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Original Article

NG2 Molecule Expression in Acute Lymphoblastic Leukemia B Cells: A Flow-Cytometric Marker for the Rapid Identification of *KMT2A* Gene Rearrangements

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Abstract. ***Background*: B-lineage acute lymphoblastic leukemias (B-ALL) harboring rearrangements of the histone lysine [K]-Methyltransferase 2A (*KMT2A*) gene on chromosome 11q23 (*KMT2A-r*) represent a category with dismal prognosis. The prompt identification of these cases represents an urgent clinical need. Considering the correlation between rat neuron glial-antigen 2 (NG2) chondroitin-sulfate-proteoglycan molecule expression and *KMT2A-r*, we aimed to identify an optimized cytofluorimetric diagnostic panel to predict the presence of *KMT2A-r*.**
***Materials and Methods*: We evaluated 88 NG2+ B-ALL cases identified with an NG2 positivity threshold >10% from a cohort of 1382 newly diagnosed B-ALLs referred to the Division of Hematology of 'Sapienza' University of Rome.**
***Results*: Eighty-five of 88 (96.6%) NG2+ B-ALLs harbored *KMT2A-r* and were mainly pro-B ALL (77/85; 91%). Only 2 B-ALLs with *KMT2A-r* showed NG2 expression below 10%, probably due to the steroid therapy administered prior to cytofluorimetric analysis.**
Compared to *KMT2A-r*– cases, *KMT2A r*+ B-ALLs showed a higher blast percentage, significantly higher mean fluorescence intensity (MFI) of CD45, CD38, and CD58, and significantly lower MFI of CD34, CD22, TdT, and CD123.
The study confirmed differences in CD45, CD34, CD22, and TdT MFI within the same immunologic EGIL group (European Group for the immunological classification of leukemias), indicating no influence of the B-ALLs EGIL subtype on the *KMT2A-r*+ B-ALLs immunophenotype.
***Conclusions*: Our data demonstrate the association between NG2 and *KMT2A-r* in B-ALLs identify a distinctive immunophenotypic pattern, useful for rapid identification in diagnostic routines of these subtypes of B-ALLs with a poor prognosis that benefits from a specific therapeutic approach.**

Keywords: NG2, Flow-cytometry; Acute lymphoblastic leukemia; KMT2A gene rearrangements.

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Introduction. Acute lymphoblastic leukemia (ALL) is a heterogeneous malignancy arising from the malignant

transformation of immature B or T-lymphocyte precursors. A rapid and accurate diagnostic process is required to support optimal risk-oriented therapy and increase the curability rate. Immunophenotypic characterization by multiparametric flow cytometry (MFC) is an essential step for ALL diagnosis and prognosis and has significant value in evaluating minimal residual disease (MRD)[1] together with molecular testing. Indeed, the leukemic cells express surface and intracytoplasmic antigens, and characterizing these antigens allows for determining the lineage, level of differentiation and maturation, lineage infidelity, and peculiar aberrations.[2]

Specific genetic aberrations underlie different subtypes of B-cell precursor ALL (BCP-ALL) and identifying them is crucial for risk group stratification.[3,4] A subset of pro-B and, less commonly, B-common ALLs are characterized by rearrangements of the histone lysine [K]-Methyltransferase 2A gene (*KMT2A*) on chromosome 11q23 (*KMT2Ar*), which have a poor prognosis.[5-8]

The *KMT2A* gene is involved in chromosomal translocations, resulting in fusions with over 100 partner genes. The most common partners are *AFF1*, *MLLT3*, and *MLLT1*, leading to the translocations t(4;11)(q21;q23), t(9;11)(q22;q23), t(11;19)(q23;p13.1), t(11;19)(q23;p13.3), respectively. The translocation t(11;19)(q23;p13.1) often results in the *KMT2A::ELL* fusion gene. Less common partners include *MLLT10* [t(10;11)] and *AFDN* [t(6;11)].[9-10]

Patients are more likely infants (< 1 year) with hyperleukocytosis, relatively frequent central nervous system (CNS) involvement, hepato-, spleno- and lymphadenomegaly.[11] It is essential to identify this subset due to its poor prognosis, characterized by early relapse, and the need to promptly allocate these cases to allogeneic stem cell transplantation (ASCT).

In the attempt to identify antigens shared by blast cells with prognostic significance, Smith FO et al. generated a monoclonal antibody moAb 7.1 that recognizes the human homologue of the rat neuron glial-antigen 2 (NG2) chondroitin sulfate proteoglycan molecule, expressed by leukemic blasts, but not by normal hematopoietic cells.[12] Moreover, a correlation between NG2 expression and *KMT2A-r* as well as poor prognosis, was found;[13] additionally, NG2 expression was reported to be associated with leukemia invasiveness and CNS infiltration.[14]

Therefore, NG2 is a crucial marker for identifying B-ALL patients with KMT2A-r cases. In our routine experience, we observed that NG2 expression is mainly associated with a $CD10^{-}/cIgM^{-}/CD20^{-}/CD34^{-/+}/CD133^{+}/CD13^{-}/CD33^{-}$ phenotype and identifies predominantly a pro-B ALL subtype. On these premises, the purpose of our study is to propose an optimized cytofluorimetric diagnostic panel of routine antigens to quickly predict the presence of *KMT2A-r* since NG2 expression may aid in the prompt identification of a specific category of B-ALLs that have a poor prognosis. Identification of transcripts also contributes to the prognostic stratification of patients and evaluation MRD by Real Time PCR during follow-up.

Materials and Methods. Between 2001 and 2023, a total of 1382 cases of newly diagnosed B-ALLs were referred for diagnostic purpose to our center at the Division of Hematology of 'Sapienza' University of Rome. Among these, 88/1382 (6.4%) were NG2+, with 78 being pro-B and 10 B-common ALLs. They included 32 males and 56 females; median age was 44 years (range 15- 92) years. Patients' bone marrow (BM) samples were obtained with informed consent in accordance with the Declaration of Helsinki.

Flow cytometry. B-ALL diagnosis was assessed by MFC using a combination of mAbs directed against myeloid (MPO), B and T lymphoid (cCD79a and cCD3) lineage antigens; after that, with a combination of mAbs against CD45/CD10/CD34/TDT/HLADR/CD19/CD22/CD20/ CD38/Igκ/Igλ/cIgM/CD3/CD13/CD33/CD58/CD123 (Becton Dickinson, San Jose, CA; Società Italiana Chimici, SIC, Life Sciences, Rome, Italy) and NG2 (Beckman Coulter, Brea, CA). In presence of NG2 positivity, with a threshold >10%, CD15 and CD133 were also analyzed. Representative plots of the flow gating strategy are reported in **Supplementary Figure S1**. Cell surface antigen expression was quantified on the same flow cytometer and with the same mAbs combination, as the mean fluorescent intensity (MFI) of values obtained with specific mAbs compared with values given by the isotype controls. A sample was considered positive for surface antigens if more than 10% of leukemic cells exhibiting fluorescence compared with negative control.

Molecular testing. Molecular analysis on BM samples was carried out using a Multiplex RT-PCR system with a nested approach. As previously described (15), the screening with Multiplex-RT-PCR was designed to detect simultaneously and in a quick time, the most common fusion genes in T-ALL rather than in B-ALL: *TCF3::PBX1*, *ETV6::RUNX1*, *SIL::TAL1*, *NUP98::RAP1GDS1*, *SET::NUP214*, *BCR::ABL1* p190 (e1a2) and p210 (e13a2, e14a2), *KMT2A::AFF1* and *KMT2A::MLLT1*.

To avoid the technical difficulty of introducing more genes in only one reaction tube and, therefore, more bands of amplification that did not interact with each other, in our multiplex approach, we had to choose only the most representative fusion genes of ALL, which had amplification bands that did not overlap, making difficult the interpretation of the data. Therefore, considering the

more frequent and representative alterations in ALL, despite the great heterogeneity of *KMT2A* rearrangements, only 2 fusions were tested – *KMT2A::AFF1* and *KMT2A::MLLT1*.

Statistics. Summary statistics (mean and standard deviation, median, and range) were reported by group. Mann-Whitney test for independent groups was used for comparisons of NG2+KMT2Ar+ B ALLs vs NG2-KMT2Ar-B ALLs, and statistical significance was assessed by a two-tailed test.

Results.

B-ALL characterization. Overall, 88 B-ALLs out of 1382 expressed the NG2 marker (6.4%). Among 88 NG2+ B-ALL cases, 78 were phenotypically classified as pro-B and 10 as B-common B-ALLs. They were characterized by a mean percentage of blasts of 80.6±14.7% (range 30-97) and NG2 expression was not present across the entire blast population but was distinctive of 47.6±26% (range 10-82%) of leukemic cells, with a mean MFI of 141.7±160.9 (range 6-980). Notably, of 88 NG2 positive cases, 85 had *KMT2A-r* (96.6%) with the following distribution: 77 harbored *KMT2A::AFF1+* (72 pro-B and 5 B-common ALLs), and 8 *KMT2A::MLLT1+* (5 pro-B and 3 B-common ALLs). The remaining 3/88 NG2+ B-ALLs (3.4%) did not harbor *KMT2A-r–* (1 pro-B and 2 B-common ALLs). As already known, *KMT2A-r* + were characterized by a higher blast percentage when compared with *KMT2A-r* – (*KMT2A-r* + *vs KMT2A-r* –: 82±14 *vs* 69±22, p=0.0001) and this difference was statistically significant even when within the same EGIL group (i.e.: *KMT2A-r+ vs KMT2A-r–* pro-B, p<0.001 and *KMT2A-r+ vs KMT2A-r–* B-common, p<0.01).

Only 2 pro-B ALLs with *KMT2A*-r showed low NG2 expression (<below the 10%) positivity cut-off, being equal to 4% and 5%, probably due to the steroid therapy administered to these patients before diagnostic work-up. The remaining 3/88 NG2+ B-ALLs (3.4%) did not harbor *KMT2A-r–* (1 pro-B and 2 B-common ALLs).

Antigen detection and surface expression intensity. To evaluate if the presence of *KMT2A-r* was associated with a distinctive immunophenotypic profile, beyond NG2+ B-ALLs, we analyzed and compared the MFI of CD45, TdT, CD34, CD19, CD20, CD22, CD38, CD58 and, CD123 antigens in NG2+ *KMT2A-r* + (n=85; 77 pro-B and 8 B-common ALLs) and NG2– *KMT2A-r* – B-ALLs (n= 39; 12 pro-B and 27 B-common ALLs). As shown in **Table 1**. In addition, to exclude any influence of the B-ALLs EGIL subtype on the peculiar immunophenotypic profile of *KMT2Ar+* cases, we compared the expression intensity of all the markers analyzed, amongst the same subtype of B-ALLs (**Table 2**). Most of the markers analyzed (7/9, 77.8%) were differently expressed between the two categories. CD45 was expressed in all cases: MFI values were significantly higher in *KMT2A-r+* than *KMT2A-r*⁻ B-ALL regardless of the EGIL immunologic subtypes. CD34 was partially expressed in *KMT2Ar+*. More specifically, *KMT2Ar+* B-ALLs were characterized by a significantly lower percentage of CD34+ blasts, as well as MFI, respect to *KMT2Ar–* samples independently from the EGIL subtype. In addition, among our cases, CD34 MFI appeared significantly lower in the presence of KMT2Ar, independently from the B-ALLs EGIL immunologic subtype (**Table 2**). TdT was expressed in all cases of B-lineage ALL. In particular, *KMT2Ar+* B-ALL showed a significant downmodulation of TdT MFI compared to *KMT2Ar–* cases. This evidence was also confirmed within the same immunologic EGIL subtypes (*KMT2Ar+* vs *KMT2Ar–* pro-B ALL) (**Table 2**).

CD19 and CD22, both targetable antigens, were expressed in all cases; interestingly CD19 was equally expressed in terms of both percentage and MFI regardless of *KMT2A-r* status; at variance, CD22 MFI values were significantly lower in *KMT2Ar+* than *KMT2Ar–* B-ALLs, thus at least explaining the suboptimal efficacy of inotuzumab in this set of patients.[16]

CD38 was positive in all B-ALLs studied, and its MFI

Table 1. Comparison of the mean fluorescence intensity (MFI) of all the markers analyzed between NG2+ *KMT2A-r+* (n=85; 77 pro-B and 8 B-common ALLs) and NG2– KMT2A-r– B-ALLs (n= 39; 12 pro-B and 27 B-common ALLs).

Antigens	**NG2+KMT2Ar+ B ALLs** (n=85) media ± SD (range)	**NG2-KMT2Ar-B ALLs** (n=39) media ± SD (range)	**p value**
CD45	188±125 (23-751)	105±53 (32-248)	<0.001
CD34	63±84 (7-562)	131±130 (13-580)	0.001
TdT	22±12 (6-75)	45±32 (14-153)	< 0.001
CD19	154±77 (21-334)	165±114 (25-518)	0.80
CD22	36±29 (9-151)	118±92 (20-261)	< 0.001
CD38	102±90 (10-480)	65±56 (4-264)	0.010
CD58	73±54 (20-217)	52±48 (11-197)	< 0.001
CD123	25±24 (7-94)	94±151 (12-581)	< 0.001

Table 2. Comparison of the mean fluorescence intensity (MFI) of all the markers analyzed amongst B-ALL cells with the same EGIL immunophenotypic subset (*KMT2A-r+ vs* KMT2Ar– pro-B ALLs).

Antigens	KMT2Ar+ pro-B ALLs (n=77) media ± SD (range)	KMT2Ar- pro-B ALLs (n=12) media ± SD (range)	p value
CD45	193+126 (23-751)	89±49 (41-182)	<0.001
CD34	62±86 (7-562)	138±69 (61-242)	<0.001
TdT	22±12 (7-75)	35±25 (14-100)	0.005
CD19	155±79 (21-334)	135±93 (25-328)	0.30
CD22	36 ± 29 (9-151)	54±28 (25-81)	0.023
CD38	102±90 (10-480)	65±41(15-138)	0.20
CD58	73±54 (20-217)	53±56 (11-195)	0.073
CD123	25±24 (7-94)	87±154 (12-520)	0.056

Table 3. Comparison of the mean fluorescence intensity (MFI) of all the markers analyzed amongst B-ALL cells with the same EGIL immunophenotypic subset (n=8 KMT2Ar+ *vs* n=27 KMT2Ar– B-common ALLs).

Antigens	KMT2Ar+ B-common ALLs (n=8) media ± SD (range)	KMT2Ar- B-common ALLs (n=27) media ± SD (range)	p value
CD45	111 ±46.8 (46-160)	100 ±49 (32-248)	0.572
CD34	120 ±72 (4-147)	134 ±70 (13-580)	0.611
TdT	53±17 (6-48)	20±49 (18-153)	0.079
CD19	136 ±34 (89-175)	154±55 (42-518)	0.391
CD22	49±27 (12-68)	67±34 (20-261)	0.180
CD38	77±51 (33-161)	64±88 (4-264)	0.695
CD58	63±44 (24-138)	51±108 (12-197)	0.767
CD123	62±26 (12-72)	80±95 (16-581)	0.590

Table 4. Comparison of the mean fluorescence intensity (MFI) of all the markers analyzed amongst B-ALL cells without KMT2Ar (n=12 KMT2Ar– pro-B *vs* n=27 KMT2Ar– B-common ALLs).

Antigens	KMT2Ar- pro-B ALLs (n=12) media ± SD (range)	KMT2Ar- B-common ALLs (n=27) media ± SD (range)	p value
CD45	89±49 (41-182)	100 ±49 (32-248)	0.520
CD34	138±69 (61-242)	134 ±70 (13-580)	0.868
TdT	35±25 (14-100)	20±49 (18-153)	0.323
CD19	135±93 (25-328)	154±55 (42-518)	0.428
CD22	54±28 (25-81)	67±34 (20-261)	0.256
CD38	65±41(15-138)	64±88 (4-264)	0.970
CD58	53±56 (11-195)	51±108 (12-197)	0.958
CD123	87±154 (12-520)	80±95 (16-581)	0.862

was significantly higher in (*KMT2A-r+ vs KMT2A-r–*: 102±90 *vs* 65±56, p=0.010). Finally, CD58 MFI appeared higher in *KMT2Ar+* B-ALL (*KMT2Ar+ vs KMT2Ar–*: 73±54 *vs* 52±48, p< 0.001) while CD123, the interleukin-3 (IL-3) receptor α-chain, another antigen useful in MRD, had a lower MFI in *KMT2Ar* (*KMT2Ar+* vs *KMT2Ar–:* 25±24 *vs* 94±151, p< 0.001).

Two additional markers were also evaluated: CD15 was evaluated in 48/88 NG2+ cases (43 *KMT2A::AFF1+* pro-B, 3 *KMT2A::AFF1+* B-common and 2 *KMT2A::MLLT1+* pro-B ALLs) and detected in in 16/48 NG2+ cases (CD15+ B-ALLs: 72.06±24.40%, range 26-90%; CD15 MFI: 78.16±53.4, range 20-190); CD133 (Prominin-1), , was again studied in 48/88 NG2+ cases (43 *KMT2A::AFF1+* pro-B, 3 *KMT2A::AFF1+* B-common and 2 *KMT2A::MLLT1+* pro-B ALLs) and was detected in all cases. with a mean percentage of positive cells of 84.37 ±11.64 (range 55-97%) and a mean MFI of 92.42±45.29 (range 18-190), suggesting a strong association of CD133 expression with NG2 positivity and the presence of *KMT2Ar*, mainly in the pro-B EGIL subtype.

Moreover, no differences in any of the antigens analysed were observed comparing *KMT2Ar+* (n=8) vs

KMT2Ar– (n=27) B-common ALLs, probably due to the low number of KMT2Ar+ cases characterized by this subtype (**Table 3**). In addition, no differences emerged in terms of antigen expression between *KMT2Ar*– pro-B ALLs and *KMT2Ar*– B-common ALLs, confirming the key role played by the presence of *KMT2Ar* in the peculiar phenotype of *KMT2Ar*+ leukemic cells (**Table 4**).

Discussion. Immunophenotypic characterization of ALL is crucial for diagnosis and prognosis. This study identifies an immunophenotypic pattern that can be useful for the rapid identification of B-ALLs with a strong correlation between NG2 positivity and *KMT2Ar* in diagnostic routines. Other studies have demonstrated the high sensitivity of NG2 in identifying *KMT2Ar*+ B-ALLs. However, most of them were performed on relatively small samples or very heterogeneous groups.[17-18] Therefore, the strength of our study lies in a homogeneous and large cohort of newly diagnosed patients referred to a single Institution. NG2 belongs to the chondroitin sulfate proteoglycan family. It is highly expressed during early embryonic development but downregulated during differentiation. Its role in normal hematopoiesis and MLL-mediated leukemogenesis is still unclear.[19-21] Originally, NG2 was found to be highly expressed in melanoma patients and associated with tumor cell adhesion, migration, and metastasis.[13,22,23] In *KMT2A-r*+ B-ALLs, NG2 expression has recently been shown to be involved in leukemia invasiveness and central nervous system (CNS) infiltration, contributing to the frequent occurrence of CNS disease/relapse in this type of B-ALLs. The clinical data from the infant cohort of *KMT2Ar*+ B-ALL showed that high NG2 expression is associated with lower event-free survival, a higher number of circulating blasts and a more frequent occurrence of CNS disease/relapse. This is because NG2 expression is highly upregulated in blasts infiltrating extramedullar hematopoietic sites and CNS. In our study, 88/1382 (6.4%) cases displayed NG2 positivity with a cut-off more than 10%. As this marker is not uniformly expressed in the leukemic cell population, different levels of positivity may have predictive value. Our threshold level for NG2 positivity was in line with the work by Zerkalenkova E et al.,[24] in which the authors suggest that while any detectable NG2 positivity strongly associates with the presence of *KMT2Ar* in infant ALLs, NG2 positivity above 10% should be considered predictive of *KMT2Ar* in other ALL patient groups. Therefore, subsequent molecular and/or cytogenetic analysis should be performed in these patients.

Additionally, we also evaluated NG2 MFI. The percentage of NG2+ blasts is a more feasible and reliable predictor of *KMT2Ar*+ B-ALLs than NG2 expression quantification. Therefore, we considered NG2 positivity expressed as a percentage of positive cells.

In our cohort, 85/88 NG2+ cases were characterized by *KMT2Ar*+, while 3/88 cases, although NG2+, appeared *KMT2Ar*– by molecular analysis. However, we could not exclude the presence of *KMT2Ar* even in the latter cases, as cytogenetic data were not available. Furthermore, only two cases of B-ALL with NG2 expression below the 10% positivity threshold (4% and 5%, respectively), were found to be *KMT2Ar*+, likely due to the steroid therapy administration before diagnosis to these patients, which is known to cause down-modulation of this antigen.

The steroid-induced downmodulation together with its characteristic heterogeneous expression on the leukemic cell population, makes NG2 a reliable marker for the identification of *KMT2Ar*+ B-ALLs in therapy-naive patients at diagnosis; on the contrary, this antigen should not be considered for MRD evaluation. Accordingly, NG2 evaluation plays a crucial role into the diagnostic workflows for leukemia immunophenotyping, due to its strong positive predictive value for *KMT2Ar*, leading to the prompt identification of this subgroup of patients[25] who often need stem cells transplantation because of their dismal prognosis. In this subset of B-ALLs, NG2 appeared also associated with CD133 antigen expression.[26]

Based on our experience, the association between CD133 and NG2 is highly specific to *KMT2Ar*+ leukemic cells. This association strengthens the role of NG2 positivity in predicting KMT2Ar+ B-ALL. In addition to NG2 expression, our data also revealed that *KMT2Ar*+ differ from *KMT2Ar*– B-ALLs also in other phenotypic characteristics, such as the presence/absence of certain antigens and their varying expression intensity. Specifically, CD45, CD38, and CD58 show higher expression intensity in the presence of *KMT2Ar*, while CD34, CD22, TdT, and CD123 show lower expression intensity compared to *KMT2Ar*– cases. On the contrary, as previously described,[27] no differences were observed in CD19 MFI between the two categories. Therefore, as the presence of *KMT2Ar* increases CD38 MFI and does not impact the intensity of CD19 antigen expression, these patients can still benefit from the specific therapy currently available (monoclonal antibodies).[28-29] On the other hand, no differences in any of the antigens analysed were observed comparing *KMT2Ar*+ *vs* *KMT2Ar*– B-common ALLs, probably due to the low number of *KMT2Ar*+ cases characterized by this EGIL immunologic subtype. However, no differences were observed in any of the analysed antigens when comparing KMT2Ar+ and KMT2Ar- B-common ALLs. This is likely due to the low number of KMT2Ar+ cases characterised by this EGIL immunologic subtype. In addition, a recent study also shows the crucial role of NG2 in leukemogenesis and its potential role as specific antigen of a target therapy. Blockade of NG2 would seem to remove the chemoprotective effect of the bone marrow

stroma through the mobilization of *KMT2Ar+* blasts in the peripheral blood, which thus become more accessible to chemotherapy.[30] Overall, our data show the important role of NG2 detection since this antigen leads to a precise and reliable prediction of high-risk adult B-ALL patients characterized by *KMT2Ar*. In conclusion NG2 should be always included in the diagnostic MFC panel in combination with CD133 for the rapid detection of this peculiar and poor prognosis B-ALL subset.

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