WT1, PRAME, and PR3 mRNA Expression in Acute Myeloid Leukemia (AML)

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Summary: Several tumor-associated antigens (TAAs) were recently identified, that could qualify as targets for immunotherapy, they could qualify (on RNA-level) for monitoring of tumor load. Here, we studied the expression levels of the immunogenic antigens PRAME (preferentially expressed antigen of melanoma), WT1 (Wilms' tumor gene), and PR3 (proteinase 3) on myeloid blasts by real-time quantitative polymerase chain reaction and correlated these data to the state and course of disease and to the defined subgroups of acute myeloid leukemia (AML). At first diagnoses, 41 of 47 patients tested showed overexpression of PRAME (87%), 38 of WT1 (81%), and 26 of PR3 (55%), with the highest expression levels for PRAME (2048-fold), followed by WT1 (486-fold) and PR3 (196-fold). Thereby, with 70%, the most frequent combination at first diagnoses was detected to be PRAME and WT1 (33/47 patients). Overall, 21 patients (45%) revealed overexpression for all 3 TAAs. Moreover, the highest expression levels of PRAME were found to be correlated with the FAB subtype M5, cytogenetic unfavorable risk groups, and AMLs arising from myelodysplasia (secondary AML; $P = 0.02$). To compare TAA expression levels in the course of disease, expression data were calculatory adjusted to 100% blasts, revealing a relative increase in the PRAME expression levels during the course of persistent disease (3/4 cases). Independent of stage of disease, by trend, higher TAA expression levels were found on blasts derived from peripheral blood than those derived from the bone marrow. In conclusion, it is suggested that vaccine strategies for cancer immunotherapy should comprise different TAA peptides anticipating the diverse TAA expression levels on blasts evolving during the course of disease or treatment.

Key Words: acute myeloid leukemia, dendritic cells, tumor-associated antigens (TAA), leukemia-associated antigens (LAA), WT1, PRAME, PR3, T lymphocytes, immunotherapy, monitoring

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Acute myeloid leukemia (AML) is a neoplastic disorder characterized by a clonal proliferation of myeloid precursor cells and associated with an impaired cell differentiation. Although most of the patients younger than 60 years of age achieve a complete remission (CR), the 2-year survival is only about 46%. With a CR rate of 52% and a 2-year survival of only 23%. the prognosis of the elderly patients is even worse.[1] Treatment options for AML patients with cytotoxic regimens and stem cell transplantation are still unsatisfying due to early relapse or persistence of the disease. In addition, therapeutic options are often limited due to the advanced age of the patients. Thus, there is an urgent need for treatment approaches addressing residual leukemic cells in residual disease or relapse with a more favorable safety profile.

In recent years, several antigens have been identified in AML patients that were overexpressed compared with healthy tissue control RNA. Those overexpressed leukemia-associated antigens (LAAs) could contribute toward the detection of specific, anti–LAA-directed cytotoxic T cells (CTL) that could be used for adoptive immunotherapy.[2,3]

The Wilms tumor gene (WT1) is located on chromosome 11p13 and encodes a zinc-finger transcription factor.[4] WT1 is necessary to control cellular growth and differentiation, and acts as an activator or suppressor depending on the cofactors and cell type.[5–8] Low expression levels are found in the central nervous system, in the urogenital system, and in tissues involved in hematopoiesis. Overexpression is found in solid tumors and leukemias.[9] The PRAME gene (preferentially expressed antigen of melanoma) is located on chromosome 22 and encodes a protein consisting of 509 amino acids.[10,11] Small amounts of PRAME transcripts were detected in normal tissues like testis, adrenals, ovary, and endometrial tissues.[12] It is overexpressed in several types of cancer including acute leukemia, lung cancer, and multiple myeloma. New studies of PRAME showed that PRAME is involved in the repression of RAR signaling[13] and modulates cell proliferation, apoptosis, and tumorigenicity.[10,14]

The PR3 gene (PR3 serine proteinase, neutrophil, Wegener granulomatosis autoantigen) is localized on chromosome 19. PR3 is expressed in normal kidney tissues.[15] It is involved in the growth and differentiation of human leukemia cells and overexpressed in AML and in chronic myeloid leukemia (CML).[16–18]

It was already shown that $CD8^+$ anti–LAA-directed T cells are able to specifically lyse LAA-overexpressing blasts[2,3]; however, the clinical significance of the in vivo generation of LAA-specific CTL (eg, after LAA vaccination) or an adoptive therapy with LAA-specific HLA-A02–restricted T cells for the further course of the disease is not clear.[19,20]

The aim of the present study was to analyze the expression levels of these LAAs in AML patients, peripheral blood (PB) samples, or bone marrow (BM) samples, compared with healthy donors, to detect potentially multiple overexpressed LAAs in PB and BM samples obtained in different stages of the disease, to correlate the expression levels with AML subtypes, different cellular compartments, and to contribute to a more differentiated diagnosis and estimation of prognosis and clinical outcome.

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MATERIALS AND METHODS

Patient Characteristics, Sample Collection, and Diagnosis

This retrospective study included 73 cases of primary (pAML) or secondary AML (sAML), diagnosed at the Department of Medicine III, Großhadern Clinic, University of Munich, Germany, between 1985 and 2008. Sample material (BM, PB) was obtained from patients at the time point of first diagnosis (n = 47), at relapse (n = 12), during the course of persisting disease (n = 25), or in CR (n = 6). Diagnosis and classification of all cases were performed on the basis of morphology and cytochemistry according to the FAB classification.[21,22] Cytogenetic analyses were carried out according to standard protocols and criteria defined by the International System for Human Cytogenetic Nomenclature.[23] So far, cytogenetic data were available; AML patients were categorized into risk groups as follows: "favorable" presenting with t(8;21), t(15;17), inv(16), or t(16,16) and "unfavorable" with −5, 5q−, −7, 7q−, t(11q23), inv(3), t(3;3), 17p, or a complex aberrant karyotype with ≥3 abnormalities. "Intermediate" comprises cases with a normal karyotype and remaining aberrations.[24–26] PB samples and BM samples from 10 healthy donors each served as controls. Patients' characteristics with FAB subtype, sex, age, material, stage of disease, percentual morphologic blast counts, cytogenetic markers, and cytogenetic risk groups are summarized in Table 1.

Cell Lines

The human CML cell line K562, the human acute lymphatic leukemia cell line Jurkat, the human Burkitt lymphoma cell lines Ramos and Raji, and the human AML cell lines HL60 (FAB M2) and Kasumi-1 (FAB M2) were obtained from the ATTC (Manassas, VA). Human leukemia cell lines were cultured in RPMI 1640 medium (Cell Concept GmbH, Umkirch, Germany) containing 10% fetal calf serum (PAN Biotech GmbH, Passau, Germany), L-glutamine (2 mM; Gibco, Germany), penicillin (100 U/mL), and streptomycin (100 U/mL; Gibco).

Sample, RNA, and cDNA Preparation

Mononuclear cells (MNCs) were isolated from PB samples and BM samples by Ficoll density gradient. The MNCs were stored in liquid nitrogen under standard conditions (FCS containing 10% dimethyl sulfoxide; WAK-Chemie Medical GmbH, Steinbach, Germany) and thawed for RNA preparation. The total RNA was extracted using the RNeasy mini kit including DNase digestion following the manufacturer's instructions (Qiagen, Hilden, Germany). The amount of RNA was measured by photometry. RNA was stored at −80°C. Total RNA was transcribed into cDNA using Random Hexamer Primers. Total RNA of 100 ng was transcribed in a total volume of 20 μL to cDNA using the reverse transcriptase containing cDNA synthesis kit according to the manufacturer's instructions (First Strand cDNA Synthesis Kit; Fermentas, St. Leon-Rot, Germany). Negative controls without reverse transcription were used for each sample. cDNA was stored at −20°C.

Relative Quantification of cDNA by Real-time Polymerase Chain Reaction (RQ-RT PCR)

Real-time PCR analyses were carried out using the Applied Biosystem 7300 Real-Time PCR System and SDSv1.3.1 Software (Applied Biosystems, Foster City, CA). For the polymerase chain reaction (PCR) reaction, 2 μL of cDNA (corresponding to 10 ng of total RNA) was added to the PCR reaction mix to reach a final volume of 20 μL containing 10 μL Taqman Universal PCR Master Mix (Applied Biosystems) and 1 μL Gene Expression Assay Mix (Applied Biosystems). PCR amplification was performed with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles with 15 seconds at 95°C and 60 seconds at 60°C, according to the manufacturer's description.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA were applied as endogenous controls and were used to normalize the expression. Results are presented as a relative quantification on the basis of $RQ = 2_t^{-\Delta\Delta C}$. Healthy donors were recruited as controls. The following equations were used: $C_{t\ \text{target gene}} - C_{t\ \text{GAPDH or 18S rRNA}} = \Delta C_t$ in AML samples and healthy donor samples and $\Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{mean of healthy donor}} = \Delta\Delta C_t$. All samples were analyzed in triplicate and the mean values were taken for further calculations. The cDNA from Jurkat cell lines were used as a positive control for all LAAs; negative controls obtained with healthy donors and controls without template were included in each experiment. The threshold level was defined as 1-fold overexpression compared with healthy controls; in some experiments, overexpression was defined as 10-fold overexpression of healthy threshold levels. For a better comparison of LAA-expression levels in different stages of the disease, we standardized the RQ data ("unadjusted," ua) to 100% morphologically detected blasts ("adjusted," a) and included these data in figures. In contrast, in cases studied in "low blast count" stages, we presented "adjusted" data of every given sample (also those with values under the control threshold levels in nonadjusted evaluations) and included "unadjusted" data in addition.

Primers

Gene Expression Assay Mixes (Applied Biosystems) were used for all RQ-RT PCR amplifications of housekeeping genes GAPDH (Assay-ID: HS99999905_m1), 18S rRNA (Assay-ID: Hs 99999901_s1), and for LAA genes WT1 (Assay-ID: Hs01103754_m1), PRAME (Assay-ID: Hs00196132_m1), and PR3 (Assay-ID: Hs01597752_m1).

Statistical Methods

All calculations were performed on a personal computer using Microsoft Excel 2003, Sigma Plot 10.0, and SPSS 17.0 program (SPSS Inc., Chicago, IL). The expression profiles in different groups of patients were compared using the Mann-Whitney *U* and the Kruskal-Wallis test (nonparametric tests). A *P*-value ≤0.05 was considered to be statistically significant.

Cutoff Analyses

On the basis of our RNA expression data, we grouped cases at first diagnosis according to most different LAA-expression profiles in sAML and pAML cases to define those "cutoff" values that allowed the most (significant) subdivision of samples in cases with more "favorable" (pAML) from "unfavorable" (sAML) phenotypes.[27]

RESULTS

Evaluation of LAA-expression Profiles in AML Samples by RQ-RT PCR

Expressions of WT1, PRAME, and PR3 in PB and/or BM samples derived from 73 patients with AML of different subtypes (eg, FAB subtypes, cytogenetic risk groups) or stages of the disease were analyzed by RQ-RT PCR and compared with samples derived from 10 healthy donors. In all cases, RNA in adequate quantity and quality could be isolated and endogenous RNA controls (GAPDH and 18S rRNA) were performed. The LAA profiles were determined for the whole MNC fraction

TABLE 1. Patients' Characteristics

						Cell Source			
No.	**FAB Subtype**	**Age (y)**	**Sex**	**Stage (+mo)**	**Blasts (%)**	**PB**	**BM**	**Cytogenetic Marker**	**Cytogenetic Risk**
#1	pM0	29	F	dgn	78		+	46,XX	Intermediate
#2	pM0	78	F	dgn	99	+		46,XX	Intermediate
#3	pM0	66	F	rel	70	+			
#4	pM0*	40	F†	pers	29	+			
	pM0*	40	F	pers	30		+		
	pM0	40	F†	pers	91	+			
#5	pM1	69	F	dgn	90	+		46,XX,del(20)(q11q13)	Intermediate
#6	pM1	70	F	dgn	95	+			
#7	pM1*	64	M	dgn	95	+			
	pM1*	64	M	dgn	90		+		
#8	pM1	71	F	dgn	88		+	46,XX	Intermediate
#9	pM1	75	M	dgn	95		+	46,XY	Intermediate
#10	pM1	67	F†	dgn‡	68	+		46,XX,del(5)(q13q31)	Unfavorable
	pM1	68	F†	pers (+11)‡	65	+			
#11	pM1	38	F	dgn	95	+		46,XX	Intermediate
#12	pM1	58	F†	rel	44	+			
#13	pM1	80	F	dgn	90	+		46,XX	Intermediate
#14	pM1	39	F	dgn	77	+		46,XX	Intermediate
#15	pM1*	58	M	rel	23	+			
	pM1*	58	M	rel	66		+		
#16	pM2	58	F	dgn	66		+	46,XX	Intermediate
#17	pM2	67	M	pers	5	+			
#18	pM2	44	F	dgn‡	54		+		
	pM2	44	F	pers (+1)‡	26		+		
	pM2	44	F	pers (+6)‡	6		+		
#19	pM2	76	M	dgn	66		+	46,XY	Intermediate
#20	pM2	39	M	dgn	77	+		46,XY,t(8;21)(q22;q22)	Favorable
#21	pM2	49	F	CR	0	+			
#22	pM2	41	F†	pers	71	+			
#23	pM3	71	F	pers	67		+		
	pM3	71	F	pers	42		+		
	pM3	71	F	pers	51		+		
	pM3	71	F	pers	1	+			
	pM3	71	F	pers	62		+		
#24	pM3	66	M	dgn	90	+		46,XY,t(15;17)(q22;q21)	Favorable
#25	pM3	50	F	dgn	90		+	46,XX,t(5;10)(q13;q36); t(15;17)(q22;q21)	Favorable
#26	pM3	60	M	pers	14		+		
#27	pM3	62	M	rel	78		+		
#28	pM3	61	M	pers	9		+		
#29	pM4	64	M	dgn	95		+		
#30	pM4	68	F	dgn	95	+		46,XX	Intermediate
#31	pM4	56	F	dgn	93		+		
#32	pM4	57	M	dgn	65		+		
#33	pM4	64	F	dgn	49		+		
#34	pM4	64	M	dgn	90		+	47,XY,+11,inv(11)(p15q13)	Intermediate
#35	pM4	51	M	rel	37	+			
#36	pM4	24	M†	pers	9	+			
#37	pM4	34	F	dgn	88	+			
#38	pM4	58	F	rel	59	+			
#39	pM4eo	14	M	dgn	70	+		46,XY,inv(16)(p13q22)	Favorable
#40	pM5	72	F	dgn	90	+			
#41	pM5	43	F	dgn	94		+	46,XX	Intermediate
#42	pM5	67	F	dgn	86		+		
#43	pM5	60	F	dgn	97		+	46,XX,t(9;11)(p21;q23)	Unfavorable
#44	pM5	35	F	dgn	95	+		46,XX,t(9;11)(p22;q23)	Unfavorable
#45	pM5	24	F	dgn†	94	+			
	pM5	25	F	CR (+3)‡	4		+		
#46	pM5*	52	F	dgn‡	59	+			
	pM5*	52	F	pers (+1)‡	14	+			
	pM5*	52	F	dgn‡	86		+		
	pM5*	52	F	pers (+1)‡	5		+		
#47	pM5	33	F	pers	19		+		
#48	pM5	33	F	dgn	67	+			
#49	pM6	42	M	pers	2	+			
#50	uncl	75	F	dgn	27	+			
#51	sAML	67	M	dgn	23	+		46,XY	Intermediate

TABLE 1. (*continued*)

No.	FAB Subtype	Age (y)	Sex	Stage (+mo)	Blasts (%)	Cell Source		Cytogenetic Marker	Cytogenetic Risk
						PB	BM		
#52	sAML	69	F	rel	50		+		
#53	sAML	74	M	dgn	51		+		
#54	sAML	71	F	dgn	65		+	46,XX	Intermediate
#55	sAML	64	M	dgn	75	+		46,XY, 45 XY,−7	Unfavorable
#56	sAML	83	F	dgn	36		+	50,XX,+1,+4,del(5)(q12q33),+9,+11	Unfavorable
#57	sAML	66	M	CR	4		+		
#58	sAML	66	M	CR	1		+		
#59	sAML	66	M	CR	4		+		
#60	sAML	65	M	pers	36		+		
#61	sAML	67	M	rel	23		+		
#62	sAML	62	F	dgn	55		+	46,XX	Intermediate
#63	sAML	77	F	dgn	88		+		
#64	sAML	50	F	dgn	47		+		
	sAML	51	F	CR (+2)	1		+		
#65	sAML	54	F	dgn	22	+		41,XX,der(6p−),−7,der(7p+),−11,−17,−20	Unfavorable
#66	sAML	67	M	pers	52	+			
#67	sAML	65	F†	pers	3	+			
#68	sAML	37	M†	rel	95	+			
#69	sAML	64	M†	rel	60	+			
#70	sAML*	49	M	dgn	59	+		46,XY	Intermediate
	sAML*	49	M	dgn	36		+	46,XY	Intermediate
#71	sAML	46	M	pers	3	+			
#72	sAML	51	F†	rel	82	+			
#73	sAML	66	F†	pers	63	+			

AML subtypes, stages of the disease, age, sex, cell sources, cytogenetic markers, and risks are given.
*Cases with PB and BM studied in parallel.
†Cases treated with FLASMA protocol.
‡Cases studied in different stages of disease.
AML indicates acute myeloid leukemia; blast%, morphologic blast percentage; CR, complete remission; dgn, diagnosis; f, female; FAB, M0; m, male; pers, persisting disease; pM0, primary acute myeloid leukemia; rel, relapse; sAML, secondary acute myeloid leukemia; uncl, unclassified acute myeloid leukemia; +, cell source peripheral blood (PB) or bone marrow (BM).

containing 54% blasts on average. Thus, for better comparability, the LAA-expression values were also adjusted to 100% blasts (adjusted values). After 40 amplification steps, the LAA-expression levels were low or undetectable in control samples, and expression levels were set according to threshold levels found in healthy controls. The WT1 C_t range was 29–36 for BM and 35–40 for PB. The PRAME C_t range was 38–40 for BM and 37–40 for PB, and the PR3 C_t range was 21–23 for BM and 27–39 for PB. All analyzed cell lines showed overexpression for the analyzed LAAs, with the exception of the cell lines Raji and Ramos for gene PR3 (data not shown).

Expression Levels of LAAs in Different AML Subtypes at First Diagnosis

At first diagnoses (n = 47), we could detect an RNA overexpression for the WT1 gene in 81% (n = 38), for the PRAME gene in 87% (n = 41), and for the PR3 gene in 55% (n = 26) of all AML cases tested. Adjusting these results to the

TABLE 2. Overexpression of WT1, PRAME, and PR3

FAB Subtype	WT1 (n/N)		PRAME (n/N)		PR3 (n/N)	
	Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted
pM0 (n = 2)	2/2	2/2	2/2	2/2	0/2	0/2
pM1 (n = 10)	9/10	9/10	8/10	9/10	6/10	6/10
pM2 (n = 4)	4/4	4/4	4/4	4/4	2/4	2/4
pM3 (n = 2)	1/2	1/2	2/2	2/2	1/2	1/2
pM4 (n = 7)	6/7	6/7	5/7	5/7	3/7	3/7
pM4eo (n = 1)	1/1	1/1	1/1	1/1	1/1	1/1
pM5 (n = 9)	4/9	4/9	9/9	9/9	6/9	6/9
pAML uncl (n = 1)	1/1	1/1	1/1	1/1	1/1	1/1
Σ pAML (n = 36)	28/36	28/36	32/36	33/36	20/36	20/36
sAML (n = 11)	10/11	10/11	9/11	10/11	6/11	7/11
Σ (N = 47) [n/N (%)]	38/47 (81)	38/47 (81)	41/47 (87)	43/47 (94)	26/47 (55)	27/47 (57)

Samples unadjusted and adjusted to 100% morphologically detected blasts.
Σ indicates sum; FAB, M0; pM0, primary acute myeloid leukemia; sAML, secondary acute myeloid leukemia; uncl, unclassified primary acute myeloid leukemia.

calculated assumption of 100% blasts, the overexpression rate was 81% for WT1, 94% for PRAME, and 57% for PR3 (Table 2). If threshold levels for LAA overexpression were defined as more than a 10-fold RNA overexpression compared with healthy controls, in 62% of the cases for WT1, in 55% for PRAME, and in 32% for PR3 for the unadjusted data group, and in 64% for WT1, 55% for PRAME, and in 34% for PR3 in the adjusted data group, respectively, LAA overexpression was detected (data not shown). Selecting only cases with LAA overexpression of PRAME showed the highest overexpression rate, with a mean 2048-fold overexpression (adjusted to 100% blasts: 3312-fold overexpression), followed by WT1, with a mean 486-fold overexpression (adjusted to 100% blasts: 1133-fold overexpression), and PR3 with a mean 196-fold overexpression (adjusted to 100% blasts: 326-fold overexpression, Fig. 1A). A detailed analysis of all cases with overexpression at first diagnoses subdivided into different FAB subtypes is shown in Fig. 1B: with the exception of PR3 in FAB subtype M0, WT1, PRAME, and PR3 were overexpressed in all analyzed subtypes. The highest overexpression was shown in 1 case of AML M4eo for WT1, with an 1127-fold overexpression. The lowest overexpression for WT1 was observed in M0, with a mean 47-fold overexpression (adjusted to 100% blasts: 53-fold overexpression, Fig. 1B_1). The highest overexpression for PRAME was detectable in subtype M5, with a mean 6744-fold overexpression (adjusted to 100% blasts: 7155-fold overexpression, Fig. 1B_2), and the lowest in subtype M4eo, with 6-fold overexpression (adjusted to 100% blasts: 9-fold overexpression). The highest overexpression for PR3 was detectable in subtype M5, with a mean 482-fold overexpression (adjusted to 100% blasts: 760-fold overexpression), and the lowest in subtype M4, with a mean 59-fold overexpression (adjusted to 100% blasts: 119-fold overexpression, Fig. 1B_3).

With the exception of PRAME, we found comparable rates of overexpression in cases separated in pAML or sAML. The highest overexpression rate was found for PRAME, with a mean 2012-fold overexpression (adjusted to 100% blasts: 2160-fold overexpression) in pAML compared with 2177-fold overexpression (adjusted to 100% blasts: 7409-fold overexpression, Fig. 2A) in sAML. Differences in PRAME expression in pAML compared with sAML were significant (Fig. 2A).

An analysis of cases subdivided into cytogenetic risk groups showed a higher overexpression of WT1 and PRAME in "unfavorable risk" compared with "favorable risk" groups. WT1 showed an overexpression in the "unfavorable risk" group, with a mean 677-fold overexpression (adjusted to 100% blasts: 1566-fold overexpression), and PRAME showed an overexpression in the "unfavorable risk" group, with a mean 4918-fold overexpression (adjusted to 100% blasts: 5196-fold overexpression), respectively. This prominent difference was not found for PR3 (Fig. 2B).

Parallel/Simultaneous Overexpression of Multiple LAAs at First Diagnosis

We observed a parallel/simultaneous expression of different LAAs in single patients at first diagnosis. In detail, > 49% (adjusted to 100% blasts: 53%) of the cases showed a relative overexpression of 2 LAAs. The highest simultaneous overexpression was found for the combination of WT1 and PRAME with an overexpression of 70% of the cases at first diagnosis (adjusted to 100% blasts: 79%). In 45% of the cases at first diagnosis, we detected a relative overexpression in three LAAs (adjusted to 100% blasts: 53%; Table 3).

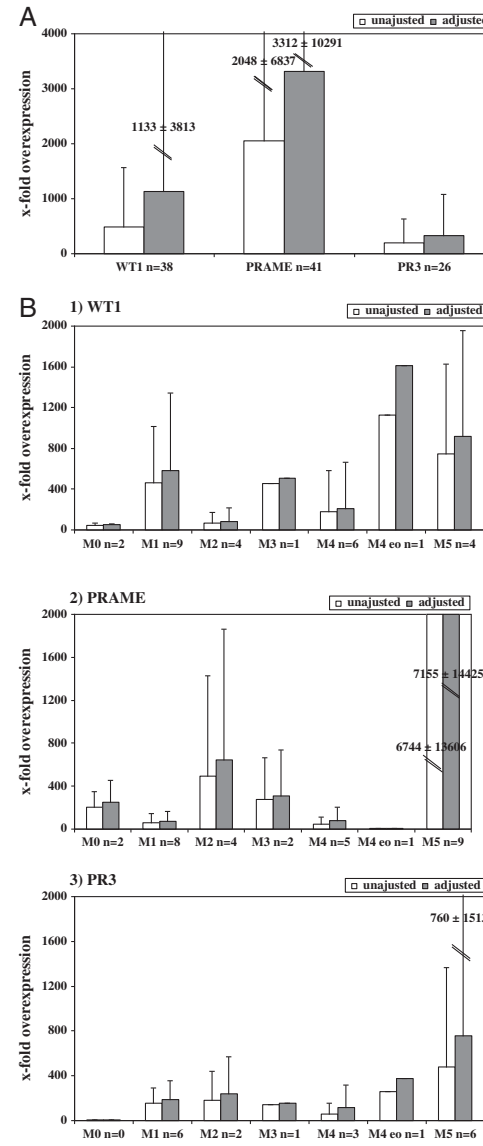

FIGURE 1. RNA expression levels of LAAs in cases with overexpression at first diagnosis. Total RNA was extracted using the RNeasy mini kit, quantified photometrically, stored at –80°C, and finally transcribed into cDNA using Random Hexamer Primers (LAA genes: WT1, PRAME, and PR3). Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA were used to normalize the expression. Results are presented as a relative quantification on the basis of $RQ = 2_t^{-\Delta\Delta C}$. Healthy donors were recruited as controls. The following equations were used: $C_{t\ \text{target gene}} - C_{t\ \text{GAPDH or 18S rRNA}} = \Delta C_t$ in AML samples and healthy donor samples; $\Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{mean of healthy donor}} = \Delta\Delta C_t$. All samples were analyzed in triplicate and the mean values were taken for further calculations. For a better comparison of LAA-expression levels in different stages of the disease, we standardized the RQ data ("unadjusted," ua) to 100% morphologically detected blasts ("adjusted," a) and included these data in figures. In contrast, in cases studied in "low blast count" stages, we presented "adjusted" data of every given sample (also those with values under the control threshold levels in nonadjusted evaluations) and included "unadjusted" data in addition. Mean LAA values with SDs in all AML cases with overexpression at first diagnosis for the unadjusted values and values adjusted to 100% blasts (A) and of cases subdivided in FAB subtypes (B) are given (values for BM and PB of all cases pooled). AML indicates acute myeloid leukemia; BM, bone marrow; LAA, leukemia-associated antigen; PB, peripheral blood.

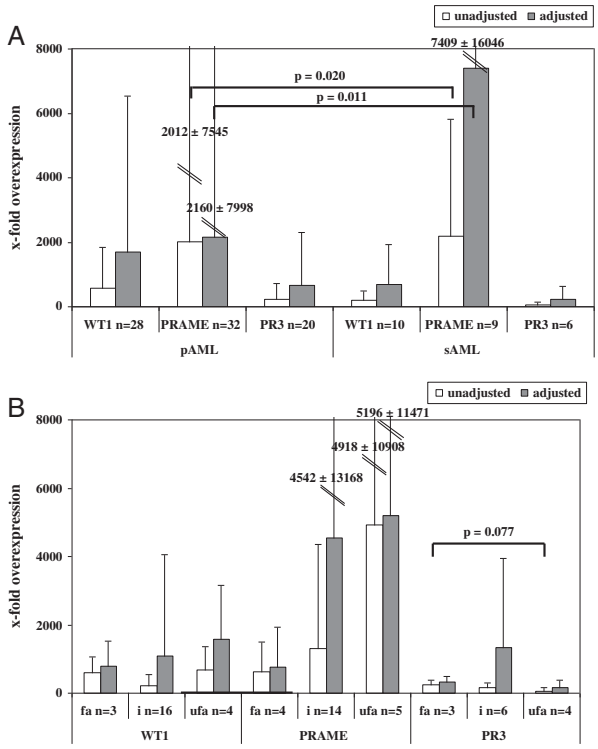

FIGURE 2. RNA expression levels of LAAs in cases with overexpression at first diagnosis. The polymerase chain reaction strategy is given in Figure 1. Expression profiles in groups were compared using the Mann-Whitney *U* test. A $P \leq 0.05$ was considered to be statistically significant. Mean LAA values with SDs and statistical data of pAML compared with sAML cases with overexpression at first diagnosis for the unadjusted values and values adjusted to 100% blasts (A) and of cases subdivided into cytogenetic risk groups are shown (B). fa indicates favorable; i, intermediate; LAA, leukemia-associated antigen; n, number; pAML, primary acute myeloid leukemia; sAML, secondary acute myeloid leukemia; ufa, unfavorable.

TABLE 3. Between 43% (51%) and 70% (79%) of Cases With AML at First Diagnosis (n = 47) Presented With Multiple LAA Overexpression

LAAs	Unadjusted [n/N (%)]	Adjusted [n/N (%)]
LAA overexpression in cases at first diagnosis		
WT1+PRAME	33/47 (70)	37/47 (79)
WT1+PR3	24/47 (51)	25/47 (55)
PRAME+PR3	23/47 (49)	26/47 (55)
WT1+PRAME +PR3	21/47 (43)	25/47 (53)

Subtypes	LAAs	Unadjusted [n/N (%)]	Adjusted [n/N (%)]
LAA overexpression in cases subdivided into pAML vs. sAML			
pAML	WT1+PRAME	25/36 (69)	27/36 (75)
sAML	WT1+PRAME	8/11 (73)	10/11 (91)
pAML	WT1+PR3	18/36 (50)	18/36 (50)
sAML	WT1+PR3	6/11 (55)	7/11 (64)
pAML	PRAME+PR3	19/36 (53)	19/36 (53)
sAML	PRAME+PR3	4/11 (36)	7/11 (64)
pAML	WT1+PRAME +PR3	17/36 (47)	18/36 (50)
sAML	WT1+PRAME +PR3	4/11 (27)	7/11 (55)

Samples unadjusted and adjusted to 100% morphologically detected blasts.

AML indicates acute myeloid leukemia; LAA, leukemia-associated antigen; pAML, primary acute myeloid leukemia; sAML, secondary acute myeloid leukemia.

LAA-expression Levels in Different Stages of AML

Another focus of our work was to study the value of LAA analysis to quantify (residual) blasts in different stages of the disease and to monitor expression levels in the course of the disease. Because blast counts in different stages of the disease are variable, we present "adjusted" data of all available samples (and not only those with LAA overexpression) and, in addition, the corresponding "unadjusted" data. The highest "unadjusted" overexpression rates were found in cases at first diagnosis, followed by cases with persistent disease and remission. Samples of cases with relapse showed similar expression profiles as samples in persistent disease (Fig. 3). It is interesting to note that, the highest overexpression rate of the unadjusted data was found for PRAME (1787-fold) compared with only a 393-fold overexpression of WT1-RNA and a 109-fold overexpression of PR3. On adjusting expression values to blast counts, overexpression of LAA was detectable in varying degrees, depending on blast counts. The highest overexpression rate was found in cases with persistent disease (Fig. 3). It is interesting to note that, cases with relapse showed reduced overexpression rates adjusted to blast counts. This observation prompted us to study LAA overexpression during the course of the disease in individual cases with AML (Fig. 4). These individual analyses for WT1, PR3, or PRAME overexpression in parallel analyses of 6 cases with AML confirmed our finding, that LAA overexpressions are higher in persisting disease compared with first diagnosis in the adjusted and unadjusted data. In remission, data were variable: very low LAA expressions and overexpressions could be found in individual patients.

Expression Levels in Different Cellular Compartments

We analyzed the LAA-expression patterns of 90 cases with AML in different cellular compartments (PB and BM) in different stages of the disease. In a first analysis of all available PB samples or BM samples, we detected a higher relative overexpression rate of WT1, PRAME, and PR3 in PB compared with BM. A detailed analysis of the overexpression patterns in different compartments showed a (statistically significant) higher grade of overexpression in PB compartments compared with BM compartments of WT1 (Mann-Whitney test, $P < 0.001$), PR3 ($P < 0.001$), and PRAME ($P =$ not significant) for the adjusted and unadjusted data. An analysis of only cases with overexpression confirmed our findings (Fig. 5A). In case threshold levels for LAA overexpression were defined as more than a 10-fold RNA overexpression, a statistically significant overexpression in PB compartments compared with BM compartments for all 3 analyzed LAAs in the adjusted and unadjusted data group, with the exception of PR3 for unadjusted data, was detected (data not shown).

The results of an analysis of patients' PB samples and BM samples, harvested in parallel at the same time point, are shown in Figure 5B. For all analyzed cases, a (significant) higher expression was found for unadjusted/adjusted PR3-RNA ($P = 0.016/0.025$), but not for WT1-RNA

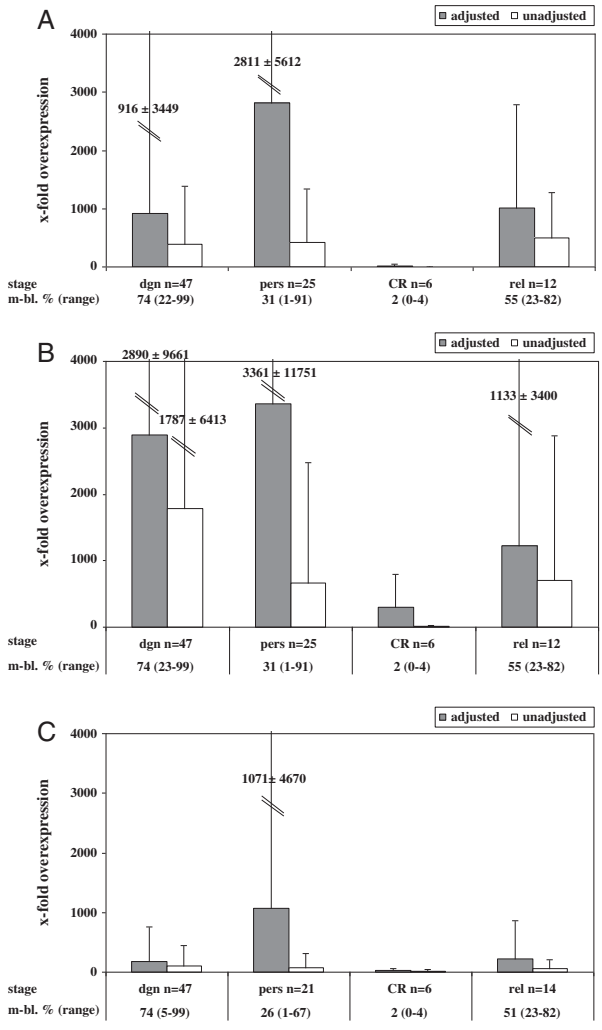

FIGURE 3. RNA expression levels of distinct LAAs in different stages of the disease. The polymerase chain reaction strategy is given in Figure 1. Mean LAA values with SDs of acute myeloid leukemia cases in different stages of the disease for the unadjusted values and values adjusted to 100% blasts of WT1 (A), PRAME (Bb), and PR3 (C) are given. CR indicates complete remission; dgn, diagnosis; LAA, leukemia-associated antigen; m-bL, mean % blasts; pers, persisting disease; rel, relapse.

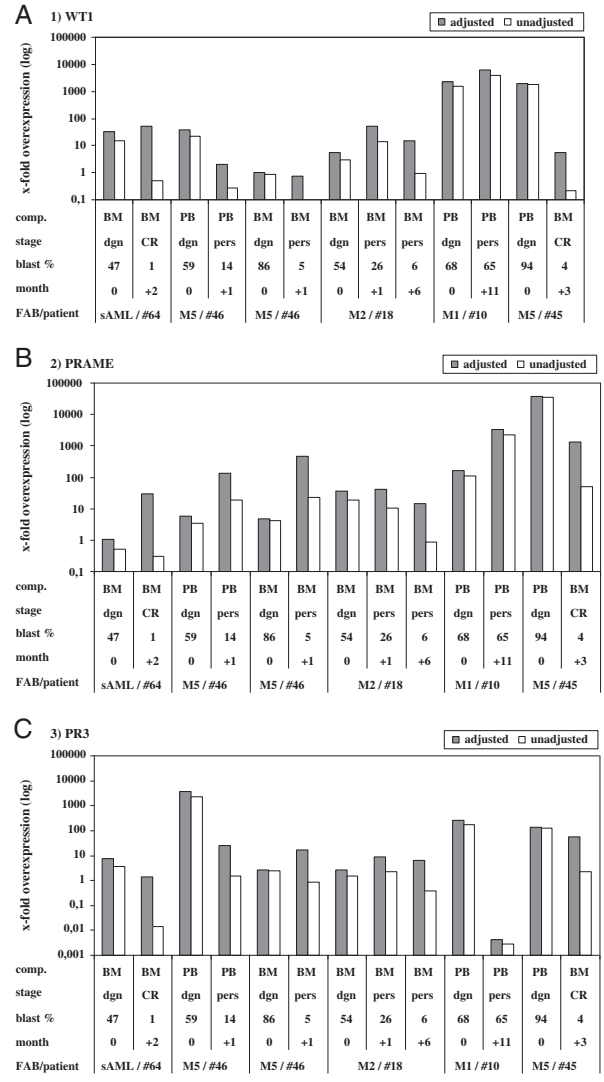

FIGURE 4. Leukemia-associated antigen–expression levels in 5 individual patients studied in different stages of the disease. The polymerase chain reaction strategy is given in Figure 1. Individual expression levels of 6 patients in different stages of the disease for the unadjusted values and values adjusted to 100% blasts of WT1 (A), PRAME (B), and PR3 (C) are presented. BM indicates bone marrow; CR, complete remission; dgn, diagnosis; PB, peripheral blood; pers, persisting disease; sAML, secondary acute myeloid leukemia.

($P = 0.337/0.109$) and PRAME-RNA ($P = 0.631/0.873$), in PB compared with BM. The highest overexpression for PRAME was found in BM samples for the adjusted and unadjusted data, with the exception of patient #70, in PB compared with BM.

Prognostic Significance

In AML, several prognostically relevant factors contribute toward estimation of patients' outcome. We analyzed LAA-expression levels in defined risk groups: we could correlate a "favorable" cytogenetic risk and a pAML appearance with a (significantly) lower expression of PRAME, as demonstrated in Figure 2. Moreover, we could correlate a tendentially significant "favorable" cytogenetic risk and a pAML with a higher expression of PR3 and a lower expression of WT1 as demonstrated with adjusted and unadjusted data (Fig. 2).

Refined studies applying cutoff analyses revealed that 100%/67% of cases with a >400-/2500-fold PRAME overexpression in BM/PB samples, but only 15%/13% of cases with a <400-/2500-fold overexpression, presented with a pAML appearance. Results with adjusted and unadjusted cases were comparable (Fig. 6A). PR3 expression in BM was very low; therefore, no predictive cutoff could be defined. However, for PB samples, a cutoff for unadjusted (100-fold) or adjusted samples (80-fold overexpression) could be defined: 90% of cases with a >100-/80-fold overexpression (in adjusted/unadjusted data) presented with a pAML appearance (Fig. 6B). For WT1, no predictive cutoff could be defined. This means that a low expression of PRAME in BM and PB and a high overexpression of PR3

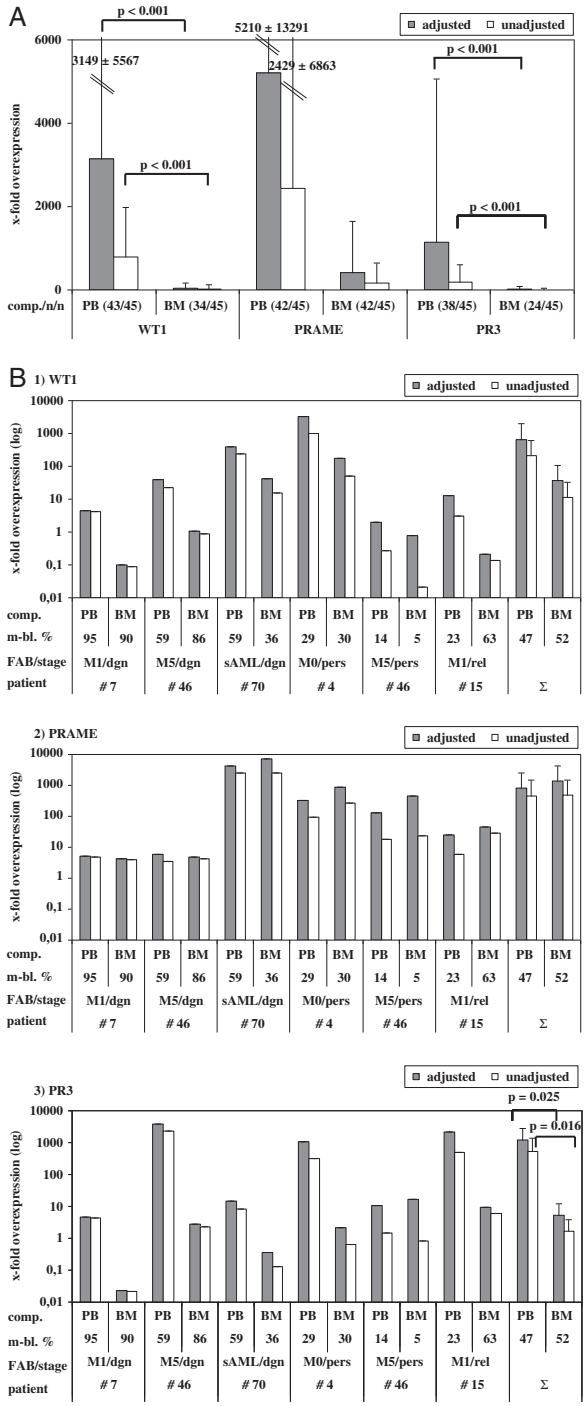

FIGURE 5. RNA expression levels of LAAs in PB samples versus, BM samples with all cases with overexpression included (A) and in 6 cases with LAA expression (B). The polymerase chain reaction strategy is given in Figure 1. Expression profiles in groups were compared using the Mann-Whitney *U* test. A $P \leq 0.05$ was considered to be statistically significant. Mean LAA values with SDs and statistical significance in PB samples compared with BM samples for the unadjusted values and values adjusted to 100% blasts are shown (A). Unadjusted and LAA-expression values adjusted to 100% blasts in 6 cases studied in different stages of the disease are given (B). BM indicates bone marrow; comp., compartment; dgn, diagnosis; LAA, leukemia-associated antigen; m-bL, mean % blasts; PB, peripheral blood; pers, persisting disease; rel, relapse; sAML, secondary acute myeloid leukemia.

in PB are associated with a favorable outcome. In addition, we correlated a response to immunotherapy with the FLAMSA protocol with levels of LAA overexpression. We could show that those patients who had successfully responded to FLAMSA therapy (n = 3) were characterized by higher mean PR3 values (adjusted/unadjusted data, 525-/56-fold overexpression) and lower mean PRAME values (adjusted/unadjusted data, 1135-/307-fold overexpression) compared with those cases with no response (n = 9), mean PR3 values (adjusted/unadjusted data, 197-/80-fold overexpression), and mean PRAME values (adjusted/unadjusted data, 2443-/1588-fold overexpression) (Table 4).

DISCUSSION

LAA Potential Targets for Immunotherapy

T lymphocytes are central mediators of an immune response against leukemic cells. Infusion of donor lymphocytes can even restore CRs in relapsed patients after allogenic stem cell transplantation.[28] In the last few years, several antigens were identified that could qualify as candidates to develop targeted therapies directed against single targets. The transcription factor WT1, the differentiation factor PR3, and proliferation and apoptosis factor PRAME were shown to be expressed in small cell numbers in healthy postnatal life, but are highly overexpressed in several solid tumors and leukemias.[12,29–31] It was shown that these antigens can induce T-cell proliferation and specific CTL responses and that those LAA-specific T cells can be isolated from HLA-A02–restricted donors for patients undergoing cell transplantation.[30] The aim of our study was to further shed light on the role of an overexpression of 3 LAA in detail and to contribute toward the development or refinement of LAA-directed therapies.

Strategies to Define and Apply LAA Overexpressions in AML Subtypes and Contributions to Prognosis

The expression of differentiation or transcription factors like WT1, PRAME, and PR3 normally is highly regulated. Their function in healthy organisms is to regulate the normal cell differentiation, proliferation, or apoptosis, but they can also be involved in tumorigenesis.[5–8,10,14,17,18,32,33] Here, we contribute more detailed data for all of these LAAs: for AML, several studies have been published that revealed that WT1,[16,30,34–36] PR3,[16,30] and PRAME[10,12,14,16,30,37,38] are regularly overexpressed in 30%–80% of the cases. These varying results obtained by different groups can be explained by different methodological strategies using different primers, absolute, or relative quantification systems, internal controls, or threshold levels for the definition of "overexpression": Spanaki et al[10] defined a PRAME overexpression after RQ-RT PCR in acute leukemia compared with GAPDH expression in the K562 leukemic cell line. Jacobsohn et al[39] defined a WT1 overexpression in cases with higher than a threshold 1 expression in 1 μg of K562 RNA detected by a 2-step real-time PCR. Ostergaard et al[9] used the β2M (β2-microglobulin) and ABL (Abelson) as internal control genes. Steinbach et al[12] defined a PRAME threshold for overexpression in cases with a 10 times higher LAA expression compared with healthy BM. This means that, in general, a comparison of data obtained by different working groups is difficult because no standardized strategies to estimate overexpression are applied by different groups.

We used an RQ-RT PCR method with GAPDH as internal RNA control and defined an overexpression in

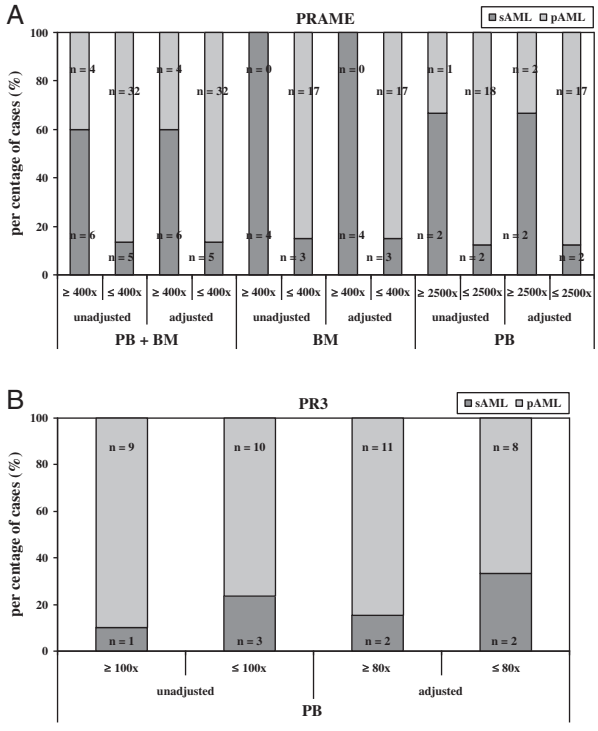

FIGURE 6. Predictive cutoff analyses values for PRAME (A) and PR3 (B) could be evaluated that enable an allocation to pAML or sAML cases at first diagnosis. The polymerase chain reaction strategy is given in Figure 1. Cases with and without overexpression subdivided into BM and/or PB are included. BM indicates bone marrow; pAML, primary acute myeloid leukemia; PB, peripheral blood; sAML, secondary acute myeloid leukemia.

cases with higher than a "threshold 1" in healthy donors. Applying our strategy, we found an overexpression of WT1-RNA in 81%, for PRAME-RNA in 87%, and for PR3-RNA in 55% of the analyzed cases at first diagnosis, thereby confirming data already published: Lapillonne et al[34] detected an overexpression for WT1 in 78% of pediatric AML at first diagnosis; Spanaki et al[10] described an overexpression of PRAME in 53% of acute lymphatic leukemia and AML patients; and Greiner et al[16] described an overexpression for WT1 in 67%, for PRAME in 64%, and for PR3 in 67% of AML patients at first diagnosis. To refine the evaluation strategies, we adjusted values obtained calculatory to individual (morphologically evaluated) blast counts at the date of sample collection. Adjustment of our PCR results to blast proportions enables a correlation analysis of LAA overexpression with the tumor load and contributes to differentiation between cases with low blast counts and high LAA overexpression and cases with high blast counts and low LAA overexpression. Applying these refined criteria, we found an overexpression rate for WT1 in 81%, for PRAME in 94%, and for PR3 in 57%.

Another aim of our work was to analyze a parallel/simultaneous expression of 2 or more LAAs. We detected a multiple overexpression of 2 or 3 LAAs in about 50% of the cases at first diagnosis, an observation that was also observed by other groups.[40] This could mean that the *same* blast population presents with multiple overexpressions or, alternatively, that *different* blast populations present with different LAA overexpressions. As a consequence, this could mean that *different* blasts might be more or less sensitive to chemotherapy, resulting in different blast populations detectable in the course of the disease, as already demonstrated by others and us.[41] Although, in general, the prognostic significance of LAA-expression levels at first diagnosis of AML is controversially discussed in our patient cohort, we found some prognostically interesting correlations of overexpressions of PR3 and PRAME (but not of WT1) with AML subtypes. Separating our cases into *prognostically different subtypes,* we found higher overexpressions of WT1 and PR3 and significantly lower overexpressions of PRAME in *pAML* compared with *sAML* in the adjusted and the unadjusted data. With respect to prognosis, we could demonstrate that prognostically unfavorable subtypes like *FAB-type M5*, cases with sAML, or *cytogenetic "unfavorable risk"* cases presented with (significant) overexpression of PRAME, which was even more pronounced by presentation of "adjusted" results. In part, our results could be confirmed by other groups: Greiner and colleagues demonstrated an overexpression of PRAME in sAML compared with pAML and in cytogenetic "unfavorable risk" compared with "favorable risk," although refined analyses in subgroups revealed that this was not true for all of the distinct risk types.[10,37] This could explain the different results described by other groups on clinical correlations of LAA overexpressions.

In terms of the prognostic significance of LAA overexpressions, Greiner and colleagues[19,40] observed that patients with a high overexpression of *single* LAA like G250/CA9, PRAME, or RHAMM/HMMR were characterized by a more favorable clinical outcome compared with cases with *multiple* (low) overexpression. We could in part confirm these data: we found a higher rate of cases with multiple overexpressions (but not for co-overexpression of WT1 with PRAME) in pAML compared with sAML. We could speculate that the reasons for these unfavorable outcomes could be the different susceptibilities of various LAA-expressing clones to chemotherapy, as described before. Patients who had responded to FLAMSA immunotherapy had a lower mean overexpression of PRAME compared with the nonresponder patient cohort.

In contrast, refined analyses of our group subdivided into different *LAA-expression types* showed that cases with high *PRAME* overexpressions could clearly be assigned to prognostically unfavorable sAML cases. This could not be demonstrated by Steinbach et al[12] for cases with *childhood* AML, who described a significantly higher rate of disease-free and overall survival in cases with PRAME overexpression. We could not define clear associations of *WT1* expression levels with prognostic subtypes: we could not evaluate predictive cutoff values for WT1 to estimate associations with prognostic subtypes and levels of WT1 expressions were comparable in cases with favorable/unfavorable cytogenetic risk, but were higher in pAML compared with sAML cases. Data presented in the literature resemble similar evaluations: Lapillonne and colleagues found a correlation of high WT1 expressions with "favorable risk" karyotypes in childhood AML, whereas Bergmann and colleagues described WT1 overexpressions for cases with a poor clinical outcome and Ostergaard and colleagues could not correlate a WT1 overexpression at first diagnosis with distinct cytogenetic risk groups.[9,30,34,42,43] In terms of *PR3* expression, we found that higher overexpressions are associated with a more "favorable"

TABLE 4. Cases Treated With the FLAMSA Protocol and Their Expression Profiles (x-Fold Overexpression)

No.	Stage	Response to FLAMSA	WT1 Adjusted	WT1 Unadjusted	PRAME Adjusted	PRAME Unadjusted	PR3 Adjusted	PR3 Unadjusted
Responders to FLAMSA therapy								
#67	pers, refractory	Yes, in rem 1.5 y later	20,335	610	512	15	950	28
#4a	pers, refractory	Yes, for 3 y	1162	825	1041	739	133	94
#36	pers, refractory	Yes, in rem 2 y later	3	ND	1852	167	493	44
Mean			7167	478	1135	307	525	56
Nonresponders to FLAMSA therapy								
#4b	pers, refractory, rel after SCT	No	423	385	2677	2436	ND	ND
#4c	pers, rel after SCT	No	3363	975	322	93	1070	310
#73	Second rel	No	3460	2180	13,734	8653	36	23
#68	pers, rel after SCT	No	165	115	136	95	23	16
#72	rel after SCT	No	1719	1410	4	3	ND	ND
#12	rel after second SCT	No	1418	624	1528	672	244	107
#10b	pers, refractory after SCT	No	6240	4056	3424	2225	ND	ND
#69	rel	No	37	22	2	1	152	91
Mean			2103	1221	2728	1772	191	68

Unadjusted data and values adjusted to 100% blasts are shown.
#Cases treated with the FLAMSA Protocol (as given in Table 1).
Samples unadjusted and adjusted to 100% morphologically detected blasts; mean, a, b, c samples from the same patient in different stages included.
dgn indicates diagnosis; LAA, leukemia-associated antigen; ND, not detectable; pers, persisting disease; rel, relapse; rem, complete remission; SCT, stem cell transplantation.

prognosis with respect to cytogenetic risk and, in addition, that cases with higher expression levels had a higher chance of belonging to the pAML appearance group. This could be confirmed by Yong and colleagues, who also found a correlation of higher PR3 expression levels with "favorable" and lower levels with "unfavorable" cytogenetic risk groups in CML patients.[19,44] Patients who had responded to FLAMSA immunotherapy are associated with a higher overexpression rate of PR3 and confirmed previously described observations.

LAA Overexpressions to Monitor Minimal Residual Disease (MRD) or Oligoclonality in the Course of AML

The potency of the analyzed LAAs as a marker for MRD and a prognostic marker is controversially discussed; whereas WT1 was described as an efficient MRD marker for the majority of AML patients, other groups described WT1 as a marker strictly correlated with blast counts, but not as a useful marker for the detection of MRD.[9,10,42,45,46] We detected variable LAA overexpressions in patients' samples in different stages of the disease. In terms of unadjusted data, the highest average LAA overexpression was found at first diagnosis, followed by persisting disease and relapse. Low levels were found in remission. In general, we confirm data of the literature: some groups described decreased WT1 or PRAME expression levels in remission and increased levels at relapse.[31,37,47] Adjusting detected expression profiles to 100% blasts, our data showed the highest expression rates in cases with persisting disease. This could reveal a higher transcriptory activity of LAA genes in those cases, possibly as an influence of chemotherapy or a selection of distinct (therapy resistant) clones with high LAA transcription, resulting in a clonal shift from polyclonal leukemic cells to an oligoclonal status, probably based on single-cell events.

Adjusting detected expression profiles to 100% blasts, we could moreover detect overexpressed LAA genes in some BM samples in remission (PB samples had to be excluded from analyses because, in PB, per definition, no blasts are detectable in remission). Those cases could probably point to immanent relapses. On the basis of our observations, we recommend an analysis of LAA expression adjusted to blast counts for a refined analysis of the tumor load, although more data in the course of the disease have to be generated and correlated with the clinical course of the disease to shed light on the role of LAA-expression profiling for MRD-diagnosis in more detail. Our approach could contribute to further differentially estimate prognosis and possibly to evaluate the effect for vaccination strategies with different or single LAA antigens.

LAA Overexpression in Different Compartments

Principally LAA–peptide-based vaccines are able to induce immune responses: Rezvani and colleagues described a $CD8^+$ T-cell reaction against PR3 and WT1 after peptide vaccination with decreased leukemic load and WT1 expression, although at this point, no data on the clinical response and duration of remission are available.[3,16,48] An interesting observation of our study was that LAA-expression levels were (significantly) different in PB samples compared with BM samples. Quantitatively different LAA-expression profiles of WT1 were already described in some healthy PB samples compared with BM samples, but not studied and discussed in detail.[9,46] In contrast to our findings, new studies could not describe significant differences of LAA-expression levels in both compartments of AML patients.[46] This could be explained by an

asymmetric distribution of the LAA-expressing blasts in the 2 compartments. As a consequence, this could mean that immunotherapies addressing (single) LAA targets, which are differently expressed in PB compartments or BM compartments, might not be equally efficient against PB or BM blasts.

Immunotherapy Against LAA and Concluding Remarks

In conclusion, we detected comparable overexpression rates of the LAAs as described in the literature. In addition, we can add that our calculatory system adjusting the LAA-expression levels to blast counts contributes to a refined analysis: we can differentiate between few cells overexpressing high LAA levels or many cells overexpressing low LAA levels. Possibly, the clonality of leukemic cells could shift during therapy from a polyclonal to an oligoclonal status, which could result in different responses or sensitivities to chemotherapies or immunotherapies. Moreover, we can contribute toward estimation of the prognosis of the patients with PRAME or PR3 (but not WT1) overexpression, and we detected an overexpression of >2 LAAs in $>45\%$ of samples at first diagnosis in unadjusted data and in $>55\%$ of the adjusted data and confirmed the results of the literature, that simultaneous overexpressions of LAAs occur: Greiner et al[40] described a simultaneous expression of 2 or more antigens in 80% of AML patients. These observations and especially our findings on LAA expressions in the different cellular compartments might contribute to refined LAA-directed immunotherapies, which are efficient to eliminate targets in BM and in PB or targets expressing different LAAs—for example, applying bivalent or polyvalent vaccines in clinical trials[3,49] to minimize the risk of immune escape.

In summary, we recommend the establishment of standardized methods to define overexpressions using standardized procedures with defined control (housekeeping) genes to produce comparable data and to estimate the tumor load, especially in remission of the disease. The European Leukaemia Net Study has already defined a standardized WT1 assay to compare the results obtained with different WT1 expression assays and to estimate the diagnostic relevance.[46,50] We recommend to include, in addition, PRAME-addressing and PR3-addressing strategies, to include evaluation strategies with data adjusted to 100% blasts, and especially to monitor LAA levels in different cellular compartments.

It must be shown in the future whether immunotherapeutic strategies targeting LAA are sufficient tools to eliminate leukemic cells.[51,52]

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Conflicts of Interest/Financial Disclosures

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