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Research paper

Prognostic importance of Aurora Kinases and mitotic spindle genes transcript levels in Myelodysplastic syndrome

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ABSTRACT

Myelodysplastic syndrome (MDS) are a heterogeneous group of clonal disease characterized by insufficiency of bone marrow, increase of apoptosis and increased risk of acute leukemia progression. Proteins related to the mitotic spindle (*AURKA*, *AURKB*, *TPX2*), to the mitotic checkpoint (*MAD2, CDC20*) and the regulation of the cell cycle (*p21*) are directly related to chromosomal stability and tumor development. This study aimed to evaluate the mRNA expression levels of these genes in 101 MDS patients using a real-time PCR methodology. We identified that *CDC20* expression are increased in patients with dysmegakaryopoiesis ($p = 0.024$), thrombocytopenia ($p = 0.000$) and high-risk patients ($p = 0.014$, 0.018) *MAD2* expression are decreased in patients with 2 or 3 cytopenias ($p = 0.000$) and neutrophil below 800/mm^3. *TPX2* is also overexpressed in patients presenting dysmegakaryopoiesis ($p = 0.009$). A decrease in *AURKA* and *AURKB* expression were observed in patients with altered karyotype ($p = 0.000$), who presented dysplasia in 3 lineages ($p = 0.000$; 0.017) and hemoglobin inferior to 8 g/dL ($p = 0.024$). The expression of *AURKA*, *AURKB* and *MAD2* ($p = 0.000$; 0.001; 0.025) were decreased in patients with hypoplastic MDS, associated with high frequency of chromosomal alterations and high mortality rate. This study reaffirms the importance of aurora kinases and mitotic spindle genes to the pathogenesis and clinical evolution of MDS.

1. Introduction

The Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal diseases characterized by bone marrow insufficiency and an increase of apoptosis, leading to an ineffective hematopoiesis with dysplasias, peripheral cytopenias and increased risk of progression to acute myeloid leukemia (AML) [1,2].

Chromosomal abnormalities are presented in up to 50% of the MDS patients. The cytogenetics analysis contributes to the diagnosis, prognosis, assessment of response to therapy and clonal evolution [3,4]. The development of chromosomal alterations, which leads to the development of neoplasias, is related to malfunctioning of the cell cycle, resulting from mutations in genes encoding cell cycle proteins and signaling pathways due these genetic lesions [5].

The Aurora kinase family (Aurora-A, -B) is an important group of serine/threonine kinases involved in several functions during mitosis. They are involved in regulation of chromosomal alignment and segregation, maturation separation of the centrosomes and formation of the mitotic spindle [6–8]. Dysfunction of these kinases can cause chromosomal instability and is associated with tumorigenesis of several neoplasias, as acute myeloid leukemia (AML) [9]. We previously demonstrated that tissue samples of MDS patients with abnormal karyotype presented higher AURKB protein expression than patients with normal karyotype [12]. Several modulating factors have been studied as activators of Aurora A activity, location and stability. The most reported AURKA regulator is TPX2 [10], a protein that plays an important role in the formation of mitotic spindle [10]. Many studies have associated the increased expression of *TPX2* to chromosomal instability in and tumor development [11,12]. Nevertheless, we have not identified studies evaluating the mRNA expression of *TPX2* in patients with MDS.

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The maintenance of chromosomal stability during the cell division is also related to the Spindle Assembly Checkpoint (SAC) that monitors the kinetochore-microtubule attachment, to prevent segregation errors [13,14]. When SAC is activated, the Anaphase Promoting Complex (APC) is inhibited by MAD2, which inactivates the protein CDC20 (responsible for the activation of APC), preventing the cell from entering at anaphase until the microtubules are correctly attached to the kinetochores [13–15]. Several studies have reported the overexpression of *MAD2* and *CDC20* in the development and progression of human cancers, as lung tumor and gastric cancer [6–8]. Genga and colleagues [8] demonstrated for the first time that higher CDC20 and MAD2 expressions were associated with presence of thrombocytopenia and complex karyotype in MDS patients.

The regulation of the cell cycle depends on several proteins, like CDKN1A (a.k.a. p21), which is a cyclin kinase dependent inhibitor that regulates the transitions from G1 to S and G2 to mitosis. *p21* has been related to the carcinogenesis of several tumors and has shown influence on the expression of genes related to the cell cycle, DNA repair and regulation of apoptosis [16,17].

The aim of this report was evaluate the correlation and prognosis of mRNA expression of genes related to the mechanisms of cell cycle regulation (*p21*), mitotic checkpoint (*MAD2* and *CDC20*) and mitotic spindle (*AURKA*, *AURKB* and *TPX2*) in MDS patients.

2. Patients, materials and methods

2.1. Patients

We evaluated 101 bone marrow samples MDS patients, diagnosed at Federal University of Ceará according to the WHO classification [18], from 2008 to 2015. The 101 samples were collected at the time of diagnosis and bone marrow samples of 10 healthy volunteers were used as healthy control.

The study was approved by the Ethics Committee of the Federal University of Ceara and informed written consent was obtained from eacindividual.

2.2. Cytogenetic analysis

Conventional G-Banded karyotype analyses were prepared from bone marrow cells of 101 MDS patients. Briefly, cultures were established in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 30% fetal calf serum. After a 24 h incubation, colcemid was added to cell culture for blocking of mitotic fuse (final concentration 0.05 mg/ml). After harvesting procedure, the cells were exposed to a KCl hypotonic solution (0.068 mol/L) and fixed with *Carnoy's* buffer (methanol/acetic acid in 3:1 proportion). The slides were prepared and stained with Giemsa solution. A minimum of 20 metaphases were analyzed whenever possible using CytoVision Automated Karyotyping System (Applied Imaging, San Jose, CA, USA).

2.3. Total RNA extraction

Total RNA extractions from isolated mononuclear cells (bone marrow), obtained from MDS patients, were performed with TRizol Reagent™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

2.4. cDNA synthesis

cDNA was generated from total RNA using the High Capacity cDNA Reverse Transcription kit® (Applied Biosystems, San Jose, CA, USA), according to the manufacturer's protocol. cDNA synthesis was generated using Mastercycler Pro Vapor Protect Technology® machine (Eppendorf, Hamburg, Germany). cDNA samples were stored at −20 °C until further use.

2.5. Quantitative real-Time PCR

Quantitative real-time PCR (qPCR) reactions were based on TaqMan methodology® (Applied Biosystems, Carlsbad, CA, USA) and performed on a 7500 Fast System® (Applied Biosystems, Carlsbad, CA, USA). Predeveloped TaqMan gene expression assays (Assays-on-Demand, Applied Biosystems, Carlsbad, CA, USA) for *AURKA* (Hs01582072_m1), *AURKB* (Hs00945858_g1), *TPX2* (Hs00201616_m1) *MAD2* (Hs01554513_g1), *CDC20* (Hs00426680_mH) and *CDKN1A* (Hs0035782_m1), as well as the TaqMan Universal Master Mix II® (Applied Biosystems, Carlsbad, CA, USA) were used to quantify mRNA expression.

To normalize input cDNA, three reference genes were used: beta-2-microglobulin gene (*B2M*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ubiquitin C (*UBC*). The standard deviation of each reference gene was calculated based on the Cq value. After analysis of the standard deviation, a Pearson correlation coefficient was calculated for each pair of candidate reference genes. Then, the geometric mean of all reference genes was made (also known as the central tendency average or INDEX) and Pearson's correlation of each gene was separately obtained with the index. The reference genes that had the lowest standard deviation and the highest correlation were considered the most stable. Thus, *B2M* and *GAPDH* were selected.

Each sample was performed in duplicate and the expression ratios were calculated using the $2^{-\Delta Cq}$ method [19] from the Cq values provided by the 7500 Real-Time PCR System software (Applied Biosystems, Inc., Foster City, CA, USA).

2.6. Statistical analysis

Data on relative mRNA expression (ΔC_q values – *quantitative cycle*) were expressed as mean and range (maximum and minimum) in order to determine the possible association between relative gene expressions and variables. Normality was evaluated by Shapiro-Wilk test. Outliers were removed. The Student's *t*-test or one-way ANOVA with Tukey/Games Howell *post-hoc* test were used to analyze the influence of relative expression regarding these variables: age, WHO classification [18], low risk vs. high risk, bone marrow cellularity, dysplasias (number of dysplasias, dyseritropoiesis, dysgranulopoiesis, dysmegakaryopoiesis), IPSS-R variables [20] (blast count, hemoglobin count, absolute neutrophil count - ANC, platelets), number of citopenias, karyotype (normal vs. abnormal, aneuploidy, del(5q), del(7q), number of alterations, IPSS-R prognostic), risk classification (IPSS-R), transfusion dependency, death and AML evolution. The variance homogeneity for all variables analysis was evaluated by Levene's test.

The overall survival of MDS patients regarding expression was evaluated by the Kaplan-Meier method. For this analysis, the gene expression variable was readjusted to cutoff points established by the *Cutoff Finder* algorithm [21]. The best cutoff point predicting MDS survival based on the gene expression was calculated by this software, through Kaplan Meyer curve analysis based on the log-rank test. Also, a multiple regression were run to predict the overall survival from MAD2, CDC20, TPX2, AURKB, AURKA, P21 mRNA expression and other factors. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were tested.

The *Pearson's* correlation test was used for obtaining the r and the r-square (r^2) values, that demonstrates the influence of mRNA expression level of a given gene over other one.

Statistical analyses were performed using the SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Prism software, La Jolla, CA, USA) softwares. Probability level (p-value) < 0.05 was adopted.