PAX5 fusion genes are frequent in poor risk childhood acute lymphoblastic leukaemia and can be targeted with BIBF1120

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Summary
Background Despite intensive risk-based treatment protocols, 15% of paediatric patients with B-Cell Precursor Acute Lymphoblastic Leukaemia (BCP-ALL) experience relapse. There is urgent need of novel strategies to target poor prognosis subgroups, like PAX5 translocated.

Methods We considered 289 childhood BCP-ALL cases consecutively enrolled in Italy in the AIEOP-BFM ALL2000/R2006 protocols and we performed extensive molecular profiling, integrating gene expression, copy number analyses and fusion genes discovery by target-capture NGS. We developed preclinical strategies to target PAX5 fusion genes.

Findings We identified 135 cases without recurrent genetic rearrangements. Among them, 59 patients (43.7%) had a Ph-like signature; the remaining cases were identified as ERG-related (26%), High-Hyperdiploid-like (17%), ETV6::RUNX1-like (8.9%), MEF2D-rearranged (2.2%) or KMT2A-like (1.5%). A poor prognosis was associated with the Ph-like signature, independently from other high-risk features.

Interestingly, PAX5 was altered in 54.4% of Ph-like compared to 16.2% of non-Ph-like cases, with 7 patients carrying PAX5 fusions (PAX5t), involving either novel (ALDH18A1, IKZF1, CDH13) or known (FRAS1, AUTS2, DACH2) partner genes. PAX5t cases have a specific driver activity signature, extending to multiple pathways including LCK hyperactivation. Among FDA-approved drugs and inhibitors, we selected Dasatinib, Bosutinib and Foretinib, in addition to Nintedanib, known to be LCK ligands. We demonstrated the efficacy of the LCK-inhibitor BIBF1120/Nintedanib, as single agent or in combination with conventional chemotherapy, both ex vivo and in patient-derived xenograft model, showing a synergistic effect with dexamethasone.

Interpretation This study provides new insights in high-risk Ph-like leukaemia and identifies a potential therapy for targeting PAX5-fusion poor risk group.

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Research in context

Evidence before this study

The discovery of the Philadelphia-like (or BCR/ABL-like) subgroup of patients affected by acute lymphoblastic leukaemia is the most relevant recent advancement in the definition of the ALL genetic landscape, and even more relevant, a step forward in the identification of new patients’ subgroups targetable with precision therapy. In addition to the pioneer studies by Mullighan’s and Den Boer’s groups (in 2009), we considered several independent studies which further characterized Ph-like patients and reviewed both by Pui in 2017 and Shiraz in 2020. Moreover, further new genetic subgroups of ALL emerged, with association with outcome and potential for targeting, in a continuous path for the definition of ALL heterogeneity and a chance for improving the cure rate by emerging therapies. More specifically in this context, we focused our attention on the ALL subgroup with PAX5 gene alterations, summarized by the two most recent studies by Gu 2019 and Jung 2020, and by our own previous studies on both i) PAX5 fusions in infant ALL patients and ii) on the functional characterization of the activated pathways downstream to PAX5 fusions, as a potential target for therapies.

Added value of this study

In this paper, in addition to the description of the Ph-like subgroup in the Italian population of childhood ALL, we extend the genomic landscape with further details of other ‘like’ subgroups and additional copy number abnormalities. A main advantage of this study is the long follow up of the populations which allows for a more stable outcome profile.

Compared to previously published studies, our focus on PAX5 gene rearrangements allowed us to detail the characterization of the PAX5 gene fusion with several different partners. Herewith, we show that PAX5 fusion genes are more recurrent among Ph-like patients and they have their own specific signature, which is here detailed. Moreover, we show that PAX5 fusions can be targeted by Dasatinib, Bosutinib and Foretinib, in addition to the kinase inhibitor BIBF1120/Nintedanib, which is directed against the Lymphocyte-specific tyrosine kinase (LCK), which is over-activated in PAX5 translocated cases.

Implications of all the available evidence

The ex vivo and in vivo testing of the LCK inhibitor BIBF1120/Nintedanib sustains the rationale for developing novel preclinical strategies to target PAX5 fusion genes, a step forward in the precision medicine for childhood ALL. In the future, patients carrying PAX5 fusions may benefit from a novel therapeutic approach.

Introduction

Despite intensive risk-based treatment protocols, 15% of paediatric patients with B-Cell Precursor Acute Lymphoblastic Leukaemia (BCP-ALL) experience disease relapse. Technological improvement progressively refined the molecular characterization of childhood ALL, identifying among other features the “Ph-like” subgroup (also known as “BCR/ABL1-like”). Ph-like patients are characterized by poor outcome regardless of treatment protocol, with a high incidence of relapse in children, adolescents and adult cohorts. Ph-like ALL was also recognized as a provisional entity in the 2016 revision to the World Health Organization (WHO) classification of haematologic neoplasms and there is general agreement on the importance to identify the Ph-like subtype. Those patients are characterized by recurrent molecular alterations, mainly fusion genes, classifiable as either ABL1-class (involving ABL1, ABL2, PDGFRα, PDGFβR, CSF1R, LYN) or JAK/STAT-class (affecting JAK2, CRLF2, EPOR). The pathogenetic involvement of kinase pathways sustained the use of kinase inhibitors already included in trials for Ph-positive ALL.

In addition, other molecular lesions have been discovered in the Ph-like subgroup, whose prognostic impact still needs to be elucidated, also in association with cooperative lesions, e.g., IKZF1-plus profile. Namely, PAX5 gene alterations have been frequently detected in Ph-like ALL, however, their relation with the Ph-like signature, association with outcome and targeting have been poorly elucidated. The PAX5 gene, which encodes for a B-cell related transcription factor, is altered in about 30% of BCP-ALL as being involved in deletions, amplifications, mutations and translocations that determine the formation of fusion genes encoding aberrant transcription factors. Recently, Gu et al. characterized PAX5 alterations (PAX5alt) as an heterogeneous group in childhood and adult cohorts, including cases carrying either PAX5 fusion genes, P80R variant and others, with P80R representing a subgroup with intermediate prognosis. Importantly, the PAX5alt signature cluster is very tight and partially overlaps with

Key words: Childhood ALL; PAX5 fusion genes; Ph-like ALL; BIBF1120; Nintedanib
the Ph-like one, whereas PAX5 P80R has a completely separated pattern.\(^\text{12}\) Overall, PAX5alt and PAX5 P80R account for 9.7% of B-other ALL and have a significant role in leukaemia initiation, prognosis and risk stratification, thus requiring an early identification and targeted therapy.\(^\text{14}\)

Our previous studies, in murine pre-BI cells as well as in primary patient cells, showed that PAX5 fusions sustain survival of leukaemic cells through overexpression of the Lymphocyte kinase (LCK) gene\(^\text{24,15}\) a directly repressed target of PAX5.\(^\text{16}\) We demonstrated that LCK hyperactivation upon PAX5 fusion expression leads to STAT5 activation and survival advantage, and that LCK phosphorylation can be specifically targeted by the kinase inhibitor BIBF1120, also known as Nintedanib (Boehringer Ingelheim, Ingelheim am Rhein, Germany),\(^\text{17}\) a small molecule which acts as a triple kinase inhibitor with anti-angiogenic anti-tumour effect.\(^\text{17}\) Nintedanib is approved for the treatment of patients with idiopathic pulmonary fibrosis,\(^\text{18}\) in use in phase 3 and 4 trials for solid tumors\(^\text{19}\) (e.g. pancreatic, hepatocellular, ovarian cancers and lung carcinomas), as well as in three clinical studies for relapsed/refractory adult AML (www.clinicaltrial.gov). It binds the ATP pocket domain of many molecules and it blocks the activity of many receptor-tyrosine kinases,\(^\text{20}\) such as the VEGF/FGF/ PDGF receptors and the non-receptor tyrosine kinases Src-family members, including LCK.\(^\text{17,20}\)

The present study aimed at the characterization of PAX5 rearranged cases among the Ph-like subgroup of children with BCP-ALL diagnosed in Italy. We herewith tested \textit{ex vivo} and \textit{in vivo} the efficacy of the LCK inhibitor Nintedanib/BIBF1120, setting the rationale for developing novel preclinical strategies to target PAX5 fusion genes.

**Methods**

**Patients' cohort**

We considered a cohort of 289 consecutive childhood BCP-ALL cases enrolled in Italy in the AIEOP-BFM ALL2000/R2006 protocols (Eudract number: 2007-004270-43).\(^\text{21}\) Among them, we identified 135 cases defined as “B-others” by excluding patients with the recurrent t(9;22), t(12;21), t(4;11) or t(11;19) translocations, or cases either with High Hyperdiploidy state (DNA index\(\geq\)1.16) or carrying constitutional trisomy 21. To confirm that the study cohort was representative of the whole B-other population, the features of the study cohort of \(N = 135\) cases were compared with the whole B-other population of 837 patients enrolled in the same period in Italy in the AIEOP-BFM ALL2000/R2006 protocols. Analyses did not reveal any difference considering gender, age or MRD stratification. Although the study cohort was, likely by chance, enriched for high-risk features (i.e., prednisone response and WBC and consequently stratification to the final High-Risk group), nonetheless, the estimated 5 years EFS did not differ between analysed and not-analysed patients (74.2±3.8 vs 76.5±1.6, respectively, \(p = 0.48\)) (data not shown).

**Ethics**

Investigation has been conducted in accordance with the ethical standards of the Declaration of Helsinki and following national and international guidelines. The study is approved by each institutional review board (Eudract number: 2007-004270-43). A written informed consent was obtained from patients or legal representatives. Regarding animal work, ethical approval was obtained by the Animal Welfare Office of the University of Milano-Bicocca prior to submit the request to the Ministry of Health. Animal testing was conducted in accordance with current European and National legislation (authorization n° 09/2018, protocol FB7CC-38 released by the Italian Minister of Health).

**Gene expression profiling and Leukaemia diagnostic classification model**

Patients were analysed using HG-U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA, USA) to assess Gene expression profiles. Leukaemia subtypes were classified accordingly to the previously published diagnostic GEP categories using The Diagnostic Classifier (DC) model. The model is based on all-pairwise linear classifiers for the 18 distinct classes of leukaemia, MDS and healthy control, developed for the Microarray Innovation LEukemia (MILE) study, as described in previous studies.\(^\text{22}\) Data were normalized using Robust Multiarray Average (RMA) method using Bioconductor R package (r-package.org). ComBat function in the sva package was used to correct the batch effect of different protocol for microarray preparation. Unsupervised analyses were generated using the top 1000 variable (based on variance) probe sets and processed by t-SNE algorithm with a perplexity score of 30. Data are available at GEO (accession numbers GSE97547, GSE13164, GSE13159, GSE1204). A data-driven network inference algorithm (NetBID, https://jyyulab.github.io/NetBID/)\(^\text{3}\) was applied to identify drivers in PAX5-translocated BCP-ALL patients, and a scalable solution of the Algorithm was used for the Reconstruction of Accurate Cellular Networks (SARACNe).\(^\text{24}\) The top 44 drivers for PAX5t were selected using default parameters reported in the package and \(p\) value<0.05 on the primary comparison PAX5t versus BCP-ALL excluding Ph-like/BCR-ABL-like group. Genes resulted with a positive driver activity in PAX5t were subjected to DGIdb4.2.0 software analysis to identified drug approved interactions. Cytoscape v3.9.0 was used to build gene-drug interaction network.
Copy number alteration analysis in B-others cohort

DNA material of BM at diagnosis was available from 131/135 patients for Copy Number Alteration (CNA) analysis using Multiplex Ligation-dependent Probe Amplification (MLPA)25 or the Affymetrix Cytogenetics Whole Genome 2.7M Array or Cytoscan-HD (Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol.

DNA and RNA target capture NGS analysis and bioinformatics

DNA or RNA target capture Next Generation Sequencing analysis has been performed for 86/135 patients, following a bioinformatic pipeline previously setup in our laboratory.26 Total RNA was extracted during diagnosis from bone marrow mononuclear cells by the guanidine thiocyanate-phenol-chloroform method and the RNA Capture ‘TruSight RNA PanCancer’ (Illumina, San Diego, CA, USA) was applied. If RNA was not available, we applied a DNA target panel including 17 genes, by Nextera Rapid Capture Custom panel (Illumina). We used the Illumina MiSeq platform. FASTQ files are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-11319. Fusion genes were validated by RT-PCR (experimental details are provided in Supplementary Table S7).

In vivo expansion of leukemic cells and drug experiments

Primary patient-derived xenograft (PDX) samples were obtained by intravenous injection of BM cells at diagnosis of PAX5 translocated BCP-ALL patients into female NSG mice (NOD/SCID gamma-/- mice, Charles River Laboratories, Calco, Italy) after at least two weeks of acclimatization and then after sublethal irradiation (125 Rad, X-ray) with Radgil (Gilardoni, Mandello del Lario, Italy). Leukaemia engraftment expansion was periodically evaluated by intra-femoral BM aspiration and assessed by FACS as positive to hCD10 (eBioCB-10M, three days prior to reach confluence and then MW96, three days prior to reach confluence and then p4), or dexamethasone [0.1 mg/kg, by oral gavage (o.g.)] or dexamethasone [0.5 mg/kg, by intraperitoneal injection (i.p.)], or in combination, for 2 weeks and wash out during the weekend. No animals have been excluded. The vehicle group has been treated both by o.g. BIBF1120 vehicle (Kolliphor/ethanol/water) and by i.p. physiological solution. At the end of the treatment, we sacrificed animals and analysed data in hematopoietic tissues and organs, such as Bone Marrow (BM), Peripheral Blood (PB), Spleen (SP), and meninges of Central Nervous Systems (CNS). Spleen weight was recorded prior to cell recovery. We collected the absolute number of cells for organs and we assessed the percentage of human cells (hCD10/CD19/CD45) vs. murine cells (mCD45) by FACS, to assess leukaemia engraftment (hCD45, Clone 2D1, #347464, RRID: AB_400307, BD Biosciences; mCD45, 30-F11, #12-0451, RRID AB_465668, eBioscience). According to experimental needs and international guidelines, we estimated to include N = 5 animals to each group (groups of animals, included in a single cage), N = 4 groups in each experiment, for a total of N = 20 animals in each experiment. The experimental staff was aware during the conduction of the experiment and analysis, not during the allocation and a strategy for potential confounders was not applied. We performed the power analyses according to Freedman, using a log-rank statistic test and the 3Rs rules. In details, the number of animals was calculated by considering either the final number of animals, the total number of events, the proportion of surviving in the control group and the estimated proportion of surviving in the experimental group, calculating a difference of 30% in survival outcome. We obtained to use 20 mice per single experiment, composed by 4 independent treatment groups of 5 mice each.

Ex vivo drug screening experiments of leukemic blasts

Ex vivo extended drug screenings were performed as previously described.27–30 Briefly the DMSO dissolved compound library (MedChemExpress, NJ, USA) was dispensed with increasing concentrations of the inhibitors in 6 dilution steps (0.008 - 25 μM) using digital dispenser (Dyooe, Tecan, Mannedorf, Switzerland), which ensures precise and robotic compound application in randomized fashion. Differential responses were monitored with ATP-dependent CellTiter-Glo Luminescent cell viability kit (Promega, Madison, USA) after 72 h of inhibitor exposure using Microplate reader (Spark® 10M, Tecan). Drug sensitivity scores (DSS) for the inhibitors were determined, followed by heat map visualization and unsupervised hierarchical clustering of the DSS scores were performed using R package gplots.31

Ex vivo apoptosis experiments of leukemic blasts in co-culture with human bone marrow stromal cells

Blasts derived from xenografts were plated on a layer of human bone marrow stromal cells (HBMS). Stromal cells were seeded at the concentration of 2x10⁴/well in MW96, three days prior to reach confluence and then leukemic blasts were plated at 2x10⁵/well in 200μl of...
AIM V (ThermoFisher). At 48h we tested three different concentrations (including IC50) for each drug, in mono-therapy or combination. Apoptosis/viability was evaluated by Annexin V-PE and 7-AAD staining (kit GFP CERTIFIED Apoptosis/Necrosis detection, Enzo Life Science Inc., Lausen, Switzerland) on leukemic cells positive by anti-hCD10-APC (BD Biosciences). Vincristine (stock 1mg/mL), dexamethasone (4mg/mL), L-asparaginase (kidrolase, stock 4000 IU/mL) and BIBF1120/Nintedanib (stock 10nM) were tested. In order to determine the efficacy of drug treatments and their combinations, the following parameters have been used: IC50 to determine the efficacy of drugs to inhibit the 50% of their targets and BLISS score: formula used to determine synergic (OE>EE), additive (OE=EE) or antagonist (OE<EE) activity between the drugs used in experiments Ey=Ex+Ey-(Ex*Ey), considering the expected effect (EE) of single drugs together Ex and Ey, and the Observed Effect (OE) really observed in experiments. Furthermore, we analysed data by Compusyn software, based on the Chou-Talalay’s combination index theorem (CI) to evaluate the effect of interaction between n drugs: CI<1 stands for synergy, CI=1 for additivity, CI>1 for antagonism.

Phosphoflow analysis
As previously setup and validated in our lab,23 the phosphoflow test was used to evaluate the levels of phosphorylation of PDPK1, Akt (Ser473), Akt (Thr308), S6 (S235/S236), 4EBP1 (Thr37/46), in leukemic cells (CD10+) derived from mice treated with vehicle or Nintedanib mice. After 1.5h serum starvation in X-VIVO at 37°C leukemic cells were then fixed for 10 minutes at room temperature by 1.5% PFA/mL and then permeabilized with 90% cold methanol, kept on ice for 30 minutes then washed with Staining Buffer. Further, cells were dark stained for 30 minutes at room temperature with antibodies directed to PDPK1 (Anti-PDPK1 pS441 #560092 RRID: AB_1645523 BD Biosciences), pAkt (Ser473) (Phospho-Akt Ser473 #4075 Cell Signaling), pAkt (Thr308) (Anti-Akt pT308 #5327 RRID: AB_2225329 BD Biosciences), pS6 (Anti-S6 pS235/pS236 #560435 RRID: AB_1645523 BD Biosciences), and 4pEBP1 (Phospho-4E-BP1 Thr37/46 #2860 Cell Signaling). Cells were also stained for CD10 (CD10 PE-Cy7™ #565282 RRID: AB_273913 BD Biosciences), in order to clearly detect B leukemic cells.

FACS experiments
BD FACS CANTOII or LSRX20 FORTESSA cytometer (BD Biosciences) and FACS DIVA software were used.

Statistical analysis
Analysis of clinical characteristics of patients were performed by the chi-square test for association, when appropriate. Event-free survival (EFS) was calculated from the date of diagnosis to the date of the first event. Events considered were resistance, relapse, death or second malignant neoplasms, whichever occurred first. EFS curves were estimated according to the Kaplan-Meier method, with standard errors (SE) according to the Greenwood formula; differences between groups were evaluated using the log-rank test. Cumulative incidence of relapse (CIR) curves accounting for competing events, were estimated according to the Chou-Talalay’s combination index theorem (CI) to evaluate the effect of interaction between n drugs: CI<1 stands for synergy, CI=1 for additivity, CI>1 for antagonism.

Role of funders
This work was supported by Ricerca Finalizzata-Giovani Ricercatori (Italian Ministry of Health) for reagents used in ex vivo and in vivo experiments. Moreover, AIRC and Transcall grants contributed to expenses for patients’ data collection, molecular profiling and characterization. Fondazione Cariparo supported costs due for data analysis. The funders did not have any role nor in study design, data collection, data analyses, interpretation or writing of report.

Results
With the aim to define targetable subgroups, we considered consecutively diagnosed 289 BCP-ALL patients and defined 135 cases referred to as ‘B-others’ according to the workflow (outlined in figure S1) which excluded recurrent aberrations and specific gene expression signatures. B-others were scrutinized for specific GEP profiles and fusion transcripts by RNA-seq, and unsupervised t-distributed stochastic neighbour embedding (tSNE) analysis (Figure 1a) allowed to recognized several BCP-ALL subtypes based on the expression of the first 1000 most variable genes and fusion transcripts. Patients with a Ph-like profile formed the largest group of 59/135 of B-other patients. The remaining cases clustered together with known groups and were denominates accordingly.

A significant poor risk was confirmed for our Ph-like patients’ cohort. In fact, Ph-like cases had a significantly inferior EFS (5-year figure (SE) of 59.9% (6.9) for Ph-like versus 85.2% (4.1) for non-Ph-like, p = 0.001), mainly due to a higher CIR (Table 1; Figure 2, panel a,
Figure 1. (a) Two dimensional tSNE plot of 289 paediatric BCP-ALL cases based on the top 1000 variable genes of gene expression data. The BCP-ALL subgroups are represented with different colours. Colour label indicated the different subgroups. Yellow highlights a subgroup of Ph-like patient carrying PAX5-translocations (PAX5t). Groups recognized as “like” cluster together with samples carrying the associated translocations, which is attributed to a similar gene expression profile. (b) Plot summarizing the molecular characterization of B-other cohort in childhood ALL: assessment of specific genetic subtypes, as reported in details in Supplementary Table S2.
Table 1: Clinical Characteristics and outcome of Ph-like vs. non-Ph-like subgroups, in AIEMO ALL 2000/R2006.

|                    | Ph-like |  | Non-Ph-like |  |
|--------------------|---------|---------|-------------|---------|
|                    | N       | %       | N           | %       |
| **Total n. of patients** | 59 | 43.7 | 76 | 56.3 |
| **GENDER** | | | | |
| Male | 36 | 61.0 | 37 | 48.7 |
| Female | 23 | 39.0 | 39 | 51.3 |
| *p*-value=0.15 | | | | |
| **AGE** | | | | |
| 1-5 yrs | 28 | 47.5 | 42 | 55.3 |
| 6-9 yrs | 11 | 18.6 | 23 | 30.2 |
| 10-17 yrs | 20 | 33.9 | 11 | 14.5 |
| *p*-value=0.02 | | | | |
| **WBC** | | | | |
| <20000 | 25 | 42.4 | 47 | 61.8 |
| 20-10000 | 28 | 47.4 | 20 | 26.3 |
| ≥20000 | 6 | 10.2 | 9 | 11.9 |
| *p*-value=0.04 | | | | |
| **Prednisone RESPONSE** | | | | |
| Good | 52 | 88.1 | 61 | 80.3 |
| Poor | 7 | 11.9 | 15 | 19.7 |
| *p*-value=0.22 | | | | |
| **MRD stratification** | | | | |
| Standard | 15 | 28.8 | 17 | 27.4 |
| Medium | 28 | 53.9 | 38 | 61.3 |
| High | 9 | 17.3 | 7 | 11.3 |
| Not known | 7 | 14 | | |
| *p*-value (NK excluded)=0.60 | | | | |
| **FINAL RISK** | | | | |
| Standard | 14 | 23.7 | 16 | 21.0 |
| Medium | 31 | 52.6 | 43 | 56.6 |
| High | 14 | 23.7 | 17 | 22.4 |
| *p*-value=0.89 | | | | |
| **Non Ph-like subgroups** | | | | |
| ETV6::RUNX1-like | 12 | 15.8 | | |
| HHD-like | 23 | 30.3 | | |
| KMT2A-like | 2 | 2.6 | | |
| ERG-related | 35 | 46.0 | | |
| MEF2C | 3 | 4.0 | | |
| Class-tie (not evaluable) | 1 | 1.3 | | |
| **Events** | | | | |
| Resistant | 0 | 0 | | |
| Death in Induction | 1 | 1.7 | 0 | |
| Relapses | 20 | 33.9 | 12 | 15.8 |
| - BM | 17 | 5 | | |
| - CNS | 1 | 0 | | |
| - BM-other | 2 | 7 | | |
| Death in CCR | 3 | 5.1 | 0 | |
| - After CHEMO | 2 | | | |
| - After HSCT | 1 | | | |
| SMN | 0 | 1 | 1.3 | |
| Alive in CCR | 35 | 59.3 | 63 | 82.9 | |

The Overall Survival is inferior in Ph-like vs. non-Ph-like (5-year, 75.4% vs. 94.6%, *p* = 0.003). The significantly poor EFS and OS were confirmed when excluding cases already classified as high risk (HR) (*p* = 0.02 and *p* = 0.049, Figure 2, panel b left and right), with a trend for higher CIR (Figure 2, panel b middle). In HR patients the 5-year EFS (SE) was 35.7% (12-8) for Ph-like vs. 76.5% (10-3) for non-Ph-like (*p* = 0.04, Figure 2, panel c left), with a trend for a difference in CIR (50-0% (13 4) vs. 17-6% (9-6), *p* = 0.08) (Figure 2, panel c right). Notably, the survival in Ph-like is 42-9% vs. 82-4% in non-Ph-like (*p* = 0.029). When medium risk (MR) and standard risk (SR) patients were considered separately, the Ph-like outcome was associated with worse prognosis only in MR (60-1% vs. 85-4%, *p* = 0.02, Supplementary Figure S2a and S2b). The EFS analysis of the most represented B-other subgroups (Ph-like, ERG-related, ETV6::RUNX1-like and HHD-like, whose clinical characteristics are described in Supplementary Table S1) confirmed that Ph-like patients show the worst outcome (Supplementary Figure S3a), even excluding HR patients (Supplementary Figure S3b). Events according to subgroups are shown in Supplementary Table S2. Of relevance, among 32/135 B-other cases experiencing relapse (23-7%), 20 were Ph-like (34%), as indicated in Table 1, and their clinical characteristics are described in Supplementary Table S2. Moreover, a Cox model was fitted on our cohort of 135 patients; given the relatively small sample, we adjusted for the main characteristics known as generally associated to outcome. A highly significant impact on EFS was confirmed for Ph-like (hazard ratio (HR)=2.93, *p*-value=0.003) as well as for assignment to final High-Risk group (HR=2.25, *p*-value=0.03), while age and WBC at diagnosis did not have a significant impact on outcome (Table 2).

In addition, we collected data from immunophenotype analysis of the complete cohort (N = 135) and we classified data according to phenotype profiles, according to Ph-like signature: Non-Ph-like: pre-pre-B: 9%; call: 60%; pre-B: 18%; preB/B: 1%; Biclonal B: 4% and B-lineage: 8%; whereas Ph-like were distributed as follows: pre-pre-B: 2%; call: 81%; pre-B: 14% and B-lineage: 3%. Thus, overall, the majority of cases were classified as having call phenotype in both groups.

In Ph-like patients, CNA’s were recurrent in leukaemia-associated genes, such as CDKN2A and/or CDKN2B (33-6%), PAX5 (25-2%), IKZF1 (24-4%), ETV6 (15-3%), RB1 (10% and CRFL2 (8-4%) (Figure 1b), with the IKZF-plus profile accounting for 7% of cases (Supplementary Table S4). The mean number of CNA events per Ph-like patient is almost double (2-77 events/pt) compared to ERG-related patients (1-53 events/pt) or non-Ph-like B-other patients overall (1-57 events/pt).

Fusion genes were identified in 30/38 (52%) Ph-like cases analysed (see heatmap of Figure 1b and Supplementary Table S4). Among those 30 fusion genes, 21
a. Ph-like vs non Ph-like: overall analysis

b. Ph-like vs non Ph-like: No high risk patients

c. Ph-like vs non Ph-like: High risk only patients

d. Ph-like patients accordingly to different class fusions

Figure 2. Outcome analysis of AIEOP B-Other cohort, in terms of EFS (on the left column) and CIR (in the middle column) and OS (in the right), comparing Ph-like vs. non-Ph-like patients overall (a), in no High Risk (b), in HR patients (c), respectively; (d) EFS, CIR and OS analyses according to the fusion classes reveal statistical significance in Ph-like cases, classified accordingly to different class fusions, such as ABL/JAK-class, PAX5t, other fusions and negative after NGS analysis.
accordingly to different class fusions, such as reveal statistical significance in Ph-like cases, classified NGS analysis. In fact, the PAX5t cases have a poor EFS, class, PAX5-class, other fusions and negative cases after (S6. As showed in Figure 2d, EFS (Validation primers are reported in Supplementary Table found in the Ph-like group (Supplementary Table S5). Notably, all the 7 PAX5t were Interestingly, also PAX5t gene lesions were mostly associated with the Ph-like signature. Considering CNAs and translocations, the PAX5 gene was involved in 43/131 B-other patients (32-8%), with 31/57 (54.3%) in Ph-like and 12/74 (16%) in non-Ph-like cases (Supplementary Tables S4 and S5). Notably, all the 7 PAX5t were found in the Ph-like group (Supplementary Table S5). Validation primers are reported in Supplementary Table S6. As showed in Figure 2d, EFS (p = 0.001) and OS (p = 0.009) analyses according to the fusion classes reveal statistical significance in Ph-like cases, classified accordingly to different class fusions, such as ABL/JAK-class, PAX5-class, other fusions and negative cases after NGS analysis. In fact, the PAX5t cases have a poor EFS, similar to ABL/JAK-class patients (53% and 50%, respectively) and lower than Ph-like cases negative for any fusion gene (78%). Moreover, PAX5t OS was inferior to ABL/JAK-class and negative cases (about 53% vs. 83-3% and 88-9%). However, CIR analysis was not different among the subgroups (p = 0.21). In details among PAX5t had 3 events out of 7, corresponding to BM relapse and they died later; among N = 12 ABL/JAK, they had one death post HSCT and 5 relapses (4 BM and 1 CNS) with only one death (the 4 non-deceased all have an adequate post-relapse follow-up).

These features of PAX5t cases prompted us to explore the feasibility of targeting patients with this aberration. By applying the NetBID2 data-driven network interference algorithms (Figure 3a), we constructed the BCP-ALL interactome and identified the hub drivers’ activity (DA) based on network gene interaction on either PAX5 translocated patients versus all BCP-ALL excluding Ph-like (Figure 3b, left column) or versus Ph-like (Figure 3b, middle column). We also assessed a Differential Expression (DE) of genes belonging to these pathways (Figure 3c). Interestingly, in the top NetBID2 drivers we identified the LCK signalling factor showing a significantly higher activity in PAX5t compared to other BCP-ALL subgroups (Figure 3d). Furthermore, analysis of LCK activity in BCP-ALL patients with known PAX5 status (N = 131) revealed that it is an exclusive signature of PAX5t cases, with statistically significance compared to all the categories, such as patients with amplification or deletion or being wild type for PAX5 gene (Figure 3e). Moreover, among the 22 genes resulted with a positive driver activity in PAX5t vs. Ph-like by NetBID2 analysis (p < 0.05) were subjected to DGIdbv4.2.0 software analysis to identified drug approved interactions and among them we found 7 genes that can be targeted, including LCK by

| Table 2: Cox model analysis on study cohort of 135 B-other patients. |
|----------------|----------------|----------------|
|                  | Hazard Ratio | p-value          |
| Ph-like          |                |                  |
| Non Ph-like      | 1              |                  |
| Ph-like          | 2.93           | 0.003            |
| Final risk       |                |                  |
| Non High Risk    | 1              |                  |
| High Risk        | 2.25           | 0.03             |
| Age at diagnosis |                |                  |
| 1-9 years        | 1              |                  |
| 10-17 years      | 1.10           | 0.80             |
| WBC at diagnosis |                |                  |
| <100000         | 1              |                  |
| ≥100000         | 1.16           | 0.78             |

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Figure 3. PAX5 translocated cases (or PAX5t) have a specific interactome signature. (a) Schema of NetBID2 analysis in BCP-ALL at diagnosis to identify driver in PAX5t BCP-ALL patients. To construct the BCP-ALL interactome the SJARCe algorithm was employed on gene expression profiles of BCP-ALL patients \((n = 289)\), and driver gene networks in PAX5t patients were identified in comparison with Ph-like or all other BCP-ALL subtypes. (b) Heatmap of the top 44 predicted driver’s activity (DA) and (c) differentially expressed (DE) in the BCP-ALL interactome based on PAX5t patients. Drivers were ranked according to \(p\)-value. Colour code was generated by z-score. (left column= PAX5t vs. other BCP-ALL subtypes; middle column= PAX5t vs. Ph-like, right column= Ph-like vs. other BCP-ALL subtypes). (d) Boxplot of LCK activity by NetBID2 analysis of PAX5 translocated patients compared between Ph-like and all other BCP-ALL subtypes using gene expression data. \(p\)-value was calculated using Welch T-test. (e) Boxplot of LCK activity in BCP-ALL patients with known PAX5 status \((N = 131)\). \(p\)-value was calculated by Welch t-test. PAX5amp: patients with amplification of PAX5 gene; PAX5del: patients with deletion of PAX5 gene; PAX5t: patients with translocation of PAX5 gene; PAX5wt: patients with PAX5 gene without aberrations. (f) Comparative cellular viability of different subgroups of leukemia (PAX5t, MLLt or CRLF2t) samples and control-B-lymphoblastoid cell lines (CTR-B-LCLS), measured by ATP-Glo based luminescent based assay after exposure of the depicted drugs, whereas Staurospsorin was taken as a positive control. Drug sensitivity scores (DSS) are plotted as a clustered heat map, followed by unsupervised hierarchical clustering. The horizontal and the vertical axis of the dendrogram illustrates the dissimilarity between clusters, whereas the color of the cell is related to its position along with a DSS gradient. The \(p\)-values are calculated with the one-way Anova test related to PAX5t group, **** \((p < 0.0001)\), *** \((p < 0.001)\), ** \((p < 0.01)\); * \((p < 0.05)\); ns = not significant.
Nintedanib (Supplementary Table S7 and Supplementary Figure S5a). Further, a positive enrichment of upregulated genes has been determined interacting with LCK signalling in these patients (Supplementary Figure S5b). These data pointed LCK-signalling as a potential signalling target in this subgroup of patients. Thus, in agreement with our previous data on Pax5 targeting by the kinase inhibitor BIBF1120/Nintedanib in murine preB1 cells and in primary patients’ cells,13 and sustained by independent studies in which Nintedanib is reported to be effective in LCK targeting as summarized in the Drug Gene Interaction Database (https://www.dgidb.org GENES/LCK_interactions), we developed a LCK targeting strategy in a preclinical model of Pax5 fusion PDX. Moreover, we applied a wider ex vivo drug screening with FDA-approved drugs and inhibitors in early to late clinical trials, and we selected Dasatinib, Bosutinib and Foretinib, known to be among the top most potent LCK ligands, as assayed by Kinome DiscoversRx study (http://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling/kinomescan)13-15 in addition to Nintedanib/BIBF1120. Here we demonstrated their potential therapeutic efficacy and specificity to (N = 3) Pax5t cases (carrying Pax5::AUTS2, Pax5::DACH2 and Pax5::SOX5, respectively), by evaluating the differential drug sensitivity score of Pax5t samples and including both MLLr (ALL-PO, KOPN8, RS4;11 and SEM) and CRLF2r cell lines (MUTZ5) and control B-cell lymphoblastoid cell lines (derived from healthy donors). As shown in Figure 3f, in Supplementary Figure S6 and Table S8, Nintedanib demonstrated to be the highest specific and effective compound to Pax5t, considering the statistics (One-way Anova test), compared to leukaemia cell lines and controls. We also tested other kinase inhibitors, not directly related to LCK but to BCR::ABL targeting, were not effective on Pax5t cases, whereas conventional chemotherapy drugs were effective, e.g., Dexamethasone, Vinristine among other (Supplementary Figure S6).

To investigate drug sensitivity and efficacy, we assessed apoptosis in leukemic cells. We performed ex vivo cell incubation by BIBF1120 alone or in combination with chemotherapeutic drugs used as standard therapy in ALL treatment protocols, such as Dexamethasone, Vinristine or Asparaginase. Figure 4 shows a representative experiment in four different Pax5-fusion BCP-ALL samples at 48h (2 Pax5::AUTS2 pt.1 and pt.2, Pax5::DACH2 pt.3 and Pax5::SOX5 pt.4). After treatment viability has been determined, as difference from apoptosis and necrosis, by Annexin V-PE and 7-AAD staining. BIBF1120 had a synergistic effect with either Dexamethasone or Vinristine, rather than additive with Asparaginase (Combination index values are reported in Supplementary Figure S7 a-b, as Bliss score), specifically in cases carrying Pax5 fusions, whereas it did not show any activity in Pax5-deleted or -wild type cases, regardless of their Ph-like profile (N = 4 cases, Supplementary Figure S7 panel c).

According to the synergistic effect of dexamethasone and BIBF1120 in ex vivo experiments, their efficacy was further assessed in the in vivo PDX mouse model. The BIBF1120 dose finding experiments were carried out in secondary transplants of Pax5::AUTS2 pt.1, determining 40mg/kg as the most effective dose of BIBF1120 in absence of toxicity, phenotypic symptoms or weight loss (data not shown). Further, as schematically shown in Figure 5a, we performed secondary transplants with cells carrying either Pax5::AUTS2 (pt.1) or Pax5::DACH2 (pt.3). At bulk disease detection (hCD10/CD19 positive cells >10%, by BM aspiration), animals were randomized into four groups (vehicle, BIBF1120 alone, dexamethasone alone, combination) and we started treatment.

In Pax5::AUTS2 pt.1, the daily treatment was started at day14 with 42% hCD10 positive cells mean engraftment detection in BM (ranging from 23-3 to 60-63%, Figure 5b). After two weeks, we sacrificed animals and assessed the disease levels as percentage of tumour cells in each organ, as reported in Figure 5 panels from c to g and the mean engraftment in vehicle BM was 82-6% (range 75-86.7%). As shown in Figure 5c, in the Pax5::AUTS2 PDX mice we detected a mild effect in the BM of BIBF1120 alone (disease reduction of 24%, p = 0.057), further enhanced by the combination with dexamethasone (-49%, p = 0.005, with a mean engraftment in vehicle mice of 82-6%). In the spleen (Figure 5d and S8c), the efficacy was highly significant both for BIBF1120 (-52%, p = 0.025) and the combination (-91%, p = 0.015, mean engraftment vehicle 69-5%). We also observed a macroscopic effect on spleen weight decrease by the combination treatment (Figure 5e). A similar statistically significant effect was observed also in peripheral blood (Figure 5f and S8d), whilst, BIBF1120 alone showed a specific significant efficacy in CNS meninges, considering both percentages and absolute number of leukemic cells, as showed in Figure 5g and S8e, respectively. Moreover, data were further confirmed by evaluation of the tumour burden, defined as the absolute number of human CD10 positive (hCD10+) leukemic cells over the total number of cells in each organ (BM, spleen, CNS and PB), as shown in Supplementary Figure S8 panel from b to e.

The efficacy of treatment was further confirmed in the Pax5::DACH2 PDX mice. We started treatment at day21, with 15% BM Engraftment (range 10-34.3%, Figure 5h). After two weeks, the number of human leukemic cells in BM decreased of 47% using BIBF1120 alone (p = 0.02 as % and p = 0.004 as n° cells, Figure 5i and S8f, respectively), further diminished by the combination (70% vs. vehicle, p = 0.001 as % and p = 0.00001 as n° cells, with a mean engraftment in vehicle mice of 65%, ranging from 53-3 to 70%) (Figure 5i and S8f). As shown in Figure 5i and...
Supplementary Figure S8g, in the spleen, the efficacy was highly significant both with BIBF1120 (−45.6%, $p = 0.0003$ as % and $p = 0.03$ in cells) and the combination (−97.5% tumour burden, $p = 0.0008$, mean engraftment vehicle 72.4%), with considerable effects on spleen weight (Figure 5m). Strikingly, BIBF1120 treatment alone showed similar efficacy in PB and CNS, with leukaemia decrease as much as −45% ($p = 0.04$) and −76% ($p = 0.007$), respectively. Dexamethasone alone was not effective in the BM and spleen (Figures 5i and 5l), whereas it decreased the leukaemia bulk both in PB (−65%, $p = 0.004$, Figure 5n) and CNS (−52.8%, $p = 0.03$, Figure S8j). Importantly, the combination with BIBF1120 nearly achieved remission in PB (−94%, $p = 0.0001$, Figure 5n and S8h) and it was significant in CNS (−84.7%, $p = 0.005$, Figure 5o and S8i).

Since the AKT signalling pathway was activated downstream LCK via the PIP3 signalling pathway,34 we hypothesised a molecular regulation mechanism involving both kinases, as depicted in Figure 6 panel a. To sustain this molecular regulation, we investigated the in vivo Nintedanib/BIBF1120 treatment both in four

**Figure 4.** *Ex vivo* treatment by BIBF1120 either alone or in combination with Dexamethasone (a), Vincristine (b) or Asparaginase (c). Experiments have been performed in presence of different PAX5 fusion genes, such as PAX5::AUTS2 in Pt. n°1 and n°2, or alternatively carrying PAX5::DACH2 as in Pt. n°3 or PAX5::SOX5 in Pt. n°4. After drug treatment, viability has been determined, as difference from apoptosis and necrosis, by Annexin V-PE and 7-AAD staining. Untreated samples (ut) have been analysed as internal control.
Figure 5. In vivo treatment by BIBF1120 either administered alone or in combination with Dexamethasone. The experimental plan is depicted in panel (a): NSG mice were transplanted with human leukemic cell carrying different PAX5 fusion genes, namely PAX5::AUTS2 as in Pt. n°1 (panels b-g) or PAX5::DACH2 as in Pt. n°3 (panels h-o). Human leukaemia engraftment was evaluated in hematopoietic tissues prior to start treatment in Bone Marrow and Peripheral Blood (panels b and h, in both patients, respectively) and after two weeks of treatment, in all hematopoietic organs, such as Bone Marrow for Pt.1 (panel c) and Pt.3 (panel l), in Spleen as percentage (panel d and i) and weight (panels e and m), in Peripheral Blood, f and n) and in Central Nervous System Meninges (g and o). Data are expressed as percentages of human CD10+ cells. Statistical analyses have been performed by GraphPad Prism software and T test is shown as *p < 0.05, **p < 0.01, ***p < 0.001.
patients’ primary cells and in PAX5::AUTS2 mice (Supplementary Figure S9). In PDX cells phoshoflow analysis showed a marked inhibition of pAKT-Thr308 (-29%, p < 0.01) and the downstream effectsors pS6 (-64%, p < 0.001) and 4pEBP1 (-26%, p < 0.05), comparing BIBF1120 vs. vehicle group (Figure 6b). Inhibitor treatment mildly affected pAKT-Ser473 (-9%) and PDK1 (-13%, p < 0.05) phosphorylation levels. As shown in Figure 6c, we confirmed results in PAX5::DACH2 mice, demonstrating a reliable AKT inhibition, with consistent decreased phosphorylation levels of both pAKT-Thr308 (-34%, p < 0.01) and pAKT-Ser473 (-61%, p < 0.001), respectively. Moreover, also PDK1 (-37%, p < 0.05), 4pEBP1 (-38%, p < 0.001) and pS6 (-78%, p < 0.0001) were decreased.

Discussion

After a first definition,1,2 several studies identified the BCR/ABL1-like or Ph-like group both in paediatric and in adult cohorts of BCP-ALL, demonstrating an association with poor MRD response and overall outcome.3 Thus, the terms ‘BCR::ABL1-like’ or Ph-like have been included as provisional entity in the 2016 WHO classification of ALL.3 In the present study, we identified and characterized the Ph-like subgroup in an AIEOP cohort of BCP-ALL patients, enrolled into the AIEOP-BFM ALL2000/R2006 protocol.21 A Ph-like signature was characterized the Ph-like subgroup in an AIEOP cohort (around 50%), and an inferior OS (about 53% vs. 83%) unacceptable for childhood ALL. Likewise, we recently reported that PAX5 fusions are recurrent and associated with poor outcome in infant ALL patients (<1 year at diagnosis), not carrying a KMT2A/MLL rearrangement.17

Importantly, and in agreement with our previous studies,14,15 we demonstrated that PAX5 fusions are characterized by LCK activity upregulation and are targetable by the Nintedanib/BIBF1120 inhibitor.14,15,17,20 Ex vivo treatment with Nintedanib/BIBF1120 demonstrated to promote apoptosis of leukemic cells, both in monotherapy and in combination with chemotherapy agents (Vincristine, Dexamethasone or Asparaginase). Strikingly, Dexamethasone and BIBF1120 had a synergistic effect, as further assessed in vivo assays in PDX NSG mice with two different PAX5 fusions (PAX5::AUTS2 and PAX5::DACH2), in which we observed a significant efficacy in different organs by BIBF1120 alone and in drug combination. Overall BIBF1120 alone was more effective than Dexamethasone in reducing tumour burden. Furthermore, ex vivo drug screening revealed differential sensitivity of Nintedanib and Dasatinib against PAX5t leukemia samples when compared to MLLt/CRLF2t samples. Our findings are also in agreement with the role and targeting of LCK in T-ALL setting, in which they demonstrate both the role of LCK in pathogenesis and how it is possible to target with specific inhibitors such as Dasatinib and Nintedanib, through gene silencing LCK in Patient-Derived Xenograft model.15,19

Moreover, we demonstrated that BIBF1120 was also effective on the AKT signalling in PAX5t, by inhibiting both the pAkt-Thr308 and pAkt-Ser473 residues, essential to activate the pathway.16 This finding reveals a role for alternative mechanisms of PAX5t leukaemia, considering that AKT is involved in apoptosis, metabolism and multidrug resistance in CLL and other malignancies.34

In conclusions, PAX5 lesions are the most recurrent aberrations in Ph-like BCP-ALL, they have a specific signature and an unacceptable prognosis; PAX5t involve
multiple pathways that can be targeted by Nintedanib/BIBF1120. The drug could be inserted in a second line of treatment, both to allow the identification of the patient’s profile and to integrate this information with the MRD analysis data. In this way it could be possible to assign these patients to an experimental group, where they receive this inhibitor in addition to the chemotherapy drugs already in use. This drug has an acceptable tolerability profile and is already approved in clinical trials for idiopathic pulmonary fibrosis by the US Food and Drug Administration (FDA) since October 2014 and by the European Medicines Agency (EMA) since January 2015, and it is tested in several cancers, with anti-angiogenic and anti-tumour effects. These results strongly encourage further studies for a tailored treatment of patients with PAX5 fusions, implementing the deployment of inhibitors in combination with chemotherapy.

Contributors
G.F., S.B. and G.C. initiated the project; G.F. and S.B. performed experiments; D.S. and M.G.V. collected patients data and performed survival analyses; E.V. and S.B. developed the signature classification; A.G. and A.P. performed bioinformatic analyses; S.R., C.S. and M.Q. performed NGS analyses and RT-PCR validation tests; C.P. and M.G. completed the CNA analyses; G.F., C.S., M.Q., C.P., M.B., S.M., A.M.S. developed the in vivo work; C.S. and M.Q. setup the ex-vivo and

**Figure 6.** LCK and Akt pathway involvement in leukemic cells with PAX5 fusion genes. (a) A schematic view of molecular hypothesised mechanism of LCK and Akt interaction. After in vivo BIBF1120 treatment vs. vehicle mice, in (b) pt.#1 PAX5::AUTS2 and (c) pt.#3 PAX5::DACH2, respectively. PDK1, pAKT-Ser473 and -Thr308, pS6 and 4eEBP1 are analysed by FACS. Data are expressed as ratio of Phosphorylation levels, considering vehicle phosphoprotein levels equal to 1 (dotted line) and symbols are correspondent to single BIBF1120-treated mice. Statistical analyses have been performed by GraphPad Prism software and T test is shown as *p < 0.05, **p < 0.01, ***p < 0.001.
phosphoflow experiments; B.B. performed immunophenotypic analyses; S.Bh., J.W.T. and A.B. developed and analysed drug screening; C.M., L.L.N., F.L., V.C., C.R. and A.Bi. provided access to patients’ data, treated in their centres; G.F., S.B., G.tk. and G.C. analysed data and wrote the paper, with contributions from all authors. G.F., S.B., D.S., M.G.V., G.tk. and G.C. have verified the underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement

GEP data are available at GEO (accession numbers GSE79547, GSE13164, GSE13159, GSE13204).

Target capture Next Generation Sequencing FASTQ files are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-11319.

Declaration of interests

The authors declare no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104224.

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