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High expression of WT1 gene in acute myeloid leukemias with more predominant WT1+17AA isoforms at relapse

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ABSTRACT

Real-time quantitative reverse transcriptase polymerase chain reaction method was established for detecting the expression levels of WT1 gene and WT1+17AA isoforms in 226 acute myeloid leukemia (AML) bone marrow (BM) cells. The results showed that WT1 gene was 2–3 logarithms expressed more in AML BM cells at initial diagnosis or relapse than in normal BM cells ($p < 0.001$), with predominant WT1+17AA isoforms expression (the ratio of WT1+17AA/WT1 more than 0.50). Interestingly the ratio of WT1+17AA/WT1 was statistically higher in relapsed AMLs than in initially diagnosed ($p = 0.01$), speculating that WT1+17AA isoforms might participate in AML relapse.

1. Introduction

Wilm's tumor gene (WT1) which produces four major distinct isoforms due to two alternative splicing events within this gene was firstly defined as tumor suppressing gene associated with Wilm's tumor, and it is suggested to play an important role in leukemogenesis. The first alternative splice corresponding to exon 5 causes the presence or absence of a 17-amino acid insertion between the trans-regulatory and ZF domain while a second splice results in gain or loss of a 9 bp insertion encoding 3 amino acids (KTS) between the third and fourth ZF DNA-binding domain; this produces four distinct isoforms designated as −17AA/−KTS(WT1A), +17AA/−KTS(WT1B), −17AA/+KTS(WT1C) and +17AA/+KTS(WT1D) [1–6]. WT1 isoforms are proposed to have distinct functions [4]. The changes in the ratio of these four isoforms in cells are thus thought to render different phenotypes.

WT1 is preferentially expressed during embryogenesis, playing a pivotal role in the development of the urogenital system. In human 20-week-old kidney the relative ratio of the four splice variants WT1A, WT1B, WT1C, and WT1D is 1:2.5:3.8:8.3, suggesting WT1+17AA isoforms is dominantly expressed in human embryonic kidney [7]. In adults WT1 is expressed to a very low extent in kidney, ovary, endometrium, testis, spleen, and normal hematopoietic progenitor cells [1,3]. Many reports showed that WT1 is expressed at a high level in hematopoietic malignancies including acute leukemia, chronic myelogenous leukemia, and myelodysplastic syndromes [1,2,5,8–17], as well as in various kinds of solid cancers including malignant mesothelioma, lung, breast, and renal cell cancer [8,18], whereas normal blood cells and CD34+ hematopoietic progenitors have been found to express WT1 at a far lower level or not at all [8,9]. Moreover, growing evidence has suggested that the high expression of WT1 in many acute leukemia patients may indeed be employed for MRD monitoring during patient follow-up [2,5,8–17]. WT1 has therefore been suggested to play a role as an oncogene participating in leukemogenesis [1,8,9], and is considered as a therapeutic target for leukemias and solid tumors [12,19–22], although its functional significance within the leukemogenesis process still

Abbreviations: WT1, Wilm's tumor gene; AML, acute myelocytic leukemia; RQ-RT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; BM, bone marrow; WT1+17AA, WT1 gene with 17 amino acids insertion; FAB, French-American-British; MNCs, mononuclear cells.

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remains to be elucidated. As regard to expression of WT1 isoforms in leukemias, the ratio of +KTS:−KTS was found to be approximately 1.6:1.0, and maintained within a very small range in all cell types by cloning and sequencing, indicating that WT1 KTS splicing is not regulated in a tissue-specific manner [7]. In contrast to the KTS splice variants, very little information exists on the functional effects of the presence of exon 5. Recent data suggested that WT1 have roles in the control of cell cycle regulatory and apoptotic pathways, and WT1+17AA isoforms played antiapoptotic roles at some points upstream of the mitochondria in the intrinsic apoptosis pathway [23].

Acute myeloid leukemia (AML) is characterized by a variety of complex molecular genetic abnormalities of partly still unresolved significance for gene expression and regulation. Obviously it is important to quantitatively determine the WT1 gene and its isoforms expression levels and to study its relevance to leukemogenesis in various AML subtypes during the course of the disease. Because there are few reports on the WT1+17AA isoforms expression in AMLs, in the present study, we focused on the expression levels of total WT1 gene and WT1+17AA isoforms in AML blasts of different French-American-British (FAB) subtypes at the time of diagnosis and at relapse by using RQ-RT-PCR method. We then discuss the possible function of WT1+17AA isoforms during the leukemogenesis and leukemic cell differentiation.

2. Materials and methods

2.1. Bone marrow sample sources

After ethical committee approval and informed consent, diagnostic bone marrow (BM) samples from 226 AML patients (182 at diagnosis and 44 at relapse) were collected from the three Department of Hematology, The First People's Hospital of Changzhou, China (79 patients, diagnosed between March 2003 and December 2007), The Department of Hematology and Oncology, Children's Hospital of Suzhou University, China (60 paediatric patients, diagnosed between February 2006 and March 2008) and The First Affiliated Hospital of Suzhou University, Jiangsu Institute of Hematology, China (87 patients, diagnosed between March 2003 and February 2005). No patients of complete remission status were included in the study. Diagnosis of AML was based on morphology, immunophenotype, and cytogenetic karyotype. Patients were classified according to the FAB classification. The proportion of blasts in the samples was determined by microscopy to ensure that mononuclear cells (MNCs) from leukemia patients at the time of diagnosis or relapse were predominantly (>60%) blast cells. Of the 226 BM samples of acute leukemia patients, 60 were derived from paediatric patients (age <14 years), 124 were from adult patients (age range from 15 to 60 years) and 42 were from old patient (age >61 years). 141 were males and 85 females. Meanwhile, 44 BM samples from either healthy volunteers, donors for allogeneic bone marrow transplantation, or non-hematological patients undergoing orthopaedic surgery were collected as normal controls. K562 cell line which highly expresses WT1 gene was taken as a positive control. Informed consent was obtained from all patients and healthy donors.

2.2. Sample processing

Bone marrows were collected into sterile tubes with anticoagulant (ACD, K2 or K3EDTA) and immediately transported to the lab at room temperature. The initial processing of the samples was performed within 12 h after their collection, in most cases within the first 4 h. MNCs were separated from the samples by centrifugation on Ficoll density gradient medium (gravity 1.077 g ml^{-1}, Chemical Reagent Ltd., Shanghai, China) and all the prepared samples were cryopreserved on liquid nitrogen for further experiments.

2.3. RNA extractions and cDNA conversion

Total RNA was extracted from MNCs of bone marrows, using TRIzol one-step procedure according to manufacturer's instructions (Gibco Ltd.) and dissolved in diethylpyrocarbonate (DEPC)-treated water. Reverse transcription was performed using random hexamer primers 100 ng for total RNA (2 μg/40 μl) and 100 U of reverse transcriptase (Promega Ltd.). The cDNA was stored at −20 °C or immediately used for real-time polymerase chain reaction (PCR), as described previously [13,14].

2.4. Primers and probe designation

All primers and the TaqMan probe for WT1 and WT1+17AA isoforms were designed by Primer Priemer software (version 5.0) and their positions referred to WT1 sequence were as described previously [24]. The primers and probe of ABL gene were shown as follows, SP: 5′-GAT ACG AAG GGA GGG TGT ACC A-3′, AP: 5′-CTC GGC CAG GGT GTT GAA-3′, Probe: 5′-FAM-TGC TTC TGA TGG CAA GCT CTA CGT CTC CCT-TAMARA-3′.

2.5. Quantitative RT-PCR

Real time RT-PCR analyses were performed using MJ Opticon TM2 fluorescence detection system (MJ. Research Inc., USA). The composition of PCR reaction mixture for WT1+17AA isoforms was as follows: 5× TaqMan PCR buffer 5 μl, 250 mM $MgCl_2$ 0.3 μl (final concentration 3 mM), 10 mM dNTP 0.75 μl (final concentration 0.3 mM), 5 U/μl Taq DNA Polymerase 0.25 μl (final concentration 0.05 U/μl, TaKaRa Ltd., China), 10 mM primers SP2 and AP1 0.5 μl each (final concentration 0.2 μM), 10 mM probe 0.3 μl (final concentration 0.12 μM), and cDNA 2 μl (resemble 100 ng RNA). Sterilized water was added to a final volume of 25 μl. The PCR was initiated for 3 min at 50 °C to heat activate the Uracil-DNA Glycosylase (UNG enzyme) and followed at 95 °C for 5 min to inactivate the UNG enzyme and heat start, then running for 45 cycles at the following condition: denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 60 s. All sample analysis was performed in duplicate. WT1+17AA isoforms copy number were calculated as the average.

For quantitative assessment of WT1+17AA isoforms, a calibration curve was used. Briefly, the standard curve was obtained by serial dilutions ranging from 10^6 to 10^1 molecules of a standard recombinant T vector. Standard preparation was as described previously [13]. Given a 100% PCR efficiency, 3.32 cycles are needed to reach 10-fold amplification of the initial molecule number. Corresponding standard curve generated the best slope of −3.32. We optimize our management to obtain a slope at −3.20 to −3.50, which underlines the consistency of PCR with this theoretical assumption and the accuracy of the experimental system. Quantitative assessment of WT1 was performed in the same way as for WT1+17AA isoforms. For each individual samples, WT1 gene and WT1+17AA isoforms were detected simultaneously during the same PCR amplification.

ABL gene was utilized as housekeeping gene for internal controlling the RNA quality. The PCR reaction mixture and procedure for ABL gene were the same as WT1+17AA isoforms except that 250 mM $MgCl_2$ 0.4 μl (final concentration 4 mM) was added into the PCR reaction mixture.

To reduce the risk of contamination, thermocycling were performed in separate laboratories. PCR mixtures were disposed inside a super-clean bench (Air Tech Ltd., SuZhou, China). Negative controls were performed for all RT-PCR steps, including a reverse transcriptase negative control for every sample.

2.6. Normalisation of WT1 and WT1+17AA isoforms expression

The housekeeping ABL gene expression levels in K562 cell lines and BM cells of acute leukemias were about 10^6 copies/100 ng RNA. If the ABL gene expression level in any of the samples was lower than 10^4 copies/100 ng RNA, this sample was regarded as bad quality of RNA and excluded from the final statistic analysis. The copy values of WT1 and WT1+17AA isoforms obtained by RQ-RT-PCR were normalized with respect to the number of ABL. Normalized WT1 expression (WT1/ABL) was represented as a ratio of WT1 to ABL levels in each individual sample, WT1+17AA isoforms were normalized in the same way. The proportion of WT1+17AA isoforms in each individual sample was calculated as a ratio of WT1+17AA isoforms copy value to WT1 gene copy value, determined as WT1+17AA/WT1.

2.7. Statistical methods

Statistical analysis was performed with SPSS software (version 10.0). All experiments were performed three times in each individual sample and the results were presented as the mean value of the three. *T*-test was used to compare the means between two groups and one-way ANOVA was used to compare the means among more than two different groups. The Mann–Whitney *U*-test was used to compare the median between two different groups and the Kruskal–Wallis H test was used to compare median among more than two different groups. *p*-Values <0.05 were considered to be significant.

3. Results

3.1. WT1+17AA isoforms and WT1 gene expression levels in normal BM cells

WT1 gene was very lowly expressed in 44 normal BM cells with median WT1/ABL of 0.0037, ranged from 0 to 0.0158, which was 2–3 logarithms lower than that in K562 cell line with amount to 1.111 ± 0.128. Here the WT1/ABL in seven samples were lower than 1E−05, we considered the WT1 gene expression negative (value of 0), and the ratios of WT1+17AA/WT1 in normal controls were not calculated for statistic analysis.