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Proteins related to the spindle and checkpoint mitotic emphasize the different pathogenesis of hypoplastic MDS

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ABSTRACT

Some studies show that alterations in expression of proteins related to mitotic spindle (AURORAS KINASE A and B) and mitotic checkpoint (CDC20 and MAD2L1) are involved in chromosomal instability and tumor progression in various solid and hematologic malignancies. This study aimed to evaluate these genes in MDS patients. The cytogenetics analysis was carried out by G-banding, *AURKA* and *AURKB* amplification was performed using FISH, and *AURKA*, *AURKB*, *CDC20* and *MAD2L1* gene expression was performed by qRT-PCR in 61 samples of bone marrow from MDS patients. *AURKA* gene amplification was observed in 10% of the cases, which also showed higher expression levels than the control group ($p = 0.038$). Patients with normo/hypercellular BM presented significantly higher expression levels than hypocellular BM patients, but normo and hypercellular BM groups did not differ. After logistic regression analysis, our results showed that HIGH expression levels were associated with increased risk of developing normo/hypercellular MDS. It also indicated that age is associated with *AURKA*, *CDC20* and *MAD2L1* HIGH expression levels. The distinct expression of hypocellular patients emphasizes the prognostic importance of cellularity to MDS. The amplification/high expression of *AURKA* suggests that the increased expression of this gene may be related to the pathogenesis of disease.

1. Introduction

Myelodysplastic syndromes (MDS) represent a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, cytopenia, unilineage or multilineage dysplasia and susceptibility to leukemia. Biologically, it is the result of stem cell dysfunction, genetic instability and the deregulation of apoptosis [1]. Despite the similarities, there is a great heterogeneity among the subtypes. The type and degree of impairment of the different mechanisms involved may determine how the disease manifests clinically, including the intensity and number of cytopenias, and if the disease has an indolent or aggressive behavior (rapid progression to acute myeloid leukemia) [2]. Clinical heterogeneity of MDS is therefore a reflection of the diversity of molecular mechanisms involved in the pathogenesis of this disease.

In hypoplastic MDS response to different treatment as antithymocyte globulin (ATG) with considerable response emphasizes the different pathogenesis of this subtype of MDS [3]. Immunologic abnormalities seems to play a major role. Increased oligoclonal T-cell expansion and suppression of hematopoietic stem cell by cytotoxic T cells have been detected and ATG is believed to block these T cells, restauring normal haematopoiesis [3].

Aurora kinases (AURORA A and AURORA B) comprise a family of serine/threonine kinases that play a critical role in regulating mitosis and cytokinesis during G2/M. Their altered expression has been associated with chromosomal instability, centrosome amplification and chromosomal abnormalities. Besides, its increased expression has been related to grim prognosis in colorectal cancer, hepatocellular carcinoma and acute myeloid leukemia [4–7]. Recently we reported no association between Aurora B expression and prognosis in MDS [8].

CDC20 is an activator of complex promoter of anaphase (APC) that participates of the degradation of proteins triggering the metaphase-anaphase transition. MAD2 (mitotic arrest deficient 2) is also an important protein related to mitotic checkpoint arresting

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the cell in metaphase when it is located to unattached kinetochores. When MAD2 dissociates from kinetochores, the inhibitory effect above CDC20 is relieved and APC becomes active, promoting anaphase. Recently, CDC20 has been reported as an oncoprotein promoting the development and progression of human cancers [9]. It has also been reported that overexpression of MAD2 in transgenic mice leads to a wide variety of tumors and MAD2 overexpression has been reported in lung tumor and in human osteosarcoma [10]. To the best of our knowledge, these proteins (MAD2/CDC20) have not been reported in myeloid malignancies.

The aim of this report is to study the genes related to the spindle (AURORA A and AURORA B) and checkpoint mitotic (CDC20 and MAD2) proteins in myelodysplastic syndrome and evaluate the association with important prognostic markers of MDS.

2. Patients, materials and methods

2.1. Patients

Sixty-one adult patients with MDS from Federal University of Ceara classified by WHO, IPSS and WPSS were enrolled into the study [11,12]. The cellularity was assessed on BM biopsies. Hypocellularity was defined as <30% in patients <70 years, and <20% in patients >70% years, according to the established criteria [13].

2.2. Cytogenetic analysis

The bone marrow cells were cultivated as previously reported for chromosome analysis [14]. Briefly, the cells were separated into two short-term cultures with 7 mL RPMI 1640 (pH 7.0), 3 mL fetal bovine serum (Invitrogen) and incubated for 24 h at 37 °C. Colcemid (50 μL) was added to the culture for 30 min, followed by 0.075 M KCl at room temperature for 20 min and Carnoy's fixative for 5 min, four times. Slides were prepared and submitted to G-banding and whenever possible at least 20 metaphases.

2.3. Fluorescent in situ hybridization (FISH)

Amplifications involving *AURORA A KINASE* (*AURKA*) and *AURORA B KINASE* (*AURKB*) genes were assessed by fluorescent in situ hybridization (FISH) according to probe manufacturer instructions (AURKA: AURKA (20q13)/20q11 and AURKB: AURKB (17/p13)/SE17; Kreatech Diagnostics, Amsterdam, The Netherlands). Amplification was defined by ≥3 red signals (*AURKA* or *AURKB* genes regions) and 2 green control signals (MPARE1 for AURKA and SE17 for AURKB) copies/cell. A minimum of 200 cells per specimen/probe were scored by two independent investigators. In the presence of discrepancy the results were reviewed, combined and averaged. Samples from 8 bone marrow donors were used as controls. The cut-off level for normal values was established as >4% for each target region.

2.4. Gene expression analysis

Total RNA from MDS patients and donors bone marrow cells were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a High Capacity cDNA reverse transcription Kit (Applied BioSystems, Foster City, CA, USA), following the manufacturer's instructions. For genes analysis, TaqMan Assays were used (AURORA A: Hs00269212_m1, AURORA B: Hs00177782_m1, CDC20: Hs00426680_mH, MAD2L1: Hs01554513-g1; Applied Biosystems). β2-Microglobulin (B2M: Hs99999907_m1) and Ubiquitin C (UBC: Hs00824723_m1) were chosen as endogeneous internal control for each sample. The samples were analyzed in duplicate. The comparative cycle threshold (Ct) method was used to determine the relative expression. Their expression was calculated as a relative quantification to the average value of B2M and UBC housekeeping genes. The control group was composed by four samples from bone marrow donors.

2.5. Statistical analysis

For expression genes and FISH analysis in different groups, Kruskal–Wallis rank sum test was used. The chi-square test or Fisher's exact test was used to determine the association among the evaluated variables. Logistic regression analysis was used to estimate the odds ratios for variables contributing to the risk of MDS and to adjust the analysis for confounders contributing to MDS susceptibility.

All tests were considered to be statistically significant with a p-value of ≤0.05. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for analysis.

3. Results

The clinical and biological characteristics of the 61 MDS patients are summarized in Table 1. The patients' median age was 66 years (range 15–91 years) and 42.6% (26/61) were under 60 years old. Most cases were considered low/INT-1 risk (85%) and very low/low (45%) according to IPSS and WPSS, respectively. According to BM cellularity, 28% (14/50) were considered hypocellular, 20% (10/50) normocellular and 52% (26/50) hypercellular.

3.1. Cytogenetics by G-banding

Successful cytogenetic analyses were available for 45 patients (74%) and chromosomal abnormalities were detected in 55.6% (25/45) cases. The most common alterations were related to chromosomes 5 (−5/5q−), occurring in 44% of 25 patients with karyotype abnormalities. Other frequent anomalies were chromosome 7 (−7/7q−) (20% of cases patients with chromosomal abnormalities) chromosome 17 (−17/17p−) (16%), chromosome 8 (+8) (12%) and chromosome 20 (−20/−20q) (8%). Isolated deletions of the long arm of chromosome 5 (5q−) were found in 24% (6/25) of cases with any clonal cytogenetic alteration.

According to IPSS, 60% cases were considered karyotype of good prognosis, 22.2% and 17.8% were of intermediate and high risk cytogenetic, respectively.

3.2. Fluorescent in situ hybridization (FISH) of AURKA and AURKB genes

We performed FISH analysis in 41 cases from the 61 MDS patients. Amplification of *AURKA* gene was detected in 9.8% (4/41) patients (Cases 6, 32, 35 and 37) (Fig. 1a). All cases of *AURKA* amplification showed transfusion dependence, a marker unfavorable prognosis in MDS (Hb range: 2.6–7.4 g/dL) [15]. *AURKA* gene expression of patients with amplification showed expression levels 2.8 times higher ($p = 0.038$) when compared to the control group (Fig. 1b). If separately analyzed, the case 37 (which presented numeric chromosome abnormalities) were 3.3 times higher expressed than control group.

AURKB gene amplification was detected in 5.4% (2/37) patients (Cases 11 and 56).

No deletions of *AURKA* and *AURKB* genes were detected by FISH.

3.3. Gene expression analysis

The expression gene values of the 61 MDS patients are showed in Table 2. Initially, we compared MDS gene expression with healthy donors and among the MDS patients according to clinical and laboratory parameters (sex, age, bone marrow cellularity, number of cytopenias, transfusion dependence, cytogenetics, WHO classification, IPSS and WPSS). There was no difference between the groups (Table 2), except for the BM cellularity in all genes analyzed, and age for checkpoint mitotic genes (*CDC20* and *MAD2L1*).

Patients with hypercellular and normocellular bone marrow presented significantly higher expression levels than patients with hypocellular bone marrow, but normo and hypercellular BM groups did not differ. Furthermore, there were no difference between expression of cases with hypocellular BM and controls.

It should be pointed out that the gene expressions varied widely, maybe because of the high heterogeneity of the disease into each group. Once it was not observed difference of expression among the groups regarding the others variables, the relative expression of the genes was grouped into two groups ("lower" and "higher" expressed). By adopting the mean value from ΔCt of control group, it was possible to identify two subgroups of patients according to their expression: LOW GROUP when the gene expression was lower than or equal to the mean, and HIGH GROUP when gene expression was higher than the mean.

After applied a logistic regression analysis, our results showed that HIGH expression levels of all genes (*AURKA*,