Leukemia Research 39 (2015) 749–756

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

CD47 protein expression in acute myeloid leukemia: A tissue microarray-based analysis

Serena Galli[a,b], Inti Zlobec[a], Christian Schürch[a,c], Aurel Perren[a], Adrian F. Ochsenbein[b,c], Yara Banz[a,c,*]

[a] Institute of Pathology, University of Bern, Bern, Switzerland
[b] Department of Oncology, University Hospital Bern, Bern, Switzerland
[c] Department of Clinical Research, University of Bern, Bern, Switzerland

ARTICLE INFO

Article history:
Received 3 March 2015
Accepted 13 April 2015
Available online 20 April 2015

Keywords:
CD47
Acute myeloid leukemia
Tissue microarray
Bone marrow

ABSTRACT

Binding of CD47 to signal regulatory protein alpha (SIRPα), an inhibitory receptor, negatively regulates phagocytosis. In acute myeloid leukemia (AML), CD47 is overexpressed on peripheral blasts and leukemia stem cells and inversely correlates with survival.

Aim of the study was to investigate the correlation between CD47 protein expression by immunohistochemistry (IHC) in a bone marrow (BM) tissue microarray (TMA) and clinical outcome in AML patients. CD47 staining on BM leukemia blasts was scored semi-quantitatively and correlated with clinical parameters and known prognostic factors in AML. Low (scores 0–2) and high (score 3) CD47 protein expression were observed in 75% and 25% of AML patients. CD47 expression significantly correlated with percentage BM blast infiltration and peripheral blood blasts. Moreover, high CD47 expression was associated with nucleophosmin (NPM1) gene mutations. In contrast, CD47 expression did not significantly correlate with overall or progression free survival or response to therapy.

In summary, a BM TMA permits rapid and reproducible semi-quantitative analysis of CD47 protein expression by IHC. While CD47 expression on circulating AML blasts has been shown to be a negative prognostic marker for a very defined population of AML patients with NK AML, CD47 expression on AML BM blasts is not.

1. Introduction

CD47 is a transmembrane glycoprotein, which is broadly expressed in most human tissues and functions as a ligand for several receptors, including signal regulatory protein alpha (SIRPα). CD47-SIRPα signaling on macrophages or dendritic cells results in inhibition of phagocytosis by immunoreceptor tyrosine-based inhibition motif (ITIM)-mediated recruitment of protein tyrosine phosphatases Src homology region 2 domain-containing phosphatase-1/2 (SHP-1/2) [1]. Serving as ligand for thrombospondin, CD47 has an inhibitory effect on the nitric oxide (NO)/cyclic guanine monophosphate (cGMP) signaling pathway and restricts NO-mediated vasodilatation and disinhibition of platelet aggregation [2]. Interaction of CD47 on myeloid cells with endothelial CD31 allows for endothelial transmigration [3].

In the context of acute myeloid leukemia (AML), CD47 expression, as measured by flow cytometry, is increased on AML leukemia stem cells (LSCs) as compared to normal hematopoietic stem cells (HSCs) [4,5]. Upregulation of CD47 expression is primarily a physiological mechanism: Administration of cyclophosphamide, granulocyte colony stimulating factor (G-CSF) and lipopolysaccharide, respectively induces mobilization of HSCs, leading to significantly elevated CD47 expression on circulating HSCs in comparison to their bone marrow (BM)-resident counterparts. This CD47 up regulation presumably protects mobilized HSC from subsequent macrophage-mediated phagocytosis [4]. Additionally, CD47 was shown to be of prognostic value in AML as well as in myelodysplastic syndrome (MDS), where CD47 was expressed at higher levels in high-risk as compared to low-risk MDS [6]. In a cohort of 132 AML patients with normal karyotype, high CD47 mRNA levels in peripheral blood blasts were significantly associated with a lower event-free and overall survival [5]. Evasion of macrophage-mediated phagocytosis by CD47-SIRPα signaling resulted in reduced blast clearance by the innate immune system and conferred a survival advantage to the LSCs as compared to

* Corresponding author at: Institute of Pathology, University of Bern, Murtenstrasse 31, 3010 Bern, Switzerland. Tel.: +41 31 632 88 75; fax: +41 31 632 99 38.
E-mail address: yara.banz@pathology.unibe.ch (Y. Banz).

http://dx.doi.org/10.1016/j.leukres.2015.04.007
0145-2126/

the normal HSC counterparts. Thus, CD47 may not only be a useful biomarker to identify LSCs but may also represent a clinically relevant pathogenetic factor of disease.

Alternative pathogenetic mechanisms in addition to inhibition of phagocytosis have been proposed to contribute to a worse clinical outcome: CD47-SIRPα signaling on dendritic cells (DCs) impairs their T-cell allo-stimulatory capacity by blocking DC maturation [7], whilst the role of SIRPα-independent triggering of CD47 to induce caspase-independent programmed cell death as not been unanimously confirmed [4,8].

Blocking CD47 on disease-sustaining LSCs may therefore represent a promising therapeutic approach. Indeed, previous studies have shown that blocking the CD47-SIRPα interaction using anti-CD47 antibodies promotes phagocytosis and clearing of LSCs [9–11].

In light of the potential of CD47 as an independent prognostic marker in AML, the aim of this study was to investigate the correlation between CD47 protein expression on BM blasts and clinical outcome using immunohistochemistry (IHC) in a tissue microarray (TMA) of formalin-fixed, paraffin-embedded BM specimens.

2. Methods

2.1. Case selection

The cases for the tissue microarray (TMA) were selected from 248 patients who were diagnosed with acute myeloid leukemia (AML) and were identified retrospectively by data base search from the archives of the Institute of Pathology, University of Bern, Switzerland, between 2006 and 2013. Formalin-fixed, paraffin-embedded (FFPE) bone marrow biopsies were available from all patients. The main primary exclusion criteria were poor material quality or quantity (initial biopsy material inadequate, insufficient remaining tumor tissue volume or tissue preservation). Whilst this TMA included all AML subtypes, a second TMA was constructed exclusively with normal karyotype (NK)-AML patients as a second test cohort, in particular in order to increase patient numbers in the NK subgroup (Fig. 1). Detailed clinical information for each patient was available from the files of the Department of Medical Oncology, University Hospital of Bern, Switzerland. The study was approved by the Cantonal Ethics Committee of Bern and was carried out according to national and international guidelines, in accordance with the ethical principles of the Declaration of Helsinki using Good Clinical Practice.

2.2. TMA construction

For each case, the hematoxylin and eosin (H&E) slides were reviewed in order to confirm the previous diagnosis and to identify the most representative and blast-rich areas, which were punched out from the FFPE tissue blocks using a fully automatic arraying device (TMA Grandmaster, 3D Histech, Budapest, Hungary). Additionally, nine control punches were integrated, equally distributed over the recipient array block, consisting of cell blocks of three leukemia cell lines: HT93A: AML FAB M3 [12], U937: diffuse histiocytic lymphoma [13], HL-60: AML FAB M2, all revealing CD47 expression in the FACS analysis, stratified into low, medium and high expression. The final first TMA block consisted of 222 single-punches of 0.6 mm in diameter. In order to allow for comparison of the general staining intensity between the two TMAs, three punches from the first TMA, which exhibited strong CD47 staining were included in the second TMA. The final second TMA block constructed analogous to the first one consisted of 29 single-punches of 0.6 mm in diameter.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) of CD47 protein was performed on FFPE tissue using an automated immunostainer (Leica BOND-III, Leica Biosystems). As a pretreatment for antigen retrieval, the TMA section was placed in citrate buffer at pH 6.0 at 100 °C for 30 min. Subsequently, the TMA was incubated at room temperature with the monoclonal mouse anti-human CD47 antibody (clone B6H12, Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a working concentration of 1:25 for 30 min. Antigen detection was performed using a commercial detection kit (Bond Polymer Refine Detection) with diaminobenzidin as the chromogen.

2.4. Immunohistochemical scoring

For assessment of CD47 expression the stained TMA slides were scanned with Aperio (Vista, USA) and evaluated at 40× magnification. Each spot was independently assessed twice by S.G. and Y.B. in a blinded fashion. In discrepant cases, the individual spots were reviewed by both S.G. and Y.B. in order to reach a concordant score for subsequent statistical analysis. The staining intensity was stratified from 0 to 3 (0: no membranous staining; 1: weak staining; 2: moderate staining; 3: intense staining), using four determined samples within the TMA (Fig. 2). The external controls (cell lines) were used to assure equal and adequate staining across the TMA itself. In cases with more than one (morphologically or immunophenotypically different) blast population the most intensely stained subpopulation defined the score, based on the presumption that the tumor cells with the highest CD47 expression are ultimately relevant for the biological behavior. The morphology of the BM cells and blast population was compared with the matching hematoxylin and eosin stained section.

2.5. Statistical analysis

The inter-observer agreement of CD47 scores was assessed using the simple and weighted kappa (κ) statistic and 95% confidence interval (CI). The simple κ takes into account the exact agreement between scores (how many cases were scored as 0, 1, 2 and 3 by both observers), while the weighted κ determines agreement based on how far apart the scores are. The association of CD47 with continuous clinical parameters was determined using Student's t-test and for categorical variables using the Chi-square or Fisher's exact test, where applicable. Univariate survival analysis was carried out using the log-rank test and Kaplan–Meier method for plotting survival curves. All tests were two-sided. Missing values were considered to be missing at random. p-Values <0.05 were considered statistically significant. Analyses were conducted using SAS v9.2 (SAS Institute, Cary, NC).

3. Results

AML cases were classified in accordance with the 4th edition of the 2008 WHO Classification of Tumors of the Hematopoietic and Lymphoid Tissues [14]. The clinico-pathological characteristics of the two study groups are summarized in Table 1. The cohort of TMA 1 consisted of 171 samples of AML patients amenable to analysis with a median age of 62 years, including 101 male and 70 female patients. 60 patients were diagnosed with secondary AML. The cohort of TMA 2 included 24 patients with normal karyotype AML (NK-AML) (Table 1).

3.1. Immunohistochemistry is a reproducible method for semi-quantitative CD47 detection

Staining inter- and intra-observer agreement was analyzed for both TMAs. Reviewing each TMA punch twice in two independent assessment rounds by two persons (Y.B. and S.G.) an inter-observer agreement of 75% (simple κ) and 81% (weighted κ), respectively, and an intra-observer agreement of 87% (simple κ) and 90% (weighted κ), respectively, was obtained. When staining results were grouped into low expression (scores 0–2) and high expression (score 3) the inter-observer agreement was 87% and the intra-observer agreement 97% (Table 2). Discrepant cases were re-reviewed by both Y.B. and S.G. to achieve a consensus grade for subsequent statistical analysis.

3.2. CD47 protein is highly expressed in a quarter of primary AML cases

CD47 staining was completely negative in 9 cases (score 0), 59 cases showed weak (score 1), 71 cases showed moderate (score 2) and 32 cases showed high (score 3) staining intensity (Fig. 3). Subsequent analyses were performed using the two-sided scoring system (0–2 = low expression and score 3 = high expression) based on the results of the excellent inter- and intra-observer agreement. A high CD47 expression was found in 25% of all the de novo AML patients (Fig. 3). Interestingly, secondary AML cases exhibited a strong CD47 staining in a statistically significantly ($p = 0.0450$) lower percentage of cases as compared to primary AML. Whilst in primary AML 28 out of 111 patients (25%) showed a high CD47 expression, only 4 out of 60 patients with secondary AML (7%) did so (Fig. 3 and Table 3).

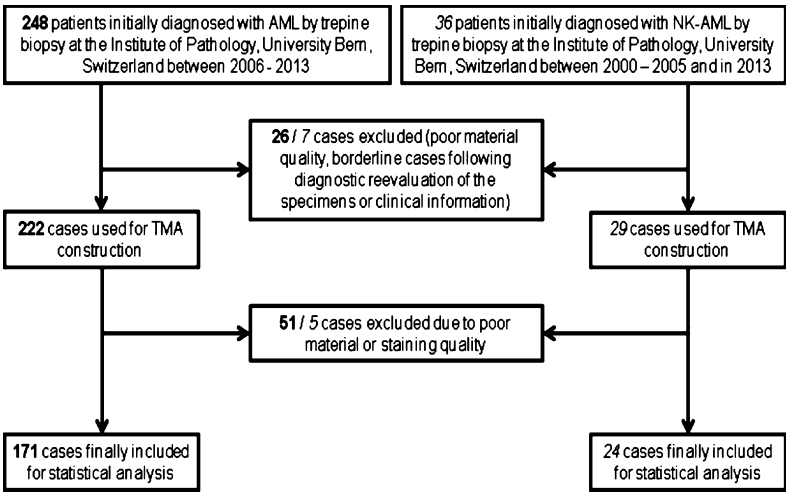

Fig. 1. Flow chart showing the retrospective case selection procedure from the University Hospital Bern with construction of the first tissue microarray (TMA, left side) with an final number of 171 samples as well as additional test TMA containing only normal karyotype acute myeloid leukemia (NK-AML) patients (right side) with an end total of 24 samples.

3.3. High CD47 expression correlates with a higher tumor load

In the entire cohort, patients with high CD47 expression (score 3) exhibited higher median peripheral blood blast percentages (65%) than patients with low CD47 expression (scores 0–2; 26%, $p = 0.0007$, Fig. 4A and Table 3). Similarly, median BM blast percentage at the time of diagnosis also showed a statistically significant correlation with CD47 expression (low: 55%; high: 85%; $p = 0.0001$; Fig. 4B and Table 3). In accordance with the above-mentioned lower CD47 expression in secondary AML, median blast BM infiltration in primary AML was 80%, whereas in secondary AML it was 25% ($p = 0.0341$) Similar results were observed for peripheral blast numbers, with blast a count three times higher in primary AML in comparison to secondary AML (primary: median blast count of 50% versus 16%, $p = 0.0375$). The absolute blast numbers were not statistically significantly different between the two groups.

3.4. Higher CD47 expression in patients with NPM1 mutation

In the entire cohort, an NPM1 mutation was found in 23% (33/144) of the patients. In the subgroup of NK-AML this proportion was higher at 43% (26/61). Patients with an NPM1 mutation revealed a significantly higher level of CD47 expression (score 3) as compared to patients without NPM1 mutation ($p = 0.0234$, Fig. 4C). This correlated with the fact that whilst 12% of non-NK patients were scored as 3+, this figure was 30% for patients with NK-AML ($p = 0.0034$).

3.5. No correlation of CD47 with t(8;21), FLT3-ITD, age, gender, FAB classification, therapy or remission

Neither an inverse correlation between high CD47 expression and the presence of t(8;21) nor a direct correlation with the presence of FLT3-ITD was observed ($p = 1.0$ and $p = 0.3544$,

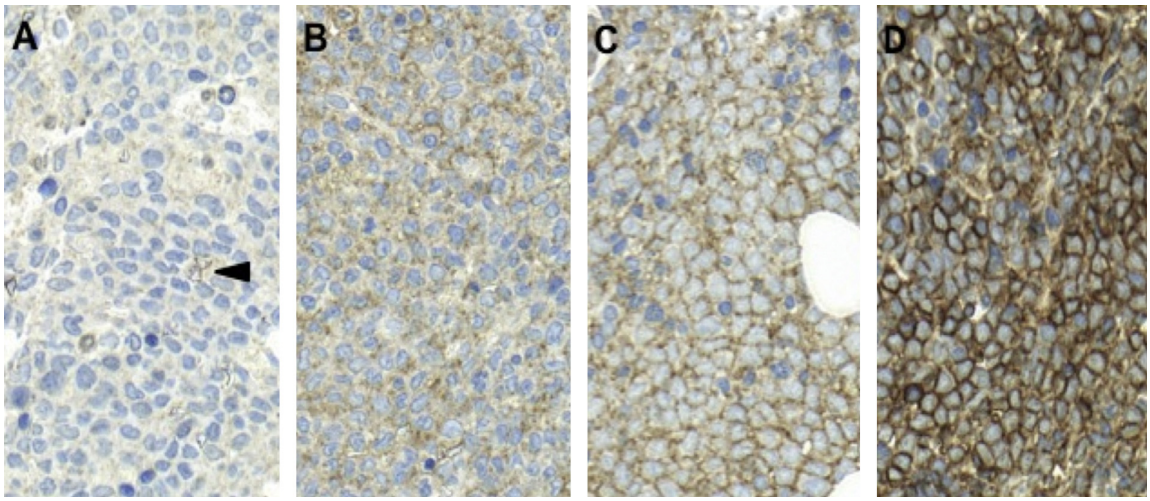

Fig. 2. Representative images from immunohistochemical staining of CD47 on bone marrow (BM) biopsies of patients with acute myeloid leukemia from the archives of the Institute of Pathology of the University of Bern. Representative examples are shown for the grading system of staining intensity 0–3. Grade 0 (A): complete lack of immunoreactivity in the blast population with minimal staining restricted to erythrocytes (arrowhead), without relevant background staining. Grade 1 (B): weak, partly finely granular membranous staining of BM blasts with clear delimitation of the cell borders. Grade 2 (C): moderate staining of BM blasts with continuous, intermediate intensity staining allowing for clear depiction of cell borders. Grade 3 (D): strong and diffuse membranous staining of bone marrow blasts with sharp demarcation of single cells.

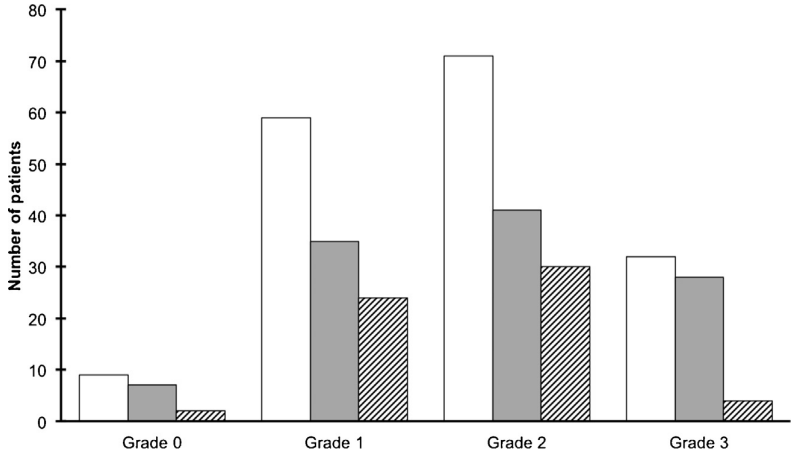

Fig. 3. Distribution of CD47 staining intensity in the whole cohort (white columns), in patients with primary acute myeloid leukemia (AML; gray columns) and in patients with secondary AML (striped columns). High expression (grade 3) was found in 19% (whole cohort: 32/171), 25% (primary AML: 28/111) and 7% (secondary AML: 4/60) of the patients, respectively.

respectively). Furthermore, no statistically significant correlation was observed between CD47 expression levels and age ($p = 0.2970$), gender ($p = 0.0507$), first-line therapy ($p = 0.7949$), allogeneic stem cell transplantation ($p = 0.1102$) or remission status ($p = 0.4216$), as summarized in Table 3.

3.6. High CD47 expression in BM blasts does not predict worse clinical outcome in the AML cohorts

When analyzing both cohorts (TMAs 1 and 2), there was no statistically significant differences in overall survival ($p = 0.1272$,

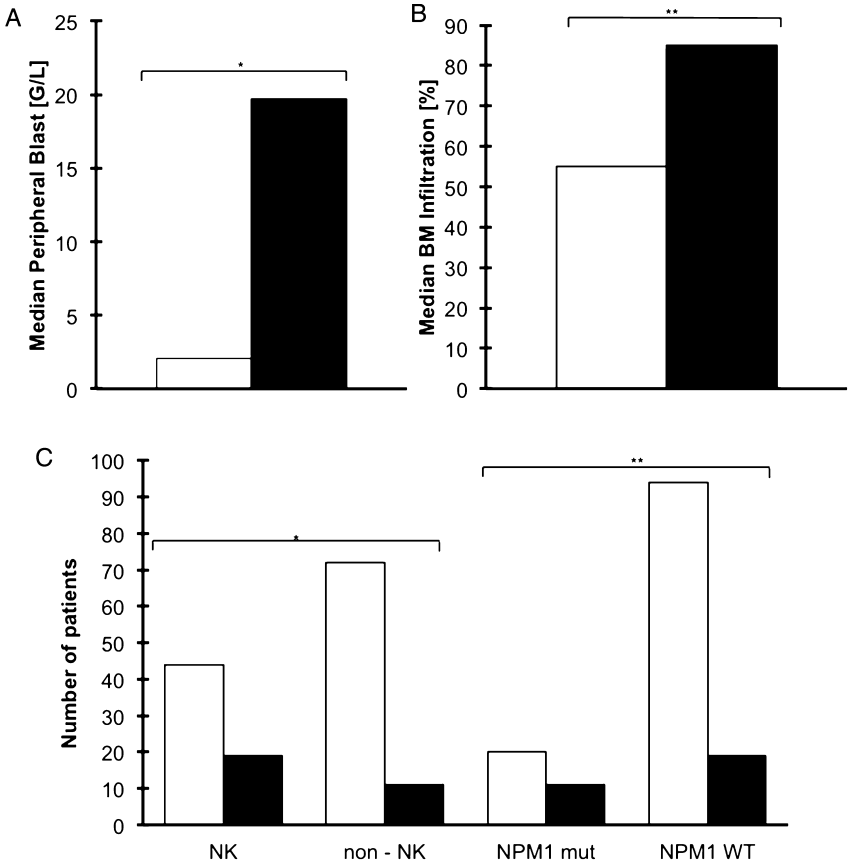

Fig. 4. Median blast numbers in peripheral blood (A) and blast percentage in bone marrow (BM) (B) as stratified by low (scores 0–2, white columns), or high (score 3, black columns) CD47 expression. High BM infiltration of myeloid blasts ($^{*}p = 0.0001$) and high peripheral blast count ($^{**}p = 0.0007$) correlates with high CD47 expression in AML patients. Distribution of CD47 expression in AML subtypes (C) in patients with normal karyotype (NK), patients with non-normal karyotype (non-NK), patients with NPM1 mutation (MPN1mut) and patients with NPM1 wild type (NMP1WT). White columns represent low expression (scores 0–2), black columns high expression (score 3). High CD47 expression was more frequent in patients with NK compared to non-NK-AML (30% versus 12%, $^{*}p = 0.0034$) and in patients with NPM1mut compared to patients with NPM1WT (35% versus 17%, $^{**}p = 0.0234$).

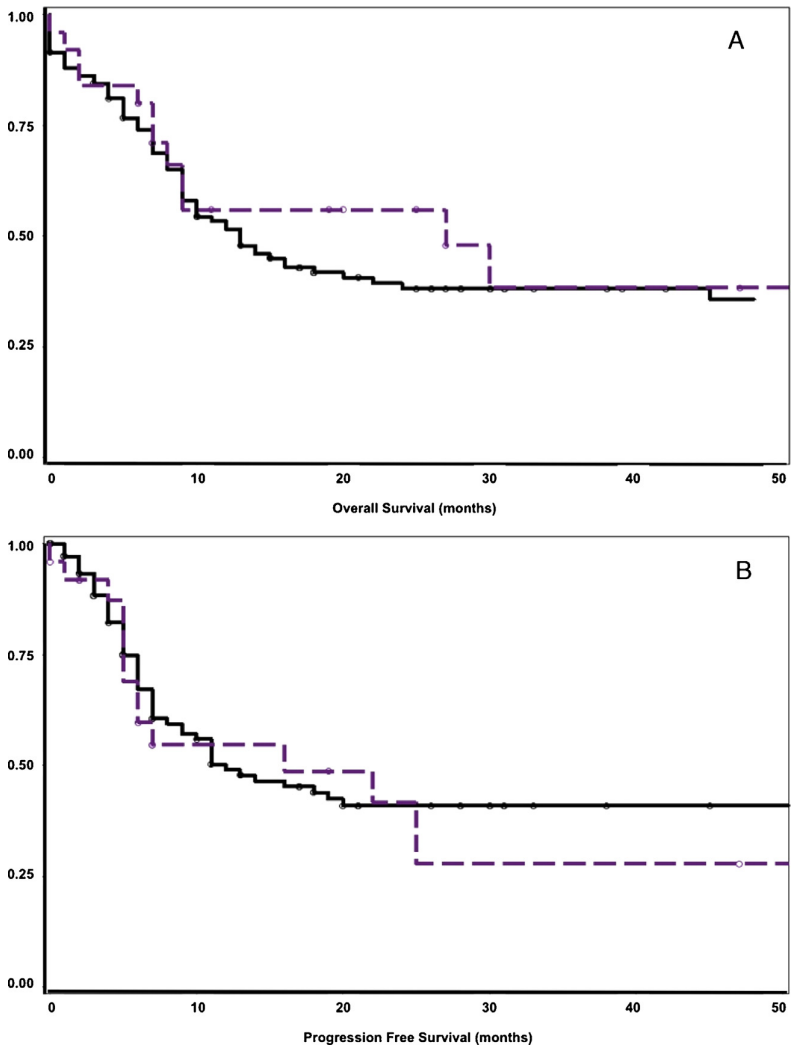

Fig. 5. Overall survival (OS, A) and progression free survival (PFS, B) for the entire cohort of acute myeloid leukemia (AML) patients (TMA 1 and 2). Low expression of CD47 (scores 0–2) in black drawn outlines and high expression (score 3) in interrupted purple. No significant differences were observed between the two groups ($p = 0.1272$ for OS and $p = 0.0531$ for EFS).

Fig. 5A) or progression free survival between low CD47 expression (grades 0–2) and high expression (grade 3), although there was a clear, but statistically just not significant trend toward worse progression free survival in the high expression group ($p = 0.0531$, Fig. 5B). Looking at the entire cohort of patients of TMA 1, excluding patients treated symptomatically or palliatively, no statistically significant difference regarding long-term overall survival (OS) or progression free survival (PFS) between low and high CD47 expression was found ($p = 0.0634$, $p = 0.0574$, respectively, Fig. 6A and B). A similar picture was seen in the cohort of TMA 2 consisting of normal karyotype AML patients ($p = 0.1189$ for OS, $p = 0.1432$ for PFS, Fig. 6C and D). Subgroup analyses did not reveal any statistically significant group differences in survival following stratification into CD47 low versus high expression (grades 0–2 and grade 3, respectively) in the group of secondary AML, in the different risk categories (high, intermediate or low) or for gender (data not shown, all p-values >0.500).

4. Discussion

The aim of the present study was to establish a TMA for CD47 analysis in the BM and to test its prognostic relevance in AML. Whilst previous studies have highlighted the possible significance of CD47 as a prognostic marker in AML [4], these analyses were based on gene expression arrays and flow cytometric analysis. Not all patients manifest with overt leukemic presentation and a “dry tap” may limit flow cytometric blast analysis from the BM. Furthermore, blast phenotype within the BM space as compared to the peripheral blood is not necessarily congruent and antigenic changes are known to increase over time following chemotherapy [15]. Immunohistochemical analysis of CD47 expression on the BM biopsy sample may offer a relatively simple and quick tool to stratify patients and in the future initiate targeted antibody strategies in newly diagnosed AML.

In our cohort, a high CD47 protein expression was found in a quarter of all patients with de novo AML. This percentage is in accordance with the previous findings of Majeti et al. where high CD47 mRNA levels were found in 28% and low levels in 72% of AML patients (arbitrary study-specific cut-off) [4].

Low CD47 expression showed a statistically highly significant correlation with a lower mean and BM marrow infiltration as well as percentage peripheral blast count. These findings are in accordance with the proposed mechanism of action of CD47-SIRPα signaling. Leukemic blasts with low CD47 surface expression may

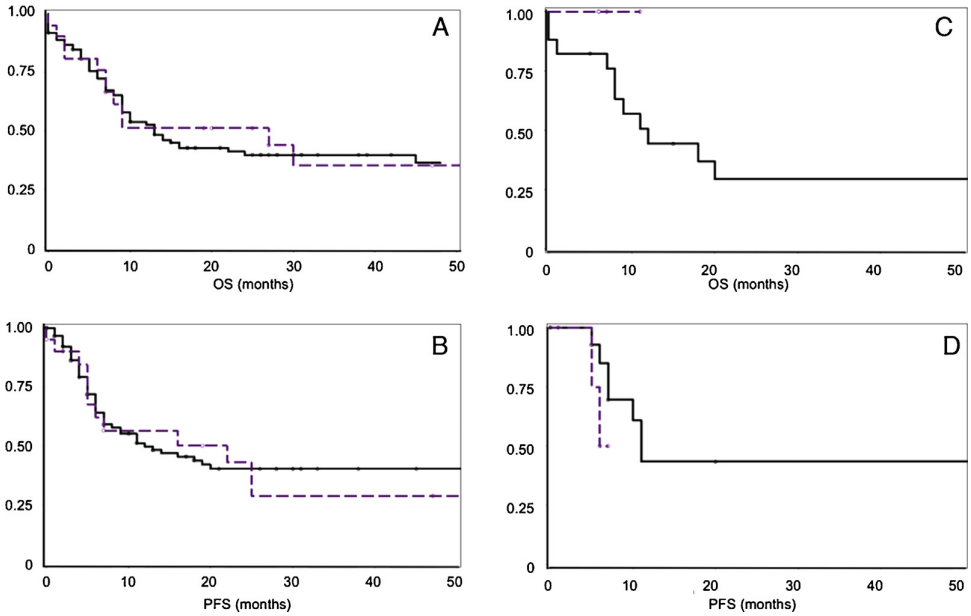

Fig. 6. Overall survival (OS) and progression free survival (PFS) divided into the two tissue microarrays examined: TMA 1 (OS in A and EFS in B) and TMA 2 of NK AML only (OS in C and EFS in D). No significant differences were observed between the two groups in either TMA ($p = 0.0634$ for OS and $p = 0.0574$ for EFS, TMA 1 and $p = 0.1189$ for OS and $p = 0.1432$ for EFS, TMA 2, respectively).

be more prone to clearance by the innate immune system compared to cells with high CD47 expression, thus resulting in a lower tumor load in the BM as well as in the periphery. In secondary AML, only 7% of all patients exhibited a high CD47 staining intensity on BM blasts. However, patients with secondary AML showed a significantly lower median BM infiltration than patients with de novo AML (primary: 80% versus secondary: 25%). It can therefore not entirely be excluded that assessment of the immunohistochemical staining pattern may have been in part limited by the low blast counts in these samples.

It has repeatedly been shown that patients with high-risk myelodysplastic syndrome (MDS) who likely progress to overt AML in the course of their disease express CD47 at higher levels than patients with low-risk MDS [6,16]. Thus, up-regulation of CD47 at the transition from low-risk to high-risk MDS may be one event in the multistep leukemogenesis of MDS. Currently, patients with AML and a history of MDS showed lower CD47 expression than in de novo AML (MDS-AML: 10%; de novo AML: 25%). Hypothetically, up-regulation of CD47 may continue in the context of leukemia progression. Interestingly though, CD47 expression was not found to be elevated in patients with myeloproliferative neoplasia (MPN). In accordance with this, none of the patients of the current AML cohort with documented antecedent MPN exhibited a high CD47 expression (data not shown).

It has previously been shown, that high CD47 expression is directly associated with FLT3-ITD mutation status and correlates negatively with the presence of t(8;21) [5]. Contrary to these findings, in the current cohort no such a correlation between FLT3-ITD or t(8;21) and CD47 protein expression on BM blasts was found. Also, no increased CD47 expression was detected in patients with NPM1 mutation or in NK-AML patients.

One possible explanation for the differing results may be the methodological differences. In the current study CD47 expression was analyzed semi-quantitatively on the protein level by immunohistochemistry, while the investigations of Majeti et al. were based on quantitative mRNA detection [5]. However, although the case numbers were small, Majeti et al. showed that mRNA levels appeared to directly correlate with flowcytometric CD47 surface expression. Of note is the fact that whilst the previous studies investigated CD47 mRNA levels in peripheral blood mononuclear cells and not sorted blasts, the current study examined the CD47 expression profile on leukemic blasts within the BM space itself. This difference is of direct relevance, as CD47 has been shown to be up regulated on HSC released into the peripheral circulation [4] in order to protect them from phagocytosis. As this was a retrospective study, no direct comparison between CD47 expression from the blasts in the peripheral blood and blasts within the bone marrow biopsy could be made. However, previous studies in AML investigating relevant survival factors such as survivin, have observed a differential protein expression in the two populations [17]. This is not surprising and may be explained by the very different microenvironment in the BM, where close contact of the blasts to the stromal tissue and the accompanying secreted cytokines may prompt a distinct protein profile as compared to the leukemic blasts in circulation. It therefore remains to be investigated how CD47 protein expression in BM blasts correlates with protein expression in circulating blasts and how potential changes over time may be associated with outcome.

5. Study limitations

As this was a retrospective study, the correlation between CD47 protein expression in the bone marrow blasts and protein levels on circulating blasts could not be investigated. Future analysis would need to determine if the postulated difference echoes the differing environments between the bone marrow and peripheral blood or whether it purely reflects a methodological phenomenon. Due to general limitations in biopsy size and punchable area due to the presence of bony trabeculae, the TMA was constructed as a single-punch per sample TMA. Whilst this may pose a problem in solid tumors, where tumor cell phenotype may differ significantly according to the localization in relation to the tumor center and tumor front [18], distribution and phenotype of the leukemic blasts within the individual marrow spaces in the biopsy should not differ significantly. Indeed, expression of CD47 was initially analyzed on whole tissue sections for establishment of the staining protocol

Table 1
Clinico-pathological features of the patients' samples included in the tissue microarrays 1 and 2 (normal karyotype only).

Characteristics	TMA 1 (n = 171)	TMA 2 (n = 24)
Median age [y] (range)	60 (19–89)	52 (16–69)
Sex, n (%)		
Female	70 (41)	13 (54)
Male	101 (59)	11 (46)
Median blast count [G/l] (range)	3 (0–225)	9 (0–175)
Median infiltration at diagnosis [%] (range)	65 (10–95)	65 (20–90)
Classification, n (%)		
Primary	111 (65)	17 (71)
Secondary	60 (35)	7 (29)
MDS	40 (23)	7 (29)
MPN	9 (5)	0 (0)
MDS/MPN	6 (3)	0 (0)
Therapy-related	5 (3)	0 (0)
FAB classification, n/N (%)		
M0	17/104 (16)	2/17 (12)
M1	19/104 (18)	5/17 (29)
M2	17/104 (16)	5/17 (29)
M3	6/104 (6)	3/17 (18)
M4	18/104 (17)	2/17 (12)
M5	22/104 (21)	0/17 (0)
M6	1/104 (1)	0/17 (0)
M7	4/104 (4)	0/17 (0)
Normal karyotype, n/N (%)	63/148 (43)	24/24 (100)
Molecular abnormalities, n/N (%)		
t(8;21)	7/144 (5)	0/21 (0)
inv(16)	4/144 (3)	0/21 (0)
t(15;17)	7/144 (5)	0/21 (0)
CEBPA	6/144 (4)	0/21 (0)
FLT3-ITD	34/144 (24)	11/21 (52)
NPM1	31/144 (22)	5/21 (24)
Prognostic group, n/N (%)[a]		
Favorable	30/145 (21)	3/21 (14)
Intermediate	77/145 (53)	18/21 (86)
Adverse	38/145 (26)	0/21 (0)
First-line treatment, n/N (%)[b]		
Induction chemotherapy	70/154 (45)	12/23 (52)
Induction chemotherapy + highdose consolidation	48/154 (31)	9/23 (39)
Palliative chemotherapy	23/154 (15)	0/23 (0)
Symptomatic treatment	13/154 (8)	2/23 (9)
Allogeneic stem cell transplantation, n/N (%)[c]	22/165 (13)	8/23 (35)
Remission after first induction cycle, n/N (%)		
Complete remission	73/103 (68)	10/17 (59)
Non-complete remission	35/103 (32)	7/17 (41)
Median overall survival (95% CI) [months]	10 (8–13)	18 (8-ne)
Median progression free survival [months]	13 (7–20)	11 (7-ne)
Median follow up [m] (range)[d]		
All patients	9 (0–90)	8 (0–121)
Survivors	25 (0–90)	11 (0–121)

MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; FAB, French–American–British.

[a] Stratification in prognostic groups according to Cancer Cell 2012; 22(5):698–698.

[b] As induction therapy most patients underwent chemotherapeutic treatment with cytarabin and an antracycline, idarubicin or daunorubicin, respectively.

[c] High-dose consolidation consisted of an additional combination chemotherapy with busulfan and cyclophosphamid, followed by autologous stem cell transplantation. For palliative chemotherapy, azacitidine or hydroxycarbamide was employed.

[d] Missing clinical data at all in 13 cases (TMA 1) and in 1 case (TMA 2), respectively.

Table 2
Summary of grading results for CD47 immunohistochemical staining for observers 1 and 2. Staining was defined as low (scores 0–2) or high (3).

Obs 1	Obs 2	Low expression	High expression	Total
Low expression		149	6	155
High expression		4	36	40
Total		151	40	195

Table 3
Comparison of clinicopathological characteristics between low CD47 and high CD47 expression groups.

Feature	Grades 0–2 (n = 139)	Grade 3 (n = 32)	p-Value
Median age [y] (range)	62 (19–89)	64 (25–85)	0.2970
Sex, male/female	87/52	14/18	0.0507
Median blast count [%] (range)	26 (0–98)	65 (0–99)	**0.0007**
Median bone marrow infiltration [%] (range)	55 (10–95)	84 (20–95)	**0.0001**
FAB			**0.0003**
0	10 (8.8)	7 (23.3)	
1	8 (7.0)	11 (36.7)	
2	14 (12.3)	3 (10.0)	
3	5 (4.4)	1 (3.3)	
4	13 (11.4)	3 (10.0)	
5	4 (3.5)	0 (0.0)	
6	1 (0.9)	0 (0.0)	
7	3 (2.6)	1 (3.3)	
Secondary	56 (49.1)	4 (13.3)	
Classification, primary/secondary	83/36	28/4	**0.0434**
Genetic aberrations			
NK/non-NK	44/72	19/11	**0.0034**
t(8;21)/no t(8;21), n (%)	6/10	1/29	1.0
t(15;17)/no t(15;17)	6/108	1/29	1.0
inv(16)/no inv(16)	4/110	0/30	0.5801
$CEBPA^{mut}/CEPBA^{WT}$	3/111	3/21	0.0723
$FLT3\text{-}ITD^{mut}/FLT3\text{-}ITD^{WT}$	25/89	9/21	0.3544
$NPM1^{mut}/NPM1^{WT}$	20/94	11/19	**0.0234**
Risk group, favorable/intermediate/adverse	22/45/19	7/19/3	0.0935
Remission status, CR/non-CR	61/27	12/8	0.4216
Allogeneic stem cell transplantation, yes/no	15/110	7/23	0.1102

Significant differences between groups, where p = <0.05 are highlighted in bold writing.

and did not reveal relevant regional differences within the individual marrow spaces (data not shown). In samples with dual blast populations the higher expressing population was defined as the one relevant for grading.

Of note, the current follow-up time was relatively short in comparison with previously published data from Majeti and the median age in this cohort was considerably higher than in their patient collective [5], two factors, which may have contributed to the differing results.

In conclusion, despite establishing a robust and reproducible methodology, we could not prove any correlation between CD47 protein expression on AML blasts in the BM and patient outcome, as measured by immunohistochemistry in a TMA. The methodology, however, of analyzing leukemic blasts using a single 0.6 mm punch TMA, has proven to be feasible and a reliable and an easy way to investigate potential new markers in AML.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

Acknowledgements

Cell lines HL-60, U937 and HT-93 were a kind gift from Dr. Mario Tschan, Institute of Pathology, University of Bern. The authors would like to thank Caroline Hammer, Mary Economou, Lilian Schöni and all the members of the Translational Research Unit of the Institute of Pathology, Bern, for construction and staining of the TMA as well as the laboratory team from the Institute of Pathology, Bern, for their technical support.

References

[1] Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. J Exp Med 2001;193:855–62.

[2] Roberts DD, Miller TW, Rogers NM, Yao M, Isenberg JS. The matricellular protein thrombospondin-1 globally regulates cardiovascular function and responses to stress via CD47. Matrix Biol 2012;31:162–9.

[3] Cooper D, Lindberg FP, Gamble JR, Brown EJ, Vadas MA. Transendothelial migration of neutrophils involves integrin-associated protein (CD47). Proc Natl Acad Sci U S A 1995;92:3978–82.

[4] Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009;138:271–85.

[5] Majeti R, Chao MP, Alizadeh AA. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 2009;138:286–99.

[6] Jiang H, Fu R, Wang H, Li L, Liu H, Shao Z. CD47 is expressed abnormally on hematopoietic cells in myelodysplastic syndrome. Leuk Res 2013;37:907–10.

[7] Braun D, Galibert L, Nakajima T, Saito H, Quang VV, Rubio M, et al. Semimature stage: a checkpoint in a dendritic cell maturation program that allows for functional reversion after signal-regulatory protein-alpha ligation and maturation signals. J Immunol 2006;177:8550–9.

[8] Irandoust M, Alvarez Zarate J, Hubeek I, van Beek EM, Schomagel K, Broekhuizen AJ, et al. Engagement of SIRPα inhibits growth and induces programmed cell death in acute myeloid leukemia cells. PLOS ONE 2013;8:e52143.

[9] Majeti R. Monoclonal antibody therapy directed against human acute myeloid leukemia stem cells. Oncogene 2011;30:1009–19.

[10] Martin-Padura I, Marighetti P, Gregato G, Agliano A, Malazzi O, Mancuso P, et al. Spontaneous cell fusion of acute leukemia cells and macrophages observed in cells with leukemic potential. Neoplasia 2012;14:1057–66.

[11] Theocharides AP, Jin L, Cheng PY, Prasolava TK, Maiko AV, Ho JM, et al. Disruption of SIRPα signaling in macrophages eliminates human acute myeloid leukemia stem cells in xenografts. J Exp Med 2012;209:1883–99.

[12] Kishi K, Toba K, Azegami T, Tsukada N, Uesugi Y, Masuko M, et al. Hematopoietic cytokine-dependent differentiation to eosinophils and neutrophils in a newly established acute promyelocytic leukemia cell line with t(15;17). Exp Hematol 1998;26:135–42.

[13] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocyte cell line (U937). Int J Cancer 1976;17:565–77.

[14] Swerdlow SH, Campo E, Harris NL, et al., editors. WHO classification of tumours of the haematopoietic and lymphoid tissue. 4th ed. Lyon, France: IARC Press; 2008. p. 262–4. World Health Organization Classification of Tumours.

[15] Baer MR, Stewart CC, Dodge RK, Leget G, Sulé N, Mrozek K, et al. High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B Study 8361). Blood 2001;97:3574–80.

[16] Pang WW, Pluvinage JV, Price EA, Sridhar K, Arber DA, Greenberg PL, et al. Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. Proc Natl Acad Sci U S A 2013;110:3011–6.

[17] Carter BZ, Qiu Y, Huang X, Diao L, Zhang N, Coombes KR, et al. Survivin is highly expressed in CD34(+)38(−) leukemic stem/progenitor cells and predicts poor clinical outcomes in AML. Blood 2012;120:173–80.

[18] Buob D, Fauvel H, Buisine MP, Truant S, Marlette C, Porchet N, et al. The complex intratumoral heterogeneity of colon cancer highlighted by laser microdissection. Dig Dis Sci 2012;57:1271–80.