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*Original Article*

# Expression of the Immunoglobulin Superfamily Cell Membrane Adhesion Molecule Cd146 in Acute Leukemia

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**Background: The expression of the immunoglobulin superfamily cell membrane adhesion molecule CD146 has been reported on several normal and pathological cell types in human. The aim of this study was to investigate CD146 expression in acute leukemia using a multiparametric cytofluorimetric approach.**

**Methods: Cytofluorimetric and cytogenetic studies were performed on peripheral blood and bone marrow samples from 162 patients with acute myeloid leukemia (AML, $n = 121$) and acute lymphoblastic leukemia (ALL, $n = 41$). ALL patients were subdivided in B-ALL ($n = 38$) and T-ALL ($n = 3$). Adult ($n = 18$) and pediatric ($n = 20$) B-ALL were considered as a whole group.**

**Results: Four out of 121 (3.3%) AML cases, 14/38 (36.8%) B-ALL, and 2/3 (66.6%) T-ALL expressed CD146 on 12–98% of blasts ($p < 0.001$). CD146 expression was not observed in 10 healthy controls. Among B-ALL CD146-positive cases, 78.6% were associated with a "common"/BII-ALL and 21.4% with a pre-B/BIII-ALL immunophenotype while pro-B/BI-ALL and mature-B/BIV-ALL cases were CD146-negative. Statistical analysis showed CD146 expression strongly associated with Ph+ positivity in B-ALL with the highest percentage of CD146-positive blasts in all Ph-positive B-ALL cases ($84 \pm 22\%$ Ph-positive B-ALL SD vs. $40 \pm 24\%$ SD in Ph-negative B-ALL; $p < 0{,}001$).**

**Conclusion: In our series, CD146 was expressed in all cases of Ph-positive B-ALL and in the vast majority of T-ALL, whereas it was rarely expressed by AML blasts. We suggest that CD146 may be considered as an additional marker for acute lymphoblastic leukemia diagnosis and monitoring of minimal residual disease in those cases which are CD146-positive at diagnosis.** 

**Key terms: acute lymphoblastic leukemia; Philadelphia chromosome; immunophenotype; CD146**

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The immunoglobulin superfamily cell membrane adhesion molecule CD146, also known as melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is the product of MCAM gene at chromosome 11q23.3 (homolog of gicerin in chicken), and it is constitutively expressed on several cells of normal and malignant origin (1). This 113-kDa glycoprotein contains extracellular, transmembrane, and cytoplasmic domains with potential recognition sequences for protein kinases (2). CD146 expression has been observed on normal human cell types including endothelial, mesenchymal, smooth muscle cells, pericytes, and trophoblast cells (3–7). It has been also demonstrated that the CD146 molecule is expressed by nonhemopoietic precursors (perivascular or mesenchymal/osteoprogenitor cells) in bone marrow microenvironment (8–10). CD146 has also been identified as an endothelial biomarker playing a key role in tumor associated angiogenesis and lymphoangiogenesis, possibly through a direct interaction with VEGFR2 and activation of the p38/IKK/NF-B and Akt pathways (11,12). Interestingly, CD146 was first identified as a marker for melanoma progression and metastatic spread and subsequently observed on several types of tumors such as osteosarcoma, gall bladder, or rectal adenocarcinoma and on human prostate cancer cell lines (13–19). The CD146 is also reported as an epithelial–mesenchymal transition inducer, in particular associated to the most aggressive and lethal subtypes of breast cancer (20–22). Although currently scant information exist about CD146 in hemopoietic cells, its expression on activated T-cell populations as well as on a subset of murine NK cells was reported (23,24). CD146 expression could identify a unique subset of CD3+CD4+ T-lymphocytes that may play an important role in the pathogenesis of various musculoskeletal diseases (25). On the other hand, few reports exist regarding CD146 on B-cells (26). Thus, the biological role of CD146 in hematological malignancies remains to be clarified. Since improvements in our knowledge of the immunophenotype of leukemia cells is important in the diagnostic workup of these malignancies, we investigated by flow cytometry the expression of this multifaceted cell adhesion molecule on blast cells of different types of acute leukemia.

## MATERIALS AND METHODS

### Patients

Bone marrow (BM) and/or peripheral blood (PB) samples were obtained from 162 acute leukemia cases admitted between March 2010 and December 2012 to three Italian hematology units: Hematology Unit of S. Anna Hospital, Department of Medical Sciences, University of Ferrara; Hematology Unit of Azienda Ospedaliera "Istituti Ospitalieri", Cremona; and Onco-Hematology Division, SDB Department, University of Padova, Italy. Patients were affected by AML ($n = 121$) and ALL ($n = 41$). Adult ($n = 20$) and pediatric ($n = 21$) ALL were considered as a whole group. ALL patients were, respectively, subdivided in B-ALL ($n = 38$) and T-ALL ($n = 3$). The diagnosis of AML and ALL was performed according to WHO and EGIL criteria (27–30). In particular, AML diagnosis was obtained from PB in 31% and from BM in 69% of cases, respectively; diagnosis of ALL was obtained from PB in 7% of cases, the remaining from BM. In 20% of all cases (AML and ALL), both BM and PB from the same patient were analyzed with no significant differences.

Three B-ALL and two T-ALL were studied at diagnosis and at relapse. Experiments were performed on PB from 10 healthy controls evaluated for anemia (3 cases), neutropenia (1 case), and thrombocytopenia (6 cases) associated with BM analysis in 7 cases.

### Acute Leukemia Immunophenotyping

Briefly, $1 \times 10^6$ cells derived from whole fresh BM or PB samples were incubated with different combination of monoclonal antibodies (BD Becton Dickinson, San Jose, CA, USA and Beckman Coulter srl Europe, Milano, Italy) as suggested by the panel for conventional multiparametric flow-cytometric workup for acute leukemia diagnosis (28–30). In pediatric B-ALL patients, CD58-FITC, CD146-PE, CD45-ECD, CD10-PECy5, CD19-APC, and CD20-APCCy7 were also tested. After incubation in the dark at room temperature for 20 min, samples were lysed, and then washed and centrifuged. Cells were suspended in flow solution (BD Fluidic Solution BD, Becton Dickinson, San Jose, CA, USA and Beckman Coulter srl Europe, Milano, Italy) for the acquisition procedure.

For the detection of cytoplasmic antigens, fixation and permeabilization steps were performed with the Fix and Perm kit (An Der Grub Bio Research GmbH, Austria). In all hematological centers involved, routine daily controls as well as standardized quality controls were performed on a regular basis and instrument setup routinely verified. In particular, all centers are equipped with FACSCalibur and/or FACSCanto II instruments with two laser options (Becton Dickinson Pharmingen, Milano, Italy). Acquisitions and analysis of normal and leukemic samples were performed using these instruments. Pediatric samples were analyzed in parallel using also Beckman Coulter instrumentation. In all AML and ALL samples, CD146 analysis was optimized using additional combinations of monoclonal antibodies as follows: CD3-FITC cl. SKT/CD146-PE cl. P1H12/CD45- PercP cl. 4G7/CD34-APC cl.8G12 and anti CD19-FITC cl. 4G7 or anti CD19-PerCP Cy5.5 cl. SJ25C1 (Becton Dickinson, San Jose, CA, USA). In all cases, antigen expression was defined by the percentage of blast cells that resulted positive for the different markers in the immunological gate (31). CD146 expression was assessed on the blast immunological gate.

Adult and pediatric B-lineage ALL (B-ALL) were further classified according to EGIL criteria as follows: pro-B/BI-ALL; "common"/BII-ALL; pre-B/BIII-ALL; mature/BIV-ALL (30).

### Conventional Cytogenetics and FISH Analysis

Conventional cytogenetic analysis of BM or PB leukemic samples was performed in all patients as a part of the diagnostic workup based on standard G-banding procedure as previously described (32). Fluorescence in situ hybridization (FISH) was performed on BM and/or PB cells using a panel of probes for the detection of recurrent cytogenetic abnormalities in AML and ALL (27,31): 11q23*lMLL*, 14q32/*IGH*, 8q24/*MYC*, *RUNX1-CBFA2, CBFB/MYH11, ETV6/RUNX1, TCF3/PBX1,* and *BCR/ABL*. The cut-off level for single gene break-apart probes and translocation probes (dual color, dual fusion systems) was set at 3% and 1.5%, respectively. Briefly, target DNA was fixed to glass slide by a passage in a 19:1 methanol/acetic acid fixative solution followed by a co-denaturation procedure using directly fluorochrome-labeled probes (Abbott Molecular Co, Downers Grove, IL, USA distributed in Italy by Abbott, Rome) in a denaturation/hybridization System (ThermoBrite, Abbott Molecular Co, Downers Grove, IL, USA). Hybridization was allowed to occur overnight for 18–20 h at 37°C. After posthybridization washing, nuclei of these cells were counterstained with 4′-6′-diamidine-2-phenylindole (DAPI) antifade. For each probe, 200 nuclei were evaluated. Interphase nuclei were scored and FISH data collected on a fluorescence photomicroscope equipped with a black and white charged couple camera device and run by Genikon™ FISH Imaging Software version 3.6.13 (Nikon Instruments S.p.A. Sesto Fiorentino, Firenze, Italy). Hybridization was repeated in those slides with <80% of the nuclei showing two control-probe signals. Immunoselected CD146-positive blasts were also analyzed by FISH in order to confirm their positivity for *BCR/ABL* translocation.

### Blasts Cells Enrichment by Immunoselection

After Ficoll gradient, PB and/or BM were processed by using human immunomagnetic CD146 and CD34 Microbead Kit (Miltenyi Biotec Inc. Teterow, Germany). The purity of CD146-positive and CD34-positive blasts was assessed by flow cytometry as described in the commercial kit manual. After the immunoselection procedure, $3 \times 10^4$ immunoselected CD146-positive blasts were washed, centrifuged by a Cytospin centrifuge, fixed and stained in methanol and May-Grunwald Giemsa, respectively, and then observed under microscope. Cytospins were also used for routine FISH analysis. Selected CD34-positive from control samples were analyzed by flow cytometry in order to determine CD146 positivity.

### Statistical Analysis

Data were analyzed by STATA statistical package, version 12 (StataCorp, LP College Station, Texas, US). After a preliminary descriptive analysis, a kurtosis test was performed in order to define the distribution of our series. Fisher's exact test was applied in order to evaluate possible associations between categorical variables. The Mann–Whitney test was used for numerical variables. A variance ratio test was applied in order to evaluate if CD146 expression and Ph positivity variances could be considered equal. Then a two-sample *t* test and Mann–Whitney test were used to compare CD146 expression on leukemic blasts and Ph positivity.

## RESULTS

### Cd146 Expression in Controls and Leukemic Patients

CD146 expression in 10 healthy controls was $0.5 \pm 0.2\%$ SD on PB-derived lymphocytes and $0.8 \pm 0.4\%$ SD on BM. These values were significantly lower with respect to those observed in AML and ALL samples, where CD146-positive blasts were always >12% of the total cells. Moreover, a strong difference in CD146 expression was also demonstrated between AML and ALL: in fact, CD146 positivity was detected in 4/121 AML cases (3.3%) vs 16/41 ALL cases (39%) ($p < 0.001$).

Age, sex distribution, and CD146 status are reported in Table 1. Thirty-eight cases (31.4%) of AML had a previous history of myelodysplastic syndrome (MDS) and four cases (3.3%) previously underwent chemotherapy for Non-Hodgkin Lymphoma (average time from chemotherapy to acute leukemia diagnosis: 4.8 years; range: 2.3–9.2 years).

### Cd146 Expression in AML

In the four CD146-positive AML cases, the mean percentage of CD45dim and CD45dim/CD34+ blasts on the total number of cells analyzed (R1) was, respectively, of $43.1 \pm 28\%$ SD and $31.3 \pm 28.1\%$ SD (Fig. 1). Interestingly, among CD146-positive AML, the mean percentage of CD45dim/CD146-positive blasts on the total number of cells analyzed was $16.3 \pm 8.0\%$ SD. This value was significantly higher than that observed in PB and BM from healthy controls.

According to immunophenotype, CD146-positive AML cases were classified as M0 FAB subtype in two cases with a myeloperoxidase-negative pattern, positivity for CD117, CD13, CD34, CD33, HLA-DR (in decreasing order of expression), and atypical expression, respectively, of CD7 and CD19 antigens; the other two CD146-positive AML cases were classified as M4 subtype with positivity for myeloperoxidase, HLA-DR, CD33, CD13, CD4, and CD117 (Fig. 1).

### Cd146 Expression in B-ALL

Fourteen out of 38 B-ALL cases showed positivity for CD146. In these cases, CD146 expression on gated blasts had a mean of $67.1 \pm 33\%$ SD which was significantly higher than in the control group ($p < 0{,}001$).

CD146-positive B-ALL cases showed also a significantly higher median age with respect to CD146-negative B-ALL ($p < 0.001$; Table 1).

The mean percentage of CD146-positive and CD146-negative blasts on the total amount of cells obtained from B-lineage ALL was similar (60% and 55%, respectively). Immunophenotyic features of CD146-positive

Table 1
*General Features of 162 Cases of Acute Leukemia Studied for CD146 Expression*

| Diagnosis | CD146 negative *N* cases | CD146 positive *N* cases | *P* values |
|---|---|---|---|
| AML ($n = 121$) | 117 (96.7%) | 4 (3.3%) | <0.001 |
| Age (median, range) | 69, 26–87 | 67, 32–86 | NS |
| Male/Female | 61/56 | 2/2 | NS |
| B-ALL ($n = 38$) | 24 (63.2) | 14 (36.8) | NS |
| Age (median, range) | 6, 1–74 | 44, 3–80 | <0.001 |
| Male/Female | 11/4 | 9/14 | NS |
| T-ALL ($n = 3$) | 1 (33.3) | 2 (66.6) | NS |
| Age | Single case: 5 | Two cases: 3, 21 | ND |
| Male/female | 1/0 | 2/0 | ND |

and CD146-negative ALL blasts are reported in Figure 2. B-ALL immunophenotype showed no significant differences in the expression of TdT, CD19, CD22, and HLA-DR on CD146-positive vs. CD146-negative blasts (Fig. 2).

When using the EGIL classification, 66.7% of "common"/BII-ALL and 33.3% of pre-B/BIII-ALL cases expressed CD146, whereas no CD146-positive case was observed in pro-B/BI-ALL and mature/BIV-ALL ($p = 0.067$). Among CD146-positive B-ALL, 78.6% were associated to a "common"/BII-ALL and the 21.4% to a pre-B/BIII-ALL immunophenotype. All cases of Ph-positive B-ALL also were CD146-positive, displaying the highest percentage of CD146 (Ph-positive B-ALL mean CD146 expression: $84 \pm 22\%$ SD vs. Ph-negative B-ALL mean CD146 expression $40 \pm 24\%$ SD; $p < 0.001$) (Fig. 3).

As expected, the percentage of CD10 and CD20 positive cells was higher in the CD146-positive blast population of "common"/BII-ALL although not reaching statistical significance. We also found that the expression of CD13 and CD33 on adult B-ALL blasts was higher in the CD146-positive group, while the atypical expression of CD4, CD7, and CD56 could be rarely observed (data not shown). Moreover, no immunophenotypic shift was observed on the blast population during the follow-up of CD146-positive B-ALL patients. Besides, we observed an increase of CD146-positive blasts at relapse (100% of the blast population, Fig. 4).

### Cd146 Expression in T-ALL

In this study, three cases of T-lineage ALL cases were investigated: two-third showed the expression of CD146 on leukemic blasts. In these two CD146-positive cases, the percentage of CD146-positive blasts was very high and the mean percentage of CD146-positive blasts was 95% of the total number of blasts. The immunophenotypic pattern of CD146-positive T-ALL showed positivity for CD7, CD4, and CD8 while the only one case of CD146-negative T-ALL presented expression of cytoplasmic CD3, atypical expression of CD4, and expression of CD117, CD19, and CD13 as expected in the early T-cell precursor ALL phenotype. A complex karyotype was found associated to the 2 cases of CD146-positive T-ALL.

### Conventional Cytogenetic Analysis of Cd146 Positive Acute Leukemia

#### Aml

All four CD146-positive AML were classified as AML secondary to myelodysplastic syndrome according to WHO (25,26). As shown in Figure 1, the four CD146-positive AML cases presented variable cytogenetic lesions. One out of 4 cases without analyzable mitoses did not show any cytogenetic defect when studied by interphase FISH while a second case had a normal karyotype (with no detectable mutations in NPM1 and FLT3) and the others are reported in Figure 1.

#### B-all

All cases of Ph+ B-ALL were CD146-positive. In particular, 7 out of 14 (50%) CD146-positive B-ALL cases presented a *t*(9;22)(q34;q11)/*BCR/ABL* translocation (Fig. 3). Within the Ph+ B-ALL group, 4/7 cases (57.1%) carried the *t*(9;22) as single abnormality and displayed the highest percentage of CD146-positive leukemic blasts ($95 \pm 0.1\%$ SD), while the remaining Ph+ CD146-positive B-ALL showed associated chromosomal aberrations such as del(3)(p21), del(1)(q32), add(7)(q22), and −7 displaying a mean percentage of the CD146-positive leukemic blasts of $81.2 \pm 25\%$ SD. Interestingly, the only CD146-positive pediatric B-lineage-ALL case had a "common"/BII-ALL phenotype and carried the *t*(9;22)(q34;q11). The remaining B-ALL cases were associated with other cytogenetic lesions such as the *t*(1;19)(q23;p13)/TCF3-PBX1, t(4;11)(q21;q23)/*AF4-MLL,* hyperdiploid, or complex karyotype, and in one case with a del(22)(q11) lesion.

A variance ratio test demonstrated that variance in CD146 expression for Ph+ cases was 0.44 times greater than Ph− cases ($p = 0.6$) (Fig. 3) and, when applying variable confrontation tests, CD146 expression strongly associated with the presence of Ph+ abnormality ($p = 0.001$).

## DISCUSSION

In normal hemopoietic tissue, CD146 expression is possibly restricted to a subpopulation of activated T cells corresponding to <1% of all cells in PB and BM. In the present series, we confirm this evidence and we show the lack of CD146 expression on normal B-lymphocytes. We focused in particular on CD146 expression in acute leukemia since the biological association of CD146 expression with different types of acute leukemia is not yet elucidated. In literature, reports about CD146 expression on progenitor cells in normal bone marrow are scant while different studies have described the expression of CD146 in circulating endothelial progenitor cells (EPC) defined as CD34+VEGFR+CD133+ CD45+ or CD45- EPC (32).

We investigated CD146 expression on hematogones and CD34-positive cells. From our results on BM samples from healthy controls, 7–10% of total immunoselected

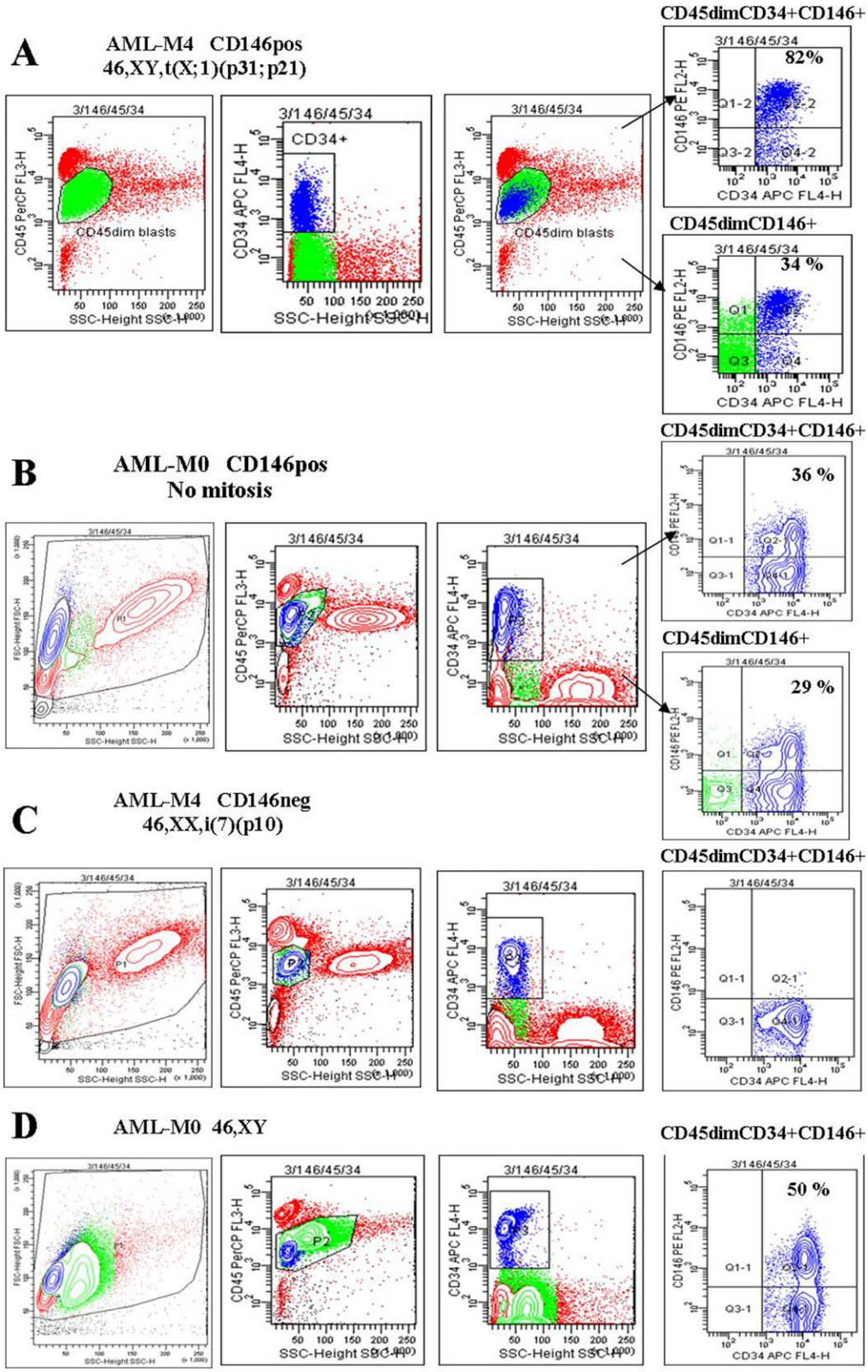


FIG. 1. Four different examples of CD146 expression pattern among 121 cases of AML are shown. Panels A, B, and D present CD146 positivity in secondary AML. Panel C shows an example of a CD146-negative case. For each case, the gating strategy on CD45dim and/or CD34+ blasts is shown. Percentage values refer respectively to CD146-positive blasts on CD45dim and CD45dim/CD34+. AML FAB subtype and cytogenetics are reported. Density and contour plots are alternatively used to better display and define CD146-positive blasts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

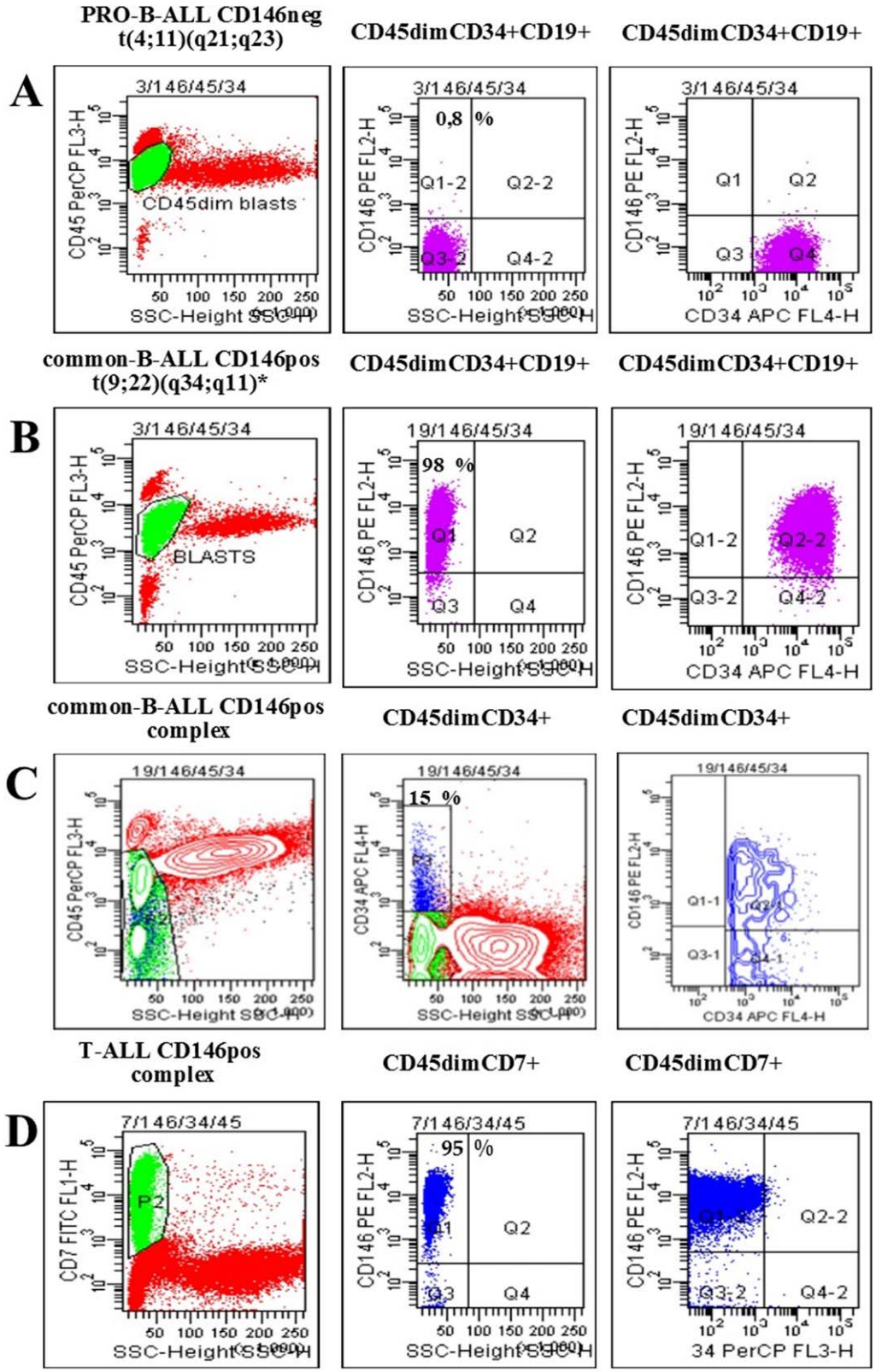


FIG. 2. CD146 expression in ALL blasts. In the figure, some examples of CD146-positive B-ALL (A–B–C rows) and T-ALL (D) cases are reported. In Panel A, CD146-negative pro-B/BI-ALL. In Panels B and C, two different cases of CD146-positive common B-ALL (density and contour plot) are shown. Panel D: an example of CD146-positive CD7+ T-ALL. *Patients carrying other chromosomal aberrations in addition to the *t* (9;22). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

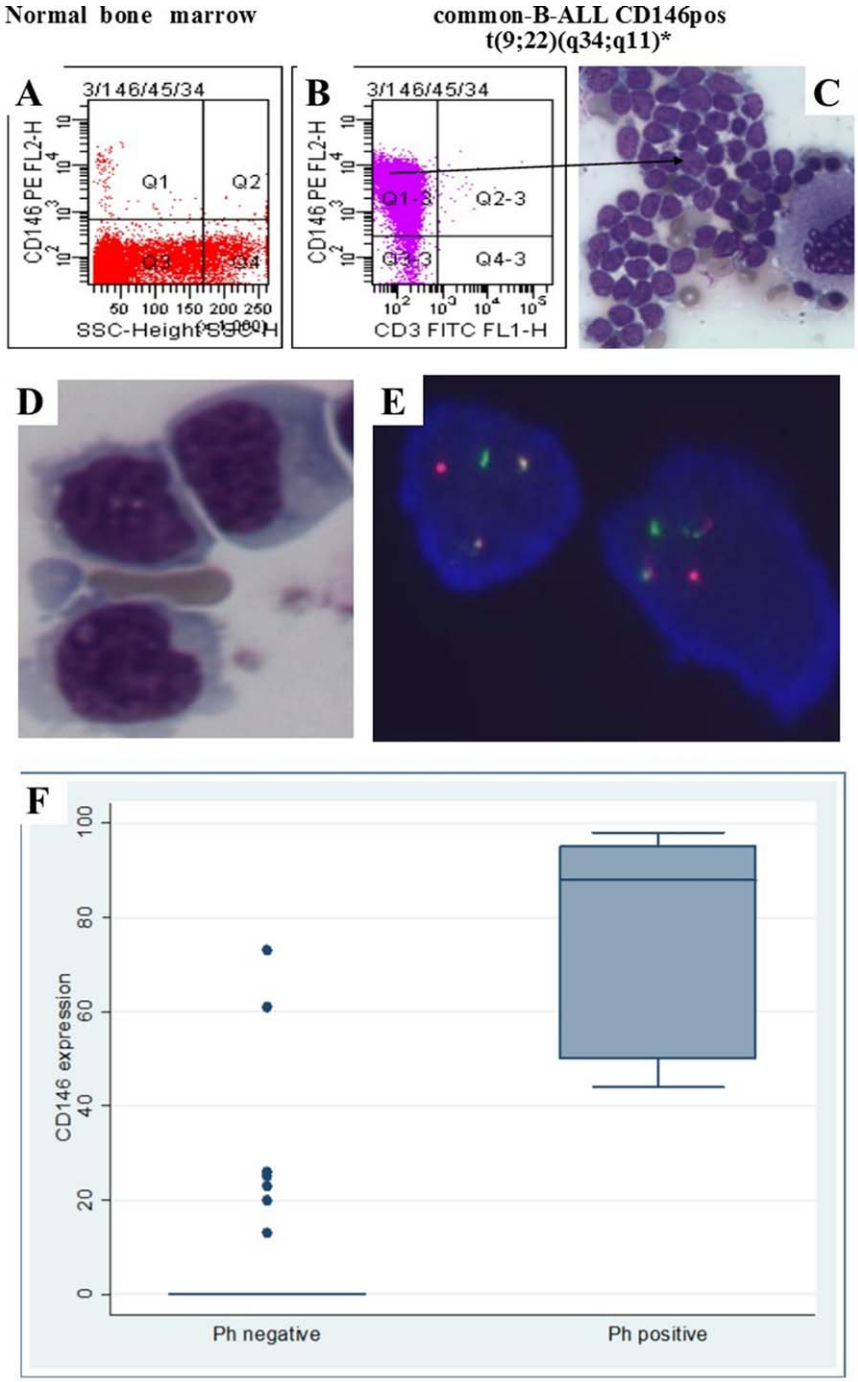


FIG. 3. Cytomorphology in CD146-positive B-ALL. Panel A: CD146-positive normal cells in bone marrow. Panels B, C, and D: cytomorphological features of selected CD146-positive leukemic blasts from a case of B-lineage ALL. Panel E: BCR/ABL rearrangement on immunoselected CD146-positive blasts in a case of common B-ALL. A significant statistical correlation was found between B-lineage ALL and the Ph chromosomal lesion as shown in the box plot (Panel F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and/or gated CD34-positive cells expressed CD146; nevertheless, restricting the gate to the CD45dim SSClow morphological area (hematogones area) and from here gating on CD10+CD19+CD45dim and CD34-positive and/or CD34-negative area, no CD146-positive cells were found (Supporting Information Figure).

Furthermore, in our experience, no circulating CD146-positive/CD45-EPC were found in patients affected by Systemic Sclerosis (33) while we demonstrated CD146 expression by a subset of circulating CD34+ hematopoietic progenitor cells as well as in PB-derived EPC after in vitro isolation in patients with acute coronary syndrome

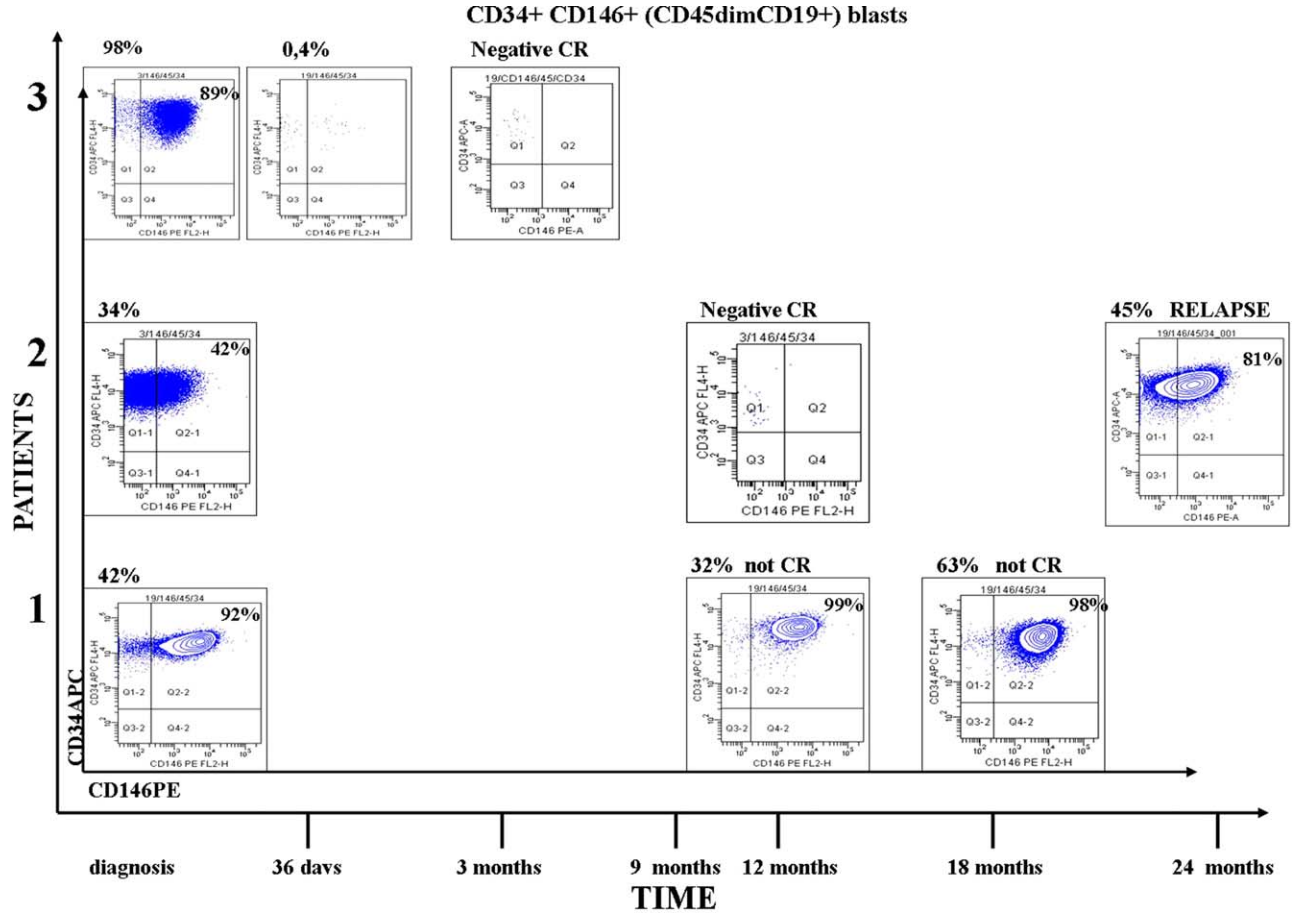


FIG. 4. CD146 expression at diagnosis and follow-up. Three Ph-positive "common"/BII-ALL cases at different phases of treatment: percentage of BM blasts is marked on the top of the dot plot. The percentage of CD146+ blasts is included in the plot/CD146 area. Patient 1 displayed a 46,XY,*t*(9;22)(q34;q11) alteration and never reached remission. Patient 2 had a complex karyotype, maintained a complete remission (CR) after 1 year from the diagnosis but relapsed at 24 months. Patient 3 had *t*(9;22)(q34;q11), obtained CR, and underwent allogeneic transplantation relapsing after 2 years from diagnosis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.].

(34). On the other hand, no reports are present in literature regarding CD146 expression on normal B-lymphocytes except for the evidence that CD146/MUC18 expression in melanoma cells correlates with the presence of a particular B-I lymphocyte subset (35). In this study, our results showed a very low number of CD146-positive AML cases (3.3% of AML) expressing CD146 in a minor proportion of blasts when compared to CD146-positive B-ALL. Interestingly, all CD146-positive AML cases were classified as secondary AML not otherwise specified. Conversely, 66% of T-ALL and 36.8% of the total B-ALL cases expressed CD146 on leukemic blasts confirming previous preliminary observations by Filshie et al. on immortalized leukemic T-cells and on a minority of B-cell lines (25). In their study, Filshie and coworkers described three mAbs (WM85, CC9, and EB4) able to identify a CD146/MUC18 expression on different types of formalin- and paraffin-embedded samples. Filshie focused on leukemic cell lines such as NALM-6 ($n = 6$) or K-562 ($n = 4$). A limited number of fresh samples were also analyzed by using an indirect immunostaining after Ficoll gradient. The samples were 4 T-ALL, 20 B-ALL, 7 AML, and an heterogeneous group of seven B-cell lymphoproliferative disorders. With this background, we demonstrated that it is possible to apply a different monoclonal antibody (such as clone P1H12-directly conjugated PE) in combination with other antibodies on fresh primary samples using a multiparametric flow cytometric approach for the assessment of CD146 expression. With this strategy, we studied a large series of 162 samples from hematological patients. Moreover, we provided and described the relative information on the immunophenotypic and cytogenetic characteristic of blasts and we described the relative expression of CD146 on B- and T-ALL and AML according to WHO/EGIL classification. We then reported on the overall expression of CD146 in different types of acute leukemia underlying for the first time that this marker appears to be expressed particularly in T-ALL (66%) as well as in all B-ALL cases bearing the t(9;22)(q34;q11)/*BCR/ABL* translocation. No obvious conclusions were possible for CD146-positive T-ALL due to the limited number of cases analyzed. Nevertheless, a very high frequency of CD146-

positive cases and high percentage of positivity was found in T-ALL as previously observed by Filshie et al. (26). Moreover, we reported that CD146 expression in T-ALL was also associated with expression of CD7, CD4, and CD8 on CD146-positive blasts. On the other hand, the only CD146-negative case was an early T-cell precursor (ETP)-ALL. These data are in agreement with previous studies that described CD146 expression on a subpopulation of activated T cells as well as on a subset of CD3+CD4+ T lymphocytes in patients affected by various musculoskeletal diseases and on pathologic lymphocytes in lymphoma cells of the chicken (25,36). These findings suggest that this molecule may also play a role in normal hemopoiesis representing a specific T marker of lymphopoiesis. Instead, among CD146-positive cases, 78.6% of B-ALL was associated with a "common"/BII-ALL and 21.4% with a pre-B/BIII-ALL immunophenotype while all pro-B/BI-ALL and mature/BIV-ALL cases were negative. In addition, a strong association between t(9;22)/*BCR/ABL* translocation and CD146 positivity in B-ALL was found since all Ph+ B-ALL were also CD146-positive. In particular, Ph+ B-ALL showed the highest proportion of CD146-positive blasts. Before considering CD146-expression as a marker of minimal residual disease monitoring, further studies are needed in order to confirm this association (37). We did not investigate the prognostic value of CD146 expression in our series; nevertheless, an increase in the proportion of CD146-positive blasts was observed at relapse suggesting that CD146-positivity could possibly represent a marker of disease aggressiveness. In fact, CD146 is a member of the immunoglobulin superfamily with an adhesive role in cell–cell and cell–matrix binding (38,39). One could speculate that when applied to acute leukemia, the expression of CD146 on leukemic cells could promote their adhesion to stromal cells in different tissues or microenvironments thus favoring leukemic relapse.

In summary, our results suggest that CD146 is highly expressed in T-ALL, whereas on B-ALL leukemia, CD146 expression is likely to represent an aberrant marker frequently associated with the *t*(9;22)(q34;q11)/*BCR/ABL* translocation. We propose that CD146 may be considered in the setting of clinical trials as additional marker to better define some specific leukemia subtypes especially in Ph+ B-ALL and T-ALL patients.

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## LITERATURE CITED

1. Lehman JM, Riethmüller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. Cell Biol 1989;86:9891–9895.
2. Ouhtit A, Gaur RL, Elmageed ZYA, Fernando A, Thouta R, Trappey AK, Abdraboth ME, El-Sayyad HI, Rao P, Raj MGH. Towards understanding the mode of action of the multifaced cell adhesion receptor CD146. Biochimica Et Biophysica Acta 2009;1795:130–136.
3. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord blood and bone marrow. Stem Cells 2007;25:1384–1392.
4. Russel KC, Phinney DG, Lacey MR, Barrilleaux BL, Meyertholen KE, O'Connor KC. In vitro high capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. Stem Cells. 2010;28:7883.
5. Sorrentino A, Ferracin M, Castelli G, Biffoni M, Tomaselli G, Baiocchi M, Fatica A, Negrini M, Peschle C, Valtieri M. Isolation and characterization of CD146-positive multipotent mesenchymal stromal cells. Exp Haematol 2008;36:1035–1046.
6. Crisan M, Corselli M, Chen WC, Péault B. Perivascular cells for regenerative medicine. J Cell Mol Med 2012;6:2851–60.
7. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P. Self – renewing osteoprogenitors in bone marrow sinusoids can organize hematopoietic microenvironment. Cell 2007;131:324–336.
8. Tormin A, Li O, Brune JC, Walsh S, Schutz B, Ehinger M, Ditzel N, Kassem M, Scheding S. CD146 expression on primary non hematopoietic bone marrow stem cells is correlated with in situ localization. Blood 2011;117:5067–77.
9. Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Péault B, Rubin P, Donnenberg AD. Stromal vascular progenitors in adult human adipose tissue. Cytometry Part B 2010;77A:22–30.
10. Bianco P, Robey P, Simmons P. Mesenchymal stem cells: Revisiting history, concepts, and assay. Cell Stem Cell 2008;2:313–9.
11. Furstenberger G, von Moos R, Senn H-J, Boneberg E-M. Real-time PCR of CD146 mRNA in peripheral blood enables the relative quantification of circulating endothelial cells and is an indicator of angiogenesis. Br J Cancer 2005;93:793–798.
12. Jiang T, Zhuang J, Duan H, Luo Y, Zeng Q, Fan K, Yan H, Lu D, Ye Z, Hao J, Feng J, Yang D, Yan X. CD146 is a coreceptor for VEGFR-2 in tumor angiogenesis. Blood 2012;120:2330–9.
13. Sers C, Riethmüller G, Johnson JP. MUC18, a melanoma-progression associated molecule, and its potential role in tumor vascularization and hematogenous spread. Cancer Res. 1994;54:5689–94.
14. Shih IM. The role of CD146 (Mel-CAM) in biology and pathology. J Pathol 1999;189:4–11.
15. Luo Y, Zheng C, Zhang J, Lu D, Zhuang J, Xing S, Feng J, Yang D, Yan X. Recognition of CD146 as an ERM-binding protein offers novel mechanisms for melanoma cell migration. Oncogene 2012;31: 306–321.
16. Schiano C, Grimaldi V, Casamassimi A, Infante V, Esposito A, Giovane A, Napoli C. Different expression of CD146 in human normal and osteosarcoma cell lines. Med Oncol. 2012;24:2998–3002.
17. Wang W, Yang ZL, Liu JQ, Jiang S, Miao XY. Identification of CD146 expression, angiogenesis, and lympahngiogenesis as progression, metastasis, and poor-prognosis related markers for gallbladder adenocarcinoma. Tumour Biol 2012;33:173–182.
18. Duda DG, Cohen KS, di Tomaso E, Au P, Klein RJ, Scadden DT, Willet CG, Jain RK. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: Implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol 2006;24: 1449–53.7.
19. Wu GJ, Peng Q, Fu P, Wang SW, Chiang CF, Dillehay DL, Wu MW. Ectopical expression of human MUC18 increases metastasis of human prostate cancer cells. Gene 2004;327:201–213.
20. Zeng Q, Li W, Lu D, Wu Z, Duan H, Luo Y, Feng J, Yang D, Fu L, Yan X. CD146, an epithelial mesenchymal transition inducer, is associated with triple-negative breast cancer. Pnas 2012;109:1127–32.
21. Mostert B, Kraan J, Bolt-de Vries J, van der Spoel P, Sieuwerts AM, Schutte M, Timmermans AM, Foekens R, Martens JWM, Gratama JW, Foekens JA, Sleijfer S. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. Breast Cancer Treat 2011;127:33–41.
22. Imbert AM, Garulli C, Choquet E, Koubi M, Aurrand-Lions M, Chabannon C. CD146 expression in humann breast cancer cell lines induces phenotypic and functional changes observed in epithelial to mesenchymal transition. PloSOne 2012;7:1–8.
23. Pickl WF, Majdic O, Fischer GF, Petzelbauer P, Faé I, Waclavicek M, Stöckl J, Scheinecker C, Vidicki T, Aschauer H, Johnson JP, Knapp W. MUC18/MCAM (CD146), an activation antigen of human T lymphocytes. J Immunol. 1997;158:2107–15.

24. Despoix N, Walzer T, Jouve N, Blot-Chabaud M, Bardin N, Paul P, Lyonnet L, Vivier E, Digent- George F, Vely F. Mouse CD146/MCAM is a marker of natural killer cell maturation. Eur J Immunol 2008;38: 2855–64.
25. Dagur PK, Tatlici G, Gourley M, Samsel L, Raghavachari N, Liu P,. McCoy P Jr. CD146 T lymphocytes are increased in both the peripheral circulation and in the synovial effusions of patients with various musculoskeletal diseases and display pro-inflammatory gene profiles. Cytometry B Clin Cytom. 2010;78B:88–95.
26. Filshie RJA, Zanettino ACW, Makrynikola V, Gronthos S, Henniker AJ, Bendall LJ, Gottlieb DJ, Dimmons DJ, Bradstock KF. MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies. Leukemia 1998;12:414–21.
27. Falini B, Tiacci E, Martelli MP, Ascani S, Pileri SA. New classification of acute myeloid leukaemias and precursor-related neoplasms: changes and unsolved issues. Discov Med. 2010;10:281–292.
28. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937–951.
29. Matutes E, Pickl WF, Van't Veer M, Morilla R, Swansbury J, Strobl H, Attarbaschi A, Hopfinger G, Ashley S, Bene MC, Porwit A, Orfao A, Lemez P, Schabath R, Ludwig WD. Mixed-phenotype acute leukemia: Clinical and laboratory features and outcome in 100 patients according to the WHO 2008 classification. Blood 2011;117:3163–3171.
30. Bènè MC, Nebe T, Bettelheim P, Buldini B, Bumbea H, Kern W, Lacombe F, Lemez F, Marinov I, Matutes E, Maynadiè M, Oelschlagel U, Orfao A, Schabath R, Solenthaler M, Tschurtschenthaler G, Vladareanu AM, Zini G, Faure GC, Porwit A. Immunophenotyping of acute leukemia and lymphoprolipherative disorders: A consensus proposal of the European LeukemiaNet work package 10. Leukemia 2011;25:567–574.
31. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995 Oct;9:1783–6.
32. Ortolani C. Flow Cytometry of Haematological Malignancies. Ed. Wiley-Blackwell. 2011.34.
33. Rigolin GM, Maffei R, Rizzotto L, Ciccone M, Sofritti O, Daghia G, Cibien F, Cavazzini F, Marasca R, Cuneo A. Circulating endothelial cells in patients with chronic lymphocytic leukemia: Clinical prognostic and biologic significance. Cancer. 2010 Apr 15;116: 1926–37.
34. Campioni D, Lo Monaco A, Lanza F, Moretti S, Ferrari L, Fotinidi M, La Corte R, Cuneo A, Trotta F. CXCR4 pos circulating progenitor cells coexpressing monocytic and endothelial markers correlating with fibrotic clinical features are present in the peripheral blood of patients affected by systemic sclerosis. Haematologica 2008;93:1233−7.
35. Campioni D, Zauli G, Gambetti S, Campo G, Cuneo A, Ferrari R, Secchiero P. In vitro characterization of circulating endothelial progenitor cells isolated from patients with acute coronary syndrome. PLoS One 2013;8:e56377
36. Staquicini FI, Tandle A, Libutti SK, Sun J, Zigler M, Bar-Eli M, Aliperti F, Pérez EC, Gershenwald JE, Mariano M, Pasqualini R, Arap W, Lopes JD. A subset of host B lymphocytes controls melanoma metastasis through a melanoma cell adhesion molecule/MUC18-dependent interaction: Evidence from mice and humans. Cancer Res. 2008;68:419–28.
37. Gaipa G, Basso G, Campana D. Detection of minimal residual disease in pediatric acute lymphoblastic leukemia. Cytometry B Clin Cytom. 2013;84:359–69.
38. Tsuchiya S, Tsukamoto Y, Furuya M, Hiroi S, Miki N, Sasaki F, Taira E. Gicerin, a cell adhesion molecule, promotes the metastasis of lymphoma cells of the chicken. Cell Tissue Res 2003;314:389–397.
39. Stopp S, Bornhauser M, Ugarte F, Wobus M, Kuhn M, Brenner S, Thieme S. Expression of the melanoma cell adhesion molecule in human mesenchymal stromal cells regulates proliferation, differentiation, and maintenance of hematopoietic stem and progenitor cells. Haematologica 2013;98:505–513.