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Expression of Surface-associated 82 kDa proMMP-9 in Lymphatic Leukemia Blast Cells Differentially Correlates With Prognosis

JOERG SCHMOHL[1], SABINE MOEBIUS[2], THOMAS GUENTHER[2], ELENA PEPELDJYIKA[2], CORINNA L. SEIDEL[3], WISHNU SUTANTO[2], FRIEDHELM SCHUSTER[4], DORIS KRAEMER[5], HELMUT SALIH[6], AMELY HARTMANN[7], JOHANNA TISCHER[2], CHRISTIAN RIES[8*] and HELGA SCHMETZER[2*]

[1]*Department of Hematology and Oncology, Diakonie Hospital Stuttgart, Stuttgart, Germany;*
[2]*Department for Hematopoietic Cell Transplantation, Medical Department 3, University Hospital of Munich, Munich, Germany;*
[3]*Department of Orthodontics and Orofacial Orthopedics, Universitätsklinikum Erlangen and Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg, Erlangen, Germany;*
[4]*Department for Paediatric Hematology and Oncology, University Hospital of Duesseldorf, Duesseldorf, Germany;*
[5]*Department of Haematology, Oncology and Palliative Care, Kath. Krankenhaus Hagen gem. GmbH, St.-Josefs-Hospital, Hagen, Germany;*
[6]*Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK), partner site Tuebingen 3, Tübingen, Germany;*
[7]*Praxis Dr. Seiler, Private Practice for Oral Surgery, Filderstadt, Germany;*
[8]*University Hospital of Munich, Institute for Cardiovascular Prevention, Munich, Germany*

Abstract. *Background/Aim: Matrix metalloproteinases (MMPs) degrade extracellular matrix and process regulatory proteins. Recently, a membrane-bound 82kDa variant of proMMP-9 identified on myeloid blasts was shown to be associated with prognosis. Patients and Methods: To investigate the role of 82kDa proMMP-9 with acute lymphoblastic leukemia (ALL) and chronic lymphoid leukemia (CLL), we performed flow-cytometry analysis of expression on ALL blasts (n=18) and CLL lymphocytes (n=21) from blood and correlated data with clinical parameters. Results: In ALL, mature B-linear blasts expressed higher levels of 82kDa proMMP-9 compared to T-linear blasts. Elevated levels of 82kDa proMMP-9 were found in elderly patients and at patients with relapse. No correlation was observed on blood cells and extramedullary disease. In CLL, the 82kDa proMMP-9 expression did not correlate with any of the clinical parameters. Conclusion: Our findings suggest that higher levels of 82kDa proMMP-9 expression on blast cells may correlate with a more unfavorable ALL-subtype. Further studies are required to clarify the prognostic role of the 82kDa pro-MMP-9 expression.*

Leukemia is characterized by uncontrolled proliferation of hematopoietic cells that lack the ability to differentiate into mature blood cells (1). Acute leukemias are diseases characterized by a clonal expansion of myeloid/ lymphoid precursor cells with impaired differentiation and a replacement of the remaining hematopoiesis resulting in clinical symptoms like fever, infections or bleeding complications. Acute lymphoblastic leukemia (ALL) occurs in children and elderly patients. Extramedullary infiltrations especially affects the central nervous system, but can also occur in other organs (2). Treatment of ALL depends on age and classification into risk groups of patients. In general, therapy is characterized by an induction- and a post-remission therapy that often includes allogeneic hematopoietic stem cell transplantation (SCT) (2, 3). Childhood ALL is treated with SCT only in case of a bad cytogenetic risk or in case of relapse (2, 4). Whereas a t(9;22) (Philadelphia chromosome) with the corresponding fusion gene BCR-ABL is the most adverse condition in adult patients(2), it is predictive for a better outcome in children between 1 and 9 years of age (5, 6).

*These Authors contributed equally to this study.

Correspondence to: Prof. Dr. Helga Schmetzer, Medicine Department 3, University of Munich, Marchioninistr. 15, 81377 Munich, Germany. Tel: +49 89440073137, Fax: +49 89440076137, e-mail: helga.schmetzer@med.uni-muenchen.de

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Chronic lymphocytic leukemia (CLL) is the most common chronic leukemia in older age. Median patient age is 65 years at first diagnosis (7). This disease is characterized by an accumulation of small, mature B-cells in blood and bone marrow due to defects in apoptosis and also a clonal proliferation of these cells (8, 9). Treatment of patients is individualized and depends on the physical condition as well as the classification according to the Binet staging system. Patients in Binet stage A and B only receive chemotherapy in case of progression and symptomatic disease, patients in Binet C stage regularly receive chemotherapy or even a SCT in refractory conditions. Del(13q14), found in approximately 55% of all patients, correlates with a favorable outcome whereas del(17p) predicts an unfavorable prognosis (10).

In addition, leukemic blast cells from patients with ALL and CLL blast crisis can be found in the bone marrow (BM) and in the peripheral blood (PB). Besides that, blasts can frequently infiltrate different organs. This cellular trafficking within different body compartments, that is normally restricted to mature functional leukocytes, requires cells to migrate through matrix barriers and penetrate blood vessel walls. This ability by the cells is facilitated by the proteolytic degradation of extracellular matrix and basement membranes that is mediated by matrix metalloproteinases (MMPs), a family of structurally and functionally related proteolytic enzymes (11). Among them, MMP-9 efficiently degrades denatured collagens (gelatin) and collagen type IV, the major component of basement membranes. In addition to its degradative potential, MMP-9 has the ability to process a variety of biologically active molecules including transformation of growth factor β, interleukin 8 and interleukin 1β into their biologically active forms (12-14), indicating importance of MMP-9 as a regulator of cellular functions. These properties have implicated MMP-9 in numerous physiological and pathological processes including leukocyte migration and tumor cell metastasis, respectively (12). Dysregulated expression of MMP-9 also contributes to the invasiveness of many hematologic malignancies such as myelomonocytic leukemias (15).

Under physiological conditions, MMP-9 is secreted from the cells in the form of a latent 94 kDa proenzyme that requires activation to exert its proteolytic activity which is counterbalanced by interaction with the natural tissue inhibitors of metalloproteinases (TIMPs) (16). Cell surface association of MMP-9 has been documented in various cell types including leukocytes and leukemic cells (17). In CLL, surface-bound MMP-9 regulates CLL cell invasion through basement membranes and trans-endothelial migration (18). In disseminating anaplastic large cell lymphomas, surface association and activity of MMP-9 contributes to the invasiveness of the lymphoma cells (19).

Our previous studies in AML cells have led to the discovery that the major MMP-9 form on the surface of the blast cells is a unique non-secreted 82 kDa variant of proMMP-9 (20). This particular 82 kDa proMMP-9 has structural and functional differences when compared to the 'regular' proMMP-9, indicating a specific role for the 82 kDa proMMP-9 in surface-associated proteolysis. Most remarkably, once activated, the 82 kDa proMMP-9 is only poorly inhibited by TIMP-1 (20). Thus, overexpression of this TIMP-1-insensitive MMP-9 variant on the surface of malignantly transformed cells may increase pericellular proteolysis and thereby promote cancer progression *in vivo*.

By use of a monoclonal antibody that specifically recognizes the aberrant 82 kDa proMMP-9 form, our studies demonstrated that surface expression of this species on blast cells from PB represents a novel independent marker of prognosis in patients with AML (21). In the present study, we analyzed the surface expression of 82 kDa proMMP-9 of mononuclear cells isolated from the BM or PB from patients with ALL and CLL to correlate our findings with disease related prognosis.

Patients and Methods

Patient characteristics, diagnosis and treatment. We studied the expression profiles of PB-cells from patients with ALL (n=18) and CLL (n=21) in blast/lymphocyte-rich phases of the disease and in addition some selected PB-samples from AML patients (n=6). The average age of ALL patients was 22 years (range=3-50 years), and of CLL patients 57 years (range=33-72 years). The female to male ratio was 1:0.6 in ALL and 1:0.9 in the group of CLL patients. According to the European Group for the immunological classification of leukemias (EGIL), ALL patients were classified in pro-B-acute lymphocytic leukemias (BI, n=1), common acute lymphocytic leukemias (BII, n=5), and pre-B-acute lymphocytic leukemias (BIII, n=2). Furthermore, 4 ALL patients characterized by an expression of myeloid surface antigens (My^+) were subdivided into My^+ cALLs (BII+My, n=3) and a My^+-pre-B-ALL (BIII+My, n=1). The group of precursor T-lymphoblastic leukemias was classified into a pro-T-acute lymphoblastic leukemia (TI, n=1), a pre-T-ALL (TII, n=1), cortical-T-acute lymphoblastic leukemia (TIII, n=3) and one mature T-ALL (TIV, n=1). 5 patients presented with a primary ALL and 13 with a secondary ALL. According to the criteria of the Study Group on Adult Acute Lymphoblastic Leukemia (GMALL), patients were stratified as standard, high and highest risk (Table I). ALL patients were treated by approved therapy standards according to GMALL therapy standards in adults and COALL standards in children (<18 years of age). All CLL patients had been classified as B-CLL and were divided by Binet classification into Binet A (n=14), B (n=6) and C (n=1) risk groups. CLL pts were treated by approved therapy standards according to comorbidity and pretreatment with Rituximab±Bendamustin, KNOSPE (Chlorambucil+Prednisolone), Rituximab-CHOP (Cyclophosphamid, Adriamycin, Vincristin, Prednisolone), Alemtuzumab or hematopoietic stem cell transplantation. The composition of mononuclear cells (MNC) in ALL samples was: 8% B-cells (range=3-11%), 14% T-cells (range=5-33%), 5% monocytes (range=1-29%), 4% Natural Killer (NK)-cells (range=1-13%) and 71% blasts (Bla) (range=17-99%). The composition of MNC in

Table I. *Patient and sample characteristics*.

ALL	Patient No.	Age at dgn/ Gender	Subtype	Stage	Cell source	Blast phenotype	Blasts %	Expression of MMP-9 on blasts[1] %	Cytogenetic marker at dgn	Risk score[2]	Response to immuno-therapy (SCT)[4]	Response to GMALL 07/03 or COALL 07/03 based therapy
	1106	24/f	p/B II+My	dgn	PB	34,13,19,33,133	81	15	46, XX	High risk	n.d.	CR
	1107	31/m	s/B II+My	dgn	PB	34,10,19,24,33,	36	64	t(9;11)qq	Highest risk	n.d.	CR
	1108	32/m	s/T III	dgn	PB	1a,2,cy3,5,15	98	9	46, XY	Standard	n.d.	CR
	1109	21/m	p/B II+My	dgn	PB	34,10,13, 19,33,38	85	8	46, XY	Standard	n.d.	CR
	1110	50/f	p/B III+My	dgn	PB	133,10,13,19, 24,33,34	59	2	t(9;22)qq, der(22)	Highest risk	n.d.	CR
	1111	26/m	s/B II	rel. a. SCT	PB	34,10,19	64	16	del (11q)	n.d	n.d.	n.d.
	1112	37/m	s/B II	rel. a. SCT	PB	34,10,19	17	53	n.d.	n.d	n.d.	n.d.
	1114	22/m	p/B III	dgn	PB	19,20,34,38	97	15	46, XY	Highest risk	n.d.	CR
	1115	45/m	p/T III	dgn	PB	7,3,5,38	99	6	46, XY	Standard	n.d.	CR
	1120	20/m	s/T II	dgn	PB	19,3,5,20,34	28	7	46, XY	n.d	CR	n.d.
	1121	25/f	s/B I	dgn	PB	34,19,33	32	91	n.d.	n.d	n.d	n.d.
	1129	11/m	s/B II	dgn	BM	10,19,22,34	85	16	46, XY	n.d	n.d	CR
	1132	12/m	s/B III	dgn	BM	10,19,22	84	64	n.d.	n.d	n.d	CR
	1133	3/f	s/B II	dgn	BM	34,10,19,22	55	29	46, XX	n.d	n.d	CR
	1135	17/m	s/T I	dgn	BM	7,4,5,7,10, 13,33,34	98	41	46, XY	n.d	n.d	CR
	1136	5/f	s/T III	dgn	PB	7,1a,2,3,5,10,34	82	9	46, XX	n.d	n.d	CR
	1137	3/f	s/B II	dgn	BM	10,19,22,34	71	10	46, XX	n.d	n.d	CR
	1146	8/f	s/T IV	dgn	PB	7,1,3,34	98	11	46, XX	n.d	n.d	CR

CLL	Patients No.	Age at dgn/ Gender	Subtype	Stage	Cell source	Blast phenotype	Blasts %	Expression of MMP-9 on blasts[1] %	Cytogenetic marker at dgn	Risk score[3]	Response to immuno-therapy (SCT)[4]
	1088	44/f	p/B-CLL	pers	PB	19,5,20	98	13	n.d.	A	n.d.
	1089	54/m	p/B-CLL	pers	PB	19, 5	95	7	n.d.	B	n.d.
	1090	43/m	p/B-CLL	dgn	PB	19,5,Kappa	95	6	add(1)q, del(9)q, del(11)qq	A	n.d.
	1091	68/m	p/B-CLL	pers	PB	19,5,20	96	13	del(13)q	A	n.d.
	1092	66/m	p/B-CLL	pers	PB	19,5,20	71	17	n.d.	B	n.d.
	1093	51/f	p/B-CLL	pers	PB	19,5,23,Kappa	94	9	n.d.	A	n.d.
	1094	67/m	p/B-CLL	pers	PB	19,5,Kappa	91	8	n.d.	A	n.d.
	1095	65/f	p/B-CLL	pers	PB	19, 5	88	16	del(13)q	A	n.d.
	1096	64/m	p/B-CLL	pers	PB	19,5,Lambda	95	11	n.d.	A	n.d.
	1097	72/f	p/B-CLL	pers	PB	19,5,20,22,Kappa	97	9	n.d.	A	n.d.
	1098	60/f	p/B-CLL	pers	PB	19,5,Kappa	93	9	n.d.	B	n.d.
	1099	67/m	p/B-CLL	dgn	PB	19,5,20,23,Lambda	89	8	n.d.	A	n.d.
	1100	36/m	p/B-CLL	pers	PB	19,5,Lambda	91	4	del(17)p, der(11)q	B	n.d.
	1101	52/m	p/B-CLL	pers	PB	19,5,20	96	10	n.d.	A	n.d.
	1102	45/f	p/B-CLL	pers	PB	19, 5	91	13	del(13)q	A	n.d.
	1103	67/f	p/B-CLL	pers	PB	19,5,20	87	9	del(17)p	A	n.d.
	1104	66/f	p/B-CLL	pers	PB	19,5,Kappa	94	10	n.d.	A	n.d.
	1116	66/f	p/B-CLL	pers	PB	19,5,Kappa	96	16	t(8;13)qq	A	n.d.
	1117	33/m	p/B-CLL	pers	PB	19,5,23,Kappa	40	10	del(13)q	B	n.d.
	1118	60/f	p/B-CLL	pers	PB	19,5,20,22, 23,Kappa	32	31	del(11)q	C	n.d.
	1119	55/f	p/B-CLL	pers	PB	19,5,20,22, 23,38,Kappa	57	16	n.d.	B	CR

Dgn: First diagnosis; rel.: relapse; rel. a. SCT: relapse after SCT; SCT: stem cell therapy; pers: persisting disease; PB: peripheral blood; BM: bone marrow; n.d.: no data; CR: complete remission; NCR: not complete remission; [1]proportions of blasts with co-expression; [2]ALL: GMALL; [3]CLL: Binet; [4]Anthracyclin/GMALL based chemotherapy in the following 3-7 days after diagnosis and response/non-response after 25-35 days.

CLL samples was: 11% T-cells (range=1-60%), 4% monocytes (range=1-25%), 1% NK-cells (range=0-6%) and 85% (leukemic) B-cells (range=32-98%). Analyses of B-cells, T-cells, monocytes and NK-cells for co-expression of MMP9 were only included if more than 2% of B-cells, T-cells, monocytes or NK-cells, respectively could be analyzed. All samples with an aberrant expression of cluster of differentiation (CD) 3, CD14, CD19 and CD56 on ALL blasts were excluded from analyses to evaluate cellular compositions (Table I).

Extramedullary infiltrations. Extramedullary infiltration in pts with acute leukemia was considered after occurrence of any biopsy-confirmed manifestation of extramedullar leukemic infiltrates. Hepatosplenomegaly and lymphadenopathy were not considered as manifestations of extramedullary diseases in this study. Leptomeningeal disease was only considered as extramedullary infiltration in case of positive cerebrospinal fluid cytology. One ALL patient showed lung and renal blast infiltrations, one patient presented with blast infiltrations in the bone and skin and in one patient the localization was not further documented.

Sample collection and preparation. After informed consent was obtained, PB or BM (in ALL cases) samples were collected from healthy volunteers and from patients with ALL or CLL at the University Hospitals of Tuebingen, Duesseldorf or Munich. MNC were separated by density gradient centrifugation using the Ficoll-Hypaque-technique [Biocoll Separating solution® (Biochrom, Berlin, Germany)] with a density gradient of 1.077 g/ml, washed in phosphate buffered saline [Dulbecco's PBS® (PAA, Coelbe, Germany)] and subsequently frozen in liquid nitrogen according to standard procedures. Shortly before analysis, cells were thawed, washed in PBS and counted using a Neubauer counting chamber. Alternatively, fresh MNC were directly analyzed.

Monoclonal antibodies recognizing the 82 kDa proMMP-9. Mouse monoclonal antibodies directed against the N-terminus of the human 82 kDa proMMP-9 were generated as described previously and labeled by conjugation with DyLight488 by the manufacturer (AbD Serotec/Morphosys, Duesseldorf, Germany) (20, 21). A detailed characterization of the antibody IF-6G10 recognizing the 82 kDa proMMP-9 variant has been performed; no cross reactivity against MMP-2, 3, 1, 4 and TIMP-1 or 2 was observed, while its specificity in complex protein mixtures as well as its capability to differentiate between 82 kDa proMMP-9 expressing or non-expressing cells has been shown previously (22).

Cell characterization by flow cytometry. Cellular compositions as well as surface marker profiles were analyzed with monoclonal mouse anti-human antibodies (moAbs) using flow cytometry. The moAbs were conjugated with Fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (PCy7) and allophycocyanin (APC). All antibodies were delivered by Becton Dickinson, Heidelberg, Germany ([a]), Caltag, Darmstadt, Germany ([b]), Immunotech/Beckman Coulter, Krefeld, Germany ([c]), Miltenyi Biotech, Bergisch Gladbach, Germany ([d]) and Bioscience, Heidelberg, Germany ([e]). We used FITC labeled antibodies for CD3[c] and CD8[a]. CD3[c] was also used as a PE-labeled moAB. MoAbs against CD3[c], CD4[c], CD5[c], CD15[a], CD19[c], CD33[c], CD34[c] were conjugated with PCy7. MoAbs against CD1[ab], CD3[c], CD4[a], CD7[e], CD10[c], CD13[b], CD14[c], CD15[a], CD19[c], CD20[c], CD45RO[b], CD56[c] and CD235a[a] were labeled with APC. The moABs IF-6G10 and IE-5E9 that detect 82 and 94 kDa proMMP-9, respectively, were conjugated with DyLight 488 (AbD Serotec/Morphosys, Duesseldorf, Germany). For analysis of fresh or thawed MNC and cell lines, the cells were suspended in 0.7-1 ml of PBS containing 10% fetal calf serum (Biochrome, Berlin, Germany) and incubated with four different moAbs at different combinations for 15 minutes in the dark, according to manufacturer's instructions. After washing, the cells were centrifuged (7 min at 370 g) and resuspended in 200 μl of PBS. After all staining procedures, 5,000 cells per tube were measured with a FACS Calibur® Flow Cytometer (Becton Dickinson, Heidelberg, Germany) and different cell subsets were quantified by using isotype controls according to a defined gating strategy with the help of Cell Quest data acquisition and analysis software (Becton Dickinson).

Statistical methods. Statistics were calculated using SPSS Statistics v. 20.0 (IBM Corp, Armonk, US). The Shapiro-Wilk's test was used to check for normal distribution. In case of normally distributed data, a student *t*-test was performed; in case of non-normally distributed data the Mann-Whitney *U*-test was applied. Categorical variables were compared by the χ^2 test. Correlations were assessed with the use of Spearman correlation coefficients. For step-wise multiple linear regression analysis, log-transformation of covariates was used to approximate a normal distribution. Values are reported as mean and standard deviation.

Results

Expression of 82 kDa proMMP-9 on ALL blast cells. We analyzed the expression of the 82 kDa proMMP-9 on blasts isolated from 18 patients with ALL by flow cytometry. In undifferentiated ALL, we found low expression of this marker on B-linear, but high expression on T-linear ALL blasts (Figure 1A). In differentiated ALL, 82 kDa proMMP-9 expression was significantly higher on blasts from pooled B-linear vs pooled T-linear ALLs (87%±64% *vs*. 26%±1%, p=0.025, Figure 1B). Expression levels of proMMP-9 on blasts were lower in children at age ≤10 years compared to children >10 years (18%±9% *vs*. 49%±33%, p=0.12, Figure 1C) and lower in adults at age ≤24 years compared to adults >24 years (12%±4% *vs*. 39%±38%, p=0.2, Figure 1D).

Upon sub-classification of cases according to GMALL risk groups, elevated proportions of proMMP-9-positive blasts were found in patients at relapse (48%±40%, n=2) followed by patients in the standard risk prognosis group (28%±27%, n=5) and in the high/highest risk group (25%±32%, n=4) (Figure 1E). In all cases classified and treated according to the COALL protocol for children (n=7), we found no significant differences in proMMP-9 expression levels on blasts (31%±26%, Figure 1E). Sub-classification of patients in male *vs*. female or into responders and non-responders to induction chemotherapy or immunotherapy [SCT, donor lymphocyte infusion (DLI)] did not reveal differences in the proMMP-9 expression levels (data not shown). Extramedullary disease in either kidney or skin was detected in three patients with ALL.

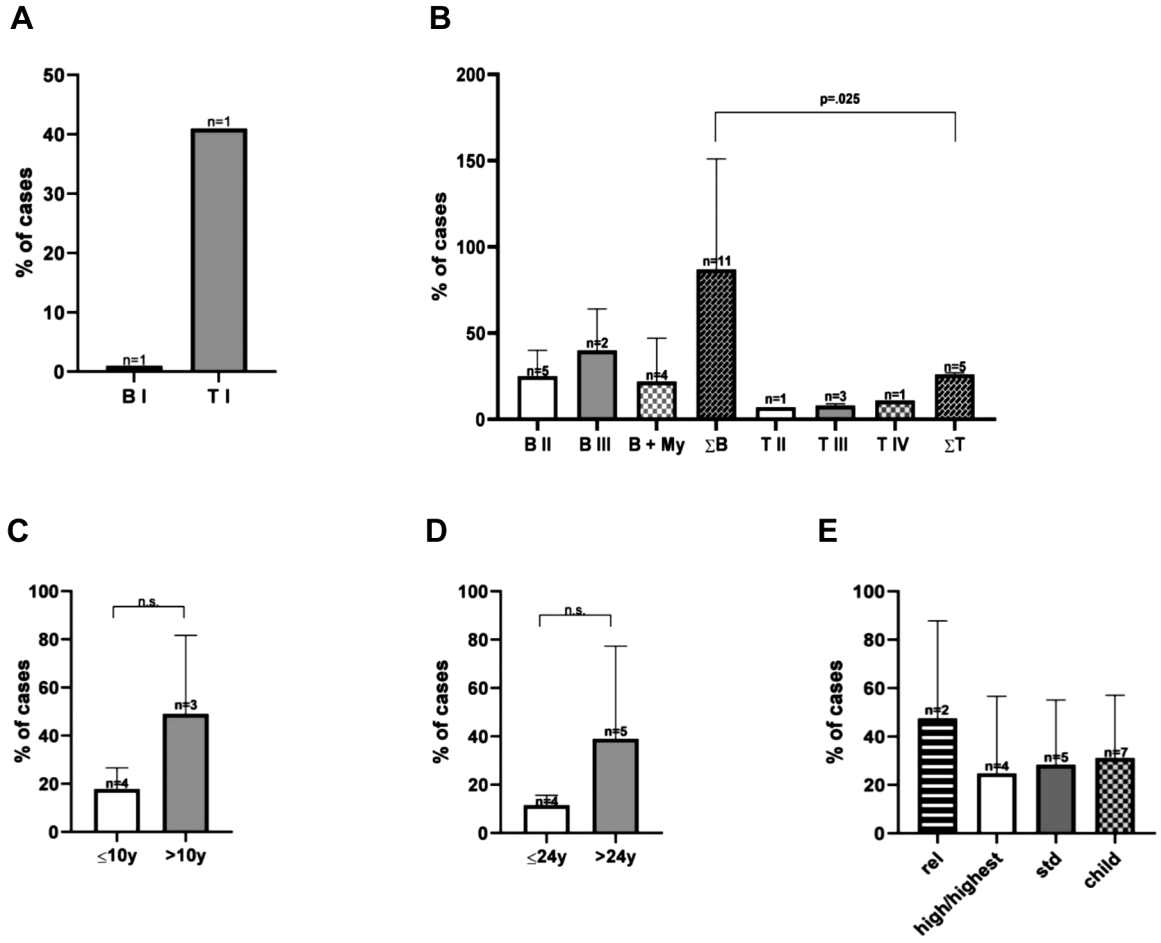

Figure 1. *Expression profiles of surface-associated the 82 kDa proMMP-9 variant on ALL blasts. Low expression of 82 kDA proMMP-9 was found in immature B-ALL and high expression on immature T-ALL. (B) Higher frequencies of the 82 kDa proMMP-9 were detected in positive B-linear (B I-III) vs. T-linear (T I-IV) ALL blasts (excluding immature cases). (C and D) Higher frequencies of 82 kDa proMMP-9 were detected in positive blasts in children as well as elderly adults. (E) Higher frequencies of 82 kDa proMMP-9 positive blasts were found at relapse. Statistical analysis was performed using Wilcoxon test. y: Years; rel: relapse; std: standard; child: children.*

These cases didn't show a different expression level of proMMP-9 compared to the other patients without extramedullary disease records. Moreover, we did not find correlations between patients' sex, proMMP-9 expression (data not shown) and analysis of overall survival (lack of occurring deaths). Together, these data suggest that expression of 82 kDa-proMMP-9 is higher on B-ALL *vs*. T-ALL blasts (excluding undifferentiated ALL cases). Moreover, expression on blasts directly correlated with the cases assigned to adverse (relapse) stages and higher age.

Expression of 82 kDa-proMMP-9 on CLL blasts. Comparative analyses of leukemic lymphocytic cells in patients with CLL did not show significant differences in surface-associated 82 kDa proMMP-9 expression upon subdivision of the pts according to sex and age (data not shown). Expression of proMMP-9 was not different in prognostically relevant CLL subtypes such as Binet risk groups (Binet A *vs*. Binet B: 10%±3% *vs*. 8%±3%, p=0.5, Figure 2A). Our cohort contained no cases with Binet C. Dividing cases in responders/non- responders to first necessary therapy (responders vs non-responders 10%±4% *vs*. 31%, p=0.23, Figure 2B) and occurrence of Richter Transformation (negative *vs*. positive: 9%±3% *vs*. 13%±4%, p=0.24), did not show significant differences in proMMP-9 blast cell expression levels (Figure 2C) suggesting that 82 kDa-proMMP-9 expression levels on mononuclear cells from patients with CLL have no diagnostic and prognostic relevance.

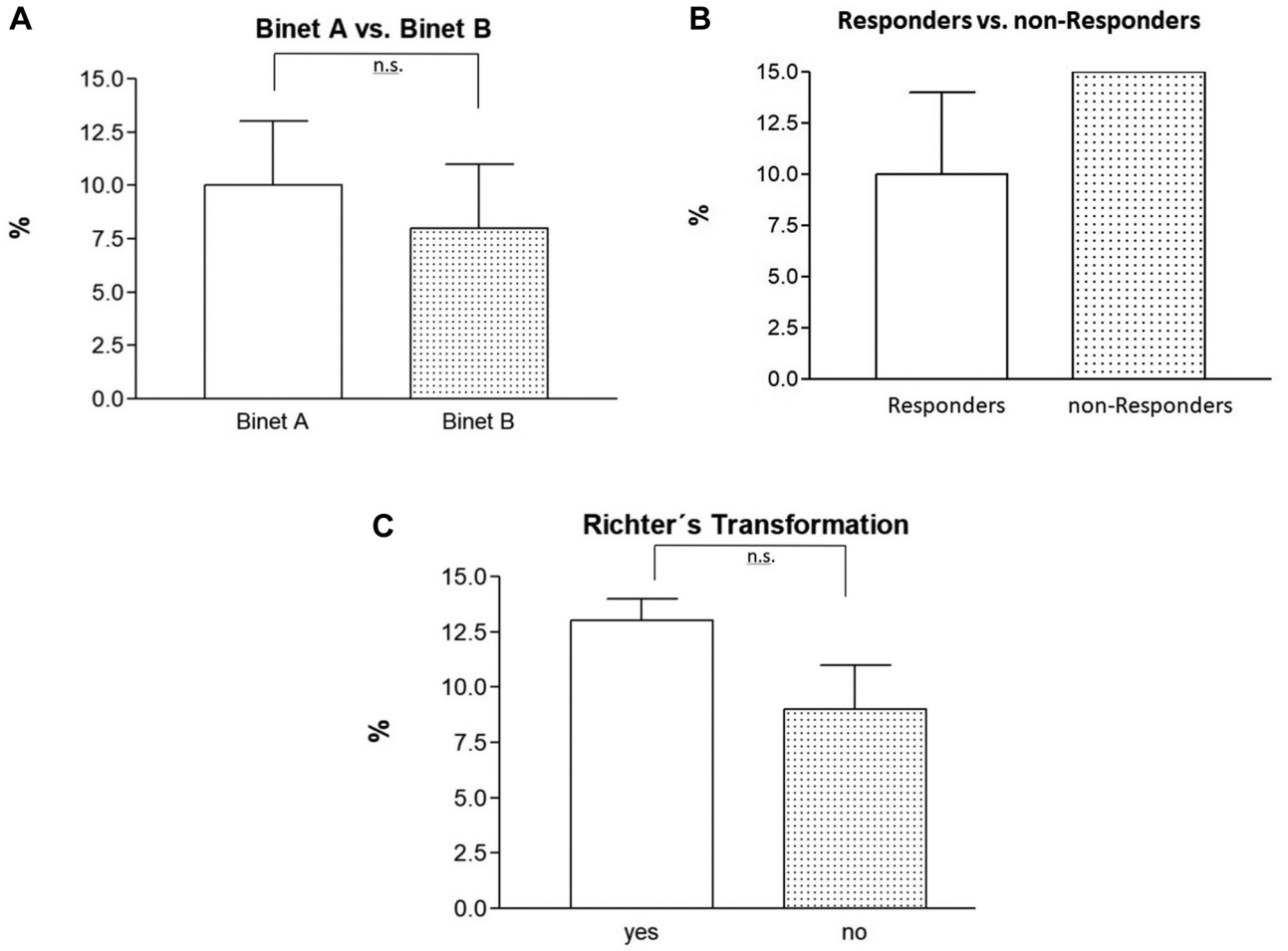

Figure 2. *Expression profiles of surface-associated 82 kDa proMMP-9 variant on CLL blasts. (A) 82 kDa proMMP-9 expression and correlation to Binet risk groups A and B. (B) 82 kDa proMMP-9 expression and correlation to patients' response to therapy. (C) 82 kDa proMMP-9 expression and its correlation to Richter's transformation. Statistical analysis was performed using Wilcoxon test.*

Discussion

We studied the expression of the 82 kDa proMMP-9 on mononuclear lymphatic cells obtained from PB and/or BM of patients with ALL and CLL. By correlating expression profiles with clinically relevant markers, our intent was to test for a potential prognostic value of the 82 kDa proMMP-9 expression in patients with lymphatic leukemia.

ALL and CLL represent heterogenous diseases with various clinical courses. The knowledge of marker expression profiles on cells correspond to defined prognostic subtypes, probabilities to respond to therapy, to survival or to characterizing the migration capacity of leukemic cells. By acquisition of those characteristics, new options can be developed helping to categorize these diseases in distinct risk groups.

In our previous work, we showed that high surface expression of the 82 kDa proMMP-9 on AML-blasts correlated with favorable disease subtypes and a beneficial patient outcome (21). Remarkably, patients with favorable risk groups (according to the NCCN® guidelines) as well as responders to therapy significantly correlated with higher expression of the 82 kDa proMMP-9. Furthermore, we were able to define a cut-off value of 11.5% of blasts with co-expression of 82 kDa proMMP-9 that allowed a significant separation of cases into those with favorable or unfavorable prognosis. In addition, Kaplan-Meier analyses showed a correlation with a higher probability of event free survival for patients with an expression rate >11.5 82 kDa proMMP-9 on blasts (21).

Previous studies by other groups have shown that in patients with ALL, high expression of MMP-9 in or on blasts correlated with a poor prognosis (23, 24). Kuittinen *et al.* described an expression of MMP-9 on BM blasts in 5 of 20 adult ALL patients by using immunoperoxidase staining and

identified a trend towards worse survival in these cases (23). Pan *et al*. found that MMP-9 expression rates were significantly higher in blast cells of patients with an extramedullary infiltration and in high-risk patients, compared to those with standard risk (24), indicating MMP-9 to be marker of poor prognosis in patients with ALL. In our present work, we used antibodies that specifically detect the 82 kDa proMMP-9 variant, in distinction to regular MMP-9. In ALL, our results demonstrate a trend towards higher expression rates of 82 kDa proMMP-9 on PB blasts in prognostically more unfavorable subgroups such as patients suffering from relapse or of higher age. We correlated prognostic parameters with different age groups (children, adults) and ALL-subgroups (immature ALL, mature ALL, B- and T-linear ALL); however, we could not define prognostically predictive cut-off values. Thus, our findings add information to previous results on MMP-9 as a marker of unfavorable prognosis in patients with ALL by suggesting implication of the 82 kDa proMMP-9 variant in this context.

Because MMP-9 is well studied for its involvement in tumor cell metastasis, we hypothesized a role of the 82 kDa proMMP-9 in the extramedullary evasion of ALL blast cells (11). However, our results did not show any correlation between 82 kDa proMMP-9 expression in blasts and their invasive behavior in patients. Obviously, the 82 kDa proMMP-9 is not crucial for degrading extracellular matrix barriers in ALL blasts but may exert a regulatory role on the surface of these cells. The results of our studies showed a low expression of the 82 kDa proMMP-9 on PB blasts in all patients with CLL at stages Binet A and B. Further subdivision into risk groups was not possible due to the low number of cases with poor risk in our patient cohort. These findings are in agreement with previous work by Kamiguti *et al*. (25) investigating MMP-9 expression in samples of 41 patients with CLL. By separating patients into prognostically relevant Binet groups, they found a significantly higher expression of MMP-9 in patients in Binet stage C compared to the ones in Binet stage A and B, correlating MMP-9 expression with poor prognosis in CLL patients. Consistently, Buggins *et al*. demonstrated a correlation of MMP-9 complexed with CD38, CD49d and CD44 on B-Lymphocytes in CLL patients with poor prognosis (26), suggesting that MMP-9 plays a central role in the pathophysiology of this disease.

Former studies by other groups have not distinguished between the regular secreted MMP-9 form and the more recently discovered non-secreted 82 kDa proMMP-9 variant associated with the surface of leukemic cells (21). According to our own present and previous results, we speculate that 82 kDa proMMP-9, when expressed on leukemic cells, plays a regulatory role by activating or inactivating cytokines/chemokines or shedding surface receptors that are essential in the growth and survival of the malignant cells. Considering the impaired inhibition by its natural inhibitor TIMP-1 (21), 82 kDa proMMP-9 activity might considerably affect the behavior of leukemic cells. However, further detailed cellular and molecular investigations are required to understand the molecular functions of this variant on the surface of leukemic cells and its contribution to the pathophysiology of leukemia.

In summary, our findings indicate a prognostic relevance for quantifying the 82 kDa proMMP-9 expression on mononuclear cells from patients with AML and ALL but not CLL. Further studies in larger cohorts of patients covering all subgroups of hematological malignancies and their various clinical courses are required to validate the 82 kDa proMMP-9 as a biomarker in leukemia.

Conflicts of Interest

All Authors declare that there are no conflicts of interest regarding this work.

Authors' Contributions

H. Schmetzer, H.S., J.S. and C.R. designed and coordinated the study; T.G., W.S., T.K., T.P. and V.E. collected samples and performed the experiments; D.S., T.G., H. Schmetzer, J.S., C.W., E.P. and C.R. analyzed the data; C.L.S. contributed to critical revision, discussion and help with submission; J.S, A.H., C.R. and H. Schmetzer wrote the paper; all Authors approved the final version of the manuscript.

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