

Brief communication

High expression of *AURKA* and *AURKB* is associated with unfavorable cytogenetic abnormalities and high white blood cell count in patients with acute myeloid leukemia

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ABSTRACT

In the present study, we analyzed *AURKA* and *AURKB* gene expression in 70 acute myeloid leukemia (AML) patients. There was no difference between leukemic samples and bone marrow mononuclear cells (BMMCs, $n = 8$) or CD34⁺ progenitors ($n = 10$) from healthy donors. High white blood cells (WBC) counts were observed in the *AURKA*⁺ and *AURKB*⁺ groups, but no significant differences regarding age, gender, platelet counts or frequency of *FLT3-ITD* mutations. *AURKA*, but not *AURKB*, expression was independently associated with high WBC counts (OR: 3.15, 95%CI 1.07–9.24, $p = 0.03$). Moreover, the majority of cases that overexpressed *AURKA* and *AURKB* presented unfavorable cytogenetic abnormalities ($p < 0.001$). In conclusion, we described a significant association between overexpression of *AURKA/B* and cytogenetics findings in AML, which may be relevant to new therapeutic approaches, based on Aurora kinase inhibitors.

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1. Introduction

Aurora kinases comprise a family of serine/threonine kinases that includes in mammals three members designated aurora A, B, and C. These proteins play a critical role in regulating mitosis and cytokinesis, with their activity peaking during G2/M. The encoding genes map to chromosomal loci that are frequently altered in human cancers, and their altered expression have been implicated in chromosomal instability, with missegregation of individual chromosomes or polyploidization accompanied by centrosome amplification and acquired chromosomal abnormalities [1]. Moreover, their overexpression and/or amplification have been reported as an independent adverse prognostic marker in colorectal cancer [2], cervical carcinoma [3], breast carcinoma [4], and hepatocellular carcinoma [5]. Nevertheless, information regarding the expression of aurora kinase A (*AURKA*) and B (*AURKB*) in hematologic malignancies is still limited. Indeed, Ye et al. [6] analyzed nine patients with acute myeloid leukemia (AML) and 20 patients with myelodysplastic syndrome (MDS) and did not detect significant correlation of *AURKA* gene expression with bone marrow blast counts, viability of CD34⁺ blast cells, cytogenetic abnormalities, IPSS score,

proliferation index assessed by Ki-67 stain, or apoptotic activity assessed by active caspase 3 staining. Accordingly, Huang et al. [7] found no significant difference between the patient age, sex, white blood cell count, or FAB classification subtype in relation to aurora kinase expression in 98 AML *de novo* cases. In contrast, Walsby et al. [8] reported that ten (90.9%) out of eleven acute promyelocytic leukemia samples, whereas only four (15.4%) out of 26 samples from patients with M4 AML were positive for *AURKA* gene expression.

Cytogenetic analysis is considered to be the most important independent prognostic parameter in AML. Most of the chromosomal abnormalities are detectable by classical cytogenetics (G-banding), and they occur in approximately 55% of adults with *de novo* AML [9]. In 10% to 20% of patients, the abnormal karyotype is complex, being defined as containing at least three chromosome aberrations, whereas in 40% to 50% of patients present normal karyotype. Large collaborative studies have proposed cytogenetic risk systems classifying patients into favorable, intermediate and unfavorable risk groups according to the specific karyotypic findings at diagnosis, providing the framework for risk stratification schemes in AML [10].

Considering the evidence suggesting that impairment of aurora kinase function led to defects in chromosome segregation and cytokinesis in normal tissues and that misexpression and/or amplification of *AURKA* and *AURKB* has been associated with markers

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of adverse prognosis in solid tumors, we have analyzed *AURKA* and *AURKB* gene expression in 70 patients with *de novo* AML and compared to other well known clinical, biological and cytogenetic prognostic factors.

2. Design and methods

2.1. Patient samples

Seventy non-consecutive *de novo* AML patients with available karyotype results were included in the present study. All patients were treated at the Hematology Service of the Medical School of Ribeirão Preto, University of São Paulo, from October 2005 to September 2008. Bone marrow (BM) cells were obtained by aspiration at diagnosis after informed consent, and isolated by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St Louis, MO, USA). The diagnosis and classification of the disease was based on morphological and immunophenotypic features and on molecular analyses described elsewhere [11].

As normal controls, eight BM mononuclear cell (BMMC) samples were isolated from healthy adult donors and processed as described for patients. Since the percentage of CD34⁺ cells may vary in BMMC suspensions, and this may affect gene expression profile, later we opted to include CD34⁺ hematopoietic progenitors isolated using immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from another 10 BM samples obtained from healthy adult donors. After isolation, samples contained more than 80% of CD34⁺ cells. It was not detected significant differences between BMMC and CD34⁺ progenitors samples, therefore their results were pooled together in order to improve the power of statistical analyzes. The study was approved by the local Ethics Committee (7147/2005) and written informed consent was obtained from all donors.

2.2. Cytogenetic analysis

Bone marrow aspirate withdrawn at the time of diagnosis was used for classical and molecular cytogenetic analysis after a 48-h non-stimulated culture in RPMI 1640 medium with 20% fetal calf serum. Karyotypes were described according to ISCN 2005 [12]. According to the cytogenetic findings, patients were classified into favorable, intermediate and unfavorable prognosis groups as described by Grimwade et al. [10]. In this large multicentric study, cases harboring *t*(15;17)(q22;q21), *t*(8;21)(q22;q22) and *inv*(16)(p13q22)/*t*(16;16)(p13;q22) were the only abnormalities found to predict a relatively favorable prognosis. In contrast, complex karyotype, *abn*(3q) [excluding *t*(3;5)(q25;q34)], *inv*(3)(q21q26)/*t*(3;3)(q21;q26), *add*(5q)/*del*(5q), *-5*, *-7*, *add*(7q)/*del*(7q), *t*(6;11)(q27;q23), *t*(10;11)(p11~13;q23), other *t*(11q23) [excluding *t*(9;11)(p21~22;q23) and *t*(11;19)(q23;p13)], *t*(9;22)(q34;q11), *-17* and *abn*(17p) predicted a significantly poorer outcome. Therefore, these subgroups were called of favorable and unfavorable prognosis, respectively. The remaining patients, including those with normal karyotype and other structural or numerical abnormalities non-mentioned comprised the intermediate risk group.

2.3. Fluorescent in situ hybridization (FISH) for *AURKA* and *AURKB*

Amplifications involving *AURKA* and *AURKB* genes were assessed by fluorescent in situ hybridization (FISH) according to Pinkel et al. [13] on interphase nuclei using commercial probes (*AURKA*: ON AURKA (20q13)/20q11 and *AURKB*: AURKB (17p13)/SE17; Kreatech Diagnostics, Amsterdam, The Netherlands). The probes for

AURKA and *AURKB* are designed as a dual-color assay to detect amplification at 20q13 and 17p13, respectively. Amplification involving the *AURKA* and *AURKB* genes regions will show multiple red signals, while the controls (MPARE1 for *AURKA* and SE17 for *AURKB*), both located in the centromeric region of their chromosomes, will provide 2 green signals. The criteria used for *AURKA* and *AURKB* genes amplifications were based on the number of spots presented during analysis. Two-hundred interphase nuclei were counterstained with DAPI and viewed with an Axio Imager M2 microscope equipped with the *FISHView* software, version 5.5 and appropriate filters (ASI, Carlsbad, CA, USA). Samples with more than two red spots per nuclei were considered to have *AURKA* or *AURKB* amplification.

2.4. RNA extraction and analysis of *AURKA* and *AURKB* gene expression

Total RNA from leukemic samples and healthy donors was 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 analysis of aurora kinase genes, primers and probe developed by Assay on Demand were used (*AURKA*: Hs00269212.m1 and *AURKB*: Hs00177782.m1; Applied BioSystems). The *AURKA* and *AURKB* genes and *GAPDH* mRNA, used as endogenous internal control for each sample, were analyzed in duplicate on the same MicroAmp optical 96-well plates using a 7500 Real-Time PCR System (Applied BioSystems).

Real-time quantitative polymerase chain reaction (RQ-PCR) assays were performed in a final reaction volume of 20 µl. The comparative cycle threshold (Ct) method was used to determine the relative expression level of *AURKA* and *AURKB* genes. On comparative analysis of leukemic samples and normal controls (BMMC and CD34⁺ cells), *AURKA* and *AURKB* gene expression was calculated as a relative quantification to the *GAPDH* housekeeping gene. The gene expression *AURKA* and *AURKB* from leukemic samples was calculated as relative quantification to normal controls ($\Delta\Delta Ct = \Delta Ct_{\text{patient}} - \Delta Ct_{\text{BMMC/CD34+}}$) and expressed as $2^{-\Delta\Delta Ct}$.

2.5. Screening for *FLT3-ITD* mutations

Genomic DNA was extracted using the Purene kit (Gentra System, Minneapolis, MN, USA) according to the manufacturer's protocol. Screening for *FLT3-ITD* was performed by PCR according to the method of Kiyoi et al. [14], followed by electrophoresis on 3% agarose gel stained with ethidium bromide.

2.6. Statistical analysis

Fisher's exact test was employed to compare differences in categorical variables and Student's *t* test was used to compare continuous variables. The Kruskal–Wallis test with Dunn's pos-test was used to compare all pairs of columns between the three prognostic groups according to karyotype results. To evaluate the impact of *AURKA* and *AURKB* expression levels on clinical and laboratory features, logistic regression models for binary outcomes were used. All statistical analyses were performed using the STATA Statistical Software 9.0 (STATA, College Station, TX, USA), with the level of significance set at $p < 0.05$.

3. Results and discussion

The clinical and biological characteristics of the 70 patients included in the present study are listed in Table 1. According to

Table 1

Comparison of clinical and biological variables of 70 untreated patients with acute myeloid leukemia according to prognostic groups based on cytogenetic findings.

Characteristics patients	Prognostic groups based on cytogenetic findings			Total	p-Value
	^b Favorable risk, n = 39	^c Intermediate risk, n = 24	^d Unfavorable risk, n = 7		
Gender					
Male	16	11	5	32	–
Female	23	13	2	38	
Age ^a , years	41 (5–90)	50 (16–88)	39.4 (13–78)	43.9 (5–90)	0.05 ^e
WBC ^a × 10 ³ /mm ³	35.3 (0.8–166)	51.2 (2.3–269)	79.5 (14.8–149)	45.2 (0.8–269)	0.05 ^{e,*}
Platelets ^a × 10 ³ /mm ³	48.5 (7–155)	112.5 (7–736)	45.2 (14–108)	69.8 (7–736)	0.05 ^e
<i>AURKA</i> gene expression ^a	0.85 (0.02–7.49)	1.85 (0.03–11.26)	39.38 (7.17–73.91)	5.04 (0.02–73.91)	0.05 ^{e,*}
<i>AURKB</i> gene expression ^e	3.81 (0.32–37.31)	2.75 (0.07–11.38)	25.03 (0.41–48.92)	5.56 (0.07–48.92)	0.05 ^{e,*}
<i>FLT3-ITD</i> status, n (%)					–
<i>FLT3-ITD</i> ⁺	8 (11.4%)	9 (12.8%)	1 (1.5%)	18 (25.7%)	
<i>FLT3-ITD</i> ⁻	31 (44.3%)	15 (21.4%)	6 (8.6%)	52 (74.3%)	

WBC: white blood cell.

^a Values represent mean (range).

^b Favorable risk group: *t*(15;17)(q22;q12–21), *t*(8;21)(q22;q22), *inv*(16)(p13q22)/*t*(16;16)(p13;q22).

^c Intermediate risk group: normal karyotype, *t*(9;11)(p22;q23), *del*(7q), *del*(9q), *del*(11q), *del*(20q), *-Y*, *+8*, *+11*, *+13*, *+21*.

^d Unfavorable risk group: complex karyotype, *inv*(3)(q21q26)/*t*(3;3)(q21;q26), *t*(6;9)(p23;q34), *t*(6;11)(q27;q23), *t*(11;19)(q23;p13.1), *del*(5q), *-5*, *-7*.

^e Kruskal–Wallis test with Dunn's pos-test.

* Significant at $p < 0.05$.

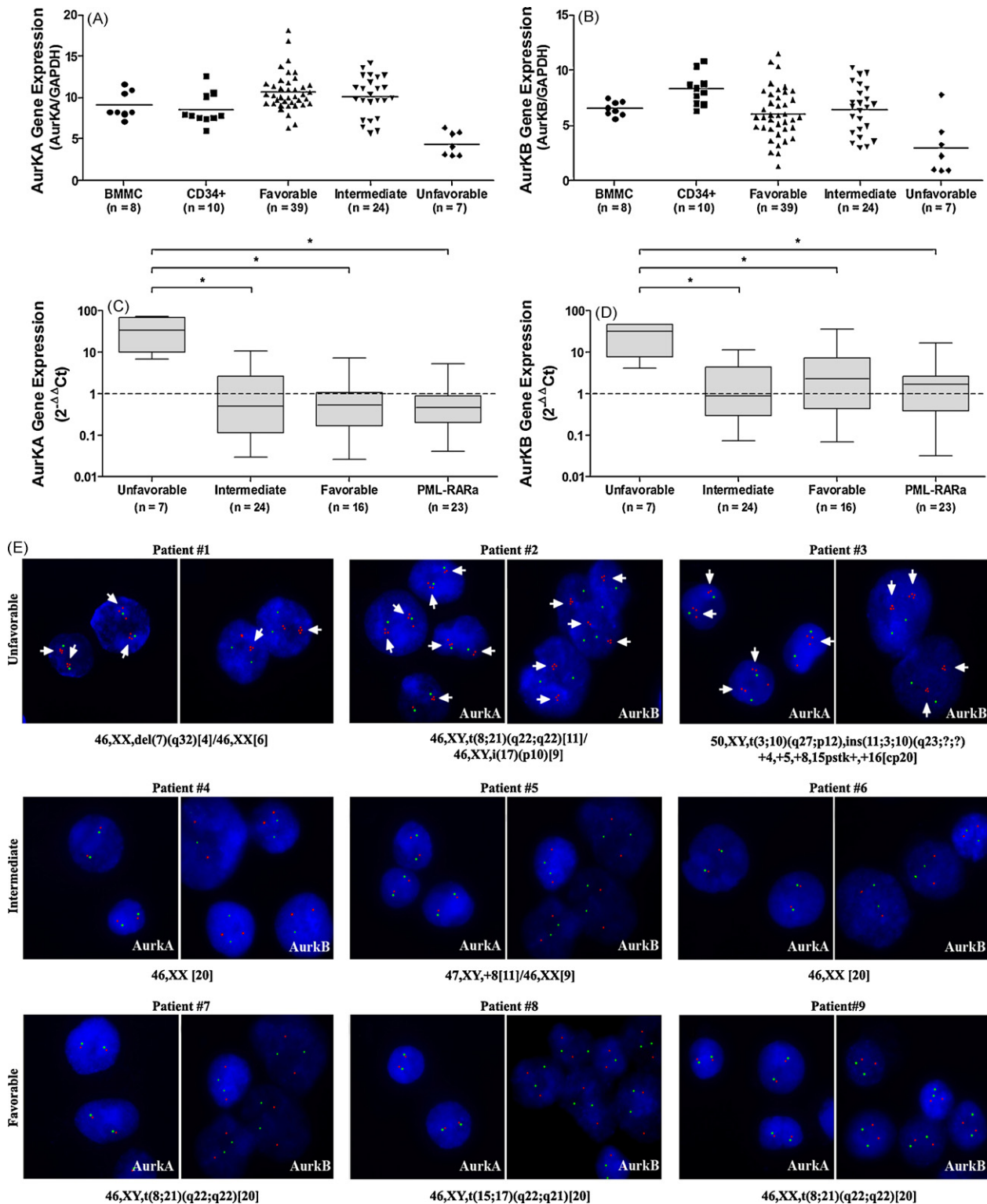


Fig. 1. *AURKA* and *AURKB* expression in AML samples according to cytogenetic findings and FISH analysis of representative samples. *AURKA* (A) and *AURKB* (B) gene expression by quantitative real-time PCR (RQ-PCR). The horizontal bars represent the mean value of gene expression relative to *GAPDH* housekeeping gene for AML patients and for BMMC and CD34⁺ cells from adult healthy donors. *AURKA* (C) and *AURKB* (D) gene expression in AML patients according to cytogenetic risk. The values of gene expression were presented as fold change ($2^{-\Delta\Delta Ct}$) using the relative expression of *AURKA* and *AURKB* genes of BMMC and CD34⁺ cells (grouped into a single group) as reference. Asterisks indicate significant differences ($p < 0.05$) between groups. (E) FISH analyses of *AURKA* and *AURKB* genes from nine distinct patients of unfavorable (patients #1, #2 and #3), intermediate (patients #4, #5 and #6) and favorable (patients #7, #8 and #9) cytogenetic risk groups. The respective karyotypes are displayed below each microphoto. The amplification of *AURKA* and *AURKB* was quantified based on the number of spots presented during analysis. The white arrows indicate the extra red signal showing *AURKA* or *AURKB* amplification.

the karyotype, patients were classified into favorable, intermediate and unfavorable prognosis groups as described elsewhere [10]. Of the 39 patients (55.7%) assigned to the favorable prognosis group, seven harbored $t(8;21)(q22;q22)$, 23 $t(15;17)(q22;q12)$, and nine $inv(16)(p13q22)/t(16;16)(p13;q22)$. Twenty-four patients (34.3%) were assigned to the intermediate prognosis group, 19 of them (27.1%) presenting a normal karyotype, one with isolated trisomy 8, two with isolated trisomy 11, one with trisomy 8 and trisomy 11, and one with the $add(13)(q10)$. The remaining seven patients (10%) were assigned to the unfavorable prognosis group. Of these, one presented a complex karyotype [15], one with $i(17)(p10)$, three with $del(7)(q32)$, one with $t(6;9)(p23;q34)$ and one with $t(6;11)(q27;q23)$.

Initially, we compared *AURKA* and *AURKB* gene expression in *de novo* AML, BMMC and $CD34^+$ samples from healthy donors. There was no difference between the three groups (*AURKA* [mean value of $\Delta Ct \pm SD$]: 9.91 ± 0.35 vs 9.17 ± 0.57 vs 8.6 ± 0.6 , $p = 0.146$; *AURKB*: 5.87 ± 0.29 vs 6.55 ± 0.23 vs 7.31 ± 0.47 , $p = 0.236$, in leukemic, BMMC and $CD34^+$ samples, respectively) (Fig. 1A and B). In contrast, Ye et al. reported increased *AURKA* and *AURKB* mRNA levels in $CD34^+$ bone marrow blasts from patients with hematological malignancies, with no association between *AURKA* and *AURKB* expression levels and clinical and/or laboratory parameters [6]. Similarly, Huang et al. described higher Aurka protein expression in BMMC obtained from 98 *de novo* AML patients compared to normal controls [7].

It should be pointed out that in the present study and others [14], *AURKA* and *AURKB* gene expressions varied widely. Once it was not observed difference of expression between BMMC and $CD34^+$ cells, the relative expression of *AURKA* and *AURKB* genes of these cells were grouped into a single group (from now on called “normal group”), set at 1 and used as reference. By adopting the mean value from ΔCt of normal group, it was possible to identify two subgroups of patients according to *AURKA* and *AURKB* expression: *AURKA*⁺ and *AURKB*⁺ when the gene expression was higher than one, and *AURKA*⁻ and *AURKB*⁻ when gene expression was lower than or equal to one.

Higher white blood cells (WBC) counts were observed in patients of the *AURKA*⁺ and *AURKB*⁺ groups, but no significant differences were found regarding age, sex, platelet counts or frequency of *FLT3-ITD* mutations. In order to determine whether *AURKA* and *AURKB* expression levels, *FLT3-ITD* mutations, age and gender were independent variables, we applied an adjusted logistic regression model for binary outcomes. In the unadjusted analysis and categorizing WBC counts as high when $WBC > 3 \times 10^4/mm^3$ and low when $WBC \leq 3 \times 10^4/mm^3$, *AURKA*⁺ patients had a 3-fold higher risk to present high WBC counts than *AURKA*⁻ patients (OR: 3.15, 95%CI

1.07–9.24, $p = 0.03$). This result did not change after adjustment for *FLT3-ITD* mutation, age and gender (Model II, III and IV, respectively – Table 2). In contrast, the overexpression of *AURKB* was not associated with high WBC counts (OR: 2.42, 95%CI 0.91–6.48, $p = 0.07$).

Interestingly, the majority of cases with unfavorable cytogenetic abnormalities presented overexpression of *AURKA* and *AURKB* (Table 1). Pearson correlation analysis showed that there was a significant association between high *AURKA* expression and unfavorable cytogenetics (relative risk: 4.5 [95% CI: 2.83–7.14], $p < 0.001$), but not between *AURKB* expression and unfavorable cytogenetics (relative risk: 1.54 [95% CI: 1.06–2.24], $p = 0.226$). Moreover, the mean of *AURKA* gene expression in the unfavorable group was 39.38 (95% CI: 11.08–67.67), whereas the intermediate and favorable groups presented means of 1.84 and 0.85, respectively (Intermediate group 95% CI: 0.54–3.14; favorable group 95% CI: 0.39–1.31) ($p < 0.001$) (Fig. 1C). Similar results were detected for *AURKB* gene expression (Fig. 1D) (mean expression of 25.03 [95% CI: 5.11–44.96]; 2.75 [95% CI: 1.26–4.24] and 1.47 [95% CI: 0.86–2.07] for the unfavorable, intermediate and favorable groups, respectively) ($p < 0.001$). No differences in *AURKA* or *AURKB* expression were detected when cases harboring $t(8;21)(q22;q22)$, $inv(16)(p13q22)/t(16;16)(p13;q22)$ and $t(15;17)(q22;q12)$ were compared.

The association between *AURKA* expression and unfavorable cytogenetics findings contrast with the results reported by Wasly et al. [8] demonstrating an association of *AURKA* expression and acute promyelocytic leukemia (APL) samples. However, we should highlight methodological differences in the two studies. In the previous study *AURKA* expression was expressed relative to the *S14* gene and cases were classified as positive or negative for *AURKA* expression according to a cut off value of >0.01 or ≤ 0.01 . In addition, only eleven cases of APL samples were evaluated, whereas in the present study, we included 23 cases. Considering the wide range distribution of *AURKA* expression, this may have influenced the results. Finally, it must be pointed out that, in the present study, a bimodal distribution of *AURKA* expression was detected in AML samples, with a minor group presenting overexpression in comparison to normal samples.

Moreover, we performed FISH analysis using *AURKA* and *AURKB* probes in nine samples (three with unfavorable, three with intermediate and three with favorable cytogenetic findings). Only the three cases with unfavorable cytogenetics presented *AURKA* and *AURKB* gene amplification (Fig. 1E). Only the three cases with unfavorable cytogenetics presented *AURKA* and *AURKB* gene amplification (Fig. 1E). Nevertheless, gene amplification may be one, but not the main mechanism leading to overexpression of aurora

Table 2
Crude and Adjusted Odds ratio of *AURKA* and *AURKB* gene expression according to WBC count.

Variables	Number of cases	WBC > 3 × 10 ⁴ /mm ³	Model I OR [95% CI]	Prognostic factors in acute myeloid leukemia		
				Model II OR [95% CI]	Model III OR [95% CI]	Model IV OR [95% CI]
Aurora kinase						
AurkA ⁻	49 (70%)	19 (38.7%)	1	1	1	1
AurkA ⁺	21 (30%)	13 (61.9%)	3.15 [1.07–9.24]	3.63 [1.19–11.05]	3.64 [1.17–11.26]	3.72 [1.18–11.71]
AurkB ⁻	29 (41.4%)	10 (34.5%)	1	1	1	1
AurkB ⁺	41 (51.6%)	23 (56.1%)	2.42 [0.91–6.48]	2.94 [1.02–8.43]	3.26 [1.1–9.64]	3.27 [1.1–9.66]
FLT3-ITD status						
FLT3-ITD ⁻	52 (74.3%)	23 (44.2%)		1	1	1
FLT3-ITD ⁺	18 (25.7%)	10 (55.5%)		1.57 [0.53–4.63]	1.82 [0.59–5.56]	1.81 [0.59–5.59]
Age						
<60	54 (77.1%)	24 (44.4%)			1	1
≥60	16 (22.9%)	8 (50%)			1.55 [0.6–4.01]	1.54 [0.59–4.01]
Gender						
Male	32 (45.7%)	17 (53.1%)				1
Female	38 (54.3%)	16 (42.1%)				1.15 [0.35–3.72]

kinases. Actually, amplification of *AURKA* was detected in only 3% of hepatocellular carcinoma [16] and 12% of breast cancer samples [17], whereas in more than 60% and 90% of the cases overexpression of *AURKA* was detected, respectively, suggesting that increased gene expression (that is, the Aurora A gene makes more Aurora A protein in cancer cells) is the most frequent mechanism leading to overexpression.

Overexpression and amplification of the Aurora kinase genes, particularly aurora kinase A, have been documented for a wide range of solid tumors, with some studies showing correlations with disease status, survival and cancer risk [18]. In human breast cancer, overexpression of these kinases induced aneuploidy, centrosome amplification and tumorigenic transformation. Altered expression of these genes was also reported to correlate with the invasiveness and chromosomal instability of the disease [19]. In agreement, our results suggest that overexpression of *AURKA* is associated with genomic instability and markers of poor prognosis in *de novo* AML.

Considering the development of several small-molecule inhibitors with varying activity against Aurora A and/or B [20], it is relevant to identify subgroups of patients as candidates for clinical trials. Huang *et al* reported that overexpression of *Aurka* in AML cells was associated with high *in vitro* sensitivity to VX-680 (small-molecule inhibitors of Aurora kinases) [7]. Recently, this molecule was shown to be effective against multiple myeloma and chronic myeloid leukemia harboring imatinib-resistant T351I and dasatinib-resistant V299L Bcr-Abl mutations [21,22]. The present report shows that *AURKA* and *AURKB* are overexpressed in approximately 10% of *de novo* AML patients with a significant association with unfavorable cytogenetic findings. The fact that a high value for the relative risk between high *AURKA* expression and unfavorable cytogenetics was obtained [RR: 4.5 (95% CI: 2.83–7.14)] suggest that despite the size of the population analyzed, this strong association should be confirmed by larger studies. Indeed, the percentage of AML cases overexpressing *AURKA* was higher in previous studies [6,7] mainly due to differences in methods and criteria adopted for the definition of overexpression. Therefore, larger multicentric studies, using standardized methodology, are required to establish which subgroup of patients may benefit from new therapeutic strategies using Aurora kinase inhibitors.

Conflict of interest

All authors have no conflict of interest to report.

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Authors' contributions: A.R.L.A. provided the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for important intellectual content, and final approval of the version to be submitted; F.M.O. performed cytogenetics analyses and drafting of manuscript; S.D.L.C. performed cytogenetics analyses; G.A.S. analyzed data, drafting of manuscript and was responsible for the article criti-

cally for important intellectual content; R.P.F. contributed to the final review of the manuscript; and E.M.R. provided the revised the article critically for important intellectual content and gave final approval of the version to be submitted.

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