

miR-939 acts as tumor suppressor by modulating JUNB transcriptional activity in pediatric anaplastic large cell lymphoma

Anaplastic large cell lymphoma (ALCL) is a distinct subtype of aggressive non-Hodgkin lymphoma, mainly affecting children and young adults. The vast majority of the pediatric cases are anaplastic lymphoma kinase-positive (ALK⁺) due to the chromosomal translocation t(2;5)(p23;q35), which leads to the constitutive expression and activation of NPM-ALK fusion protein.¹ ALK downstream signaling includes multiple oncogenic pathways and has been directly implicated in ALCL pathogenesis.² Current therapeutic approaches can cure 70% of children affected by ALCL. However, despite this, the

prognosis of patients experiencing failure of first-line treatment is very poor.³ The use of biological risk factors such as minimal disseminated disease (MDD), minimal residual disease (MRD), and anti-ALK antibody titer in the forthcoming ALCL trials aims to reduce toxicity for low-risk patients, while making it easier to identify those at higher risk of relapse, who might benefit from more intense regimes and new targeted therapies.^{4,5} We recently identified two clearly distinct subgroups of ALCL patients who were characterized by different endogenous *NPM-ALK* expression levels at diagnosis and were defined ALK-high and ALK-low.⁶ These patients displayed a bimodal distribution for *NPM-ALK* expression and the median expression values were 2,500 *NPM-ALK* copies/10,000 *ABL1* in ALK-low cases and 125,000 *NPM-*

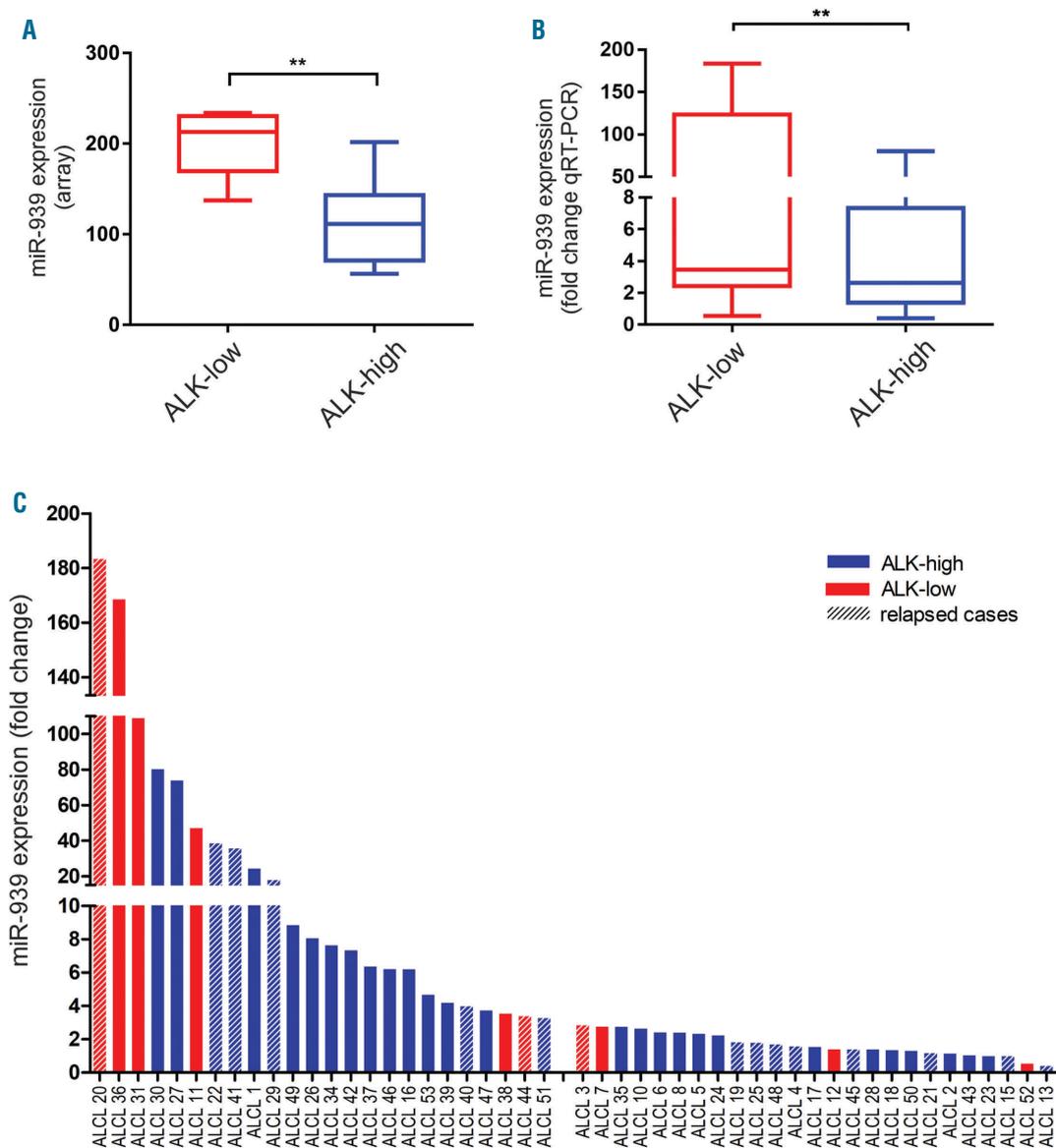


Figure 1. miR-939 is differentially expressed according to anaplastic lymphoma kinase (ALK) expression in tumor tissues. Box plots of differential miR-939 expression in anaplastic large cell lymphoma (ALCL) tumor biopsies detected by (A) human microRNA microarray v.2 (n=16: 11 ALK-high and 5 ALK-low; **P=0.0016) and (B) quantitative reverse transcription PCR (qRT-PCR) (n=49: 39 ALK-high and 10 ALK-low; **P=0.0017). qRT-PCR data have been calculated according to the comparative δ Ct method, using miR-16 as endogenous control. The horizontal line in the box indicates the median expression level of miR-939 (ALK-low median value: 3.45; ALK-high median value: 2.62). (C) Heterogeneous expression levels of miR-939 in 49 ALCL cases. Data have been measured by qRT-PCR according to the comparative δ Ct method and compared to reactive lymph nodes tissue (LN, n=11). Blue and red bars are ALK-high and ALK-low tumor biopsies, respectively. miR-939 median expression level=2.83.

ALK copies/10,000 ABL1 in ALK-high patients. Interestingly, most of the patients who relapsed belonged to the ALK-high subgroup. The median time of relapse of these ALK-high patients was 5 months, in striking contrast to the time of relapse of ALK-low cases (30 months). By means of a gene set enrichment analysis and *in vitro* functional studies, we showed a differential gene expression signature in ALK-high versus -low patients. Moreover, we identified signaling pathways preferentially associated to the different phenotype and clinical course of ALK-high and ALK-low cases. In particular, the less aggressive phenotype observed in the ALK-low group was characterized by IL-2 signaling retention, while higher ALK expression levels were shown to induce cyclins and Aurora Kinases overexpression in the ALK-high subgroup. These data also suggest that cells expressing high ALK levels could acquire a selective growth advantage as compared to ALK-low cells.⁶

To further elucidate the molecular mechanisms responsible for the heterogeneous aggressiveness of ALK⁺ ALCL, and to better characterize these two previously uncovered disease entities, we performed array-based miRNA profiling in 16 pediatric cases previously classified according to their ALK endogenous expression (n=11 ALK-high, n=5 ALK-low) (Online Supplementary Table S1). By supervised analysis, we identified 19 microRNAs significantly upregulated in the ALK-low subgroup (False Discovery Rate <0.05) (Online Supplementary Table S2).

Some of these, such as miR-155, miR-146a and miR-497, had previously been described as differentially expressed in ALK⁺ compared to ALK-ALCL.⁷

Since high ALK expression was previously associated with a more aggressive ALCL phenotype,⁶ miRNAs upregulated in the ALK-low cases could probably act as tumor suppressors. In line with this concept, Hoareau-Aveilla *et al.* found that the overexpression of miR-497 in ALK⁺ cell lines (corresponding to ALK-high patients) inhibited ALCL cell growth and induced cell cycle arrest.⁸

In this study, we focused our attention on miR-939, that was the most differentially expressed miRNA between ALK-high and ALK-low tumor cells (Figure 1A). We first evaluated miR-939 expression by quantitative reverse transcription PCR (qRT-PCR) in an extended panel of 49 ALCL primary tumor samples collected in the last 15 years in our lab from the Italian Association of Pediatric Hematology and Oncology (Associazione Italiana di Ematologia e Oncologia Pediatrica, AIEOP) centers. Unfortunately, despite a long period of recruitment, this larger cohort included only 20% of ALK-low cases, and miR-939 expression difference, observed with the array-based miRNA profiling, was less appreciable. Of note, three ALK-low cases had very high levels of miR-939 (Figure 1B). Although whether the expression of miR-939 is actually higher in ALK-low profile patients could only be confirmed by an analysis of a very large cohort of ALCL pediatric cases, it does seem evident that

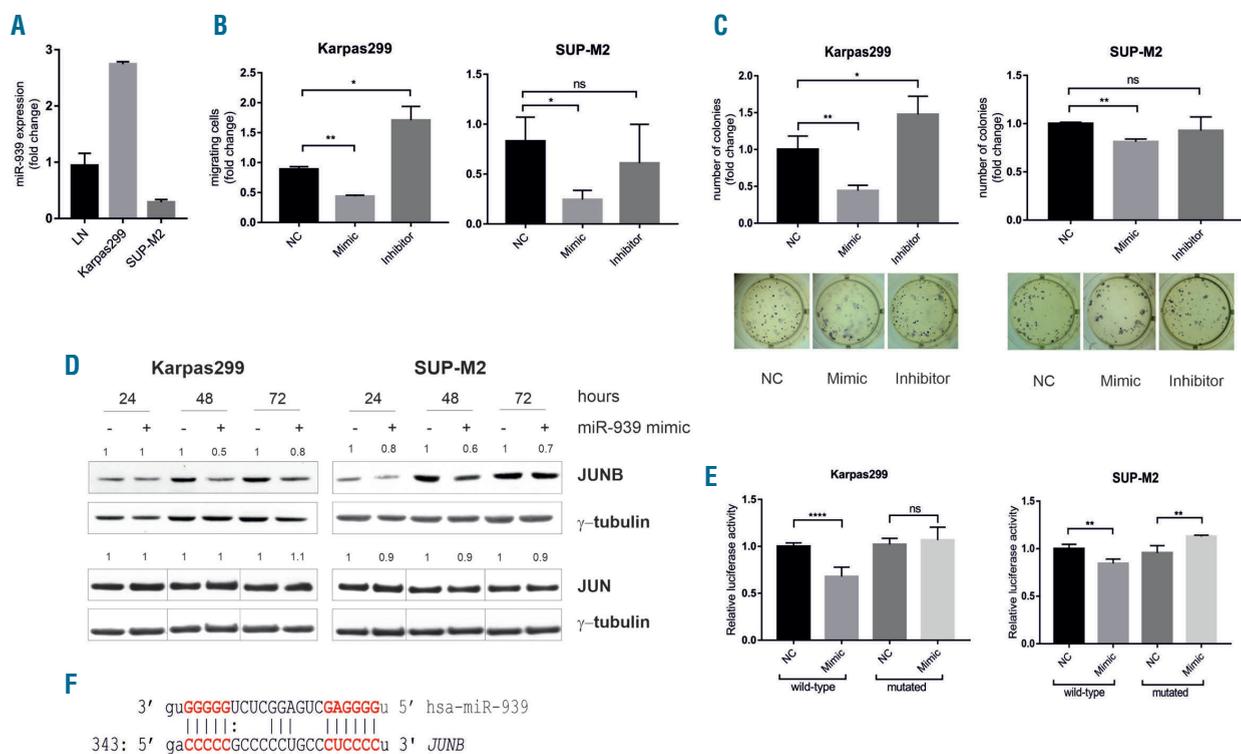


Figure 2. miR-939 impairs tumor cell migration and invasion. (A) miR-939 basal expression levels in anaplastic large cell lymphoma (ALCL) cell lines. Data have been calculated according to the comparative δ Ct method and compared to reactive lymph nodes tissue (LN, n=11). miR-16 was used as endogenous control. Karpas299 and SUP-M2 cells were transiently transfected with miR-939 mimic, or negative control (NC) or with the inhibitor and used to analyze migration (B) and clonogenicity (C). The results are shown as fold change of negative control values. Two or three independent experiments were performed in triplicate and mean results are shown. * P <0.05; ** P <0.001. (D) Western blotting analysis of JUNB and JUN proteins in Karpas299 and SUP-M2 cell lines transfected with miR-939 mimic or negative control at indicated post-transfection time points. γ -tubulin was used as loading control. Vertical lines have been inserted to indicate repositioned gel lanes. (E) The JUNB 3'UTR sequence containing the miR-939 putative binding site has been subcloned into pmirGLO reporter plasmid. Karpas299 and SUP-M2 cells transfected with miR-939 mimic and the reporter plasmid containing wild-type or mutated miR-939 binding site were tested by luciferase assay 24 hours post transfection. ** P <0.001; *** P <0.0001; ns: non-significant. (F) miR-939 binding site on 3'-UTR of JUNB. The putative binding sequences have been predicted using the on-line tool available at <http://www.microrna.org>.

ALCL tumors express different levels of miR-939 (Figure 1C and *Online Supplementary Table S3*).

To decipher the functional role of miR-939 in ALCL, we used Karpas299 and SUP-M2 cells, characterized by different basal levels of the miRNA (Figure 2A). We transiently overexpressed miR-939 or inhibited its expression in these cell lines (*Online Supplementary Figure S1*) that are more similar to ALK-high ALCL cells than to the ALK-low counterpart.⁶ The overexpression of miR-939 significantly reduced the migratory ability of both ALCL cell lines as compared to negative control cells, while its inhibition was shown to significantly increase ALCL cell migration (Figure 2B). Moreover, miR-939 overexpression reduced both Karpas299 and SUP-M2 clonogenic growth capacity (Figure 2C). In contrast, its inhibition significantly increased Karpas299 clonogenicity, while a less noticeable effect was observed on SUP-M2, which might be in line with the lower basal level of miR-939 in SUP-M2 compared to Karpas299 cells (Figure 2A). Of note, NPM-ALK expression was not affected by miR-939 overexpression, thus suggesting that miR-939 does not contribute mechanistically to the ALK-high or ALK-low status (*Online Supplementary Figure S2*).

In silico prediction of miR-939 targets and Gene Ontology analysis were performed by using miRTarBase 7.0 (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>) and Panther 14.1 (<http://pantherdb.org/data/>) online tools. Among transcription factors and nucleic acid binding proteins, which were highly represented in the miR-939 putative target list, we focused on the transcriptional factor JUNB as the most promising candidate for our analy-

ses, since its knockdown in NPM-ALK expressing cells was previously shown to impair cell proliferation.⁹ Indeed, transient overexpression of miR-939 decreased JUNB protein expression both in Karpas299 and SUP-M2 cells (Figure 2D). Moreover, direct modulation of JUNB by miR-939 was confirmed by 3'UTR reporter assay in both Karpas299 and SUP-M2 cell lines transfected with miR-939 or negative control and reporter vectors containing wild-type and mutated miR-939 binding site in JUNB 3'-UTR sequence (Figure 2E). The miR-939 recognition site was identified by using the online tools at <http://www.microrna.org> (Figure 2F).

JUNB is an activator protein-1 (AP-1) transcription factor, together with JUN, JUND and also members of the Fos/Fra, ATF and Maf subfamilies. AP-1 proteins exert their activity by forming homo- and hetero-complexes, and mainly regulate the expression of genes involved in cell proliferation.¹⁰ JUNB overexpression was shown in CD30⁺ lymphomas and its transcriptional activity was directly implicated in CD30 promoter induction in both Hodgkin lymphoma and ALCL.¹¹ NPM-ALK itself contributes to JUNB activation and CD30 expression in ALK⁺ ALCL via both ERK1/2-MAPK and mTOR pathways.^{9,12} JUNB and JUN activities were also shown to sustain PI3K-associated activation of AKT1, further suggesting the existence of a synergistic crosstalk between NPM-ALK signaling and AP-1 transcription factors.¹³ More recently, platelet-derived growth factor receptor B (PDGFRB) was confirmed as a JUNB transcriptional target in NPM-ALK-driven lymphomas and the combination of ALK and PDGFR

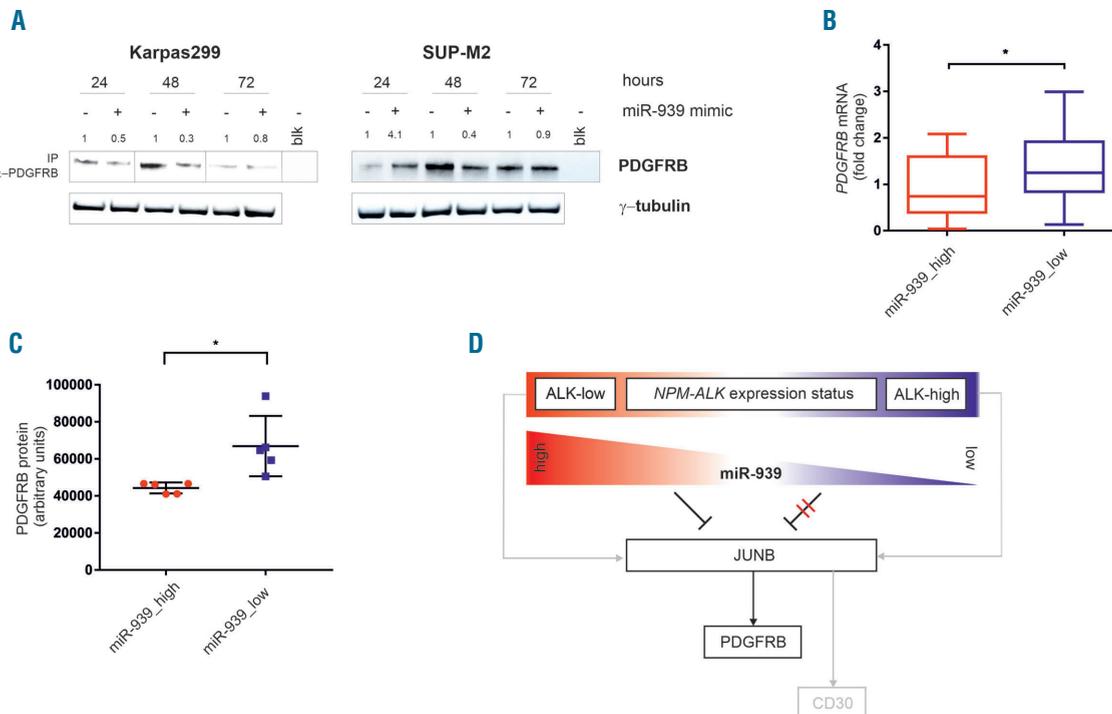


Figure 3. PDGFRB expression according to miR-939 levels in anaplastic lymphoma kinase-positive (ALK⁺) anaplastic large cell lymphoma (ALCL) cell lines and ALCL patients' samples. (A) PDGFRB protein immunoprecipitation in Karpas299 and SUP-M2 cell lines transfected with miR-939 mimic or negative control at indicated post-transfection time points; western blotting analysis of γ -tubulin was used as immunoprecipitation input control. Blk: blank. (B) Box plot of PDGFRB mRNA expression in patients expressing high or low levels of miR-939 (n=25 miR-939_{low} [\leq median value]; n=24 miR-939_{high} [$>$ median value]) detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *P=0.03. Data have been calculated according to the comparative Δ ct method and compared to reactive lymph node tissues (LN, n=11). ABL1 was used as endogenous control. (C) Dot plot representing PDGFRB protein expression assessed by RPPA in ten available ALCL tumor tissue samples (5 miR-939_{low}, 5 miR-939_{high}; *P=0.01). (D) Schematic representation of miR-939-mediated JUNB modulation in ALK-low/high ALCL. In the presence of lower levels of NPM-ALK transcript, miR-939 is able to inhibit JUNB transcriptional activity, resulting in PDGFRB downregulation (black arrows). Gray arrows are referred to previous data from the literature.¹²

inhibitors was suggested as a valuable strategy to reduce relapse rates in ALCL.¹⁴

In the presence of lower *NPM-ALK* levels, we hypothesized that miR-939 overexpression could contribute to fine-tuning the JUNB-mediated oncogenic signaling, further explaining the favorable outcome of ALK-low ALCL cases. To assess if miR-939 was able to modulate PDGFRB expression through JUNB modulation, we evaluated PDGFRB expression in Karpas299 and SUP-M2 cells after transfection with miR-939 mimic or negative control. PDGFRB was reduced in both the cell lines after 48 hours of transfection with miR-939 mimic (Figure 3A and Online Supplementary Figure S3). Even though JUN is also necessary to regulate PDGFRB transcription in ALCL,¹⁴ our results suggest that miR-939 modulates PDGFRB expression by specifically targeting JUNB, since JUN protein was not affected by miR-939 overexpression (Figure 2D). While *JUNB* mRNA did not show any significant difference between ALK-high and ALK-low groups (Online Supplementary Figure S4), *PDGFRB* transcript levels were significantly reduced in miR-939_high compared to miR-939_low ALCL cases (Figure 3B). By performing reverse phase protein arrays on ten ALCL tumor tissue samples, PDGFRB protein was also found to be significantly downregulated in miR-939_high cases (Figure 3C), suggesting that miR-939 could act as an oncosuppressor by reducing PDGFRB expression.

In conclusion, we previously showed that tumors with high or low *NPM-ALK* levels are characterized by a different gene expression profile and a different aggressiveness. In particular, when expressed at lower levels, ALK is unable to render the transformed lymphocytes completely unresponsive to pathways normally expressed in T cells, such as those involved in interleukin signaling.⁶ Here we demonstrated that miR-939 expression could contribute to PDGFRB inhibition, a crucial driver for ALCL lymphomagenesis, via JUNB downregulation (Figure 3D). Further investigations will clarify if miR-939 expression could affect anti-CD30 therapies or imatinib treatment efficacy in ALK-low ALCL patients.

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doi:10.3324/haematol.2019.241307

Disclosures: no conflicts of interests to disclose.

Contributions: AG performed the experiments, analyzed results, prepared figures and wrote the manuscript; FL analyzed results and wrote the manuscript; ABH performed microarray analyses and revised

the manuscript; CCD and IG processed clinical samples and performed qRT-PCR analyses; EC and MP collected clinical data, performed statistical analyses and revised the manuscript; BA and GV performed Reverse Phase Protein Array experiments and commented on the manuscript; MP and ESGdA performed hemato-pathological revision and commented on the manuscript; AB revised the manuscript; KB supervised microarray analyses and revised the manuscript; LM conceived and designed research, supervised experimental work and revised the manuscript. All the authors read and approved the final version of the manuscript.

Funding: this work was supported by Fondazione Città della Speranza, Padua, Italy; Fondazione CA.RI.PA.RO, Padua, Italy (grant 17/03 to LM); Camera di Commercio Venezia, Venezia, Italy; Fondazione Roche, Roma, Italy (grant to FL) and Fondazione Umberto Veronesi, Milano, Italy (fellowship to FL); Associazione Italiana contro le Leucemie, sezione provinciale di Treviso (grant to BA).

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