CD371 cell surface expression: a unique feature of *DUX4*-rearranged acute lymphoblastic leukemia

B-cell precursor acute lymphoblastic leukemia (B-ALL), the most common childhood malignancy, comprises genetically, biologically and clinically heterogeneous disease entities.[1,2] Given the concepts of risk-adapted or targeted therapies, their precise delineation is becoming increasingly important.[1-3] The recently discovered *IGH-DUX4* or, less commonly, *ERG-DUX4* rearranged subtype of B-ALL accounts for 5-7% of the cases and is characterized by the expression of C-terminally truncated DUX4 isoforms, a highly distinctive gene expression signature and a profound deregulation of ERG.[4-6] Somatic *ERG* deletions, which occur in roughly half of *DUX4*+ patients,[4,6] are indicative of a favorable outcome and attenuate the negative prognostic effect of adverse factors such as *IKZF1* deletions alone[7,8] or in combination with other deletion events.[9] Given that *ERG* deletions are secondary events driven by the overexpression of DUX4,[6] they delineate only a subset of *DUX4*+ cases and it remains to be determined whether only those patients with *ERG* deletions or the entire cohort of *DUX4*+ patients has a superior outcome. However, a reliable identification of *DUX4*+ leukemia currently requires gene expression profiling or next-generation sequencing approaches,[4-6] which are not yet feasible either for large-scale screening studies or in a diagnostic setting for many study centers. Herein we show that expression of the cell surface antigen CD371 (CLL-1), encoded by *CLEC12A* and easily detectable by flow cytometry,[10,11] is a unique feature of *DUX4*-rearranged B-ALL and identifies virtually all *DUX4*+ cases.

Our evaluation of the immunophenotype of *DUX4*+ leukemia provides solid evidence that CD371 cell surface expression is a highly specific surrogate marker to identify this otherwise difficult to ascertain genetic subgroup. This notion is based on the finding that of 46 *DUX4*+ cases, 42 showed strong and three weak CD371 antigen expression (Table 1), while all other genetic subtypes were basically negative (*Online Supplementary Tables S1-3*).

In order to detect *DUX4*+ cases we performed RNA-sequencing of two independent cohorts of patients with childhood or young adolescent leukemia (*Online Supplementary Methods; Online Supplementary Tables S1 and S2*). The Austrian cohort (cohort 1) consisted of 101 bone marrow samples from 92 patients (n=80 diagnostic, n=3 relapse samples, n=9 diagnosis/relapse matched pairs). It comprised mostly B-other cases (n=65) lacking sentinel genetic alterations as well as representative cases of all major genetic subtypes (*Online Supplementary Table S1*). Initially, most samples were subjected to RNA-sequencing without prior knowledge of their immunophenotypic details, but later on nine more CD371+ samples with unknown genetic subtype and available material were sequenced. A second cohort (cohort 2) from the Czech Republic consisted of 55 primarily B-other cases, analyzed in parallel for CD371 expression (*Online Supplementary Table S2*).

DUX4+ samples were identified by the analysis of *DUX4* expression levels (Figure 1A), the presence of *DUX4* fusion transcripts (*Online Supplementary Tables S1 and S2*) and their distinctive gene expression profile[4-6,12,13] (*Online Supplementary Methods; Online Supplementary Figure S1*). Of 48 cases classified as *DUX4*+, 44 harbored *IGH-DUX4* and one a *DUX4-ZNF384* fusion, while three lacked any *DUX4* fusion (*Online Supplementary Tables S1 and S2*); but all displayed the gene expression signature typical of *DUX4*+ leukemia (*Online Supplementary Figure S1*). As determined by single nucleotide polymorphism array analysis (*Online Supplementary Methods*) and genomic multiplex polymerase chain reaction,[8] 32% (15/47) and 65% (13/20) of the patients, respectively, showed *ERG* deletions (*Online Supplementary Tables S1 and S2; Online Supplementary Figure S2*), confirming that with either method they are detectable only in a subset of *DUX4*+ cases.[4,6] About one-third of *DUX4*+ cases displayed *IKZF1* deletions (*Online Supplementary Methods; Online Supplementary Tables S1 and S2; Online Supplementary Figure S2*), which is comparable to the frequency detected in *ERG*-deleted leukemia.[7,8]

While in the ALL-BFM 2000 clinical trial *ERG*-deleted patients were more frequently allocated to the intermediate-risk group and had a favorable outcome,[8] in the ALL-BFM 2009 study, as a consequence of more refined minimal residual disease stratification, *DUX4*+ patients were more commonly treated in the high-risk arm[14] (*Online Supplementary Tables S1 and S2*). However, to date, the follow-up of the latter cohort of patients is still too short to draw any conclusions on whether a poor initial treatment response in *DUX4*+ leukemia eventually results in a

Table 1. Summary of *DUX4*+ and CD371+ patients detected in the cohorts subjected to RNA-sequencing.

Patients (n=119)*	DUX4 (n=48)#	CD371 strong (n=43)	CD371 weak (n=9)
CD371 strong	91.3% (42/46)	–	–
CD371 weak	6.5% (3/46)	–	–
CD371 negative	2.2% (1/46)	–	–
DUX4+	–	97.7% (42/43)	33.3% (3/9)
DUX4−	–	2.3% (1/43)	66.7% (6/9)
high hyperdiploid	–	–	22.2% (2/9)
BCR-ABL1	–	–	11.1% (1/9)
near haploid	–	–	11.1% (1/9)
B-other	–	2.3% (1/43)	22.2% (2/9)

*Data were derived from 125 samples from 119 patients for whom both RNA-sequencing and CD371 data were available. #2 patients did not have material available for immunophenotyping.

poor outcome and whether *DUX4*⁺ patients might benefit further from therapy intensification or should rather be spared from additional cytotoxicity.

DUX4⁺ leukemia is characterized by high expression of distinct genes including *CLEC12A*[6,13] encoding the cell surface protein CD371. Notably, in *DUX4*-rearranged leukemia DUX4 binds to the *CLEC12A* locus, suggesting a direct transcriptional regulation.[6] CD371 is predominantly expressed on myeloid cells and, as a potential myeloid cancer stem cell marker, is considered a target for antibody-based or chimeric antigen receptor T-cell therapies.[11] Furthermore, CD371 expression is associated with 'switch' ALL,[10,14] which has a propensity to switching to monocyte-like cells upon treatment with corticosteroids.[15]

The previously reported overexpression of *CLEC12A* in

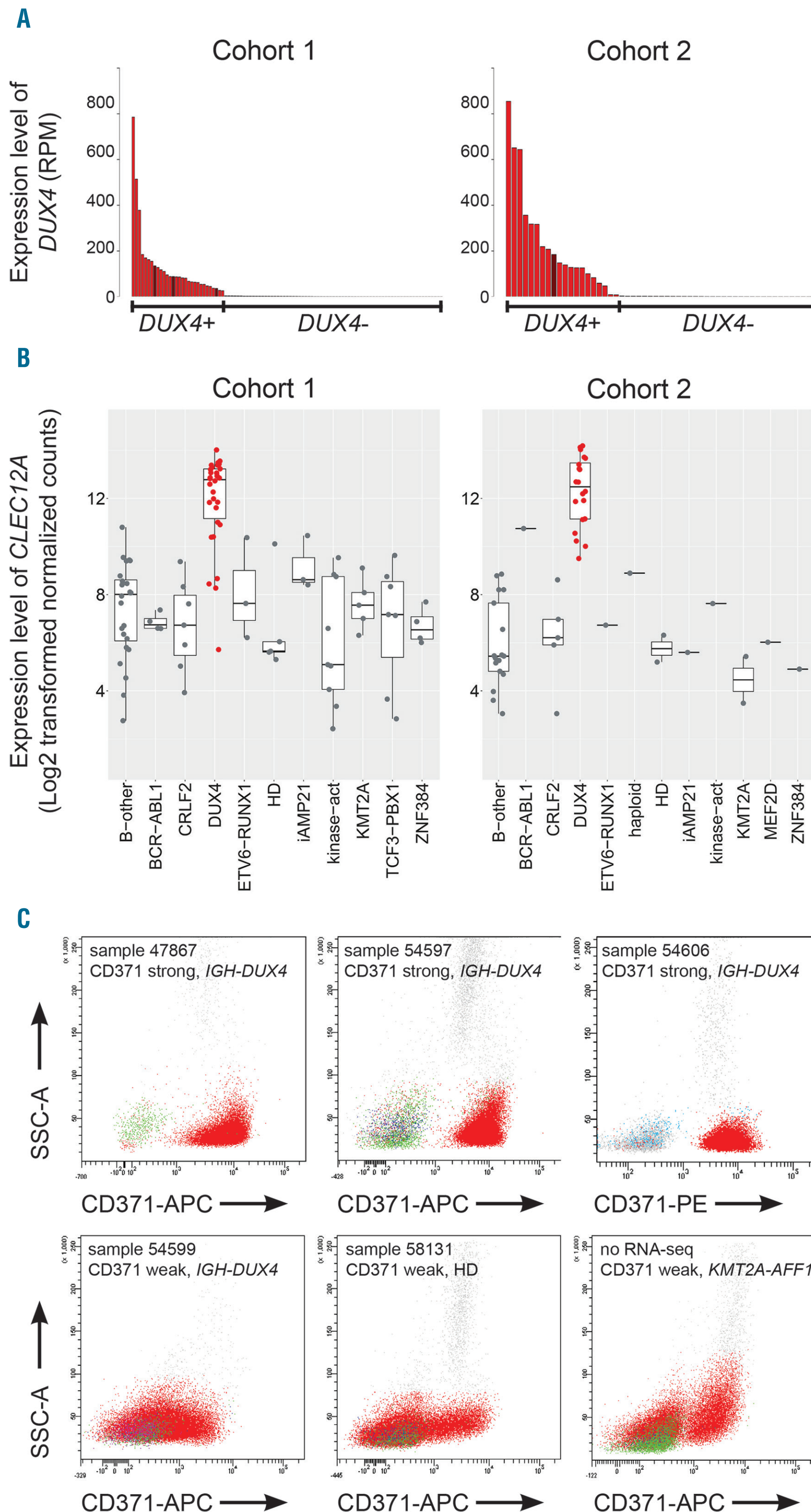

Figure 1. Identification of *DUX4*-positive leukemia. (A) The number of reads that mapped to *DUX4* cDNA (NM_001293798.2) per million total mapped reads assigned to RefSeq entries (RPM) for cohort 1 (left panel) and cohort 2 (right panel) are shown. *DUX4*⁺ cases are represented in red; cases with elevated *DUX4* expression but lacking detectable *IGH-DUX4* fusion transcripts are represented in brown. (B) Boxplots showing the expression levels of *CLEC12A* depicted as log_2-transformed normalized counts calculated by DESeq2 in cohort 1 (101 samples from 92 patients) and cohort 2 (55 patients) (left and right panel, respectively). (C) Representative FACS plots of primary bone marrow cells from patients with B-cell acute lymphoblastic leukemia (B-ALL) stained for CD371-APC or CD371-PE; blast cells are depicted in red. B-other, B-ALL cases lacking any sentinel alteration; CRLF2, cases with *P2YR8-CRLF2* or *IGH-CRLF2* rearrangement; DUX4, cases with expression of *DUX4*; haploid, masked near haploid; HD, high hyperdiploid; iAMP21, intrachromosomal amplification of chromosome 21; kinase-act, cases harboring a kinase activation fusion gene; KMT2A, *KMT2A* fusion gene; MEF2D, *MEF2D* fusion gene; ZNF384, *ZNF384* fusion gene; SSC: side scatter; APC: allophycocyanin; PE: phycoerythrin; RNA-seq: RNA-sequencing.

DUX4+ leukemia[6,13] was confirmed in both cohorts analyzed by RNA-sequencing (Figure 1B), and hence, we analyzed whether this correlates with CD371 cell surface antigen positivity. Immunophenotyping by flow cytometry and classification into strong or weak antigen expression of the respective samples with available material was performed according to the AIEOP-BFM consensus guidelines (*Online Supplementary Methods*).[10] CD371 expression was determined using phycoerythrin- or allophycocyanin-conjugated mouse anti-human CD371 (clone 50C1; BD Biosciences or BioLegend).

Of 46 *DUX4*+ cases with immunophenotypic data, 91.3% (42/46) were CD371strong, 6.5% (3/46) CD371weak and 2.2% (1/46) CD371neg (Table 1; Figure 1C; *Online Supplementary Figure S2*). The last case harbored the exceptional *DUX4-ZNF384* fusion but showed an expression signature similar to that of *DUX4*+ leukemia (*Online Supplementary Figure S1*). *Vice versa,* 97.7% (42/43) of all CD371strong cases were *DUX4*+ and only one single outlier (2.3%; 1/43) did not show either a *DUX4* or any other fusion gene that might explain the phenotype.

In most of the nine samples classified as CD371weak only a subfraction of leukemic blasts (10-38%) was antigen positive (Figure 1C). These cases were genetically more diverse and comprised three *DUX4*+, two high hyperdiploid, one *BCR-ABL1*, one masked near haploid and two B-other cases, one of which was only analyzed at relapse (Table 1; *Online Supplementary Tables S1 and S2*). There was no clear difference in the staining pattern between CD371weak *DUX4*+ samples and those with other genetic subtypes (Figure 1C).

To further substantiate the specificity of CD371 antigen expression for *DUX4*+ leukemia, we genetically subtyped 258 consecutive cases from Austrian BFM cohorts prospectively analyzed for CD371 expression (*Online Supplementary Methods; Online Supplementary Table S3*). Forty-eight of these patients, mostly *DUX4*+ and B-other cases, overlapped with cohort 1. Of 14 CD371strong cases all 12 analyzed by RNA-sequencing (no material was available for 2) were classified as *DUX4*+. Conversely, among 240 CD371neg cases a single *DUX4*+ case was found, corresponding to the one with a *DUX4-ZNF384* fusion. The few CD371weak samples again showed variable genotypes (n=1 *DUX4*+; n=2 *KMT2A-AFF1*+; n=1 high hyperdiploid). Hence, in the rare cases of weak CD371 expression, exclusion of the major genetic subtypes is required to rule out false interpretation as *DUX4*+ leukemia.

Considering that *KMT2A*-rearranged leukemia shows phenotypic heterogeneity with varying degrees of myeloid-lineage-associated antigen expression and that CD371 is primarily expressed on myeloid cells, an international prospective study is necessary to exclude occasional CD371strong expression in this rare B-ALL entity.

CD2 antigen expression, previously described in 35-45% of *ERG*-deleted cases,[7,8] was strongly associated with *DUX4*+ leukemia and present in roughly 75% of the patients, while basically absent in all other genetic subtypes (*Online Supplementary Tables S1 and S2; Online Supplementary Figure S2*). The higher frequency of CD2+ cases is most likely attributable to an enrichment of our *DUX4*+ cohort for 'switch' ALL cases commonly expressing CD371 and CD2.[14,15] In addition, the classification of the European Group for Immunophenotyping of Leukemias (EGIL) applied in the two previous studies[7,8] and the AIEOP-BFM consensus guidelines (*Online Supplementary Methods*)[10] consider cutoffs of 20% and 10%, respectively, to call a sample positive. Except for one single case with an unknown genetic subtype, all CD2 and CD371 double-positive cases were *DUX4*+. Accordingly, CD2 expression alone will detect only a proportion of *DUX4*+ cases but is also a strong indicator and in particular in combination with CD371 antigen expression may further underpin *DUX4* positivity.

Taken together our data provide compelling evidence that strong CD371 antigen expression in B-ALL is pathognomonic of *DUX4*+ leukemia. The remarkable finding that one single cell surface protein simply analyzed by flow cytometry may serve as a surrogate marker to identify *DUX4*+ leukemia will considerably facilitate the detection and further investigation of this disease entity as well as the determination of the prognostic relevance of *DUX4* positivity rather than *ERG* deletions alone,[7,8] which are present in only a subset of the cases.[4,6]

Although the international BFM-FLOW network does not consider immunophenotyping as a general gateway for genetic assessments,[10] we explicitly recommend the implementation of CD371 staining in the upfront flow cytometry-based analysis of childhood and adolescent B-cell leukemia for the identification of the *DUX4*+ subtype.

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