Validation by RQ-PCR and flow cytometry of α-defensin1-3 (*DEFA1-3*) overexpression in relapsed and refractory acute lymphoblastic leukemia

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Abstract. In spite of high cure rates and improved overall survival, 25% of pediatric patients with acute lymphoblastic leukemia (ALL) relapse after obtaining complete remission. Additionally a small proportion of patients are refractory and do not attain remission. Microarray expression analysis of matched diagnosis-relapse B-lineage ALL sample pairs identified *DEFA1-3* as a potential marker of relapse. Here, validation of *DEFA1-3* as a marker for therapy resistance is explored. *DEFA1-3* expression was analysed by RQ-PCR in patient paired samples at diagnosis and relapse of 6 early-relapse (within 18 months) and 8 late-relapse (beyond 18 months) B-lineage ALL. Diagnostic samples of 19 patients with ALL who are in continuous complete remission (median time from diagnosis 47 month) and diagnostic samples of 5 refractory patients who had not achieved remission at day 35 of therapy were also analyzed. In addition, overexpression of α-defensin1-3 proteins in blast cells at relapse was analysed by flow cytometry. *DEFA1-3* was overexpressed at relapse as compared to diagnosis in 12 of 14 samples. At diagnosis, the expression of *DEFA1-3* was significantly higher in samples from refractory patients as compared to those of patients who are in CR and to those of patients who experienced late relapse. At diagnosis, patients who relapsed early after diagnosis could not be distinguished from refractory patients based on *DEFA1-3* expression levels. Results suggest that high levels of *DEFA1-3* mRNA and α-defensin1-3 protein expression are correlated with disease progression and failure of adequate response to conventional chemotherapy.

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Introduction

In spite of high cure rates and improved overall survival, 25% of pediatric patients with acute lymphoblastic leukemia (ALL) relapse after short or long durations of complete remission (CR) (1). The mechanisms underlying the clonal evolution toward relapse and the development of drug resistance are still poorly understood. Indeed, no relevant genotypic or phenotypic differences between blast cells collected at diagnosis and at relapse have been demonstrated (2) and, most likely, only small genetic alterations are implicated in the evolution toward relapse.

To identify aberrations specific of relapse that may be considered markers of disease progression, we chose to apply high throughput microarray gene expression to the identification of genes that change expression between diagnosis and relapse. We chose a longitudinal design for the microarray expression analysis, comparing expression in clonally identical, matched diagnosis and relapse samples of the same patient. High throughput microarray expression analysis in matched diagnosis-relapse sample pairs of patients with B-lineage ALL allows to eliminate every possible bias connected with inter-individual variability in expression profiles of human subjects and between B-lineage ALL subtypes, and can identify new potential markers of relapsed leukemia. *DEFA1-3*, one of the genes encoding α-defensins, which are part of a family of anti-microbial peptides, is one of the genes that was constantly overexpressed at relapse as compared to diagnosis in matched patient samples. Although the primary role of human defensin peptides has been implicated in the innate response towards microbial invasion, evidence points to a more versatile function; defensins are now known to play also a role in antigen driven immune response (3,4) and in anti-tumour immunity (5). It has been also suggested that these peptides play a role as

Table I. Patient characteristics.

Patient no.	Sex	Age at diagnosis (months)	Phenotype	Treatment protocol	Molecular translocation (MLL-AF4; BCR-ABL)	D/R interval (months)
Early-relapse patients						
6	M	7	Prepre-B	30 AIEOP LAL 9503	MLL-AF4	5
44	M	66	Pre-B	31 AIEOP LAL 2000	Negative	10
31[a]	F	131	Pre-B	31 AIEOP LAL 2000	Negative	11
8	F	60	Pre-B	29/30 AIEOP LAL9502	Negative	11
32[a]	F	9	Pre-B	29/30 AIEOP LAL9502	Negative	13
5	M	5	Common	57 AIEOP LAL INTERFANT 99	Negative	15
Late-relapse patients						
53[a]	M	10	Pre-B	57 AIEOP LAL INTERFANT 99	Negative	31
43	M	88	-	29 AIEOP LAL9502	Negative	35
7	M	55	Common	29 AIEOP LAL 9503	Negative	37
3	F	88	Common	25 AIEOP LAL9102	Negative	40
65[a]	M	70	Common	29 AIEOP LAL9502	Negative	41
52[a]	M	53	Common	29 AIEOP LAL9502	Negative	44
4	M	168	Pre-B	29 AIEOP LAL9502	Negative	56
56[a]	M	164	Pre-B	30 AIEOP LAL 9503	Negative	63
54[a]	F	133	Pre-B	29 AIEOP LAL9502	Negative	80
De novo resistant patients						
22	M	153	Common	26 AIEOP LAL 9103	-	Resistant
23	M	108	Pre-B	30 AIEOP LAL 9503	-	Resistant
24	F	152	Common	31 AIEOP LAL 2000	Negative	Resistant
25	M	183	Common	31 AIEOP LAL 2000	Negative	Resistant
26	M	133	Common	31 AIEOP LAL 2000	Negative	Resistant
Patients in CR						
68	F	144	Common	31 AIEOP LAL 2000	Negative	46m CR
69	F	103	Common	29 AIEOP LAL 9502	BCR-ABL	94m CR
70	M	25	Common	31 AIEOP LAL 2000	Negative	47m CR
71	M	25	Common	31 AIEOP LAL 2000	Negative	38m CR
73	F	10	Prepre-B	-	MLL-AF4	41m CR
74	F	1	Prepre-B	57 AIEOP LAL INTERFANT 99	MLL-AF4	47m CR
75	F	165	Prepre-B	31 AIEOP LAL 2000	MLL-AF4	43m CR
76	F	48	Prepre-B	31 AIEOP LAL 2000	MLL-AF4	40m CR
77	M	32	Common	31 AIEOP LAL 2000	Negative	46m CR
79	F	30	-	29 AIEOP LAL 9502	Negative	90m CR
80	M	75	Pre-B	31 AIEOP LAL 2000	Negative	345m CR
81	M	43	Common	29 AIEOP LAL 9502	Negative	100m CR
82	M	36	Common	29 AIEOP LAL 9502	Negative	93m CR
83	F	60	Common	31 AIEOP LAL 2000	Negative	47m CR
84	F	214	Common	29 AIEOP LAL 9502	Negative	102m CR
87	F	96	Pre-B	31 AIEOP LAL 2000	Negative	40m CR
88	M	57	Common	29 AIEOP LAL 9502	Negative	84m CR
91	F	84	Common	29 AIEOP LAL 9502	Negative	105m CR
92	M	42	Pre-B	31 AIEOP LAL 2000	Negative	42m CR

CR, complete remission; D, diagnosis; R, relapse; Pre-B, Pre-B ALL; Pre-Pre-B, Pre-Pre-B ALL; Common, Common ALL; For phenotypic definitions of childhood ALL see van der Does-van den Berg, *et al* (16); [a]patient sample pairs used in comparative microarray gene expression analysis.

transcription factors (6). Overexpression of *DEFA1-3* might be related to the increased malignancy of relapsed leukaemia and be predictive of therapy resistance at relapse.

In order to validate the observed overexpression of *DEFA1-3* at relapse, the expression of *DEFA1-3* was analysed by RQ-PCR in sample pairs of 14 patients with relapsed B-lineage ALL. Additionally, the expression of DEFA1-3 was analysed in 19 samples of patients maintaining a state of continuous complete remission and in samples of 5 patients with refractory ALL who did not obtain CR by day 35 after the start of treatment.

We additionally validated the expression of α-defensin1-3 in leukemic blast cells by flow cytometry using a specific monoclonal antibody (mAb) for HNP1-3. We demonstrated that α-defensin1-3 is indeed expressed by leukemic blast cells and that, in pair-wise blast cell comparison, α-defensin1-3 expression is higher at relapse as compared with samples at diagnosis for the same patients.

Materials and methods

Patient characteristics. Patient information pertinent to this study is reported in Table I. The ALL scientific committee of AIEOP (Italian Association for Pediatric Hematology and Oncology) approved the study protocol. Phenotypic characterization of patients had been performed by flow cytometry using a direct immunofluorescence technique with four-color combinations of mAbs (7). The panel of monitored markers is the one routinely employed for diagnosis of B-lineage ALL at the AIEOP reference laboratory for immunophenotypic studies at the University of Padova. Specific genetic aberrations (AF4-MLL and BCR-ABL) have been identified through molecular studies (i.e. RT-PCR assays) according to the BIOMED-1 protocol (8). For all patients studied at diagnosis and relapse, the clonality profile of the blast cells was demonstrated to be unchanged (9).

Microarray analysis. We used a total of 10-20x10^6 cells to obtain RNA using the Trizol (Invitrogen) purification method, according to the manufacturer's instructions. This generally yielded 8-10 μg of total RNA. Labelling was performed using the CyScribe First Strand cDNA Labelling Kit (Amersham Bio-sciences, CA, USA) according to the manufacturer's instructions; total RNA samples at diagnosis were labeled using Cy3 dUTP (Amersham Biosciences), and total RNA samples at relapse were labeled using Cy5 dUTP (Amersham Biosciences). After reverse transcription, the templates were fragmented by addition of 2 μl of 2.5 M NaOH and incubated at 37˚C for 15 min. The degradation reaction was stopped by addition of 10 μl of 2 M HEPES. After neutralization, labelled cDNA samples were cleaned using a Microcon centrifugal filter device (Millipore, Molsheim, France). Purified samples were concentrated to a volume of 10 μl in a speed Vacufuge (Eppendorf, NY, USA) without heat and, for each sample pair (diagnosis and relapse from the same patient), the labeled Cy3 and Cy5 samples were combined and dissolved in 240 μl salt-based hybridization buffer (MWG-Biotech Laboratories, Muenchen, Germany). Hybridization was performed according to the manufacturer's guidelines (MWG-Biotech Laboratories). Hybridization occurred overnight at 42˚C using wet hybridization chambers (MWG-Biotech Laboratories). The following day arrays were washed for 5 min at room temperature in 2X SSC 0.1% SDS, followed by 1X SSC and, finally, in 0.1X SSC. Arrays were scanned using a ScanArray 4000 scanner with ScanArrayExpress software (Packard Bioscience Instruments, Billerica, MA, USA). Experimental details of microarray analysis, according to MIAME guidelines, and raw expression data can be found at Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL292.

RQ-PCR of DEFA1-3 expression. Total RNA of 6 out of 7 sample pairs of patients that were also used in the microarray experiments (pts no. 32, 53, 65, 52, 56 and 54 of Table II) together with 8 diagnosis/relapse sample pairs of additional patients (pts no. 6, 44, 8, 5, 43, 7, 3 and 4 of Table II), and of 5 refractory patients and 19 patients in continuous complete remission were used as template for reverse transcription (RT)-PCR. The Superscript™ II RNAse H-reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) was used for RT using 1 μg of total RNA and 1 μg of random primers (Invitrogen). The RT product was aliquoted and stored at -80˚C. Forward and reverse primers were designed using Applied Biosystem's Primer Express v1.0™ software (Applied Biosystems, Foster City, CA, USA). The primers for *DEFA1-3* are: F, TCT GGTCACCCTGCCTAGCT; and R, AGGAGAATGGCAG CAAGGA. The housekeeping gene, GUS, was used for normalization of *DEFA1-3* expression. The primers for GUS are: F, GAAAATATGTGGTTGGAGAGCTCATT; and R, CCGAGTGAAGATCCCCTTTTTA. For RQ-PCR each reaction mix (25 μl) contained 2x Sybr® Green Master Mix, 50 μM forward and reverse primers and approximately 5 ng of total template. Cycle conditions for the ABI 7700 were 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 sec and 60˚C for 1 min, for a total of 40 cycles.

All patient samples were analyzed in triplicate in parallel with a series of 5 dilutions (in triplicate) of standard cDNA reverse transcribed from RNA of mononuclear cells from healthy bone marrow donors. The relative quantity of the specific mRNA (*DEFA1-3* and *GUS*) for each sample was calculated based on mean Ct values and, with the corresponding standard curve, the estimated input amount was calculated by linear regression.

Flow cytometry. To independently assess α-defensin1-3 expression in blast cells of ALL patients at diagnosis and relapse, α-defensin1-3 protein expression was monitored by flow cytometry using a α-defensin1-3 specific mAb. For this purpose, mononuclear cells from bone marrow samples were isolated using a Lymphoprep™ density gradient (1.077 g/ml; Nycomed Pharma, Oslo, Norway), as recommended by the manufacturer (10). Cells were re-suspended in FACS Buffer [1% fetal calf serum and 0.1% sodium azide in phosphate-buffered saline (PBS)] at a concentration range of 2.5X10^5 and 7.5X10^5 cells per test.

For intracellular staining, cells were fixed then permeabilized and incubated with 10 μg/ml α-defensin purified IgG mAb, specific for α-defensin1-3 (Biogenesis Ltd., Poole, UK) (11) for 15 min at room temperature, washed twice with FACS Buffer and stained with FITC-conjugated AffinityPure F(ab')2

Table II. Real-time comparative quantitative PCR (RQ-PCR).

	Diagnosis					Relapse					
	Ct		Input			Ct		Input			
Patient no.	*GUS*	*DEFA1-3*	*GUS*	*DEFA1-3*	*DEFA1-3/ GUS*	*GUS*	*DEFA1-3*	*GUS*	*DEFA1-3*	*DEFA1-3/ GUS*	R/D ratio
Early relapse											
6	19.61	17.21	6.49	20.68	3.19	19.17	14.8	8.66	129.87	14.99	4.71
44	19.51	15.67	6.93	66.68	9.62	19.81	17.01	5.69	24.1	4.24	0.44
8	17.88	17.14	20.2	17.42	0.86	18.51	17.79	13.42	10.84	0.81	0.94
32	22.89	19.86	0.76	2.08	2.74	22.52	18.8	0.98	4.22	4.32	1.58
5	19.92	17.36	5.3	18.35	3.47	19.02	15.78	9.55	61.46	6.43	1.86
Late relapse											
53	26.91	29.28	0.02	0	0.04	22.2	18.43	0.6	1.23	2.06	57.86
43	31.4	33.77	0	0	0.04	18.44	15.88	14.02	43.12	3.08	87.47
7	23.22	19.98	0.62	2.5	4.04	19.8	18.67	5.73	6.79	1.19	0.29
3	19.26	21.05	8.19	1.03	0.13	21.28	18.7	2.2	5.63	2.56	20.34
65	25.11	25.68	0.19	0.05	0.29	25.06	20.81	0.19	0.95	4.92	17.00
52	23.21	17.82	0.61	8.07	13.22	23.76	16.47	0.51	19.89	38.77	2.93
4	18.5	19.79	13.45	2.56	0.19	18.48	18.53	13.63	6.38	0.47	2.46
56	26.42	29.15	0.08	0.01	0.09	26.62	20.94	0.07	0.88	12.36	141.96
54	23.99	19.4	0.36	2.82	7.92	23.76	14.63	0.42	67.97	163.02	20.58
De novo resistance											
22	20.53	15.61	3.58	59.27	16.54						
23	19.47	16.38	7.14	33.37	4.67						
24	20.8	16.57	3	29.14	9.70						
25	24.23	17.46	0.3	9.21	30.3						
26	25.77	20.57	0.12	1.1	8.93						
Continuous complete remission											
68	22.38	20.94	1.07	0.86	0.81						
69	23.94	19.32	0.4	2.18	5.51						
70	22.06	19.82	1.31	1.63	1.24						
71	23.24	23.23	0.62	0.23	0.37						
73	22.36	18.12	1.09	4.32	3.98						
74	23.19	35.78	0.64	0	0.04						
75	22.19	22.68	1.21	0.32	0.26						
76	25.37	25.22	0.16	0.07	0.46						
77	23.2	23.68	0.63	0.18	0.28						
79	24.86	19.24	0.22	2.28	10.39						
80	22.29	19.37	1.13	2.11	1.86						
81	26.51	23.17	0.07	0.24	3.58						
82	22.67	21.42	0.88	0.65	0.74						
83	24.1	19.24	0.34	2.22	6.54						
84	25.82	19.23	0.11	2.23	20.9						
87	22.2	20.7	1.21	0.97	0.81						
88	40	40	0	0	2.21						
91	24.07	20.51	0.34	1.08	3.14						
92	23.42	16.19	0.54	12.5	23.35						

All samples and series of 5 dilutions for standards have been analysed in triplicate. RNA for standards has been derived from bone marrow mononuclear cells of healthy donors The relative quantity of the specific mRNA (*DEFA1-3* and *GUS*) for each sample was calculated based on mean Ct values and, with the corresponding standard curve, the estimated input amount was calculated by linear regression. *DEFA1-3* was normalized for each patient sample using its relative *GUS* expression. Samples of patient no. 31 (Table I) were not analysed by RQ-PCR because material was unavailable.

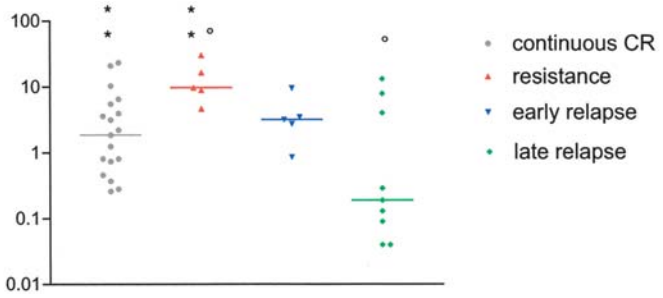

Figure 1. Scatter diagrams of normalized *DEFA1-3* expression levels from RQ-PCR experiments (Table II). Data in logarithmic scale refer to samples taken at diagnosis in good therapy responders (patients maintaining continuous complete remission) and refractory patients (resistant to therapy) and in patients who had either early or late relapse. Bars indicate the median values for each group of patients. DEFA1-3 expression statistically discriminates refractory patients from control patients who are in continuous complete remission (**p=0.03) and from patients who experienced late relapse ($^{\circ}$p=0.03). In fact, expression levels in refractory patients are significantly higher than levels in control patients who are in continuous complete remission and also different from DEFA1-3 expression levels in late relapsed patients. DEFA1-3 expression does not discriminate between refractory patients and patients who had an early relapse.

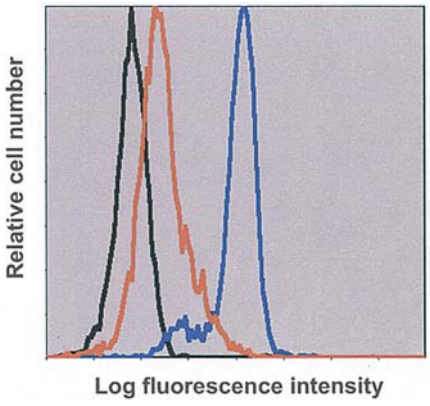

Figure 2. Representative example of flow cytometry evaluation of α-defensin1-3 expression using indirect fluorescence labelling with mAb specific for HNP1-3 (Biogenesis Ltd., catalog no. 2590-5008) and FITC-conjugated goat anti-mouse (Immunotech, catalog no. 0819). The blast cells were identified using immunological gate CD45, see Basso *et al* (7). The histogram was filtered for expression of α-defensin1-3 in blast cells. Black, isotype control; red, α-defensin1-3 at diagnosis; and blue, α-defensin1-3 expression at relapse.

Fragment goat anti-mouse IgG (H+L) (10 μg/ml) (Immunotech, Marseille, France). Cells were then washed and incubated with 50 μg/ml unlabeled goat anti-mouse F(ab')s for 30 min at 4˚C, to block the remaining free binding sites. The cells were counter-labeled with CD45-ECD (10 μg/μl) (Immunotech) for immunological gating of the blast cell population. The association of side scatter (SS) with an immunological marker, such as CD45, was the preferred method for blast cell identification (CD45 expression in blast cells is typically lower than that of normal lymphocytes). Cells were analyzed on Epics-XL MCL (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis. For statistical comparison of *DEFA1-3* expression values between patient groups a non-parametric Wilcoxon signed rank test was used to calculate p-values.

Results and discussion

DEFA1-3 appeared constantly up-regulated at relapse as compared to diagnosis; in all 7 sample pairs, *DEFA1-3* was largely overexpressed at relapse (expression ratios at relapse versus diagnosis ≥2; average ratio of 24.27±30.71). Although α-defensin1-3 is a marker of malignant cells, the gene is not commonly considered to be expressed in B-lymphocytes or leukemic blast cells. Thus, before considering it a diagnostic or disease-progression marker, we chose to analyse in more detail the *DEFA1-3* expression in leukemic blast cell samples of patients with B-lineage ALL (Table I).

The expression levels of *DEFA1-3* were validated by real-time comparative quantitative PCR (RQ-PCR) in 14 diagnosis-relapse sample pairs (Table I), including 6 of the patient sample pairs used in the microarray analysis. A higher expression at relapse (expression ratios ≥1.58 at relapse versus diagnosis, last column, Table II) was confirmed in 12 of 14 diagnosis-relapse sample pairs. This latter finding confirms the comparative microarray expression data that revealed up-regulation of *DEFA1-3* at time of relapse. Apart from the observed up-regulation of *DEFA1-3* expression between diagnosis and relapse of the same patient, *DEFA1-3* expression levels at diagnosis are highly variable among patients. Interestingly, patients with different clinical outcomes show differences in *DEFA1-3* expression levels (Table II, Diagnosis DEFA1-3/GUS). Of 14 sample pairs, five belonged to patients who had had early relapse, i.e. within 18 months after diagnosis (Table I). Four out of five early relapse samples (Table II, patient no. 6, 44, 32 and 5) at diagnosis showed expression levels of *DEFA1-3* that were significantly higher as compared to those of control patients maintaining a state of CR. In four samples of early relapsed patients, *DEFA1-3* expression levels were not clearly up-regulated at relapse (R/D ratio ≤1.58), suggesting that up-regulation is not therapy induced, but rather that relative high *DEFA1-3* expression levels correlate with a poor response to chemotherapy, resulting in early relapse.

A small proportion of pediatric patients with ALL have refractory leukemia, defined as absence of CR on day 35 after diagnosis (i.e. at the end of the first phase of induction therapy). Samples at diagnosis from 5 of these refractory patients (patient no. 22-26, Tables I and II) were also collected and analysed by RQ-PCR. All five samples exhibited high levels of *DEFA1-3* expression.

A group of 19 patients maintaining continuous CR were used as reference of *DEFA1-3* expression in good responders to therapy. As shown in Fig. 1, expression of *DEFA1-3* was significantly higher (p=0.03) in refractory patient samples as compared to the control group. *DEFA1-3* expression was also higher (p=0.03) in refractory patients than in the samples taken at diagnosis of late relapsing patients, whereas *DEFA1-3* expression levels at diagnosis were not significantly different between early relapsing patients and refractory patients (Fig. 1). These data suggest that high DEFA1-3 expression at diagnosis is predictive of therapy failure, either as resistance to therapy or early relapse.

α-defensins are known to be highly expressed in granulocytes and monocytes, while their expression in T- and B-lymphocytes is less known (11,12). Two recent microarray

expression profiling studies of peripheral blood mononuclear cells (PBMCs) (5) and leukemia bone marrow mononuclear cells (BMMCs) (14) reported a high expression of *DEFA1-3*. These studies pointed out that *DEFA1-3* overexpression was associated with overexpression of both myeloperoxidase (*MPO*) and elastase-2 (*ELA2*) genes. These latter genes are preferentially transcribed within the most immature granulocytes and it was suggested that congruent overexpression of *DEFA1-3*, *MPO* and *ELA2* was part of a granulopoiesis signature (13). In our samples, the expression ratios of myeloperoxidase and elastase-2 in samples obtained at relapse versus those taken at diagnosis are approximately 1 (average ratio of 1.22±0.48 and of 1.53±0.93, respectively), while overexpression of *DEFA3* was prominent (average ratio of 24.27±30.71), thus strongly suggesting that the *DEFA1-3* overexpression we observed was not part of a granulopoiesis signature or greater myeloid cell load. Moreover, by using flow cytometry, which allows the immunological gating of blast cells, we demonstrated that the blast cell population expresses the α-defensin1-3 protein. Importantly, flow cytometry confirmed that expression of α-defensin1-3 in the blast cell population of 3 late relapsed patients was 10-fold higher at relapse as compared to blast cells at diagnosis (Fig. 2).

The primary role of human defensin peptides has been implicated in the innate defence response towards microbial invasion. Other evidence pointed to a more versatile function; defensins are known to play a role in antigen driven immune response (3,4) and anti-tumour immunity (5), while a function as transcription factors was suggested for pmolar amounts of α-defensin1-3 (6).

The results of this study suggest that high levels of *DEFA1-3* mRNA and α-defensin1-3 protein expression at relapse are correlated with disease progression and increased malignancy associated with failure of conventional chemotherapy. In accordance with the finding that, in renal carcinoma cells, expression of α-defensin1-3 is a frequent marker of malignancy with possible direct influence on tumour proliferation (15), it may be hypothesized that α-defensins are involved in disease progression in ALL blast cells, possibly influencing blast cell proliferation and immune recognition.

DEFA1-3 expression analysis shows considerable variation among groups (Fig. 1) but also within groups. The group of samples from late relapsed patients is a clear example of the latter. A retrospective analysis of *DEFA1-3* expression in B-lineage ALL samples at diagnosis in a larger patient cohort may reveal additional clinical significance of differential *DEFA1-3* expression levels. Functional studies aimed at understanding the role of *DEFA1-3* in mechanisms of drug resistance are warranted. Cell-line transfection experiments exemplify how *DEFA1-3* overexpression has an effect on cell cycle parameters (personal unpublished data). In conclusion, our study confirms that focussed microarray expression analysis in matched diagnosis-relapse sample pairs studies can reveal new potential markers of leukemia.

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