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Rapid identification of *BCR/ABL1*-like acute lymphoblastic leukaemia patients using a predictive statistical model based on quantitative real time-polymerase chain reaction: clinical, prognostic and therapeutic implications

Sabina Chiaretti[1,*], **Monica Messina**[1,*], **Sara Grammatico**[1], **Alfonso Piciocchi**[2], **Anna Lucia Fedullo**[1], **Filomena Di Giacomo**[1,3], **Nadia Peragine**[1], **Valentina Gianfelici**[1], **Alessia Lauretti**[1], **Rohan Bareja**[4], **Maria Paola Martelli**[5], **Marco Vignetti**[1], **Valerio Apicella**[1], **Antonella Vitale**[1], **Loretta S. Li**[6], **Cyril Salek**[7], **Olivier Elemento**[4], **Giorgio Inghirami**[3], **David M. Weinstock**[8], **Anna Guarini**[9], and **Robin Foà**[1]

[1]Haematology, Department of Cellular Biotechnologies and Haematology, “Sapienza” University, Rome, Italy

[2]GIMEMA Data Centre, GIMEMA Foundation, Rome, Italy

[3]Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

[4]Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY, USA

[5]Institute of Haematology, Centro Ricerche Onco-Ematologiche (CREO), University of Perugia, Perugia, Italy

[6]Department of Paediatric Haematology/Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

[7]Institute of Haematology and Blood Transfusion, Prague, Czech Republic

[8]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

[9]Department of Molecular Medicine, “Sapienza” University, Rome, Italy

SUMMARY

BCR/ABL1 -like acute lymphoblastic leukaemia (ALL) is a subgroup of B-lineage acute lymphoblastic leukaemia that occurs within cases without recurrent molecular rearrangements. Gene expression profiling (GEP) can identify these cases but it is expensive and not widely available. Using GEP, we identified 10 genes specifically overexpressed by *BCR/ABL1*-like ALL cases and used their expression values - assessed by quantitative real time-polymerase chain reaction (Q-RT-PCR) in 26 *BCR/ABL1*-like and 26 non-*BCR/ABL1*-like cases to build a

Corresponding Authors: Sabina Chiaretti and Robin Foà, Haematology, Department of Cellular Biotechnologies and Haematology, “Sapienza” University, Via Benevento 6, 00161 Rome, Italy, Tel +39 06 441639824; Fax +39 06 44241984, chiaretti@bce.uniroma1.it; rfoa@bce.uniroma1.it.

*SC and MM equally contributed equally to this work.

DR. SABINA CHIARETTI (Orcid ID: 0000-0001-8499-4492)

DR. MONICA MESSINA (Orcid ID: 0000-0003-3531-4766)

statistical "*BCR/ABL1*-like predictor", for the identification of *BCR/ABL1*-like cases. By screening 142 B-lineage ALL patients with the "*BCR/ABL1*-like predictor", we identified 28/142 *BCR/ABL1*-like patients (19.7%). Overall, *BCR/ABL1*-like cases were enriched in *JAK/STAT* mutations ($p<0.001$), *IKZF1* deletions ($p<0.001$) and rearrangements involving cytokine receptors and tyrosine kinases ($p=0.001$), thus corroborating the validity of the prediction.

Clinically, the *BCR/ABL1*-like cases identified by the *BCR/ABL1*-like predictor achieved a lower rate of complete remission ($p=0.014$) and a worse event-free survival ($p=0.0009$) compared to non-*BCR/ABL1*-like ALL. Consistently, primary cells from *BCR/ABL1*-like cases responded *in vitro* to ponatinib.

We propose a simple tool based on Q-RT-PCR and a statistical model that is capable of easily, quickly and reliably identifying *BCR/ABL1*-like ALL cases at diagnosis.

Keywords

Acute lymphoblastic leukaemia; *BCR/ABL1*-like; adults; prognosis; tyrosine kinase inhibitors

INTRODUCTION

Among B-lineage acute lymphoblastic leukaemia (ALL), *BCR/ABL1*-like ALL is one of the most clinically relevant subsets because it is characterized by a poor outcome and could potentially benefit from the use of tyrosine kinase inhibitor (TKI) therapy (Ofran & Izraeli, 2017). Mullighan *et al* (2009a) identified a subgroup of paediatric B-lineage ALL (B-ALL) with a gene expression profile similar to that of *BCR/ABL1*-positive patients, frequent *IKZF1* deletions and poor outcome. Simultaneously, Den Boer *et al* (2009) performed gene expression profiling (GEP) analysis on a cohort of paediatric B-ALL cases and termed this subgroup *BCR/ABL1*-like; it represented 15–20% of B-ALL cases, showed an unfavourable outcome and was associated with *IKZF1, TCF3, EBF1, PAX5* and *VPREB1* deletions, and upregulation of *CRLF2*.

Subsequently, the *BCR/ABL1*-like ALL subgroup has been extensively evaluated, particularly in paediatric cohorts (Mullighan *et al*, 2009b; Mullighan *et al*, 2009c; Harvey *et al*, 2010a; Harvey *et al*, 2010b; Yoda *et al*, 2010; Chen *et al*, 2012; van der Veer *et al*, 2013; Asai *et al*, 2013) and more recently in adults (Tokunaga *et al*, 2013; Boer *et al*, 2015a; Herold *et al*, 2017; Roberts *et al*, 2017; Jain *et al*, 2017a). In addition to the association with *IKZF1* deletions and *CRLF2* deregulation, Roberts *et al* (2012) unveiled that kinase activating alterations characterize the majority (91%) of paediatric *BCR/ABL1*-like ALL cases. The most frequent alterations involve *ABL1*, *JAK2*, *PDGFRB*, *CRLF2* and *EPOR*, activating mutations of *IL7R*, *FLT3*-internal tandem duplication mutations and deletion of *SH2B3* (Roberts *et al*, 2012; Roberts *et al*, 2014a). Several of these alterations are targeted by TKIs, suggesting a potential role for tailored treatment (Roberts *et al*, 2012; Roberts *et al*, 2014a). Furthermore, all authors confirmed an association with poor outcome, while the relationship with minimal residual disease (MRD) is still debated (Roberts *et al*, 2014b; Heatley *et al*, 2017).

Despite this in-depth genetic characterization, the identification of *BCR/ABL1*-like patients is still challenging: it relies on GEP and/or a multistep approach, such as a combination of next generation sequencing (NGS) and fluorescence *in situ* hybridisation (FISH) (Roberts *et al*, 2014a). However, GEP is largely cohort/experiment-dependent and a consensus diagnostic signature has not been agreed upon (Boer *et al,* 2015b). Similarly, NGS is costly and requires bioinformatic skills. Alternatively, these cases can be recognized with Low Density Assay (LDA) of selected genes, but the full methodology has not been published (Harvey *et al*, 2013). Thus, this study aimed to: i) produce an easy, rapid and reproducible assay, based on quantitative real time-polymerase chain reaction (Q-RT-PCR) analysis, for the recognition of *BCR/ABL1*-like ALL cases; ii) define the molecular background, clinico-biological features and outcome of the cases thus identified; and iii) verify the *in vitro* response of primary *BCR/ABL1*-like ALL cells to the pan-TKI, ponatinib. The final goal is to provide a rapid, user-friendly and economically-viable diagnostic tool that can recognize these cases at presentation, a step towards a refined prognostic and therapeutic management of these poor prognosis patients.

METHODS

Identification of *BCR/ABL1*-like cases and genes

To identify the core *BCR/ABL1*-like cases and *BCR/ABL1*-like specific genes (Figure S1), we used two *in house* GEP cohorts: GEP1, run on HG-U133 plus2 (Affymetrix, Santa Clara, CA, USA) - comprising 148 B-ALL cases (70 *BCR/ABL1*-positive and 78 B-NEG, i.e. negative for *BCR/ABL1*, *ETV6/RUNX1*, *TCF3/PBX1* and *KMT2A* rearrangements) (Haferlach *et al*, 2010; Messina *et al*, 2010) - and GEP2 - including 79 cases (37 *BCR/ABL1*-positive and 42 B-NEG) (Chiaretti *et al*, 2005) - evaluated with an older version of the array (HG-U95 Av2, Affymetrix). In both GEP1 and GEP2, a t-test between *BCR/ABL1*-positive and B-NEG cases was performed to recognize the *BCR/ABL1*-like ALL cases. B-NEG cases clustering within the *BCR/ABL1*-positive cluster were regarded as the "core" *BCR/ABL1*-like samples.

To select the *BCR/ABL1*-like genes (Figure S2), the 16 "core" *BCR/ABL1*-like ALL cases of GEP1 were compared with the remaining 62 B-NEG cases by t-test. The genes selected by this approach were compared with the literature (Harvey *et al*, 2010b) and the overlapping genes, together with *CRLF2* (Yoda *et al*, 2010; van der Veer *et al*, 2013; Herold *et al*, 2017; Chiaretti *et al*, 2016), were used to build the Q-RT-PCR-based *BCR/ABL1*-like predictor. GEP analyses are detailed in Supplementary material.

Development and validation of the "*BCR/ABL1*-like predictor"

To generate the *BCR/ABL1*-like predictor, we validated the expression levels of the selected genes in the discovery cohort, including 26 core *BCR/ABL1*-like samples (16 from GEP1 and 10 from GEP2) and 26 non-*BCR/ABL1*-like selected from GEP1. The quantification of transcript levels was performed by Q-RT-PCR (Supplementary material and Table S1) and computed as $2^{-\Delta Ct}$.

Q-RT-PCR results were used to build the "*BCR/ABL1*-like predictor", extensively described in the Supplementary material. Briefly, expression values were shrunk into principal components (PCs) and a logistic regression model was used to examine the association among the PCs and *BCR/ABL1*-like ALL cases. Subsequently, a score on PCs was built and used to classify 142 additional B-NEG ALL cases, representing the screening panel.

The analysis of the genetic and clinical features was performed on a total of 194 B-NEG ALL, 52 belonging to the discovery and 142 to the screening panels (Table S2 and Supplementary material). Patients were enrolled in Gruppo Italiano Malattie EMatologiche dell'Adulto (GIMEMA) and Associazione Italiana di Ematologia ed Oncologia Pediatrica (AIEOP) protocols (Table S3).

The study was approved by the local IRB, in accordance with the Helsinki Declaration.

Analysis of genetic features

Recurrently mutated JAK/STAT and RAS pathways genes (Messina *et al*, 2016) were sequenced in 182/194 samples (Table S4). Copy number aberrations (Messina *et al*, 2017) were assessed by multiplex ligation-dependent probe amplification (MLPA) in 111/194 samples. RNA-sequencing was performed in 54 samples by the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). RNA libraries were paired-end sequenced (2×100 bp) using the Illumina HiSeq2500 platform. Identification of fusion transcripts was performed by STAR-fusion (STAR-Fusion_v0.5.1) and Fusioncatcher (v0.99.3e) (Nicorici *et al*, 2014).

Statistical analysis of clinical features and event-free survival (EFS)

Patients' characteristics were compared by chi-squared or Fisher's exact test for categorical variables and by Wilcoxon test for continuous data. Event-free survival (EFS) and overall survival (OS) was estimated by Kaplan-Meier: EFS was estimated from the time of diagnosis to the occurrence of refractoriness, relapse or death, while OS was estimated from the time of diagnosis to death. Multivariate analysis was performed using the Cox proportional model to adjust the effect of *BCR/ABL1*-like ALL cases to white blood cell (WBC) count, age and *CRLF2* on EFS. All tests were two-sided and P values <0.05 were considered statistically significant. Analyses were performed using the SAS software (release 9.4; SAS Institute, Cary, NC, USA).

In vitro experiments

To assess the sensitivity to ponatinib, the annexin V/7-aminoactinomycin D (7AAD) apoptotic test (BD Bioscience, San Josè, CA) and ^{3}H-thymidine (Perkin Elmer, Waltham, MA) proliferation assays were performed on primary cells from 7 *BCR/ABL1*-like, 6 non-*BCR/ABL1*-like and 4 *BCR/ABL1*-positive cases. Ponatinib (Selleck Chemicals, Houston, TX) at 0 μM (dimethyl sulfoxide only) or at increasing doses (0.01–10 μM) was added at time 0 and viability was measured after 48 and 72 h (Supplementary data).

RESULTS

Identification and quantification of *BCR/ABL1*-like predictor genes in the discovery cohort

The comparison of *BCR/ABL1*-positive and B-NEG cases of GEP1 (Haferlach *et al*, 2010; Messina *et al*, 2010) using a t-test recognized 16 B-NEG cases that clustered with the *BCR/ABL1*-positive cases: these 16 misclustered B-NEG cases were regarded as the "core" *BCR/ABL1*-like samples (Figure S2).

To select the *BCR/ABL1*-like genes (Figure S2) the "core" *BCR/ABL1*-like ALL cases were compared with the remaining B-NEG cases by t-test, that resulted in the identification of 285 genes (Table S5), of which 9 had been previously reported as part of the *BCR/ABL1*-like signature (Harvey *et al*, 2010b). These 9 genes (*SOCS2*, *IFITM1*, *CD99*, *TP53INP1*, *IFITM2*, *JCHAIN*, *NUDT4*, *ADGRE5* and *SEMA6A*) together with *CRLF2* (Yoda *et al*, 2010; van der Veer *et al*, 2013; Herold *et al*, 2017) were used to build the Q-RT-PCR-based *BCR/ABL1*-like predictor.

By analysing GEP2 (Chiaretti *et al*, 2005) we selected 10 additional *BCR/ABL1*-like ALLs, leading to a total of 26 *BCR/ABL1*-like cases that were included in the discovery panel of this study.

Next, the expression levels of these 10 genes were quantified by Q-RT-PCR in the discovery cohort (n=52), comprising 26 *BCR/ABL1*-like and 26 non-*BCR/ABL1*-like ALLs, classified according to GEP (Chiaretti *et al*, 2005; Haferlach *et al*, 2010; Messina *et al*, 2010). All genes were significantly overexpressed in *BCR/ABL1*-like ALL samples (Figure S3).

Development of the "*BCR/ABL1*-like predictor"

The Q-RT-PCR expression values of the 10 genes were used to build the "*BCR/ABL1*-like ALL predictor". First, we verified that in univariate analysis, all genes were risk factors for a higher *BCR/ABL1*-like ALL probability (Table S6). As a high correlation was detected among the expression levels of the 10 genes (Figure S4), it was possible to summarize the variability by means of PC analysis; by definition, the PCs thus identified are uncorrelated. In detail, expression values were shrunk into 3 PCs (accounting for >80% of the variability): the contributions of each gene to each component are expressed by factor loadings, a measure of their relationship (Table S7). Each component is mainly explained by genes with the highest loadings: PC1 is explained by the expression values of *NUDT4*, *SEMA6A*, *ADGRE5*, *SOCS2* and *JCHAIN*, PC2 by *CRLF2*, *TP53INP1*, *CD99* and PC3 by *IFITM1* and *IFITM2*.

Second, a logistic regression model was used to estimate the probability of a case being *BCR/ABL1*-like using the first 3 PCs; all components were statistically significant in multivariate analysis (Table S8).

Finally, to generate a predictive model, a score was computed by means of a linear combination of the PCs, as a result of the above mentioned multivariate logistic regression model. Figure S5 illustrates the generation of the score. The optimal cut-off was set at −0.30: cases with a score ≥−0.30 were defined as *BCR/ABL1*-like. This cut-off provides the best

distinction between *BCR/ABL1*-like and non-*BCR/ABL1*-like cases and ensures the optimal compromise between sensitivity and specificity (88.5% and 100%, respectively). The mathematical equation is provided in Supplementary material.

An online *BCR/ABL1*-like score calculator was implemented into the web-based application GIMEMA REDCap system (Harris *et al* 2009).

Identification of the *BCR/ABL1*-like ALL cases and genomic characterization

The predictive model was then validated in a screening cohort of 142 B-NEG ALL patients: 28 cases (19.7%) were classified as *BCR/ABL1*-like (min score −0.279, max score 2.176) and 114 as non-*BCR/ABL1*-like ALL (min score −1.810, max score −0.353). The comparison of the clinico-biological features of *BCR/ABL1*-like and non-*BCR/ABL1*-like ALL cases at diagnosis, carried out in the whole cohort, showed that *BCR/ABL1*-like ALLs were associated with a significantly higher WBC count at diagnosis (22.9 *vs* 12.6 $x10^9$/L, p=0.013) while no differences were observed for the other parameters (Table I).

Recurrently mutated genes were investigated in 182/194 cases. Mutations of the JAK/STAT pathway (i.e. *JAK1/2*, *CRLF2* and *IL7R*) were detected in 44.2% (23/52) of *BCR/ABL1*-like cases and only in 7.7% (10/130) of the non-*BCR/ABL1*-like cases (p<0.001), RAS pathway mutations were more frequent in non-*BCR/ABL1*-like (n=38/130, 29.2%) than in *BCR/ABL1*-like cases (n=9/52, 17.3%, p=0.068). Details are provided in Table II.

We also examined copy number aberrations in 111 cases: *IKZF1* deletions were significantly more frequent in *BCR/ABL1*-like than non-*BCR/ABL1*-like cases (82.8% *vs* 40.8%, p<0.001). We found that *EBF1* and *BTG1* deletions were significantly more frequent in *BCR/ABL1*-like cases (Table II).

Finally, *CRLF2* levels were significantly higher (p≤0.001) in *BCR/ABL1*-like (median ΔCt = 6.7, range 0.6–16.6) than in non-*BCR/ABL1*-like cases (median ΔCt = 11.3, range 3.2–17.9). Complete results are detailed in Tables S9 and S10. Comparable results were obtained when separating the discovery (Table S11) and screening cohorts (Table III).

RNA-sequencing, performed in 54 samples (28 *BCR/ABL1*-like and 26 non-*BCR/ABL1*-like), identified 13 fusion transcripts targeting tyrosine kinases (TKs)/cytokine receptors, of which 12 were detected in *BCR/ABL1*-like cases (12/28 *vs* 1/26 p=0.001). The most recurrent fusion was *EBF1/PDGFRB* found in 3 cases; *JAK2* rearrangements with different partners (i.e. *PAX5*, *EBF1*, *SSBP2*) were detected in 3other cases, *P2RY8/CRLF2* and *TSLP*-fusion genes in 2 samples; finally, *RCSD1/ABL1* and *TRIM24/FGFR1* were found in 1 case each. Within non-*BCR/ABL1*-like cases, 1 case harboured *P2RY8/CRLF2* while no fusion genes targeting TKs were documented. Figure 1 shows that 27/28 (96.4%) had at least one lesion typical of the *BCR/ABL1*-like profile and suggests that the *BCR/ABL1*-like profile is sustained by at least 2 different mechanisms: one represented by TK-rearrangements only and the other by *CRLF2* overexpression plus *JAK/STAT* mutations.

Outcome of the *BCR/ABL1*-like ALL samples

We analysed the complete remission (CR) rate and survival in the adolescents and adults with clinical data available (n=142, Table S3)

In the whole cohort, the CR rate was lower in *BCR/ABL1*-like than in non-*BCR/ABL1*-like cases (77.8% *vs* 89.6%, p=0.06).

Consistently, EFS at 36 months was significantly inferior for *BCR/ABL1*-like cases compared to non-*BCR/ABL1*-like cases (21.6% *vs* 47.2%, p<0.0001; Figure 2A).

When we considered the screening cohort only (n=95), we confirmed that the CR rate was significantly lower in *BCR/ABL1*-like than in non-*BCR/ABL1*-like cases (71.4% *vs* 91.8%, p=0.014). Similarly, EFS at 36 months was significantly inferior for *BCR/ABL1*-like cases compared to non-*BCR/ABL1*-like cases (21.3% *vs* 43.3%, p=0.0009; Figure 2B).

In line with EFS estimates, OS at 36 months was significantly inferior for *BCR/ABL1*-like cases compared to non-*BCR/ABL1*-like cases (37.3% *vs* 60.7%, p=0.05; Figure S6A) in the whole cohort and a similar trend was observed in the screening cohort only (Figure S6B). The impact of *BCR/ABL1*-like prediction retained statistical significance on EFS in multivariate analysis (Hazard ratio: 2.12, 95% confidence interval: 1.18–3.82, p=0.01) in a model adjusted for age and WBC count. We also evaluated the interaction on EFS between the *BCR/ABL1*-like signature and *CRLF2* overexpression: *BCR/ABL1*-like prediction retained statistical significance on EFS (p=0.05) in the bivariate model adjusted by *CRLF2* overexpression.

In vitro sensitivity to ponatinib

After 72 h of incubation with ponatinib (0.01 μM), a 3H-thymidine uptake assay showed that the proliferation rate of primary cells from 7 *BCR/ABL1*-like cases (2 *EBF1/PDGFRB*-positive, 1 *JAK2*-mutated and *P2RY8/CRLF2*-positive, 1 *RCSD1/ABL1*, 3 wild type WT for JAK/STAT and RAS mutations) decreased to 33.6%±15%, comparable to the sensitivity observed in 4 *BCR-ABL1*-positive cases (44.3%±12.4%). Contrarily, in the non-*BCR-ABL1*-like (non-*BCR-ABL1*-positive) samples (n=6) - all WT for JAK/STAT and RAS hotspot mutations - the proliferation rate upon ponatinib treatment decreased only to 71.5% ±28.7% (p=0.0007) (Figure 3A). Ponatinib (0.01 μM) also increased the apoptotic rate in both *BCR/ABL1*-like and *BCR/ABL1*-positive primary ALL cells (22.1%±10%, 19.9% ±8.5%, respectively), while the apoptotic response in non-*BCR/ABL1*-like/non-*BCR/ABL1*-positive ALL was significantly inferior (6.1%±8.4%, p=0.023, Figure 3B)

Discussion

Although the recognition of the *BCR/ABL1*-like ALL subset dates back to 2009, a consensus on a *BCR/ABL1*-like signature has not been reached (Mullighan *et al*, 2009a; Den Boer *et al*, 2009) and a standardized tool to identify these cases is currently not available (Ofran & Izraeli, 2017). Roberts *et al* (2014a) and Fasan *et al* (2015) proposed a combination of different methods: analysis of *CRLF2* expression, FISH targeting *ABL1* and *JAK* activating rearrangements, fusion-specific RT-PCR for the identification of the *ABL*

and *JAK* partners and MRD monitoring. However, this approach relies on multiple techniques and can only recognise cases carrying already known fusion transcripts.

Simultaneously, Harvey *et al* (2013) developed a method based on the quantification of 15 transcripts by LDA and several groups adopted this method (Heatley *et al*, 2017; Reshmi *et al*, 2017). However, the mathematical equations were not provided. Our approach took advantage of previously reported GEP data (Messina *et al*, 2010; Chiaretti *et al*, 2005) to identify a narrow list of 10 transcripts (*CRLF2*, *SOCS2*, *IFITM1*, *CD99*, *TP53INP1*, *IFITM2*, *JCHAIN*, *NUDT4*, *ADGRE5*, *SEMA6A*), capable of accurately identifying the *BCR/ABL1*-like ALLs.

The genes chosen to build the predictive model are also in common with other algorithms recently used to identify *BCR/ABL1*-like cases (Roberts *et al*, 2012; Harvey *et al*, 2013).

Using Q-RT-PCR of these 10 transcripts, we built an algorithm capable of identifying *BCR/ABL1*-like cases with a high sensitivity and specificity. Furthermore, we generated a user-friendly tool, which requires only the upload of gene expression values to assess whether a sample is *BCR/ABL1*-like or non-*BCR/ABL1*-like.

Next, the screening of 142 B-NEG ALL samples by the *BCR/ABL1*-like predictor assigned 28 cases (19.7%) to the *BCR/ABL1*-like ALL subset, in line with the reported incidence (Ofran & Izraeli, 2017).

The prediction accuracy was indirectly corroborated by the analysis of the genetic features of *BCR/ABL1*-like ALL cases. As reported in both paediatric and adult cohorts, *BCR/ABL1*-like ALL is associated with *IKZF1* deletions, *CRLF2* deregulation/rearrangements, *JAK1/2* mutations, rearrangements of genes coding for TKs and cytokine receptors (Mullighan *et al*, 2009b; Mullighan *et al*, 2009c; Harvey *et al*, 2010a; Harvey *et al*, 2010b; Yoda *et al*, 2010; Chen *et al*, 2012; van der Veer *et al*, 2013; Asai *et al*, 2013; Tokunaga *et al*, 2013; Boer *et al*, 2015a; Ge *et al*, 2016; Roberts *et al*, 2017; Jain *et al*, 2017a; Herold *et al*, 2017). Consistently, our *BCR/ABL1*-like cases frequently carried JAK/STAT pathway mutations, the most recurrent targeting *JAK2*, followed by *CRLF2* and *IL7R*. In addition, *BCR/ABL1*-like cases were enriched in *IKZF1* deletions, detected in 80% of cases, in line with Herold and colleagues (Herold *et al*, 2017).

More importantly, RNA-sequencing - performed in 28 *BCR/ABL1*-like cases - revealed that 7 carried TK rearrangements, 2 *P2RY8/CRLF2* and 2 *TSLP*-rearrangements. TK-activating fusion genes are specific to *BCR/ABL1*-like cases and may be targeted by TKIs, as shown in pre-clinical models and sporadic case reports (Roberts *et al*, 2012; Tasian *et al*, 2012; Maude *et al*, 2012; Weston *et al*, 2013; Lengline *et al*, 2013; Roberts *et al*, 2014a; Roberts *et al*, 2014c; Shi *et al*, 2014; Shi *et al*, 2015; Francis *et al*, 2016).

The integration of all the molecular features, feasible in 28 *BCR/ABL1*-like, demonstrated that 96.4% had at least one lesion typical of the *BCR/ABL1*-like profile and suggested that the *BCR/ABL1*-like profile is sustained by at least two mechanisms, either a TK-activating fusion or *CRLF2* overexpression with a concomitant JAK/STAT mutation; at variance, *CRLF2* overexpression alone seems insufficient to induce a *BCR/ABL1*-like profile.

From a clinical standpoint, the features of the *BCR-ABL1*-like cases hereby identified were in line with a *BCR-ABL1*-like profile: they displayed a significantly higher WBC count at diagnosis, a lower CR rate and a significantly worse EFS than non-*BCR-ABL1*-like patients; we observed no differences related to gender, in agreement with other reports (Boer *et al*, 2015b; Herold *et al*, 2017). An association with MRD levels was not feasible since this parameter was not available for a large set of patients. Furthermore, the incidence in paediatric cases was lower than in adolescents and adults (9.5% *vs* 29.5% and 30.6%, respectively) in our cohort.

Finally, *in vitro* experiments showed that the pan-TKI, ponatinib, the most potent inhibitor in *BCR/ABL1*-positive ALL (Jabbour *et al*, 2015), was able to reduce the proliferative rate in *BCR-ABL1*-like samples and to increase apoptosis, similarly to that observed in *BCR-ABL1*-positive ALL. Notably, the *BCR-ABL1*-like samples analysed by *in vitro* experiments comprised ABL class lesions, JAK/STAT mutated and WT cases, indicating that ponatinib is active in all cases regardless of the underlying lesion and may represent an alternative to ruxolitinib whose clinical activity remains to be determined (Jain *et al*, 2017b).

In conclusion, we hereby describe a Q-RT-PCR based assay capable of singling out *BCR-ABL1*-like patients from the B-NEG ALL cohort. This approach has many advantages: first, it requires minimal amounts of diagnostic RNA; second, it is simple and cost-effective, being based on Q-RT-PCR; third, it is rapid, because the screening can be completed within a few days. This is essential, considering the high rate of refractory cases that - if promptly recognized - could benefit from an alternative approach, contemplating the use of the pan-TKI, ponatinib. These advantages make this tool suitable to be introduced in the diagnostic workflow and appears applicable to many haematology centres, followed by further genomic screens that comprise RNA-sequencing characterization to fully elucidate the underlying molecular lesion in the patients thus identified. Finally, because this assay is not based on a specific target identification, it allows the potential recognition of all *BCR/ABL1*-like cases, including those carrying novel genetic lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SC designed research, analysed data and wrote the manuscript; MM performed experiments, analysed data and wrote the manuscript; SG performed experiments and analysed data; AP designed the *BCR/ABL1*-like predictor model and performed statistical analyses; ALF, VG, AL, NP performed experiments; FDG performed RNA sequencing experiments; MV performed statistical analyses; MPM, VA, AV and CS provided samples and clinico-biological data; OE and RB analysed RNA-sequencing data; LSL and DW provided clinical samples; GI analysed data and critically revised the manuscript; AG and RF designed the study, analysed data and critically revised the manuscript.

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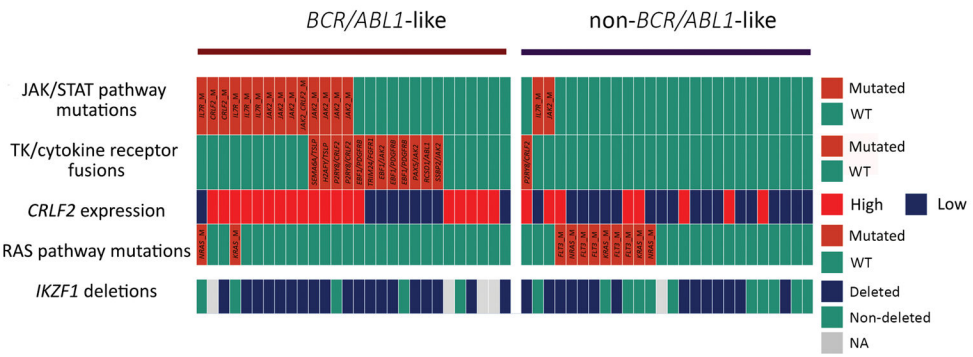

Figure 1.

Distribution of *BCR/ABL1*-like specific genetic lesions in the samples with a complete molecular characterization. Legend: green boxes for JAK/STAT pathway mutations: wild-type; red boxes: mutation detected. The mutated gene name is provided in the figure; green boxes for RAS pathway mutations: wild-type; red boxes: mutation detected; the mutated gene name is provided in the figure; *CRLF2* expression; red boxes: overexpression; TK/cytokine fusions: green boxes: no rearrangement detected; red boxes: rearrangement detected. The fusion gene is specified in the figure; *IKZF1* deletions: green boxes: no deletions; blue boxes: presence of deletions; grey boxes: sample not evaluated.

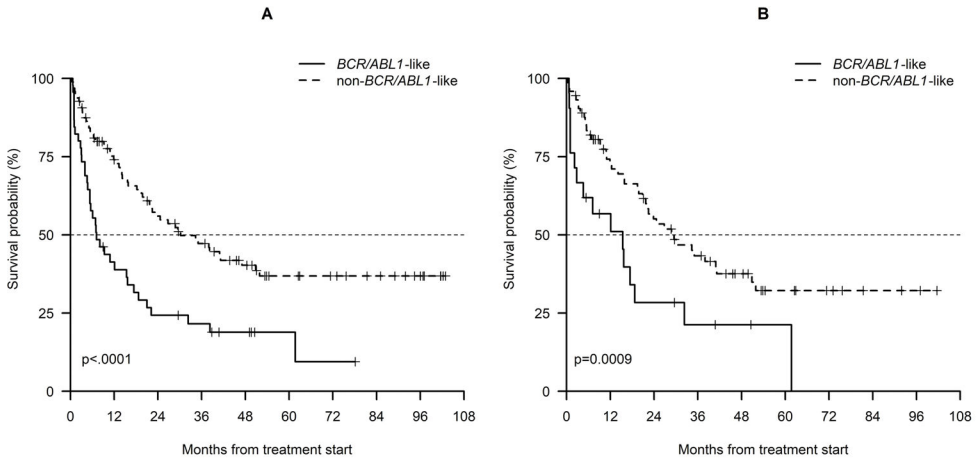

Figure 2.

Event-free survival at 36 months of adolescents and adults classified as *BCR/ABL1*-like and non-*BCR/ABL1*-like belonging to the whole cohort (A) and the screening panel only (B).

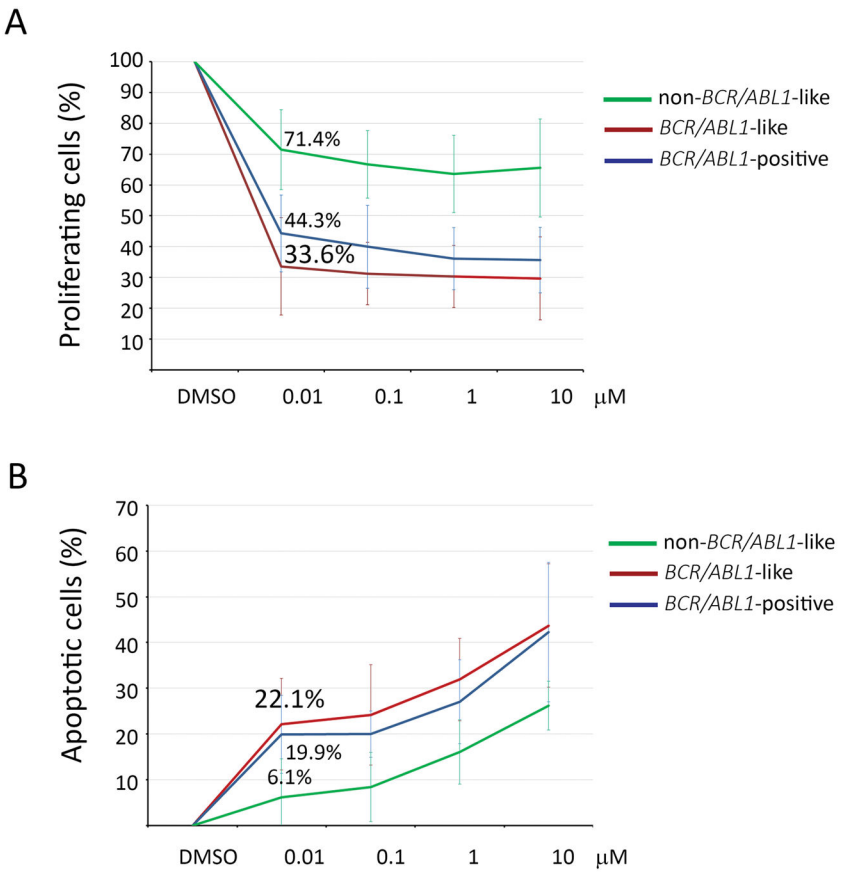

Figure 3.

In vitro response to ponatinib in *BCR/ABL1*-like and non-*BCR/ABL1*-like primary cells; *BCR/ABL1*+ cells were used as control. A) Average values of ^{3}H-thymidine incorporation of 16 primary B-ALL cells samples after 72 h of treatment with ponatinib (0.01 μM); B) Average values of Annexin V positive primary B-ALL cells samples after 72 h of treatment with ponatinib (0.01 μM). Samples are grouped according to *BCR/ABL1*-like (n=7), *BCR/ABL1*-positive (n=4) and non-*BCR/ABL1*-like (n=6).

Table I

Comparison between *BCR/ABL1*-like and non-*BCR/ABL1*-like clinico-biological features.

	***BCR/ABL1*-like n=54**	**non-*BCR/ABL1*-like n=140**	***p*-value**
Gender (male/female)	36/18	78/62	*p*=ns
Median age (range), years	32 (6–72)	28 (0–78)	*p*=ns
Age cohort 0–15 years (n=21)	2 (9.5%)	19 (90.5%)	
Age cohort 15–35 years (n=98)	29 (29.5%)	69 (70.4%)	*p*=ns
Age cohort >35 years (n=75)	23 (30.6%)	52 (69.3%)	
Median (range) WBC count, x10^9/l	22.6 (1.89–239)	12.4 (0.6–425)	*p*=0.023
Median (range) platelet count, x10^9/l	47 (0.15–283)	47 (1–308)	*p*=ns
Median (range) Hb g/l	97 (41–153)	89 (37–158)	*p*=ns

ns, not significant; WBC: white blood cell.

Table II

Comparison between *BCR/ABL1*-like and non-*BCR/ABL1*-like genetic features.

	***BCR/ABL1*-like**	**non-*BCR/ABL1*-like**	***p*-value**
JAK/STAT pathway members mutated cases	23/52 (44.2%)^	10/130 (7.7%)	*p*<0.001
JAK1/2 mutations	14/52 (26.9%)	4/130 (3.1%)	*p*<0.001
CRLF2 mutations	6/52 (11.5%)	2/130 (1.5%)	*p*=0.007
IL7R mutations	6/52 (11.5%)	4/130 (3.1%)	*p*=0.033
RAS pathway members mutated cases	9/52 (17.3%)	38/130 (29.2%) *	*p*=0.068
FLT3 mutations	0/52 (0%)	10/130 (7.7 %)	*p*=0.031
KRAS/NRAS mutations	9/52 (17.3%)	30/130 (23.1%)	*p*=ns
IKZF1 deletions	29/35 (82.8%)	31/76 (40.8%)	*p*<0.001
EBF1 deletions	14/35 (40%)	5/76 (6.6%)	*p*<0.001
BTG1 deletions	10/35 (28.5%)	5/76 (6.6%)	*p*=0.003
CRLF2 overexpressing cases$	33/54 (61.1%)	25/140 (17.8%)	*p*<0.001
CRLF2 expression levels	6.7 (0.6–16.6)	10.9 (3.2–17.9)	p<0.001
TK/cytokine receptor fusions	*EBF1/PDGFRB* (N=3) *JAK2*-fusions (N=3) *RCSD1/ABL1* (N=1) *TRIM24/FGFR1* (N=1) *P2RY8/CRLF2* (N=2) *TSLP*-fusions (N=2) 12/28 (42.8%)	*P2RY8/CRLF2* (N=1) 1/26 (3.8%)	*p*=0.001

^ Three cases carried 2 concomitant JAK/STAT pathway mutations: 2 cases harboured *JAK2* and *CRLF2* mutations, 1 case harboured *IL7R* and *CRLF2* mutations.

* Two cases carried 2 concomitant RAS pathway mutations: 1 case harboured *NRAS* and *KRAS* mutations, 1 case *FLT3* and *NRAS* mutations.

$ Overexpression was defined at ΔCt<8 as previously described by Chiaretti *et al* (2016).

Table III

Comparison between *BCR/ABL1*-like and non-*BCR/ABL1*-like ALL cases included in the screening panel.

	***BCR/ABL1*-like**	**non-*BCR/ABL1*-like**	***p*-value**
JAK/STAT pathway members mutated cases	12/27 (44.4%)^	10/107 (9.3%)	$p<0.001$
JAK1/2 mutations	7/27 (25.9%)	4/107 (3.7%)	$p=0.001$
CRLF2 mutations	2/27 (7.4%)	2/107 (1.9%)	ns
IL7R mutations	4/27 (14.8%)	4/107 (3.7%)	$p=0.05$
RAS pathway members mutated cases	6/27 (22.2%)	32/107 (29.9%)*	ns
FLT3 mutations	0	9/107 (8.4%)	ns
KRAS/NRAS mutations	6/27 (22.2%)	24/107 (22.4%)	ns
IKZF1 deletions	14/18 (77.7%)	23/62 (37.1%)	$p=0.029$
EBF1 deletions	6/18 (33.3%)	4/62 (6.5%)	$p=0.007$
BTG1 deletions	4/18 (22.2%)	4/62 (6.5%)	$p=0.071$
CRLF2 overexpressing cases	16/28 (57.1%)	21/114 (18.4%)	$p<0.001$
CRLF2 median expression levels (range)	7.6 (2–16.6)	11.3 (3.2–17.5)	$p<0.001$
TK/cytokine receptor fusions	*JAK2*-fusions (N=1) *TRIM24/FGFR1* (N=1) *TSLP*-fusions (N=2) 4/13 (30.7%)	*P2RY8/CRLF2* (N=1) 1/21 (4.7%)	$p=0.037$

ns, not significant

^ One case harboured *IL7R* and *CRLF2* mutations

* One 1 case harboured *NRAS* and *FLT3* mutations