at 99 ◦C, following by 10min at 4 ◦C.

Real-Time Quantitative RT-PCR

All the real-time quantitative RT-PCR (RQ-PCR) reactions were performed on a7700 ABI platform. 5 µL of cDNA was amplified for each reaction. Each reaction

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contained 12.5 µL of mastermix (Taqman Universal PCR Master Mix, USA), 200nM hybridization probe (WT1-probe: CTTACAGATGCACAGCAGGAAGCA CACTG), 300nM of each primer ((WT1-F: CAGGCTGCAATAAGAGATATTTTAA GCT) and (WT1-R:GAAGTCACACTGGTATGGTTTCTC A)The RQ-PCR primers and probe for ABL were:

Forward primer 5_-TGGAGATAACACTCTAAGCATAACT AAAGGT-3_

Reverse primer 5_-GATGTAGTTGCTTGGGACCCA-3_

Taq Man probe 5_-CCATTTTTGGTTTGGGCTTCACACCA TT-3_

Sterilized water was added to reach final volume of 25 µL. 50 cycles of denaturation were set at 95°C for 15 s followed by annealing/extension at 60°C for 60 s. Master mix components are described in Table I.

Generation of Plasmid Standard Curves

WT1 and ABL plasmids were made to prepare standard curve of RQ-PCR assay. Following primers and reagents in final volume of 50 µL were used to amplify WT1 and ABL RT-PCR products:

WT1- forward primer: exon 7 -5_-GGCATCTGAGACCAGTGAGAA-3_

WT1 - reverse primer: exon 10 5_-GGACTAATTCATCGACCGGG-3_

ABL-F TGG AGA TAA CAC TCT AAG CAT AAC TAA AGGT

ABL-R GAT GTAGTT GCT TGG GAC

PCR buffer 1X contained 10mM Tris-HCl, 50 mM KCl (pH 8.3), 2.5 mM MgCl2, 200 µM dNTP, 400 nM of each primer, 1 U Taq enzyme and 3µL of cDNA product.

Thermal cycler temperatures and time conditions are described as the following:

Initial melting 95°C for 30 s, 35 PCR cycles with the following conditions: 94°C for 30 s, 65°C for 1 min, 72°C for 1 min; Then the PCR products were cloned by InsTA clone PCR cloning Kit (Thermo Scientific, USA), according to the manufacturer's instructions.

The selected clones were screened for presence of the insert by PCR; sequencing was done for confirmation. The plasmids were extracted using Gene JET Plasmid Mini prep Kit (Thermo Scientific, USA), and quantified spectrophotometrically. The copy number of 1 µg of clone was estimated according to the molecular weight of the vector which was added to the sequence.

Quantitative analysis of WT1 gene expression was done by standard curve analysis. Six successful serial dilutions (1000000, 100000, 10000, 1000, 100, and 10 copies) of each gene was prepared; finally dilution series of ABL and WT1 plasmids with known concentration were used for standard curve creation. The expression ratio was compatible with WT1/ABL104 formula (15).The expression of WT1 was normalized in comparison with ABL control gene. PCR efficiency of cited genes was 97% and 98%, respectively. PCR conditions, primers and probes were set according to Visser's study for other combined genes such as PML-RARA, AML1-ETO, CBFB-MYH11 and NUP-DEK. WT1 over expression defined as ≥50 copies WT1 / 104 ABL in PB and, ≥ 250 copies WT1/104 ABL in BM samples before starting treatment in this study (15).

Statistical analyses

Statistical analysis was done by SPSS version 13 .Parametric and non-parametric tests were applied according to data distribution and the results interpretations have been described in the next section. Data were reported in form of mean ± SD in the present study.

Results

One hundered children under 15 were assessed, 50% (50) were females and the others were among males. Mean age of studied cases was 6.50± 4.22; it was 6.50± 3.33 in male and 5.60± 5.21 in female. No meaningful differences were found between age and sex parameters and gene

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expression (p>0.05). No significant relationships were found between CBC criteria and WT1 over expression. Mean expression of WT1 gene was 200.52±210.62copies of WT1/ABL104 in patients. WT1 gene over expression was observed in 82% of all patients.

Figure 1 shows patients distribution based on WHO and French American British group (FAB) categorization. According to FAB classification, minimum, and maximum mean of WT1 expression were related to M3 and M1 subtypes respectively. (TableII).

The exact amount of WT1 expression was assessed in PB and BM samples, separately. WT1 and ABL genes expression were measured for 12 normal BM and PB (6 samples of BM and 6 samples of PB from healthy control). WT1 /ABL ratio was calculated, for healthy controls. Median expression of WT1 in normal BM samples was 18 (normal range 17-21). WT1 expression was undetectable in 6 normal PB samples. Copy numbers of WT1 are mentioned in Table II. Number of control individuals was determined according to Lapillonne's study (16).

Table I: Master mix components

Materials	Required amount(microliter)
master mix	12.5
ROX stain	0.2
Forward primer(10picomolar)	1.5
Reverse primer(10picomolar)	1.5
Probe(5picomolar)	1.5
Pattern cDNA	1
Double sterile distilled water	5
Total volume	25

Table II: Copy number of WT1 over expression in FAB and WHO classification

	FAB and WHO classification								
	M1	M2	M3	M4	M5	t(8.21)	t(15.17)	Inv16	Normal karyotype
Mean of WT1/ABL ×10⁴	347 .25	137.42	85.50	230.66	178	150.25	85.50	390.00	266.6

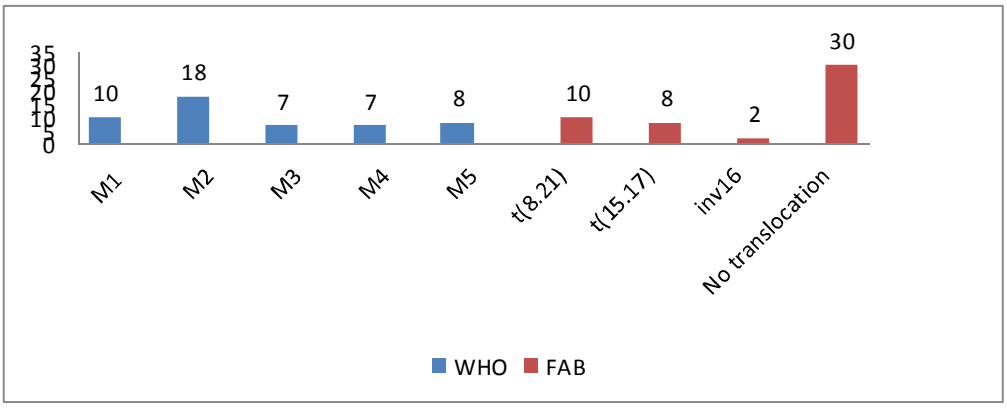


Figure 1: patient's frequency based on WHO and FAB classifications (%)

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Discussion

Translocations which happened in leukemia induce abnormal proliferation, differentiation and death by changing normal hematopoiesis. As it was mentioned recently, molecular genetics aberrations can be used as prognostic markers or treatment targets (15). Many factors can affect leukemia generation and development, including abnormal expression of tumor suppressor genes, oncogenes, Micro RNAs, mutations, chromosomal aberrations, cell signals and epigenetic changes (17,18). BAALC, ERG, MN1 and WT1 genes are known as classification factors based on genes patterns, which determine kind of intervention in AML treatment (17, 19); it is expected that additional effective molecular aberrations will be detected in AML cases. WT1 gene expression in blast cells has been reported as a poor prognostic factor and a target for MRD monitoring, frequently (20).Present study followed 100 children with de novo AML to assess WT1 expression in pediatric AML patients. Many studies have shown that WT1 is a reliable tool for detecting MRD; over expression of WT1 before BM transplantation is associated with poor event free survival (EFS) in children with AML (20). MRD is determined by many methods, such as: flow cytometry that gives informative results in about 85% of AML cases; but it needs to be personalized. Another DNA-based tests for MRD detection have similar limitations ;But WT1 gene expression as a manner for MRD determination does not need any personalizations .WT1 gene is a pan-leukemic marker, which is used to determine disease MRD (21). According to present findings, WT1 over expression happened in 82% of pediatric AML patients; so it can be used as a tool for MRD detection and a prognostic factor in mentioned cases. Over expression of WT1 gene occurs in more than 80% of AML patients and 50% of normal karyotype AML individuals. Jacobsen's study about WT1 importance on 36 pediatric AML patients, revealed that its over expression is more frequent in younger cases(21); but no significant differences between age and amount of gene expression were found in our study. According to Table II, WT1 level is higher in more undifferentiated leukemia; it was higher in M1 in comparison with M3. It is dependent to blast percentage, and it seems that age is not as effective as blast count in WT1 expression (10). In this study the most WT1 expression was found in cases with inv16, and the less was seen in patients with t (15, 17). Candoni et al., (2009) reported the most expression in inv16 disorder too (22). Trka et al. in 2002 in assessment of 47 children found that the lowest expression of WT1 was related to M5 subtype (P.value=0.0015) (23). Lapillonne's study supported this result that minimum expression of WT1 is related to M5 subtype in children; they found that meaningful over expression of WT1 was seen in M2 subtype (P= 0.04) (24). The same result has been obtained by Elisen (25) and Kreuzer (26). The less and the most mean copy numbers of WT1 expression was observed in M3 and M1 subtypes based on FAB classification respectively in present study. Hopa et al., expressed that WT1 over expression is more common in normal karyotype patients(35.3%) (27). Lapillonne's research on 92 AML children did not discover any relations between WT1 expression and WBC count (24). This finding is reported by Hopa and associates too; he and his coworkers did not observe any important relationships between gender, WBC and blast count in two groups of patients with WT1 overexpression who had normal karyotype and who did not (27). Findings of this study were quite similar and were confirmed by previous mentioned studies. No significant relationships were seen between CBC criteria and WT1 over

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expression in this study. It is recommended to do similar studies in a larger population; it is suggested to follow patients and calculate survival markers to determine WT1 significance.

Conclusion

Wilms Tumor 1 gene is a tumor suppressive gene with oncogenic role in acute leukemia. Other studies suggest high expression of this gene in acute leukemia pathogenesis especially AML. This marker can be applied as a prognostic factor which is suitable for treatment assessment.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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