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Childhood acute myelogenous leukaemia: Association between *PRAME*, apoptosis- and MDR-related gene expression

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ABSTRACT

The gene *PRAME* (preferentially expressed antigen of melanoma) encodes an antigen recognised by autologous cytolytic T lymphocytes. The mRNA level of *PRAME* is used as a tumour marker due to its overexpression in various malignancies. Furthermore, it is known that the overexpression of genes encoding antiapoptotic proteins leads to the survival of leukaemic cells via exclusion of apoptosis. On the other hand, overexpression of genes encoding ABC transporters may lead to multi drug resistance (MDR). Therefore, we investigated whether there is a relationship between *PRAME* overexpression and the expression of apoptosis- and MDR-related genes in childhood *de novo* acute myelogenous leukaemia (AML) patient samples and, furthermore, whether this is a general or an AML-subtype specific event. Microarray analysis and real time quantitative PCR revealed that clinical samples showing *PRAME* upregulation are associated with a decreasing expression of genes coding for apoptotic proteins and an overexpression of genes encoding ABC transporters. Our results indicate that patients showing *PRAME* upregulation may have an increased risk of MDR induction.

1. Introduction

In 1997 Ikeda and colleagues[1] discovered the antigen *PRAME* whose function is still being defined. The mRNA level of *PRAME* is a reliable marker for several malignancies including leukaemia, melanoma, neuroblastoma, medulloblastoma, cervical squamous cell carcinoma, renal cell carcinoma, Wilms' tumour, myeloma and others. In contrast to patients where *PRAME* was found to be widely expressed, healthy organisms normally only express detectable *PRAME* in testis and, to a much lower degree, in endometrium and ovary. However, overexpression does not occur in all patients, i.e. it is found in approximately 35–42% of adult acute myelogenous leukaemia (AML) patients[2,3] and in 62% of all childhood AML patients.[4] Similar observations have been described for acute lymphocytic leukaemia (ALL) patients, where *PRAME* was found to be expressed in 42% of investigated cases.[5] These findings might be due to individual DNA methylation patterns within the *PRAME* promoter.[6] Remarkably, AMLs carrying the chromosomal translocation t(8;21), which leads to the fusion of the genes *AML1* and *ETO*, express *PRAME* at a high level.[2] The *PRAME* mRNA level is also a useful parameter

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for monitoring the minimal residual disease (MRD), due to its significant reduction in patients upon chemotherapy[3] and PRAME is discussed to be a potential candidate for immunotherapy in AML.[7]

The overexpression of genes encoding anti-apoptotic proteins is known to lead to the survival of leukaemic cells by inhibition of apoptotic mechanisms and therefore to the failure of therapy.[8] In addition, chemotherapy is often connected with multi drug resistance (MDR), i.e. via the efflux of chemotherapeutics, mediated by ABC transporter proteins.[9] The overexpression of ABC transporter genes can be used as a measure of the response to chemotherapy and therefore is an important prognostic indicator.[10] Therefore, we sought to study whether there is a direct relationship between *PRAME* overexpression and the expression of apoptosis- and MDR-related genes in childhood *de novo* AML patient samples and furthermore whether this is a general or an AML-subtype specific event. Initial investigation using childhood AML patient samples of differing *PRAME* expression revealed a direct correlation between overexpression of *PRAME*, downregulation of apoptotic genes and upregulation of ABC transporter genes in all AML subtypes under investigation. To our knowledge, this is the first study describing a correlation between overexpression of *PRAME* and upregulation of MDR-related genes. However, cause-effect studies examining *in vitro* silencing as well as overexpression of *PRAME*, revealed that PRAME itself does not regulate the expression of MDR- and apoptosis- related genes, and that regulation likely occurs at a higher level, e.g. via a yet unidentified master regulator.

2. Materials and methods

2.1. Patients

Bone marrow samples from 50 *de novo* childhood AML patients prior to therapy were prepared as previously described[4] and their relative *PRAME* expression was determined by RTQ-PCR and calculated as previously described.[4] All patients with an intermediate relative *PRAME* expression, i.e. $>3\times10^{-4}$ and $<2.5\times10^{-3}$, were excluded from further experiments. Data of patients involved in the study are given in Table 1. Their relative *PRAME* expression values are listed in Table 2.

Table 1 – Patient data of children with *de novo* AML

Number of patients	27
Median age	11 (0–17)
Sex (male/female)	14/13
Median WBC in 10/l (range)	55 (8–355)
Auer rod (positive/negative/n.a.)	5/21/1
Median percentage of leukemic cells	
bone marrow (range)	83 (48–99)
peripheral blood (range)	70 (17–99)
Inversion inv(16) (positive/ negative/n.a.)	3/24/0
Translocation t(8/21) (positive/negative/n.a.)	3/24/0
Translocation t(9/11) (positive/negative/n.a.)	4/23/0
AML type (number of patients)	
myelocytic (FAB type M1 or M2)	9
myelomonocytic (FAB type M4)	9
monocytic (FAB type M5)	9

n.a.: not available.

Table 2 – Relative *PRAME* expression of de novo patient samples

AML subtype	Sample number		Relative *PRAME* expression
M1/2	PRAME down	1	0
		3	0
		38	0
		7	0,0002
		6	0,0003
	PRAME up	32	0,0027
		4	0,06
		25	0,096
		47	0,12
M4	PRAME down	39	0
		35	0
		30	0,0001
		33	0,0002
		31	0,0003
	PRAME up	43	0,0025
		41	0,0046
		40	0,0064
		42	0,043
M5	PRAME down	28	0
		26	0
		34	0
		36	0
		29	0,0001
	PRAME up	46	0,016
		37	0,055
		44	0,069
		45	0,088

All experiments involving patient samples have been performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from each subject or subject's guardian.

2.2. RNA preparation

Total RNA from 27 bone marrow samples (Table 1) of differing childhood AML subtypes, i.e. M1/2, M4 and M5, was isolated as previously described.[4] Quality and quantity of RNA was determined by 1% Tris-Borate-EDTA (TBE) agarose gels and photometry. Equal amounts of RNA were pooled according to *PRAME* upregulation and *PRAME* downregulation within each specific AML subtype. The *PRAME* upregulated and *PRAME* downregulated groups were matched for the presence of Auer rods (M1/M2 group) and for the presence of atypical eosinophils (M4).

2.3. Real time quantitative PCR (RTQ-PCR)

For validation of microarray data, six genes were randomly chosen to determine their relative expression ratios by RTQ-PCR. cDNA was generated by random hexamer priming and PowerScript Reverse Transcriptase according to manufacturer's instructions (Clontech, Heidelberg, Germany). For quantitative RT-PCR the qPCR MasterMix for SYBR® green I (Eurogentec, Köln, Germany) and corresponding instructions

were used. 'Primer Express' (Applied Biosystems, Darmstadt, Germany) served for primer design. RTQ-PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Glucuronidase b gene was used as endogenous control due to its reported low variability in tumour samples.[11] Relative gene expression ratios were calculated as previously described.[12] Briefly, the relative gene expression ratio of a target gene was calculated from the real-time PCR efficiencies and the threshold cycle (Ct) of an unknown sample (i.e. the sample showing *PRAME* upregulation) versus a comparison sample (i.e. the sample showing *PRAME* downregulation) and expressed in comparison to a selected reference, or housekeeping, gene.

2.4. *Microarrays*

cDNA clones were obtained from RZPD (Berlin, Germany) and fully characterised in our laboratory, i.e. sequences analysed, and problematic sequences excluded. Fabrication and usage of microarrays was performed as described in http://stanford.edu/pbrown/protocols. For normalisation, 15 different housekeeping genes were randomly distributed on the array. Fluorescence values were determined by a Gene Pix 4000 B instrument (Axon Instruments, Union City, USA). Raw data were generated using Gene Pix® Pro 4.0 Array Acquisition and Analysis software. Signal analysis for microarray experiments and calculation of relative gene expression ratios was performed according to Gene Spring software (Silicon Genetics, Redwood City, USA). The overall threshold was considered using a minimum magnitude two-fold cutoff (>2: upregulated; <0.5: downregulated).

2.5. *Cell culture*

HeLa (ATCC CCL-2) and CaSki cells (ATCC CRL-1550) were maintained in DMEM (Dulbecco's Modified Eagle Medium; GIBCO, Roskilde, Denmark) with 10% FCS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2.

2.6. *Overexpression of PRAME in CaSki cells*

The ORF of *PRAME* (total length: 1527bp) was amplified by PCR and cloned into *Bam*HI/*Not*I-digested pcDNA3 expression vector (Invitrogen, Karlsruhe, Germany). CaSki cells were grown to 80% confluence on 6 cm diameter plates and then transiently transfected with 6 μg of the fusion plasmid with TransFectin Lipid reagent (Bio-Rad, Munich, Germany). Control cells were transfected by the empty pcDNA3 expression vector. The cells were harvested for RNA isolation 24 h after transfection. The transfection efficiency was >90%. RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reverse Transcription and RTQ-PCR were performed as described above.

2.7. *Silencing of PRAME by short interfering RNA (siRNA) in HeLa cells*

Double-stranded siRNAs specific for *PRAME* mRNA were designed and manufactured using Qiagen's (Hilden, Germany) 2-for-Silencing service. HeLa cells were transfected with siRNA by using the HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two different double-stranded siRNA constructs were used in these studies designated as siRNAs 1 and 2 with the following sequences:

r(CUGUGUAGACUGUUGUAAA)dTdT and r(GCUAAGUGUCCUGAGUCUA)dTdT.

RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reverse Transcription and RTQ-PCR were performed as described above.

2.8. *Statistics*

Mean expression of *PRAME* in different groups of patients was compared using Mann-Whitney- and Kruskal-Wallis-tests.

The correlation between *PRAME* expression and other pretherapeutic findings, i.e. sex, age, FAB-type, initial WBC count, initial Hb, initial platelet counts, organomegaly, presence of Auer rods and the chromosomal aberrations t(8;21), t(9;11) and inv(16), was investigated by means of Spearman's correlation coefficient.

3. Results

In this study we examined clinical samples of *de novo* childhood AML, including subtypes (M1/2, M4 and M5), with respect to expression of *PRAME* and other genes. Our investigational interest concerned the relationship between the expression of the cancer/testis antigen *PRAME* and the expression of genes involved in apoptotic and MDR processes because such events might have direct influence on the success of chemotherapeutical treatment. In light of a direct correlation in the overall group of samples, another question emerged regarding the generalisability of these findings to specific leukaemia subtypes.

3.1. *Upregulation of PRAME in childhood AML patients correlates with a decreased expression of apoptosis-related genes and an increased expression of genes involved in MDR*

To elucidate whether there is a relationship between the presence of the cancer/testis antigen *PRAME* and the expression of genes involved in apoptotic and MDR processes we designed and fabricated special cDNA microarrays involving approximately 300 stress relevant genes out of which approximately 100 cDNA clones were known to be either involved in apoptotic or MDR processes. The remaining 200 cDNA clones represented either a direct or indirect relationship to the above two processes in terms of pathways. Each cDNA was double spotted and two independent hybridisations were performed. Only those genes showing consistent trend results across all four measures of relative expression ratio were considered for analysis in up- or downregulated categories. Microarray data revealed a total of 31 genes which were differentially expressed in each of the AML subtypes under investigation (Table 3). Only genes showing a relative expression ratio of >2 and <0.5 were considered to be up- or downregulated, respectively. The gene expression fold-changes (Fig. 1; y-axis) were calculated based on the relative gene expression

Table 3 – Relative gene expression ratios (*PRAME* up versus *PRAME* down) associated with *PRAME* upregulation in three AML subtypes (M1/2, M4, M5) as determined by microarray experiments

I.M.A.G.E.[a] ID	Gene	Protein	Relative gene expression ratios (*PRAME* up versus *PRAME* down) ±STD[b]		
			M1/2[c]	M4[c]	M5[c]
Downregulated genes					
Ribosomal Proteins					
173603	*RPS28*	Ribosomal protein	0,425 ± 0,068	0,347 ± 0,116	0,278 ± 0,101
110400	*RPLl21*	Ribosomal protein	0,438 ± 0,059	0,426 ± 0,054	0,442 ± 0,036
Apoptosis					
148052	*MDM2*	Inhibitor of p53	0,410 ± 0,048	0,405 ± 0,068	0,406 ± 0,068
138793	*TRAIL-R2*	Death receptor	0,412 ± 0,063	0,374 ± 0,092	0,469 ± 0,012
2819507	*BAK/BAK1*	Proapoptotic protein	0,429 ± 0,065	0,352 ± 0,058	0,345 ± 0,151
4578562	*BAX*	Proapoptotic protein	0,374 ± 0,055	0,368 ± 0,083	0,379 ± 0,074
Inflammation					
4868206	*IL13-RA1*	IL13 receptor	0,433 ± 0,079	0,267 ± 0,183	0,432 ± 0,025
150510	*ILRL1*	IL1 receptor	0,369 ± 0,077	0,454 ± 0,027	0,406 ± 0,083
163092	*ILF3*	IL enhancer binding factor	0,401 ± 0,048	0,374 ± 0,043	0,447 ± 0,023
Transcription activation					
5922104	*RELA*	p65 subunit of NFkB	0,298 ± 0,102	0,333 ± 0,121	0,434 ± 0,003
380719	*COPEB*	Core promotor element binding protein	0,409 ± 0,066	0,435 ± 0,047	0,396 ± 0,042
Miscellaneous					
788433	*ARL2*	ATP ribosylation factor	0,481 ± 0,018	0,387 ± 0,037	0,389 ± 0,057
34357	*ACTB*	Beta actin	0,431 ± 0,041	0,458 ± 0,039	0,430 ± 0,023
Upregulated genes					
ABC transporter					
288736	*MRP3*	Multidrug resistance-associated protein 3	6,167 ± 2,180	10,397 ± 2,690	6,947 ± 1,860
288736	*BCRP*	Breast cancer resistance protein	5,089 ± 0,797	6,450 ± 1,701	8,357 ± 1,528
Signal transduction					
121591	*MAPK4*	Mitogen-activated protein kinase 4	4,962 ± 1,228	5,733 ± 0,014	9,686 ± 0,381
470082	*MAPK7*	Mitogen-activated protein kinase 7	5,721± 0,164	6,051 ± 1,286	10,066 ± 0,229
320810	*MAPK13*	Mitogen-activated protein kinase 13	4,674 ± 1,685	7,038 ± 1,936	10,656 ± 2,960
Inflammation					
2092585	*IL5-RA1*	IL5 receptor	3,419± 0,875	4,572 ± 0,877	10,748± 1,755
2063116	*SCYA23*	Small inducible cytokine subfamily A, member 23	4,921 ± 1,201	7,187 ± 2,687	8,264 ± 0,426
768497	*SCYA1*	Small inducible cytokine subfamily A, member 1	4,905 ± 1,503	11,373 ± 0,731	14,883 ± 1,340
198699	*SCYB5*	Small inducible cytokine subfamily B, member 5	6,033 ± 2,201	5,328 ± 2,127	21,882 ± 0,245
67071	*IL22R*	IL22 receptor	7,646 ± 1,934	7,209 ± 0,164	10,113 ± 0,464
297098	*IRIRF2*	Interferon regulatory factor 2	3,989 ± 1,210	4,843 ± 0,975	8,045 ± 0,711
Apoptosis					
201890	*CIAP2*	Inhibitor of apoptosis	4,566 ± 0,049	6,271 ± 2,121	6,619 ± 0,534
360838	*AKT3*	Serine/threonine protein kinase	6,801 ± 0,658	6,798 ± 1,670	8,278 ± 3,157
Miscellanous					
2499375	*HSD11B1*	Hydroxysteroid (11-beta) dehydrogenase	7,530 ± 2,887	7,753 ± 1,835	8,449 ± 0,585
345158	*PRODH*	Proline dehydrogenase	4,049 ± 1,742	4,514 ± 1,426	5,326 ± 0,223
39044	*BECN1*	Beclin 1, myosin-like BCL-2 interacting	6,494 ± 1,896	6,794 ± 0,818	8,086 ± 1,149
2252285	*ZRF1*	Zuotin related factor 1	10,003 ± 2,303	7,346 ± 3,002	7,133 ± 0,687
1128275	*PRAME*	Tumour antigen	4,352 ± 1,157	5,316 ± 1,490	4,167 ± 1,419

All genes showing a relative expression ratio of >2 were considered to be upregulated, all genes showing a relative expression ratio of <0.5 were considered to be downregulated.

a I.M.A.G.E. = Integrated Molecular Analysis of Genomes and Expression consortium.

b STD = Standard deviation.

c M1/2, M4, M5 = AML subtypes.

ratio computed as described in Materials and methods. Relative quantitation entailed the process of computing the ratio of absolute signals for each microarray spot in two samples on the array. The results revealed the same 18 upregulated (6%) and 13 downregulated genes (4%) in each AML subtype under investigation, all correlated with *PRAME* overexpression (Table 3). Concomitant with *PRAME* upregulation, the genes *CIAP2* and *AKT3*, whose products have antiapoptotic functions,[13,14] were found to be upregulated with a relative expression ratio of >4 (Table 3). The genes *BAK1* and *BAX*, both encoding proapoptotic proteins, were moderately downregulated, i.e. they showed a relative expression ratio of <0.4 (Table 3). The gene *AKT3* showed an increased relative expression ratio, i.e. 6-8, in patients with *PRAME* upregulation (Table 3). In association with *PRAME* upregulation, the genes of both MRP3 and BCRP from the ABC transporter family were upregulated with a relative expression ratio of >6 and 5, respectively (Table 3).

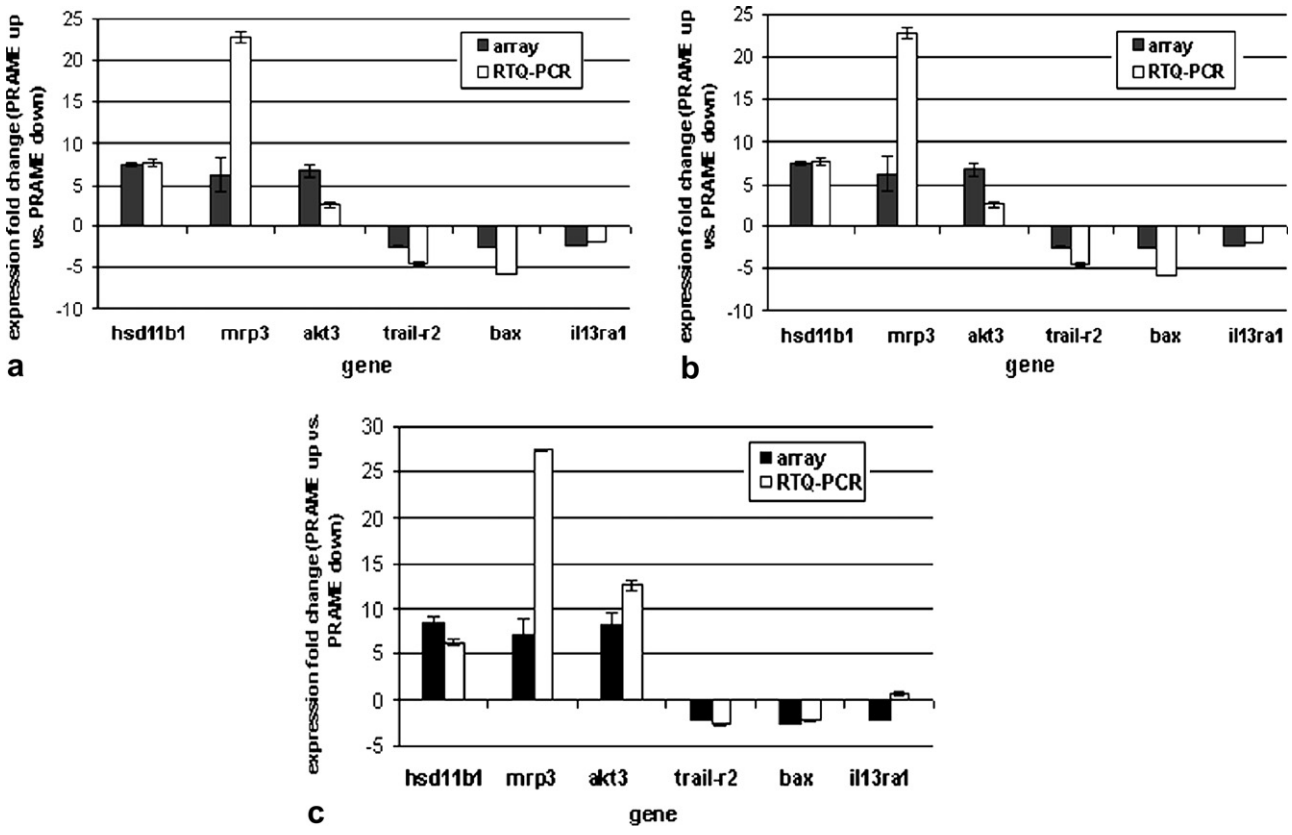

Fig. 1 – Comparison of gene expression fold changes obtained by microarray experiments and RTQ-PCR for AML subtypes M1/2 (a), M4 (b) and M5 (c). Black bars represent the data obtained by array experiments. White bars represent the RTQ-PCR data. Individual genes are represented on the x-axis.

3.2. *Confirmation of the microarray data by RTQ-PCR*

In order to confirm the array data, a number of randomly selected differentially expressed genes were subjected to RTQ-PCR experiments with pooled patient samples (Fig. 1; white bars) as described in Materials and methods and compared with the corresponding microarray data (Fig. 1; black bars). This resulted in a concordance between the two different approaches. A comparison between the RTQ-PCR data from all individuals and the above results from patients pools also revealed same trends concerning *PRAME* expression (see supplement). However, best correlation was obtained from the patient with the strongest *PRAME* upregulation.

3.3. *Correlation or cause-effect?*

Due to the above findings, the question arose whether PRAME is the direct cause of the downregulation of apoptosis-related genes and upregulation of genes involved in MDR or not. To examine this, *PRAME* was artificially silenced in cultivated HeLa cells and overexpressed in CaSki cells. HeLa cells were chosen for silencing experiments due to their endogenous constitutive expression of *PRAME*; CaSki cells were chosen for overexpression experiments due to a lack of endogenous *PRAME* expression. *PRAME* silencing experiments by siRNA led to a decrease of the expression of *PRAME* by 6.96-fold compared to control cells. *PRAME* overexpression revealed a 194130.42-fold increase compared to controls. Although the expression experiments with patient samples showed a correlative effect of *PRAME* with the expression of *MRP3*, *BCRP*, *BAX*, *BAK*, *CIAP2* and *AKT3* (Fig. 2), these six genes showed only an expression change (up or down) of less than two-fold in the *PRAME* silencing and overexpression experiments, i.e. no differential expression could be observed. Therefore, our results lead to the hypothesis of regulation occurring at a higher level, e.g. by a master regulator.

3.4. *PRAME expression and initial clinical parameters*

Statistical evaluation was performed to study potential correlations between *PRAME* expression and initial clinical parameters, i.e. sex, age, FAB-type, initial WBC count, initial Hb, initial platelet counts, organomegaly, presence of Auer rods and the chromosomal aberrations t(8;21), t(9;11) and inv(16) (data not shown). Expression of *PRAME* was negatively correlated to the WBC count ($p = 0.04$). This was in contrast to previous studies involving adult AML patient samples, where no correlation could be detected.[15] Furthermore, we found that patients with t(8;21) showed higher levels of *PRAME*

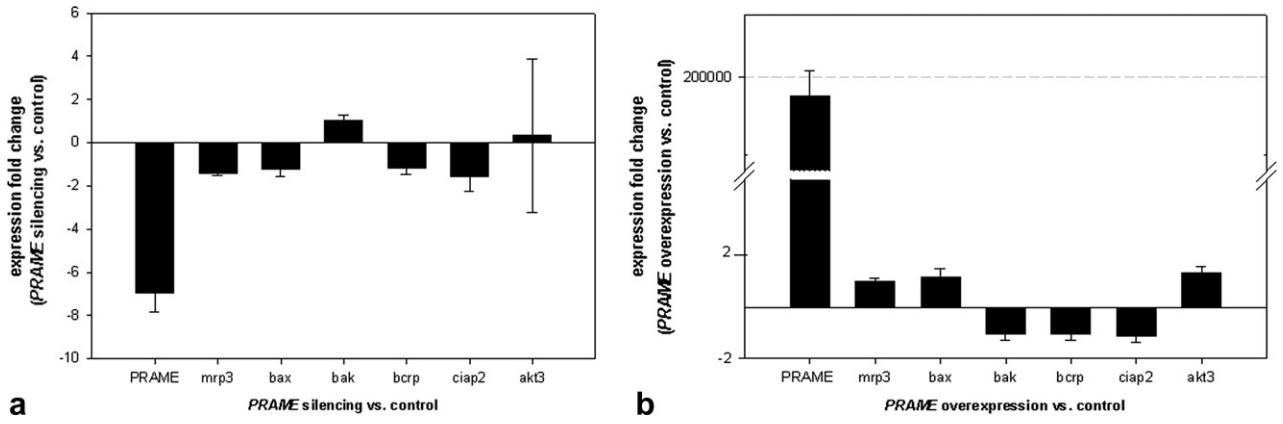

Fig. 2 – Gene expression fold changes determined for *PRAME* silencing (a) and *PRAME* overexpression (b) experiments. Black bars represent the expression fold changes (*y*-axis) between *PRAME* silencing and control (a) and *PRAME* overexpression and control (b). Individual genes are represented on the *x*-axis.

expression ($p < 0.04$).[4] All other correlations were not statistically significant.

4. Discussion

The aim of our study was to investigate different subtypes of childhood AML showing varying levels of *PRAME* expression in order to address the question of whether there is a relationship between the expression of the cancer/testis antigen *PRAME* and the expression of genes involved in apoptotic and MDR processes. Such events might have direct influence on the success of chemotherapeutic treatments and therefore on the outcome of the disease. Disregulation of apoptotic processes are known to play a significant role in drug resistance.[8] Similar effects could also arise by transcriptional and translational overexpression of MDR proteins, e.g. MRPs, which may confer therapeutic resistance in leukaemia and solid tumours.[9] Therefore, such a study might give relevant prognostic information on the success of chemotherapy. As the correlation between the expression of *PRAME* and genes involved in MDR and apoptotic processes became clear, further investigations were undertaken to answer the question of whether PRAME itself was responsible for the regulation of the above genes.

The correlation between the expression of *PRAME*, MDR- and apoptosis-related genes was investigated by our specially designed and fabricated microarrays involving approximately 300 stress relevant genes. Microarray data revealed a total of 31 genes, which were differentially expressed in the AML subtypes under investigation. Thus the number of differentially expressed genes matched the theoretical requirements for successful array experiments. For data analysis we applied stringent conditions, i.e. only genes showing a relative expression ratio of >2 or <0.5 were considered to be differentially expressed. Under these constraints, all AML subtypes showed 18 genes (6%) to be elevated and 13 genes (4%) to be diminished in expression, all concurrent with *PRAME* overexpression and in a subtype independent manner.

Concomitant with *PRAME* upregulation, the antiapoptotic genes *CIAP2* and *AKT3*[13,14] were found to be upregulated. The genes *BAK1* and *BAX*, both encoding proapoptotic proteins, were moderately downregulated. The protein CIAP2 belongs to the IAP family, the members of which are often implicated in a variety of cancer types including leukaemia.[16] Members of the IAP-family bind to the active forms of the effector caspases 3 and 7, thereby inhibiting their proteolytic activity and consequently the completion of apoptosis.[17] Upregulation of *CIAP2* argues for an increased inhibition of apoptosis in patients showing *PRAME* upregulation. This result is supported by our finding that the genes *BAX* and *BAK*, coding for proapoptotic members of the Bcl-2 superfamily, were downregulated in association with *PRAME* overexpression. The proteins BAX and BAK heterodimerise with the antiapoptotic protein BCL-2 thus promoting the release of cytochrome c into the cytoplasm. A recent study concerning *BAX* expression in AML patients revealed that patients with a high *BAX* expression at diagnosis had better outcomes in terms of disease free, event free and overall survival.[18] Upregulation of the antiapoptotic *CIAP2* and downregulation of the proapoptotic *BAX* and *BAK* might therefore be a predictor of poor outcome in patients showing *PRAME* overexpression. The gene *AKT3* showed an increased relative expression ratio in patients with *PRAME* upregulation. The serine/threonine kinase AKT plays a role not only in control of cellular growth, but also in metabolism and cell survival. AKT proteins can bolster the survival of cells under a variety of apoptotic events, e.g. the phosphorylation of BAD or procaspase-9 by AKT can inhibit apoptosis.[19] Both AKT2 and AKT3 activities have been shown in various human cancers suggesting involvement of AKT in the pathogenesis of cancer diseases.[19] The possibility of an involvement of PRAME in apoptotic processes was shown recently, in terms of a decreased expression of *HSP27*, *P21* and *S100A4* in association with *PRAME* overexpression.[20] Furthermore, other recent findings, using both *in vitro* conditions and mouse models, showed that PRAME is a dominant repressor of retinoic acid receptor signalling,[21] thus inhibiting retinoic acid (RA)-induced differentiation, growth arrest and apoptosis. Our *in vivo* data using patient material clearly support such a relationship between PRAME and suppression of apoptotic processes.

In addition to the survival of leukaemic cells due to apoptosis inhibition, chemotherapy is often connected with multi

drug resistance (MDR), i.e. efflux of chemotherapeutics, mediated by ABC transporter proteins.[9] The overexpression of ABC transporter genes is used as a measure of the response to chemotherapy and is of prognostic importance.[10] In association with *PRAME* upregulation, the genes encoding MRP3 and BCRP from the ABC transporter family were upregulated. The overexpression of these two genes can be correlated with a poor prognosis for AML patients[10,22] and has been identified as central reason for therapy failure in AML.[9,10] ABC transporters are localised in the cell membrane and can cause MDR by extruding a variety of chemotherapeutic drugs from the malignant cell. MRP3, like MRP1 and MRP2, is a transporter able to confer resistance to the anticancer drugs methotrexate, etoposide and teniposide.[23] Upregulation of *BCRP1* was found to be associated with resistance to mitoxantrone,[24] doxorubicin and verapamil in human cancer cell lines.[25] Similar to *MRP3* in ALL patients, an increased *BCRP1* expression is associated with a poor response to remission-inducing chemotherapy in children with AML.[22] Overexpression of the above genes could provide an important clue to therapy responsiveness in AML patients showing *PRAME* upregulation.

Our findings that upregulation of MDR genes is associated with decreased *BAX* expression are supported by previous studies *in vitro*.[26,27] Different MDR cell lines like HL-60/VCR, HL60/MRP or CCRF-CEM/VCR1000 are known to express less BAX and more MDR proteins compared to their chemotherapy sensitive variants.[26,27] These data suggest that patients with *PRAME* upregulation might be more resistant to chemotherapy due to similar expression features. Our results lead to the conclusion that patients showing *PRAME* upregulation might have an increased risk of MDR induction and therefore, our results broaden recently published data where *PRAME* expression was associated with a poor outcome in neuroblastoma.[28] Our microarray data were further supported by RTQ-PCR experiments using randomly selected differentially expressed genes. For these experiments we used the Glucuronidase b gene as normalisation control, because it was found to show the lowest variability in different tumour cell lines.[11] In addition, a comparison between the RTQ-PCR data from all individuals and the above results from patient pools also revealed same trends concerning *PRAME* expression, whereas the best correlation was obtained from the patient with the strongest *PRAME* upregulation.

Of great importance was the question of whether PRAME was the direct cause of the downregulation of apoptosis related genes and the upregulation of genes involved in multidrug resistance, or if the presence of an unknown upstream regulator has to be considered. To elucidate the above question, *PRAME* was silenced or overexpressed in cultivated HeLa and CaSki cells, respectively. HeLa cells showing constitutive expression of *PRAME* were chosen for the silencing experiments and CaSki cells lacking *PRAME* expression for the overexpression experiments. By this means, a causative effect of PRAME on the expression of *MRP3, BCRP, BAX, BAK, CIAP2* and *AKT3* could be excluded. Thus we hypothesise that regulation occurs on a higher level, e.g. by a master regulator. Therefore, our present investigations seek to identify the relevant regulatory factor(s) responsible for transcriptional regulation of *PRAME* and the above *PRAME*-associated genes to elucidate potential targets for AML treatment.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2006.06.018.

REFERENCES

1. Ikeda H, Lethé B, Lehmann F, et al. Characterization of an antigen that is recognized on a Melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity* 1997;**6**:199–208.
2. van Baren N, Chambost H, Ferrant A, et al. PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemic cells. *Br J Haematol*. 1998;**102**(5):1376–9.
3. Matsuhita M, Ikeda H, Kizaki M, et al. Quantitative monitoring of the PRAME gene for the detection of minimal residual disease in leukaemia. *Br J Haematol* 2001;**112**:916–26.
4. Steinbach D, Hermann J, Viehmann S, et al. Clinical implications of PRAME gene expression in childhood acute myeloid leukaemia. *Cancer Genet Cytogenet* 2002;**133**:118–23.
5. Steinbach D, Viehmann S, Zintl F, et al. PRAME gene expression in childhood acute lymphoblastic leukemia. *Cancer Genet Cytogenet*. 2002;**138**(1):89–91.
6. Sigalotti L, Fratta E, Coral S, et al. Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-aza-2′-deoxycytidine. *Cancer Res* 2004;**64**:9167–971.
7. Greiner J, Ringhoffer M, Simikopinko O, et al. Simultanous expression of different immunogenic antigens in acute myeloid leukemia. *Exp. Hematol*. 2000;**28**(12):1413–22.
8. Notarbartolo M, Cervello M, Poma P, et al. Expression of the IAPs in multidrug resistant tumor cells. *Oncol Rep* 2004;**11**(1):133–6.
9. Ross DD. Novel mechanisms of drug resistance in leukaemia. *Leukaemia* 2002;**14**(3):467–73.
10. Steinbach D, Wittig S, Cario G, et al. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. *Blood* 2003;**102**(13):4493–8.
11. Aerts JL, Gonzales MI, Topalian SL. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. *Biotechniques* 2004;**36**(1):84–6. 88, 90-1.
12. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;**29**(9):2003–7.

13. Roy N, Deveraux QL, Takahashi R, et al. The cIAP1 and cIAP2 proteins are direct inhibitors of specific caspases. *EMBO J* 1997;**16**:6914–25.
14. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999;**96**:857–68.
15. Paydas S, Tanriverdi K, Yavuz S, et al. PRAME mRNA levels in cases with acute leukemia: clinical importance and future prospects. *Am J Hematol*. 2005;**79**(4):257–61.
16. Tamm I, Kornblau SM, Segall H, et al. Expression and prognostic Significance of IAP-Family genes in Human Cancers and Myeloid Leukemias. *Clin Cancer Res* 2000;**6**:1796–803.
17. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998;**17**(8): 2215–2223.
18. Ong YL, McMullin MF, Bailie KEM, et al. High bax expression is a good prognostic indicator in acute myeloid leukaemia. *Br J Haematol* 2000;**111**:182–9.
19. Nakatani K, Sakane H, Thompson DA, et al. Identification of a human Akt3 (protein kinase b gamma) which contains the regulatory serine phosphorylation site. *Biochem Biophys Res Commun* 1999;**257**(39):906–10.
20. Tajeddine N, Gala JL, Louis M, et al. Tumor-associated antigen preferentially expressed antigen of melanoma (PRAME) induces caspase-independent cell death in vitro and reduces tumorigenicity in vivo. *Cancer Res* 2005;**65**(16):7348–55.
21. Epping MT, Wang L, Edel MJ, et al. The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling. *Cell* 2005;**122**:835–47.
22. Steinbach D, Sell W, Voigt A, et al. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukaemia. *Leukemia* 2002;**16**:1443–7.
23. Kool M, van der Linden M, de Haas M, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* 1999;**96**(12):6914–9.
24. Ross DD, Yang W, Abruzzo LV, et al. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. *J Natl Cancer Inst* 1999;**91**(5):429–33.
25. Lee JS, Scala S, Matsumoto Y, et al. Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP overexpression. *J Cell Biochem* 1997;**65**(4):513–26.
26. Tang R, Faussat AM, Majdak P, et al. Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. *Leukemia* 2004;**7**:1246–51.
27. Nuessler V, Stotzer O, Gullis E, et al. Bcl-2, bax and bcl-xL expression in human sensitive and resistant leukemia cell lines. *Leukemia* 1999;**11**:1864–72.
28. Oberthuer A, Hero B, Spitz R, et al. The tumor-associated antigen PRAME is universally expressed in high-stage neuroblastoma and associated with poor outcome. *Clin Cancer Res* 2004;**10**(13):4307–13.