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Immunohistocemichal Assessment of the Role of WT1 Protein Expression in CML and its Correlation with CD 31 as an Angiogenic Marker

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Abstract

Background Several studies have demonstrated that Wilms' tumour gene 1 (WT1) is consistently overexpressed in most forms of leukemias, and the usefulness of quantitative assessment of WT1 expression as a molecular marker for minimal residual disease (MRD). Many suggest a role of WT1 for angiogenesis in hematological malignancies, WT1 is also expressed in a large variety of tumour blood vessels, and some suggests that it might be a general marker for angiogenesis.

Objective To assess the role of WT1 protein expression immunohistochemically in chronic myeloid leukemia (CML) and to determine whether there is a correlation between WT1 protein expression and CD31 expression as a marker of angiogenesis.

Methods This study involved 16 cases of newly diagnosed CML. In addition, 20 age matched control cases were involved having no apparent bone marrow pathology. Immunohistochemistry was done on bone marrow biopsies using Anti-WT1 and Anti-CD31 Monoclonal antibodies.

Results There was a significant increase in WT1 protein expression in CML cases, as well as an increase in CD31 expression; however, there was no significant correlation between WT1 expression and hematological parameters (WBC count, platelets count, PCV level, and peripheral blood blast %) and CD31 expression.

Conclusion This study showed that WT1 is overexpressed in CML patients, while it was undetected in controls, thus we may propose that it maybe used as an auxiliary marker for the disease. *WT1* expression was not found to be of prognostic significance. Moreover CD31 as a marker for angiogenesis was significantly increased in CML but did not correlate with WT1 expression.

Key words WT1, chronic myeloid leukemia, immunohistochemistry

Introduction

The WT1 gene, located on chromosome 11p13, was first identified in patients with Wilms tumor; it encodes a transcription factor involved in normal and malignant hematopoiesis, unlike other tumor suppressor genes, such as Rb and p53, the expression of the WT1 gene is restricted to a limited set of tissues (fetal kidney, ovary, testis, and spleen) [(1)]. More recently, *WT1* overexpression was detected in several haematological and solid malignancies. Additional studies revealed that it had a role in the initiation phase of the malignant diseases [(2)]. Since WT1 is believed to be relevant in the maintenance of the malignant phenotype of the tumour cells and is mostly restricted to malignant tissues, it is an attractive target for immunotherapy [(2)].

Chronic myeloid leukemia (CML) is a malignant clonal blood disease that originates from a pluripotent hematopoietic stem cell. The

cytogenetic hallmark of CML, the Philadelphia chromosome (Ph), is formed as a result of reciprocal translocation between chromosomes 9 and 22, leading to the uncontrolled proliferation of the bone marrow cells [3].

Angiogenesis is the formation of new blood vessels from pre-existing vessels during adult life [4]. Many studies suggest a role for angiogenesis not only in the pathogenesis of solid tumors but also in hematological malignancies like acute and chronic leukemia, lymphoma, myelodysplastic syndromes, myeloproliferative neoplasms, and multiple myeloma [5]; furthermore, WT1 is also expressed in a large variety of tumour blood vessels [6], as it is involved in endothelial cell proliferation, vascular formation and migration, indicating that it might be a general marker for angiogenesis [2].

Since WT1 is a marker of angiogenesis and it is believed to be relevant in the maintenance of the malignant phenotype of the tumour cells [2], this study will assess the expression of WT1 in chronic myeloid leukemia, and investigate if there is a correlation between the WT1 expression and angiogenesis (as marked by CD31 expression) in CML which might help for future therapeutic trials.

Methods

Patients and sampling

This cross sectional case control study was conducted from March 2011 to July 2012 on the trephine biopsy of 16 newly diagnosed CML patients including 10 in chronic, 3 in blastic, and 3 in accelerated phases. In addition to 20 age matched control cases with benign reactive marrow with no evidence of hematological malignancy, the cases were collected from The Hematology Ward of Baghdad Teaching Hospital. This study was ethically approved by the Ministry of Health. Clinical and laboratory information regarding age, sex, packed cell volume (PCV), white blood cell (WBC) count, platelets count, blasts percent, were obtained directly from the patient through taking history and examination at time of diagnosis during the clinical course and before taking chemotherapy.

From each formalin fixed paraffin embedded bone marrow biopsy used in this study, 3 sections of 4 µm thick were taken; one representative section was stained with Hematoxylin and Eosin (H&E) stain and was reviewed, while the other sections were stained immunohistochemically with WT1 and CD31 monoclonal antibodies respectively.

Immunhistochemistry

The primary antibodies used in this study were: monoclonal mouse anti-human Wilms' Tumor 1 (WT1) protein, clone 6F-H2 (Dako Cytomation), prediluted monoclonal mouse antiendothelial cell marker (CD31) antibody, clone JC70A (Dako Cytomation); while the immunohistochemistry (IHC) secondary detection kit used was immunoperoxidase secondary detection kit (DakoCytomation IHC kit LSAB2 System-HRP, code K0679) which was purchased from DAKO, Denmark. The immunohistochemical staining procedure was done according to the manufacturer's instructions. Positive staining is expressed as a brown color, in which brown cytoplasmic staining of endothelial cells is considered positive reaction for CD31 [7]; and staining of either the nucleus and/or the cytoplasm indicated a positive result for WT1 [8].

For IHC technical quality control: tonsils tissue which was taken from a healthy young patient who had no other known disease other than inflamed tonsils that required tonsillectomy were used as a positive control tissue for CD31, while Wilms Tumor tissues were used as a positive control tissue for WT1 staining. Technical negative control was performed by omission of the primary antibody.

Scoring of immunohistochemical staining

Scoring of immunohistochemical staining was performed using specialized automated cellular image analysis system, Digimizer software, version 3.7.0, that allows precise manual measurements as well as automatic object detection with measurements of object characteristics (Fig. 1) [9].

For purpose of statistical analysis, the following variables were used:

A. Color Intensity: the average intensity of the brown color for the selected objects depending on the expression of antigens in the cells.

B. Fractional area stained: which equals to *[(mean area* Number of objects)/area of a single image field]*100*

C. Digital Labeling Index: for better estimation of the immunohistochemical expression of the WT1, CD31, we used an arithmetic tool named as Digital Labeling Index. This tool is calculated according to the following formula: (Fractional area * reverse Intensity). This parameter combines both the Fractional area and the Intensity [9].

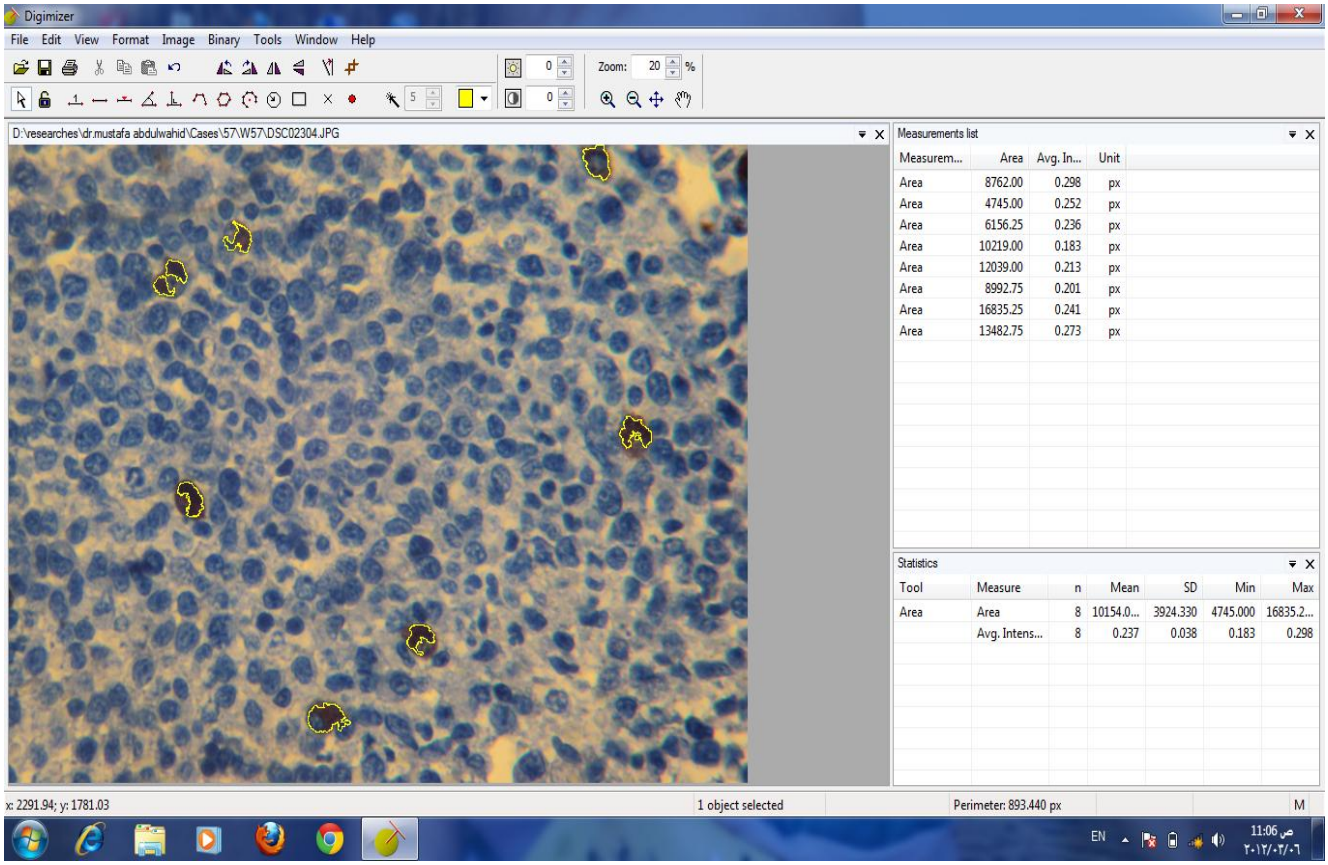

Fig. 1. Image analysis in Digimizer software

Statistical Analysis

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean± SEM, frequency was used to express discrete data. ANOVA was used to analyze numeric data while Chi-square was used to analyze discrete data, and benferroni test was used for multiple comparisons. Spearman rank correlation was used to determine relation between various markers. *P* Value of < 0.05 was considered significant.

Results

This study included 16 cases of CML, divided into 10 cases in chronic phase, 3 cases in accelerated phase, and 3 cases in blastic phase, in addition to 20 control cases; in CML. Eleven of the patients were males (69%) and 5 of them were females (31%) with a male to female ratio of 2.2:1; on the other hand, 12 of 20 control persons were males (60%), while 9 were females (40%) with a male to female ratio of 1.3:1.The age range of patients with CML was between 32-57 years with a mean of 46.5±7.4 years, by dividing the patients according to 10 year intervals, the largest number of patients (7) fall

in the age group 40-49. Regarding the comtrol group, the age range was between 24-71 years and the mean was 49.60±14.17 years, with the largest number of cases falling in the 50-59 years age group.

Regarding Immunohistochemical staining results, we depended on Digital Labelling Index (DLI) parameters in considering what is positive and what is negative; 11 out of the 16 CML cases were positive for WT1 DLI, while none of the control cases were positive; regarding CD31, 15 cases were positive in CML, while none of the control cases were positive. WT1 DLI was significantly higher in CML than controls 4.17±3.67 (P = 0.003).

WT1 DLI was significantly higher in blastic and accelerated phases of CML than Chronic phase (6.46±4.34, 9.49±1.47 respectively versus 2.27±2.81 in chronic phase, P = 0.032 and 0.001 respectively) as seen in Table 1.

Table 1: Comparison of WT1 DLI between CML phases Subclasses

Parameter	CML phase	Mean (DLI)±SD	*Vs*	CML phase	Mean±SD	P	SE
WT1	CML chronic	2.27±2.81	*Vs*	CML blastic	6.46±4.34	0.032	1.53
				CML accelerated	9.49±1.49	0.001	1.69
	CML blastic	6.46±4.34	*Vs*	CML accelerated	8.49±2.49	0.154	1.94

WT1 DLI was not significantly correlated with age and gender of the patient. WT1 DLI was significantly positively correlated with blast % BMA in CML (r = 0.619, p = 0.011), while it was not significantly correlated with PCV, WBC count or Platelets count; on the other hand. Angiogenesis parameter used in this study, CD31 DLI, was significantly higher in CML than in controls (8.38±2.51 versus 0.1±0.01, P = 0.004). There was no significant correlation between WT1 DLI with CD31 DLI in CML.

Discussion

The concrete role of WT1 in hematopoiesis and leukemogenesis remains unclear. Studies on the oncogenic activity of WT1 have led to conflicting results demonstrating cell proliferation in some and cell growth arrest in others [10]. In the presented cross sectional case control study, WT1 DLI was positive in 11 CML cases (68.75%) while none of the control cases were positive for WT1. These results were in accordance to other studies such as Rosenfeld et al [11], who found, using Real Time PCR technique, that WT1 gene was overexpressed in all cases of CML; while Huang et al [12] found, using conventional nested PCR not QR-PCR, that 17 out of 37 CML showed WT1 expression.

Interestingly, we have found that WT1 protein expression level was significantly higher in CML accelerated and CML blastic phases than CML chronic phase, while there was no significant difference in WT1 expression level between CML accelerated and CML blastic phases. This goes with Kreuzer et al study [13], which showed WT1 overexpression, using Real Time- PCR, in all CML patients studied, but revealed differences in WT1 expression levels within this patient population; similarly Huang et al [12] showed that 5/18 (27.7%) CML blastic crisis patients, 1/5 (20%) CML patients in accelerated phase, and 1/10 (10%) CML patients in chronic phase have had high WT1 expression level; on the other hand, using conventional PCR, Menssen [14] revealed overexpression of WT1 in all blast crisis cases but not in chronic phase cases. These data support the notion that increased levels of *WT1* expression are indeed specific to leukemic blasts with respect to normal hematopoietic progenitors and not a simple consequence of the differentiation degree.

In this study, WT1 protein expression in CML was not significantly associated with gender and age of the patients, and WT1 protein expression was not significantly associated with various hematological parameters (WBC count, platelets count, PCV level, and peripheral blood blast %)

which goes with Sadek et al [1], Karakas et al [15], Gu Wy et al [16]; on the other hand, it was positively correlated with Blasts % in bone marrow aspirate. Interestingly, Cao et al [17] found that WT1 expression levels in CML patients in accelerate phase or blast crisis were strikingly higher than those in non-leukemic patients or CML patients in chronic phase; thus, it appears that WT1 gene expression is associated with immature cells from which leukemic cells in CML originate.

In CML, CD31 DLI was significantly higher than in controls; which also goes with Alvaro et al [18] and Hans et al [19], which have found a significant increase in angiogenesis in CML compared with healthy control cases.

In this study, there was no significant correlation between WT1 protein expression and CD31 in CML, this does not go in line with Wagner et al [6], who found that WT1 might be involved in tumour angiogenesis, in which endothelial WT1 expression was detected in 95% of 113 AML cases of different origin and that transcriptional activation of ETS-1 by the Wilms' tumour suppressor WT1 is a crucial step in tumour vascularization via regulation of endothelial cell proliferation and migration; moreover Trka et al [20], have suggested that WT1expression can be stimulated by hypoxia, which involves activation of the WT1 promoter by HIF-1. The discrepancy between our finding and other studies may be due to the fact that others have used more sensitive methods (PCR) for evaluating WT1 than Immunohistochemical staining procedure we used in addition to the smaller sample size.

There are several controversies surrounding reported data on the prognostic significance of WT1 expression, which is mainly because of the limited number of patients and the diversity of methods used; while some groups have shown that high levels of WT1 coincide with worse prognosis [21-24], suggesting that WT1 levels could be useful for predicting prognosis in such patients, no evidence was found that the level of WT1 at diagnosis was an independent prognostic factor for survival, just as some studies failed to show any correlation between initial WT1 levels and outcome of the disease at all [25,26]. These discrepancies may be due to differing methodologies, for example, real-time PCR versus end-point analysis or due to patient selection. Moreover, based on results similar to those found above, it is strongly believed that WT1 can become a target for immunotherapeutic approaches as suggested by Rosenfeld et al [11], upcoming data support this hypothesis, as sera from many AML, CML, and MDS patients have anti-WT1 antibodies [11].

In conclusion, this study showed that WT1 was overexpressed in 68.75% of CML patients; taken together with longitudinal analyses of WT1 expression in healthy donors, which was undetectable. CD 31 expression (as a marker of angiogenesis) was significantly higher in CML in comparison with control cases but there was no significant correlation between its expression and WT1 expression.

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