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IL32 expression in peripheral blood CD3$^+$ cells from myelodysplastic syndromes patients

Matheus Rodrigues Lopes[1,2], João Kleber Novais Pereira[1], Fabiola Traina[1,3], Paula de Melo Campos[1], João Agostinho Machado-Neto[1,3], Sara Teresinha Olalla Saad[1] and Patricia Favaro[1,4*]

Abstract

Background: Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders characterized by ineffective hematopoiesis and risk of leukemia transformation. There is evidence to suggest the participation of immune system deregulation in MDS pathogenesis. Interleukin-32 (IL-32) is a newly described multifunctional cytokine reported as an important mediator in autoimmune and inflammatory disorders. In the present study, we reported the expression of *IL32* and *IL32* transcript variants (*α, β, γ* and *δ*) in peripheral blood CD3$^+$ cells from healthy controls and MDS patients.

Methods: CD3$^+$ cells were isolated by immunomagnetic cell sorting from thirty-nine untreated MDS patients and twenty-nine healthy donors. Gene expression was evaluated by quantitative PCR. For statistical analysis, Mann–Whitney test, Kruskal-Wallis test with Dunns post test and Log-rank (Mantel-Cox) were used, as appropriate. A p value <0.05 was considered statistically significant.

Results: *IL32* expression and *IL32* transcript variants *IL32α*, *IL32β*, *IL32γ*, and *IL32δ*, were similar in peripheral blood CD3$^+$ cells from healthy donors and MDS patients. Increased IL-32α expression was an independent predictor for MDS disease progression by univariate and multivariate analysis.

Conclusions: We observed that *IL32* expression is not differently expressed in CD3$^+$ cells from MDS patients; nevertheless *IL32α* has a potential role in disease progression.

Keywords: IL-32, Myelodysplastic syndromes, Disease progression, CD3$^+$ cells, Immunology

Background

Myelodysplastic syndromes (MDS) are a heterogeneous group of neoplasms characterized by dysplastic, ineffective blood cell production and risk of transformation to acute leukemia [1]. Clinical and immunological evidence suggest an association between immune system dysfunction and the pathogenesis of MDS [2, 3]. Autoimmune features are described in the early stage (low-risk) of disease, such as activated cytotoxic T lymphocytes [4], lower number of regulatory T cells (Tregs), increased Th17, B-cell dysfunction, elevated levels of TNF-α, IFN-γ and pro-apoptotic cytokines [5, 6]. On the other hand, in advanced stage (high-risk) disease, evasion of the immune system, reduction of function NK cells, increased Tregs, and low levels of apoptosis are found [7, 8], and an immune deregulation could participate in the progression of MDS [9].

IL-32, a newly described multifunctional cytokine produced mainly by T, NK and epithelial cells, has been reported as an important mediator in several autoimmune and inflammatory disorders [10, 11]. There are more than nine isoforms of IL-32 described in the GenBank Database [12]. The distinct effects of IL-32 have been reported for several isoforms. IL-32α has been reported as the most abundant transcript and the IL-32γ isoform as the longest transcript with most prominent

* Correspondence: favaropb@gmail.com; patricia.favaro@unifesp.br
[1]Hematology and Blood Transfusion Center, University of Campinas, Hemocentro-Unicamp, Instituto Nacional de Ciência e Tecnologia do Sangue, Campinas, Brazil
[4]Present Address: Department of Biological Sciences, Federal University of São Paulo, Rua São Nicolau, 210, CEP 09913-030 Diadema, SP, Brazil
Full list of author information is available at the end of the article

Table 1 Patients' characteristics

Patients	Number
MDS	39
Gender	
Male/Female	18/21
Age (years), median (range):	69 (27–89)
WHO classification	
RA/RARS/RCMD	04/07/20
RAEB-1/RAEB-2	07/01
Cytogenetic risk[a]	
Very good/good	01/32
Intermediate	02
Poor/very poor	01/01
No growth	02

Abbreviations: *MDS* myelodysplastic syndromes, *WHO* World Health Organization, *RA* refractory anemia, *RARS* refractory anemia with ringed sideroblasts, *RCMD* refractory cytopenia with multilineage dysplasia, *RAEB-1* refractory anemia with excess blast-1, *RAEB-2* refractory anemia with excess blast-2, *BM* bone marrow. [a]In MDS cohort, karyotype findings included very good : –Y ($n = 1$); good: normal ($n = 32$), intermediate: +8 ($n = 1$); other ($n = 1$); poor: 3 abnormalities ($n = 1$), and very poor: >3 abnormalities ($n = 1$)

biological activity [13]. Apart from the proinflammatory role of IL-32, the association of IL-32α with PKCε and STAT3 [14] or with FAK1 has been reported [15], suggesting a possible role of this cytokine as an intracellular signaling mediator. Furthermore, the role of IL-32 in the production of regulatory cytokines has been described: IL-32β interacts with PKCδ and C/EBPα, which results in the production of IL-10 [16], and IL-32γ is correlated with enhanced production of proinflammatory cytokines, such as IL-1β and IL-6 [17]. IL-32γ is also implicated in HIV immunosuppression [18] and tumoral growth inhibition [19]. The interaction between IL-32δ and IL-32β isoforms inhibits IL-10 production by IL-32β [20], which suggests that other interactions between IL-32 isoforms may explain their multifunctional role [21].

The expression of *IL32* isoforms has not yet been described in MDS. Herein, we report *IL32* and *L32* transcript variant (α, β, γ and δ) mRNA levels in peripheral blood $CD3^+$ cells from healthy controls and MDS patients.

A

Gene	Sequence	Concentration
IL32	FW: 5' GAACTTTTGGCCGCCATGT 3' RV: 5' GGGCCTTCAGCTTCTTCATGT 3'	150 nM
IL32α	FW:5' GACAGTGGCGGCTTATTATGAG 3' RV: 5' GCTCCGTAGGACTTGTCACAAA 3'	150 nM
IL32β	FW:5' CTGTCTCTCTCGGCTGAGTATTTGT 3' RV: 5' TCTTCATGTCATCAGAGAGGACCTT 3'	300 nM
IL32γ	FW:5' GTAATGCTCCTCCCTACTTC 3' RV: 5' GCAAAGGTGGTGTCAGTATC 3'	300 nM
IL32δ	FW:5' GACGTGGACAGGACGACTTCA 3' RV: 5' CCTCGGCACCGTAATCCAT 3'	150 nM
HPRT1	FW: 5' GAACGTCTTGCTCGAGATGTGA 3' RV: 5' TCCAGCAGGTCAGCAAAGAAT 3'	150 nM

B

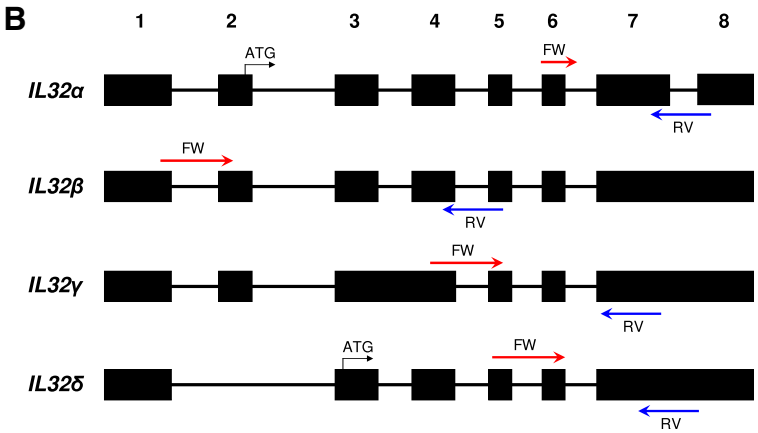

Fig. 1 **a** Sequences and concentrations of primers used in quantitative PCR experiments. **b** Schematic representation of the design of primers for the different transcript variants of *IL32* (α, β, γ and δ). The *red* arrows indicate the forward primer and the *blue* arrows indicate the reverse primer

Methods

Patients and donors

Peripheral blood samples, collected from thirty-nine newly diagnosed MDS patients (18 males, 21 females) with an age range of 27–89 years (median age = 69) and 29 unrelated, random, and healthy individuals (median age = 39, range, 28–60) were analyzed. Patients that regularly attended the clinic with a confirmed diagnosis of MDS and that were untreated at the time of the study were included. Patients' characteristics are described in Table 1. All healthy controls and patients provided informed written consent and the study was approved by the ethics committee of the University of Campinas (reference number 124/2005).

Peripheral blood $CD3^+$ T isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation (Sigma, St Louis, MO, USA). $CD3^+$ T cells from PBMC were sorted using anti-CD3 monoclonal antibody and MACS® Magnetic Cell sorting technique (Miltenyi Biotec, Bergisch Gladbach, Germany).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from sorted $CD3^+$ cells using Illustra RNAspin Mini Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and cDNA was generated using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany), according

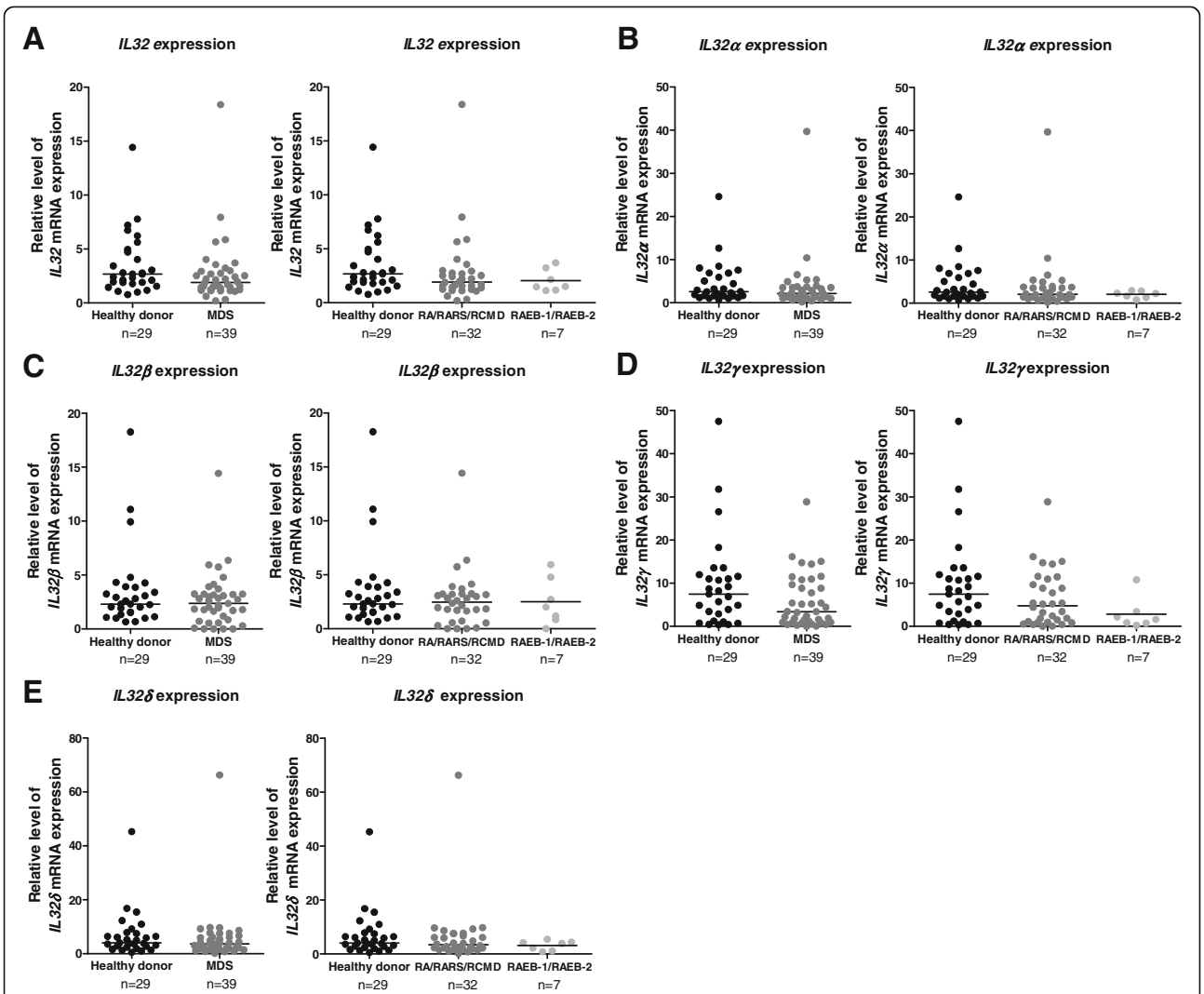

Fig. 2 *IL32* expression and *IL32* transcript variants in myelodysplastic syndromes. qPCR analysis of total *IL32* **a**, *IL32α* **b**, *IL32β* **c**, *IL32γ* **d** and *IL32δ* **e** mRNA levels in $CD3^+$ cells from healthy donors and from patients with the diagnosis of myelodysplastic syndromes (MDS). MDS patients were stratified using the WHO 2008 classification intro refractory anemia (RA)/refractory anemia with ringed sideroblasts (RARS)/refractory cytopenia with multilineage dysplasia (RCMD) groups and refractory anemia with excess blast-1 (RAEB-1)/refractory anemia with excess blast-2 (RAEB-2) groups. The "y" axis represents the relative gene mRNA expression. The numbers of subjects studied are indicated in the graph

manufactures instructions. Quantitative PCR (qPCR) was performed with SYBR Green Master Mix PCR (MBI Fermentas) in an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with specific primers for *IL32* and transcript variants (α, β, γ and δ) and *HPRT1*. Primers sequences and concentrations are described in Fig. 1. The relative gene expression was calculated using the equation, $2^{-\Delta\Delta CT}$ [22]. A negative 'No Template Control' was included for each primer pair. The dissociation protocol was performed at the end of each run to check for non-specific amplification. Three replicas were run on the same plate for each sample.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San. Diego, CA, USA) or SAS System for windows 9.2 (SAS Institute, Inc., Cary, NC, USA). For comparisons, Mann-Whitney test was used for comparison between two groups and Kruskal Wallis test with Dunns post test was used for comparison between three groups. Univariate and multivariate Cox regression were used to estimate overall survival (OS) and event free survival (EFS). OS was defined as the time (in months) between the date of sampling and the date of death (for deceased patients) or last follow-up (for censored patients). EFS was defined as the time (in months) between the sampling and the date of the first event (death or MDS progression to a higher risk MDS category by WHO or to acute myeloid leukemia (AML) with myelodysplasia-related changes) or last follow-up (for censored patients). A p value <0.05 was considered statistically significant.

Results

Expression of *IL32* in peripheral blood $CD3^+$ T cells of patients with MDS

The expression of *IL32* and *IL32* transcript variants (α, β, γ and δ) were analyzed in peripheral $CD3^+$ cells from healthy donors and patients with MDS. There were no differences in *IL32* and IL-32 transcript variants expression between control group and MDS patients (*IL32*: median 2.68 *versus* [*vs.*] 1.89; *IL32α*: 2.57 *vs.* 2.17; *IL32β*: 2.30 *vs.* 2.36; *IL32γ*: 7.46 *vs.* 3.39; *IL32δ*: 4.06 *vs.* 3.66; respectively, all $p > 0.05$). Similar findings were observed when MDS patients were stratified by WHO 2008 classification intro refractory anemia (RA)/refractory anemia with ringed sideroblasts (RARS)/refractory cytopenia with multilineage dysplasia (RCMD) group *vs.* refractory anemia with excess blast-1 (RAEB-1)/refractory anemia with excess blast-2 (RAEB-2) group (*IL32*: median 1.92 *vs.* 1.51; *IL32α*: 2.07 *vs.* 2.25; *IL32β*: 2.48 *vs.* 2.01; *IL32γ*: 4.77 *vs.* 1.60; *IL32δ*: 3.39 *vs.* 3.92; respectively, all $p > 0.05$) or each group *vs.* healthy donors (all $p > 0.05$, Fig. 2).

IL32α expression impact upon MDS progression

Despite not having observed a difference in *IL32* expression between MDS patients and healthy donors, we investigated whether *IL32* expression could impact the clinical outcome of patients with MDS. Using Cox regression analysis, we observed that high *IL32α* expression negatively impacted EFS by univariate analysis, along with male gender and increased age (all $p < 0.05$). Furthermore, multivariate analyses indicated that high *IL32α* expression, along with age,

Table 2 Univariate and Multivariate analyses of survival outcomes for MDS patients

Factor	Univariate analysis						Multivariate analysis					
	Event free survival			Overall survival			Event free survival			Overall survival		
	HR[a]	(95% CI)	*p*	HR[a]	(95% CI)	*p*	HR[a]	(95% CI)	*p*	HR[a]	(95% CI)	*p*
WHO 2008 classification												
RAEB-1/RAEB-2 *vs.* RA/RARS/RCMD	2.73	0.92–8.04	0.07	3.93	1.35–11.47	**0.01**	8.24	2.20–30.84	**0.003**	5.58	1.75–17.77	**0.003**
Gender												
Male *vs.* female	4.74	1.52–14.76	**0.007**	3.33	1.15–9.64	**0.03**	9.43	2.57–34.48	**0.02**	4.17	1.35–12.82	**0.01**
Age	1.05	1.01–1.09	**0.02**	1.05	1.01–1.09	**0.02**	-	-	-	1.06	1.01–1.11	**0.03**
IL32 expression	1.14	0.98–1.33	0.09	1.08	0.96–1.22	0.23	0.49	0.25–0.94	**0.006**	-	-	-
IL32α expression	1.08	1.01–1.15	**0.02**	1.04	0.98–1.09	0.18	3.25	1.69–6.25	**0.02**	-	-	-
IL32β expression	1.04	0.81–1.32	0.77	1.03	0.85–1.24	0.80	-	-	-	-	-	-
IL32γ expression	1.04	0.96–1.14	0.32	1.03	0.96–1.11	0.42	-	-	-	-	-	-
IL32δ expression	1.04	0.99–1.08	0.06	1.02	0.98–1.05	0.34	0.63	0.47–0.85	**0.02**	-	-	-

Abbreviations: *MDS* myelodysplastic syndromes, *WHO* World Health Organization, *HR* hazard ratio, *CI* confidence interval, *RA* refractory anemia, *RARS* refractory anemia with ringed sideroblasts, *RCMD* refractory cytopenia with multilineage dysplasia, *RAEB-1* refractory anemia with excess blast-1; RAEB-2, refractory anemia with excess blast-2

Statistically significant *p*-values are highlighted in bold

[a]Hazard ratios >1 indicate that the first factor has the poorer outcome. For continuous variables, increase or decrease in the risk is proportional percentage for each one unit increase in the variable

male gender and RAEB-1/RAEB-2 WHO 2008 classification was independently prognostic for worse EFS (all $p < 0.05$). Of note, increased *IL32* and *IL32δ* expression had a positive impact on EFS. As expected, the usual prognostic factors, including WHO 2008 classification, male gender and increased age remained independent predictors for OS (all $p < 0.05$) (Table 2).

Discussion

We herein observed that the expression profile of *IL32* and *IL32* transcript variants *IL32α*, *IL32β*, *IL32γ,* and *IL32δ*, was similar in peripheral blood $CD3^+$ cells from healthy donors compared to those from MDS patients. Notably, we described in our cohort that increased *IL32α* expression was an independent predictor for MDS disease progression. IL-32 overexpression has been described in head and neck squamous cell carcinoma and has been related to shorter survival, possibly due to the potential role of IL-32 in the metastatic process [23]. IL-32 has also been proposed as a lung adenocarcinoma prognostic biomarker, in which high IL-32 expression was associated with high grade disease and metastasis incidence [24, 25]. In clear cell renal cell carcinoma patients, IL-32 overexpression was associated with disease progression and poor overall survival, indicating that IL-32 may be a novel prognostic factor for predicting outcomes in this disease [26]. Similarly, IL-32 has been reported as a poor prognostic marker for gastric cancer [27, 28]. Using the lung cancer A549 cell line, IL-32 inhibition reduced cell viability, migration and invasion, whereas IL-32 overexpression increased migration and invasion, indicating that this protein participates in the malignant phenotype of lung cancer cells [24].

In contrast, we also observed that total *IL32* and *IL32δ* expression presented an opposite impact on EFS, highlighting the functional differences between IL-32 isoforms. Bak et al. [29] reported that IL-32θ attenuated the invasive and migratory potential by suppressing the epithelial-mesenchymal transition in colon cancer HT29 cell line, and provided evidence that IL-32θ apparently inhibits the progression and recurrence of colon cancer.

Conclusion

In conclusion, we observed that *IL32* expression is not differently expressed in $CD3^+$ cells from MDS patients; nevertheless *IL32α* has a potential role in disease progression. Future studies are necessary to verify the specific functions of IL-32 and to better characterize the different IL-32 isoforms in immune system deregulation during MDS development and progression.

Abbreviations

AML: Acute myeloid leukemia; EFS: Event free survival; IL-32: Interleukin-32; MDS: Myelodysplastic syndrome; OS: Overall survival; PBMC: Peripheral blood mononuclear cells; q-PCR: Quantitative polymerase chain reaction; RA: Refractory anemia; RAEB-1: Refractory anemia with excess blast-1; RAEB-2: Refractory anemia with excess blast-2; RARS: Refractory anemia with ringed sideroblasts; RCMD: Refractory cytopenia with multilineage dysplasia; Tregs: Regulatory T cells; WHO: World Health Organization

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

MRL performed all the experiments, statistical analyses, patient database, manuscript preparation, completion and final approval. JKPN participated in the sample preparation, quantitative PCR experiments, manuscript editing and final approval. PMC and FT participated in the interpretation of manuscript data, clinical data collection, revised the diagnoses, manuscript editing, and final approval. JAMN participated in statistical analyses, manuscript preparation, completion and final approval. STOS participated in patient follow up, manuscript editing and final approval. PF participated in the overall design of the study and experiments, statistical analyses, patient follow up, manuscript preparation, editing, completion and final approval.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All healthy controls and patients provided informed written consent and the study was approved by the ethics committee of the University of Campinas (reference number 124/2005).

Publisher's Note

Author details

[1]Hematology and Blood Transfusion Center, University of Campinas, Hemocentro-Unicamp, Instituto Nacional de Ciência e Tecnologia do Sangue, Campinas, Brazil. [2]Present Address: Federal University of Vale São Francisco (UNIVASF), Campus Paulo Afonso, Bahia, Brazil. [3]Present Address: Department of Internal Medicine, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, São Paulo, Brazil. [4]Present Address: Department of Biological Sciences, Federal University of São Paulo, Rua São Nicolau, 210, CEP 09913-030 Diadema, SP, Brazil.

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