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NPM-ALK expression levels identify two distinct subtypes of pediatric Anaplastic Large Cell Lymphoma

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Conflict of Interest
The authors declare no conflict of interest.

Running Title:
GEP in childhood ALCL

Letter to the Editor

Anaplastic large cell lymphoma (ALCL) is a distinct form of non-Hodgkin lymphoma (NHL) which accounts for 10-15% of all childhood lymphomas. Differently from ALCL in adults, where about 30% of cases are ALK+, ALCL in children is nearly universally ALK+ and in almost all of cases it is characterized by the t(2;5)(p23;q35) translocation which results in the expression of the hybrid oncogenic tyrosine kinase NPM-ALK [1]. NPM-ALK fusion protein is responsible for ALCL development, and throughout its constitutive tyrosine kinase activity it regulates signaling pathways critical for proliferation, cell-cycle control and survival of ALCL cells [2]. So far, few studies have investigated ALCL gene expression profiling, and most of them have used heterogeneous populations of pediatric and adult cases [3]. Main aim of such studies was to identify whether ALK+ and ALK- ALCL had a different transcriptional program, and although differences were found, similarities were also detected [3,4]. Based on the fact that pediatric ALCL are almost exclusively ALK+, our study focused on investigating the potential heterogeneity inside ALK+ ALCL rather than differences between ALK+ and ALK- tumours.

In order to investigate the transcriptional characteristics of ALK+ ALCL, we performed a global microarray-based transcriptional analysis on 23 paediatric tumour samples, 12 reactive lymph nodes and 4 ALCL cell lines (three ALK+ and one ALK-) (Supplementary Materials). As previously reported, we found that ALK+ tumours displayed down-regulation of both B-cell and T-cell receptor signalling molecules (such as *ARNT2*, *CD3G*, *CD24*, *CD27*, *CD40*, *CD79A*, *NFATC2*, *PIK3CD*, *TNFRSF10A*), in addition to T Helper Cell surface molecules (such as *CD19*, *CD28*, *CTLA4*), (Supplementary Table S1), consistent with the notion that this malignancy originates from undifferentiated normal CD4+ T lymphocytes [5].

To explore the molecular differences between clinically homogeneous ALK+ ALCL, we performed unsupervised clustering analysis of the tumour samples, identifying two clearly distinct subgroups of patients. Indeed, supervised analysis identified two different gene expression signatures, both related to the diverse amount of NPM-ALK in ALCL cells (ALK-low and ALK-high). Of note, ALCL cell lines clustered together and had a gene expression signatures more similar to ALK-high than ALK-low samples (Figure 1A) (Supplemetary Table S2). The ALK-high/low signatures were not affected by sample or patient selection bias, as our novel ALK-group classification was independent of the lymph node infiltration rate, since, the percentage of ALK+ cells was about 40% in the large majority of the cases and it could be applied to indipendent ALCL studies

(Supplemetary Figure S1) [3]. *NPM-ALK* levels were assessed by quantitative RQ-PCR in the same set of patients showing a significant correlation with GEP data (Pearson correlation coefficient 0.79, $p<0.0001$) (Figure1B and C, respectively). Additional 19 pediatric ALCL cases were tested by RQ-PCR and overall the full dataset, including 42 samples, displayed a bimodal distribution for *NPM-ALK* expression consistent with the presence of an ALK-low and an ALK-high group. The median expression values were 2.500 *NPM-ALK* copies/10.000 *ABL* in ALK-low cases and 125.000 *NPM-ALK* copies/10.000 *ABL* in ALK-high patients (Welch's t-test, $p<0.0001$ Figure 1D). In addition, when NPM-ALK protein expression was measured in a subset of our cases, selected based on tissue availability (Figure 1E), we found that expression and phosphorylation of NPM-ALK kinase correlated with mRNA levels of the two groups (Figure 1F), supporting the concept that ALK+ ALCL can be distinguished based on *NPM-ALK* endogenous expression level.

We did not observe significant differences between the two subgroups based on clinical characteristics, although patients with central nervous system involvement (n=4) belonged to the ALK-high subgroup (Supplementary Table S3). Interestingly, 9 out of 12 patients who experienced relapse were ALK-high cases, and had a median time of relapse of 5 months compared to the median time of relapse of 30 months of the 3 ALK-low relapsed patients.

Gene set enrichment analysis (GSEA) using the MSig DB database (Molecular Signature Database, Oncogene signature C6, http://www.broad.mit.edu/gsea/) showed a positive enrichment for genes involved in the Interleukin signalling pathway (specifically *IL-2*, *IL-15* and *IL-21*) in ALK-low compared to ALK-high ALCL samples, while genes set associated with cell growth and division (*CyclinD1*, *MYC* and *E2F1*) were significantly up-regulated in ALK-high cases (Figure 1G).

The interleukin 2 signalling pathway is critical for CD4+ T cell maturation and function [6,7], and its downregulation occurs in ALCL cell lines in response to NPM-ALK expression and activity [8]. Consistently, we showed that IL-2Rγ was expressed in ALK- (FE-PD) ALCL cell line but not in ALK+ (KARPAS-299, SUD-HL-1 and SUP-M2) cell lines (Figure 2A). In order to evaluate the IL-2Rγ expression in ALCL primary tumour cases, we analysed, by flow cytometry, 9 patients (4 ALK-low and 5 ALK-high), for whom tissue samples were available. The results showed that tumour cells in ALK-low patients ubiquitously expressed IL2Rγ (ALK+/IL2Rγ+ cells>95%), whereas ALK-high patients displayed a more heterogeneous pattern of expression (Figure 2B and C). Taking into account that ALK-high patients relapsed more frequently and rapidly than ALK-low patients, it seems that in ALK-low cases NPM-ALK is unable to make transformed

lymphocytes completely "deaf" to interleukins, and interleukin signaling retention, in turn, contributes to a less aggressive tumour phenotype.

Functional annotation analysis performed by GSEA in ALK-high tumour samples, revealed increased expression of genes involved in cell growth and survival, such as cyclins and Aurora Kinases A (AURKA) and B (AURKB). These genes are known to affect cell proliferation, but also they regulate chromosomes segregation and mitosis. Hence, to validate our bioinformatics analysis, we took advantage of commercially available aurora kinase inhibitors, which were used to treat a panel of ALK+ ALCL cell lines (KARPAS-299, SU-DHL-1 and SUP-M2) representative of the ALK-high subgroup. As expected, marked growth inhibition of ALCL cells was observed upon administration of AURKA and AURKB inhibitors, Alisertib (MLN8237) and Barasertib (AZD1152) respectively and this was associated with cell cycle arrest and induction of apoptosis (Figure 2D). In particular, the exposure of ALCL cells to increasing concentrations of MLN8237 and/or AZD1152 led to time-dependent inhibition of cell growth, which, consistent with Aurora kinases involvement in mitotic entry and cytokinesis, was characterized by a dose-dependent "growth plateau" typical of a cytostatic cell response (Figure 2D). In contrast Aurora kinases inhibitors did not cause changes in healthy PBMCs proliferation (stimulated with or w/o phytohaemagglutinin), when used in the same experimental conditions (Figure 2E). Previous data have shown that Aurora inhibitors exert their activity leading to accumulation of polyploid cells with an endoreplication phenotype before mitotic catastrophe or apoptosis occur [9]. Indeed, when drug inhibitory effects were investigated in more detail, we found that both compounds, administered for 48 h as single agents or in combination, led to a dramatic increase of the polyploid cell content (8N) over the diploid (2N) and tetraploid (4N) cell population (Supplemetary Figure S2A); whereas at earlier time points (24h) they induced a provisional arrest in G2/M phase and accumulation of tetraploid cells only (data not shown). Prolonged drug exposure, instead, affected cell survival, since the fraction of the cells undergoing apoptosis (Annexin V-positive, AV^+) or necrosis (Propidium Iodide positive, PI^+) increased over time, after 72h of treatment (Supplemetary Figure S2B). AURKA and B have been recently detected in NHL, however, their involvement in ALCL tumourigenesis, as well as their importance as therapeutic targets has not been investigated [10]. Herein, we provide evidence that AURKA and AURKB may be considered novel drug targets in ALCL, and that Aurora kinase inhibitors are effective agents in pre-clinical ALCL models. Their efficacy depends on their general mode of action and results in inhibiting proliferation, survival and cell cycle progression of ALCL cells.

Overall, our data suggest that the heterogeneous levels of aggressiveness observed in ALCL tumours may be explained by a *NPM-ALK* distinct gene expression and transcriptional program. High-level ALK+ cells seems to acquire a selective growth advantage compared to ALK-low cells. This concept merits attention to exploit novel therapeutic strategies in this malignancy which may increase curability and survival of children with ALCL.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: ***ALK* expression levels identifies two distinct signature in ALCL. A)** Supervised hierarchical clustering performed on 23 paediatric ALCL tumour biopsies and 4 cell lines gene expression profiles using the 591 probe sets differentially expressed between ALK-high and ALK-low cases. **B)** Boxplot of differential *ALK* expression in ALCL tumour biopsies detected by GEP (Welch's t-test, p=0.026). The upper border of the box indicates the upper quartile (75th percentile) while the lower border indicates the lower quartile (25th percentile) and the horizontal line in the box the median expression level of *ALK* probeset in the two subgroups of ALCL cases. **C**) *NPM-ALK* expression level assessed by quantitative RQ-PCR in the same set of patients shows a significant correlation with GEP data (Pearson correlation coefficient 0.79, p<0.0001). **D)** *NPM-ALK* expression by RQ-PCR in 42 ALCL tumour biopsies. Blue and yellow dots represent tumour biopsies evaluated also by GEP analysis. Red dots represent new 19 tumour biopsies (Welch's t-test, p<0.0001). The horizontal lines indicate the median expression level. **E)** ALK staining in two ALCL specimens, with about 40% of tumour cells, belong to ALK-low and ALK-high group respectively (x400). **F)** Western blot analysis of total (NPM-ALK) and phosphorylated (NPM-ALK^{Y664}) NPM-ALK kinase in ALK-low and –high tumour samples, using γ-tubulin (Tubulin). ALK-high A_21 lysate was used as positive control for NPM-ALK expression. Box plot represents the analysis of total (*left*) and phosphorylated (*right*) NPM-ALK protein levels in ALK-low and -high specimens. P-values were calculated using the Mann-Whitney rank sum test (**P<0.05). **G)** Gene Set Enrichment Analysis (GSEA) was performed using GSEAv2.0 with probe sets ranked by signal-to-noise and statistical significance determined by 1000 permutations. Phenotype permutations were used to enable direct comparison between ALK-high and -low ALCL patients.

Figure 2: **IL-2Rγ expression in ALCL cell lines and tumour biopsies, and Aurora kinase inhibitors activity in ALK+ ALCL cell lines. A)** Flow cytometry analysis of IL-2Rγ expression in ALK+ (KARPAS-299, SUD-HL-1 and SUP-M2) and ALK- (FEPD) ALCL cell lines. **B)** Flow cytometry analysis of ALK and IL-2Rγ expression in 2 ALK-high (A_28 and A_29) and 2 ALK-low (A_19 and A_34) patients. **C)** Percentage of ALK+ cells with or without co-expression of IL-2Rγ, as measured by flow cytometry, in ALCL tumour biopsies classified by GEP in ALK-low or ALK-high. **D)** Growth inhibition of ALCL cell lines (KARPAS-299, SU-DHL-1 and SUP-M2) exposed to varying concentrations of AURKA (Alisertib, MLN8237), AURKB (Barasertib, AZD1152) or both for 48 h. Cell viability was assayed by MTT assay. Points are triplicates of biological replicates and are representatives of three independent experiments. Results are expressed as fold of control ± SD. **E)** Growth inhibition of healthy peripheral blood mononuclear cells (PBMCs) were exposed to the same conditions with or w/o phytohaemagglutinin (PHA) exposed to varying concentrations of AURKA (Alisertib, MLN8237), AURKB (Barasertib, AZD1152) or both for 48 h. Cell viability was assayed by MTT assay. Points are triplicates of biological replicates and are representatives of two independent experiments. Results are expressed as fold of control ± SD.

Supplementary information

Supplementary Figure S1: **ALK-low/high signature validation in an independent cohort of ALCL cases.** Heatmap of 16 paediatric ALCL gene expression profiles published by Lamant *et al.* [3] based on our ALK-low/high signature. We adopted a gene "matching" approach selecting only probe sets with high correlation between the two different platforms (i.e HG-U133A and HG-U133Plus 2.0, Affymetrix Santa Clara, CA, USA) to create the core common genes signature. Lamant samples were labelled according to ALK expression; orange: low expression; blue: high expression.

Supplementary Figure S2: **Effect of Aurora kinase inhibitors on cell cycle in ALK+ ALCL cells. A)** Cell cycle profiles of ALCL cell lines grown in the presence of 0.5µM Alisertib (MLN8237), 0.5µM Barasertib (AZD1152) or both (MLN+AZD) for 48 h. After treatment the cells were collected, fixed and stained with propidium iodide (PI) to determine their DNA content by flow cytometry. Position of diploid (2N), tetraploid (4N) and polyploid (8N) DNA content is indicated by arrows. **B)** Cell survival analysis of ALCL cells treated with 0.5µM Alisertib (MLN8237), Barasertib (AZD1152) or both (MLN+AZD) for 72 h, determined after annexin-V and propidium iodide (AV/PI) staining by flow cytometry. Early apoptotic (AV^+/PI^-), late apoptotic (AV^+/PI^+) and necrotic (AV^-/PI^+) cells are reported in graph as fold(s) of control (untreated cells).

Supplementary Table S1: **Differentially expressed genes between ALCL and normal reactive lymphoid tissue.**

Supplementary Table S2: **Differentially expressed genes between ALK-high and ALK-low ALCL samples.**

Supplementary Table S3: **Clinical and biological characteristics of ALCL studied population based on *ALK* expression level.**

Figure 1

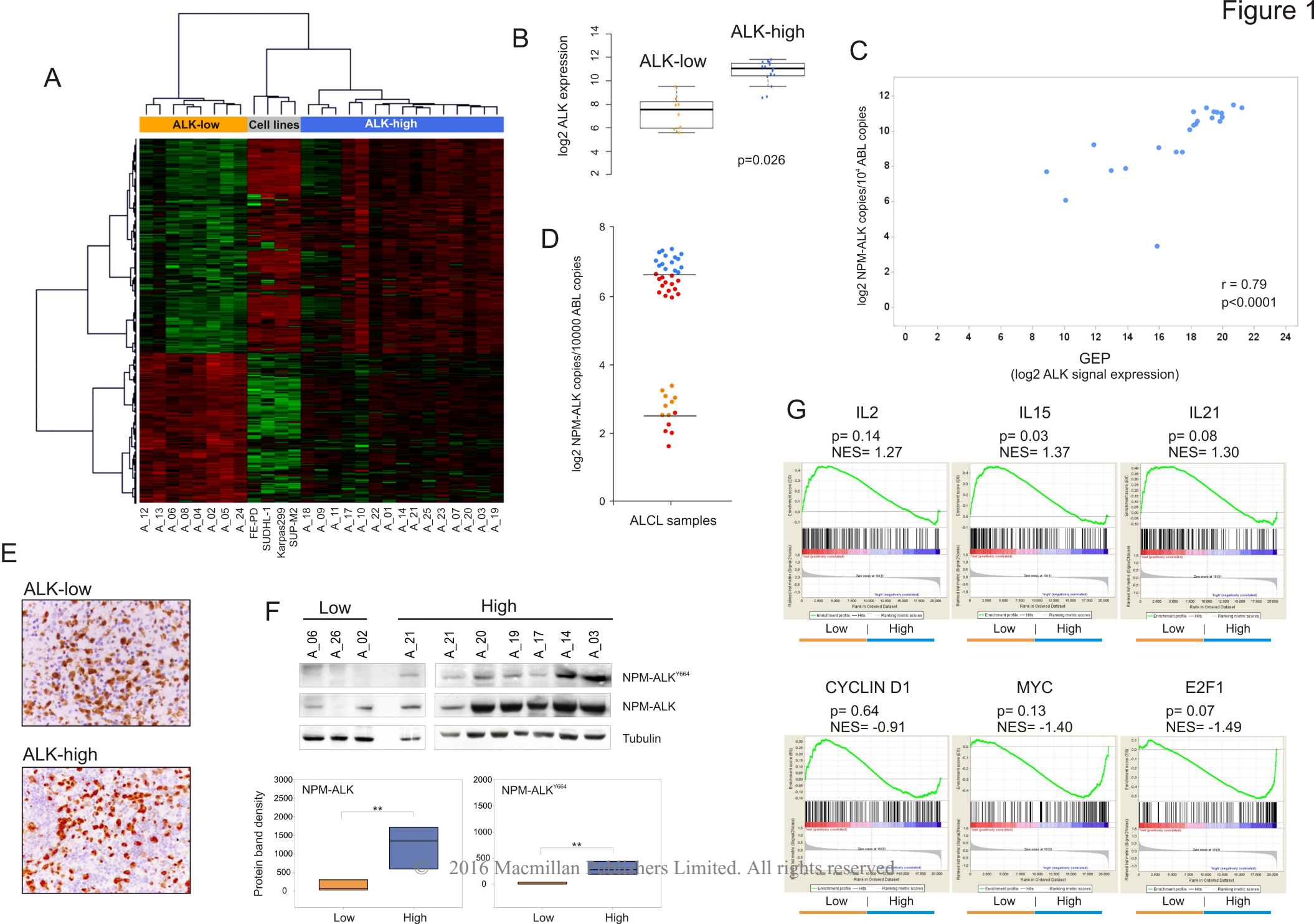

Figure 2