HEMATOLOGY, TRANSFUSION AND CELL THERAPY

Letter to the Editor

ARHGAP6 transcript levels are associated with molecular risk and impact survival outcomes in acute myeloid leukemia

Dear Editor,

Acute myeloid leukemia (AML) comprises a molecularly heterogeneous group of diseases characterized by the proliferation and accumulation of myeloid blasts in the bone marrow, peripheral blood, and other organs, with suppression of normal hematopoiesis.[1] Some recurrent genetic alterations play a central role in the pathogenesis, diagnosis, and prognosis of the disease.[2] However, the identification of new prognostic markers may improve risk stratification and therapeutic decision in AML. Recently, our research group identified four genes associated with cytoskeletal regulation that impact the prognosis of patients with AML.[3] In the present study, we sought to understand ARHGAP6 expression in association with clinical—biological features of AML.

Taking advantage of public available resources, we performed multiple transcriptomic analyzes to verify the potential contributions of ARHGAP6 to AML. ARHGAP6 mRNA expression data (probe 206167_s_at) from the different hematopoietic cell populations were obtained from the GSE24759 data set using the GEO2R platform (https://www.ncbi.nlm.nih.gov/geo/geo2r). ARHGAP6 transcript levels data (probe 206167_s_at) from samples obtained from healthy donors (CD34+ samples [n = 8] and total bone marrow cells [n = 5]) and AML patients (n = 577) were derived from AmaZonia! database 2008 (http://amazonia.transcriptome.eu).[4] Gene expression, genetic and clinical data from The Cancer Genome Atlas (TCGA) AML study[5] (n = 173, Table 1) were obtained from cBioPortal (https://www.cbioportal.org/).[6] Gene expression data were used as continuous or categorical variables. Dichotomization was realized according to the ROC curve and its respective area under the curve and the C-index using a maximization metric provided by the R package

Table 1 – Acute myeloid leukemia (AML) patients' characteristics from TCGA AML[1] study and their association with ARHGAP6 gene expression[2]

Factors	Total	ARHGAP6 expression		p-value
		Low	High	
AML patients	**173**	**71**	**102**	
Gender Male/Female	92/81	36/35	56/46	0.64
Age (years), median (range):	58 (18 - 88)	54 (18 — 77)	61 (21 — 88)	**0.001**
Bone marrow blasts (%), median (range)	72 (30 -100)	72 (35 — 100)	72 (30 — 100)	0.71
White blood cell count $\times 10^9$/L	17 (0.4 - 297.4)	33.2 (0.4 - 223.8)	8.6 (0.5 — 297.4)	**0.0006**
Molecular risk[3]				
Favorable	33	23	10	**0.001**
Intermediate	95	33	62	
Adverse	42	14	28	

Abbreviations: TCGA, The Cancer Genome Atlas; AML, acute myeloid leukemia.

[1]The clinical and laboratorial data of TCGA AML cohort were obtained from cBioPortal for Cancer Genomics (http://www.cbioportal.org).

[2]Gene expression values were dichotomized by ROC curve and C statistic.

[3]Molecular risk was stratified according TCGA study; 3 AML patients were not classified.

[4]For statistical analyzes, Mann—Whitney test was used for measured factors, and Fisher's exact test or Chi-squared test was used for categorical factors.
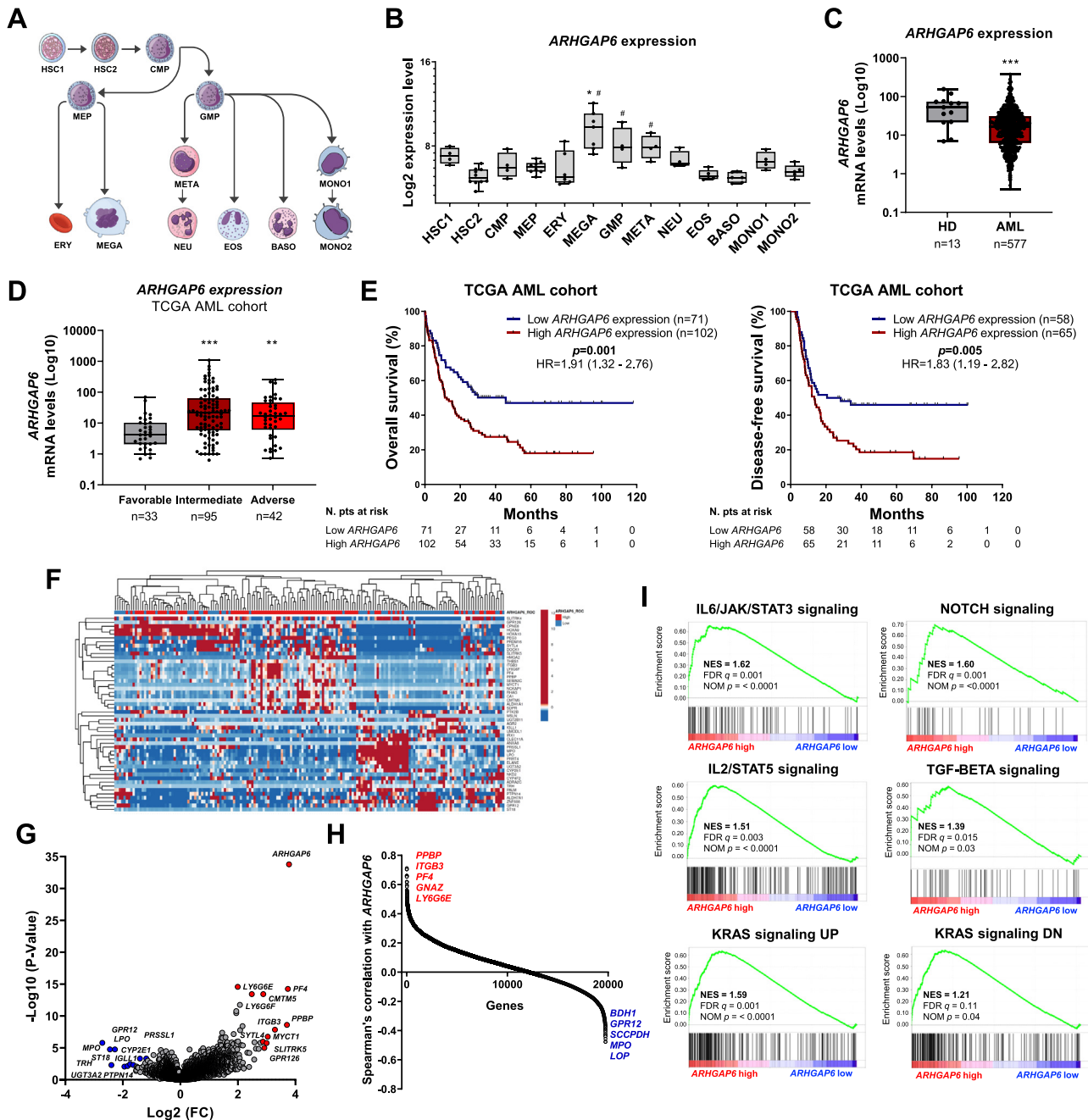
Figure 1 – *ARHGAP6* transcript levels are associated with molecular risk and impact survival outcomes and biological characteristics in acute myeloid leukemia (AML) patients. (A) **Schematic representation of the myeloid differentiation hierarchy illustrating the analyzed cell subpopulations used in the analysis (https://mindthegraph.com/). Abbreviations: HSC, hematopoietic stem-cells; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor; MEP, Megakaryocyte/erythrocyte progenitor; ERY, erythrocytes; MEGA, megakaryocytes; META, metamyelocytes; NEU, neutrophils; EOS, eosinophils; BASO, basophils; MONO, monocytes. (B) Gene expression profile of ARHGAP6 (probe 206167_s_at) in myeloid cell subpopulations (GSE24759). The p-values and cell lineages are indicated in the graphs: * $p < 0.05$ cell lineage vs. HSC1, # $p < 0.01$ cell lineage vs. HSC2; ANOVA and Bonferroni post hoc test. (C) ARHGAP6 (probe 206167_s_at) mRNA levels were compared between samples from normal hematopoietic cells (normal bone marrow n = 5, CD34+ cells n = 8) and AML patients (n = 577). The "y" axis represents mRNA expression levels at arbitrary values. The data sets were cross-referenced using tumor-specific identification numbers. *** $p < 0.001$; Mann−Whitney U test. (D) ARHGAP6 mRNA levels were compared among AML patients from TCGA cohort stratified by molecular risk. Numbers of subjects for each group are indicated. ** $p < 0.01$, *** $p < 0.0001$; Kruskal−Wallis test and Dunn post-hoc test. (E) Kaplan-Meier curves represent overall survival for AML patients dichotomized according to high or low ARHGAP6 expression (using the ROC curve as the cut-off point). Hazard ratio (HR), 95% confidence interval, and**

Cutpointr.[7] For internal data validation, the bootstrap resampling tool was used.[8,9]

For functional genomic analysis, all transcripts were pre-ranked according to their differential expression by comparing samples with high and low *ARHGAP6* expression using the limma-voom package at Galaxy (https://usegalaxy.org/). Gene Set Enrichment Analysis (GSEA) was performed with GSEA v.4.0,[10] as previously described.[3] A false discovery rate of 25% (FDR q-value < 0.25) and a p - value < 0.05 were considered statistically significant. A heatmap was constructed using ClusterVis (https://biit.cs.ut.ee/clustvis/) to represent the top 50 differentially expressed genes between high and low *ARHGAP6* expression samples. Volcano plot correlating the adjusted -log10 p-value and log2-fold-change and Spearman correlation plot were constructed in GraphPad Prism 8.0 (GraphPad Software, Inc., CA, USA).

Statistical analyses were performed using GraphPad Prism 8, Stata Statistic/Data Analysis 14.1 (Stata Corporation, USA), and SPSS Statistics for Windows, version 21.0 (SPSS, Chicago, IL, USA). Overall survival (OS) was calculated as the difference in months between diagnosis and the last follow-up or death, and disease-free survival (DFS) between the date of complete remission and relapse or the date of the last follow-up or death (which occurred first). Survival measures were estimated using Kaplan-Meier curves and compared using the Log-rank test and/or Cox regression. ANOVA and Bonferroni post-test, Kruskal Wallis and Dunn's test, or Mann-Whitney test were used for measurable factors, as appropriate. A value of $p < 0.05$ was considered statistically significant.

Firstly, since there are no descriptions of *ARHGAP6* in processes associated with normal or malignant hematopoiesis, we characterized the expression of this gene in different sub-populations of myeloid cells (Figure 1A). Megakaryocytes and granulocytic progenitors had the highest levels of *ARHGAP6* when compared to hematopoietic stem cells ($p < 0.05$, Figure 1B). Next, *ARHGAP6* expression was compared between samples derived from healthy donors and patients with AML and a significant reduction in *ARHGAP6* levels was observed in the group of patients with AML ($p \leq 0.05$, Figure 1C). Among AML patients, *ARHGAP6* expression was associated with age, white blood cell count, and molecular risk ($p < 0.05$, Table 1). Corroborating these findings, AML patients with intermediate and adverse molecular risk had higher transcriptional levels of *ARHGAP6* compared with favorable risk group ($p < 0.01$, Figure 1D). Of note, high *ARHGAP6* expression was associated with poor survival outcomes (p < 0.05, Figure 1E). The bootstrap resampling procedure revealed bias-independent accuracy of the *ARHGAP6* prediction as well as the absence of overfitting in the cohort for overall survival analysis (Supplementary Table 1). In the multivariate analysis, *ARHGAP6* transcript levels were an independent prognostic factor for overall and disease-free survival in the TCGA AML cohort ($p < 0.03$, Table 2).

Finally, the stratification of AML patients into high versus low *ARHGAP6* expression identified distinct gene signatures (Figure 1F-H) associated with molecular and biological processes relevant to leukemogenesis (Supplementary Table 2). Of these, we highlight that the high *ARHGAP6* expression was positively correlated with IL6/JAK/STAT3 signaling, NOTCH signaling, IL2/STAT5 signaling, TGF-BETA signaling, and KRAS signaling (all FDR < 0.25 and $p < 0.05$, Figure 1I).

The *ARHGAP6* gene was cloned and characterized for the first time in a screening that sought to identify genes in the critical region in Xp22.3 associated with microphthalmia with linear skin defects syndrome is an X-linked dominant, a male-lethal disorder.[11] Later, its functional characterization identified its action as GTPase-activating for RhoA and in the regulation of actin polymerization.[12] In the field of oncology, the number of studies on *ARHGAP6* is still very limited. In solid tumors (*i.e.*, cervical carcinoma, lung cancer, breast cancer, and bladder cancer), *ARHGAP6* acts as a tumor suppressor, inhibiting cell cycle progression, proliferation, survival, migration, invasion, and *in vivo* tumorigenesis.[13−16] On the molecular level, *ARHGAP6* function has been associated with the modulation of RhoA, MMP9, VEGF, STAT3, and β-catenin.[14−16] These findings corroborate the lower expression of *ARHGAP6* observed in AML patients. However, once the disease is established, *ARHGAP6* may act as an oncogene, suggesting a paradoxical role for this gene in the development and progression of leukemia. The functional genomic analysis supports this hypothesis as high *ARHGAP6* expression is positively associated with gene signatures of signaling pathways relevant to the proliferation and survival of leukemic blasts.

In summary, our results indicate that high expression of *ARHGAP6* is associated with unfavorable molecular risk, poor survival outcomes, and molecular processes associated with leukemogenesis. Taken together, these findings suggest that *ARHGAP6* may be involved with the pathogenesis and progression of AML and deserve future functional studies in experimental models.

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p values are indicated (log-rank test). (F) Heatmap constructed using ClusterVis that summarizes the expression of the top 25 upregulated and 25 downregulated genes for high versus low ARHGAP6 expression. Color intensity represents the ᴢ-score within each row. (G) Volcano plots depicting the extent (x-axis) and significance (y-axis) of differential gene expression for each gene, comparing high versus low ARHGAP6. (H) Spearman correlation showing the genes positively and negatively correlating with the ARHGAP6 in AML TCGA patients. (I) Gene Set Enrichment Analysis (GSEA) plots for biological processes associated with ARHGAP6 expression in AML patients. The top portion of the plot shows the running enrichment scores (ES) for the gene set. The point with the maximum deviation from zero is defined as the ES for the gene set. The leading-edge subset (the subset of genes with the most significant contribution to the ES) is shown as a vertical bar accumulating before the peak score for a positive ES or after the peak score for a negative ES. FDR-adjusted p values (NOM p-value) and enrichment scores normalized for gene set size (NES) are indicated.

Table 2 – Univariate and multivariate analysis for overall survival and disease-free survival of acute myeloid leukemia patients from TCGA cohort according to clinical and laboratorial characteristics and ARHGAP6 expression.

Factors	Overall Survival						Disease-free survival[3]					
	Univariate			Multivariate			Univariate			Multivariate		
	HR[1]	(95% C.I.)	p[3]	HR[1]	(95% C.I.)	p[3]	HR[1]	(95% C.I.)	p[3]	HR[1]	(95% C.I.)	p[3]
Gender Male vs female	0.96	0.6 – 1.39	0.84	0.84	0.57 – 1.22	0.37	0.91	0.59 – 1.41	0.700	0.75	0.48 – 1.17	0.20
Diagnosis age	1.04	1.02 – 1.05	**<0.0001**	1.03	1.02 – 1.05	**<0.0001**	1.00	0.99 – 1.02	0.201	1.00	0.99 – 1.02	0.32
White blood cell count[2]	1.004	1.0008 – 1.008	**0.01**	1.00	1.02 – 1.01	**<0.0001**	1.00	1.00 – 1.01	**0.002**	1.01	1.00 – 1.01	**<0.0001**
Molecular risk[3] Adverse vs intermediate vs. favorable	2.01	1.51 – 2.68	**<0.0001**	1.94	1.40 – 2.68	**<0.0001**	1.61	1.16 – 2.22	**0.004**	1.54	1.07 – 2.22	**0.01**
ARHGAP6 expression[4] High vs. low expression	1.92	1.29 – 2.86	**0.001**	1.86	1.20 – 2.89	**0.005**	1.61	1.02 – 2.53	**0.03**	1.96	1.16 – 3.30	**0.01**

Abbreviations: TCGA, The Cancer Genome Atlas.

Significant statistical differences are highlighted in bold.

[1] Hazard ratios (HR)> 1 indicates that the increase in values for continuous variable or the first factor for categorical variable has a worse outcome.

[2] Fatters were analyzed as continuous variables.

[3] Absent values were excluded in the calculation of the p values.

[4] The factors were dichotomized by ROC curve and C statistic.

Conflicts of interest

The authors declare no competing financial interests.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2023.06.004.

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Jean Carlos Lipreri da Silva [a], Juan Luiz Coelho-Silva [b], Hugo Passos Vicari [a], Keli Lima [c], Eduardo Magalhães Rego [c], Fabiola Traina [b], João Agostinho Machado-Neto [a,*]

[a] *Institute of Biomedical Sciences, University de São Paulo (USP), São Paulo, SP, Brazil*

[b] *Hematology and Clinical Oncology, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (FMUSP), Ribeirão Preto, SP, Brazil*

[c] *Laboratory of Medical Investigation in Pathogenesis and Targeted Therapy in Onco-Immuno-Hematology Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, (LIM-31 - HCFMUSP) Sao Paulo, SP, Brazil*

*Corresponding author at: Department of Pharmacology, Institute of Biomedical Sciences of University of São Paulo, Av. Prof. Lineu Prestes, 1524, CEP 05508-900, São Paulo, SP, Brazil.

E-mail address: jamachadoneto@usp.br (J.A. Machado-Neto).