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ORIGINAL ARTICLE

The Fat1 cadherin is overexpressed and an independent prognostic factor for survival in paired diagnosis–relapse samples of precursor B-cell acute lymphoblastic leukemia

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Improved survival of patients with acute lymphoblastic leukemia (ALL) has emerged from identifying new prognostic markers; however, 20% of children still suffer recurrence. Previously, the altered expression of Fat1 cadherin has been implicated in a number of solid tumors. In this report, *in vitro* analysis shows that Fat1 protein is expressed by a range of leukemia cell lines, but not by normal peripheral blood (PB) and bone marrow (BM) cells from healthy donors. *In silico* analysis of expression of array data from clinical leukemias found significant levels of Fat1 transcript in 11% of acute myeloid leukemia, 29% and 63% of ALL of B and T lineages, respectively, and little or no transcript present in normal PB or BM. Furthermore, in two independent studies of matched diagnosis - relapse of precursor B-cell (preB) ALL pediatric samples ($n = 32$ and $n = 27$), the level of Fat1 mRNA expression was prognostic at the time of diagnosis. High Fat1 mRNA expression was predictive of shorter relapse-free and overall survival, independent of other traditional prognostic markers, including white blood cell count, sex and age. The data presented demonstrate that Fat1 expression in preB-ALL has a role in the emergence of relapse and could provide a suitable therapeutic target in high-risk preB-ALL.

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INTRODUCTION

Children with acute lymphoblastic leukemia (ALL) continue to suffer a 20% incidence of relapse after treatment with the best available therapy. High-resolution genomic profiling, including analysis of single-nucleotide polymorphisms and copy number abnormalities, has greatly aided an understanding of the molecular mechanisms underlying treatment outcome, therapy response and the biology of relapse.[1,2] For precursor B-cell (preB) ALL, genomic studies have shown that copy number abnormalities in genes involved in lymphoid differentiation and cell cycle control are common, with deletions, or part thereof, found in *PAX5*, *EBF1*, *IKZF1*, *TCF-4*, *CDKN2A* and *RB1*.[2-5] Recent reports also indicate that deletions and nonsense mutations of the *IKZF1* gene are significantly associated with poor relapse-free and overall survival rates in preB-ALL.[6] However, in light of these studies, questions remain on the biology of relapse, with marker analysis complicated by the fact that phenotypic shifts in preB-ALL blasts can occur between diagnostic and post-chemotherapy or relapse samples.[7] Those cells that give rise to relapse in some cases appear to be selected during treatment, with clonal evolution occurring of a minor subclone present at diagnosis rather than simply being the development of chemotherapeutic resistance of the original leukemic clone.[8,9] The inherent genetic heterogeneity has more recently been described within subpopulations of leukemia-initiating cells, which also undergo dynamic and branching evolution. This evolution leads to shifts in subclone dominance during progression and treatment relapse, further highlighting the clinical challenge in delivering targeted therapies against differential markers expressed by the majority of clones if minor subclones then survive and undergo further evolution, leading to relapse.[10,11] Overall, the use of genomic profiling technology has been very informative on identifying novel genetic alterations in ALL, but it has been noted that a proportion of ALL cases with no discernable cytogenetic changes fail therapy.[12] Hence, there remains a requirement for the analysis of more phenotypic markers to clarify candidate genes involved in clonal evolution to provide new targets for therapy.

Human Fat1 was serendipitously cloned from a T-leukemia cell line in 1995 and shown to encode a type I transmembrane protein with 34 extracellular cadherin repeats, and named after an orthologous drosophila gene called *fat* that functions as a tumor suppressor.[13] *In situ* hybridization showed that Fat1 mRNA expression was present in some epithelial and mesenchymal compartments, but high expression was found only in fetal as opposed to adult tissues. Subsequent cloning of the *Fat1* gene in the rat,[14] mouse[15] and zebrafish[16] showed that this molecule was

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also highly conserved in vertebrates, and confirmed that its expression is developmentally regulated and largely restricted to fetal tissues. A number of studies have analyzed Fat1 expression in cancer, with loss of membranous Fat1 expression correlated with more aggressive tumors for intrahepatic cholangiocarcinoma,[17] whereas work from our laboratory has shown overexpression of Fat1 in breast carcinoma[18] and overexpression combined with aberrant post-translational processing in melanoma.[19] *In silico* analysis of Fat1 expression has shown expression in gastric, pancreatic, colorectal, breast, lung and brain cancers.[20] The present report is the first description of Fat1 expression in leukemia, where we demonstrate its expression in a range of leukemia subtypes, including acute myeloid leukemia (AML), B-ALL and T-ALL, and also determine that high Fat1 expression is prognostic at the time of diagnosis in matched diagnosis–relapse biopsies from children with preB-ALL.

MATERIALS AND METHODS

Cell culture

Human leukemic T-cell ALL (Jurkat, JM, HPB-ALL and MOLT-4), preB-ALL (NALM-6 and LK63), B-ALL (BALM-1) and AML (THP-1 and R2CA) were all maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Trace Biosciences, Castle Hill, NSW, Australia), 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM Glutamax and 2% penicillin–streptomycin (all from Invitrogen, Carlsbad, CA, USA) and were cultured in a humidified incubator at 37° with 5% CO2.

Western blot analysis

Suspension cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline, lysed in NDE lysis buffer (10 mM Tris-HCl, 1% NP40, 0.4% sodium deoxycholate and 66 mM EDTA). Electrophoresis on NuPAGE 3–8% Tris-Acetate gels (Invitrogen) and western blotting were carried out as previously described.[19]

In silico and statistical analysis of microarray data sets

The CEL files from publically available microarray gene expression data sets from NCBI's gene expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) were analyzed using MAS5.0 algorithm (Expression Console software V1.1, Affymetrix, Santa Clara, CA, USA). Raw data were analyzed using the MAS5.0 algorithm to generate an absolute call and associated *P*-value. The presence of the Fat1 transcript was defined as a *P*-value <0.06, and absent with a *P*-value >0.06, reflecting the *P*-values. Correlation of relative Fat1 expression within applicable data sets with patient outcome was carried out using R software with additional Bioconductor packages (http://www.r-project.org and http://www.bioconductor.org). The primary end points for the survival analyses was either disease-specific survival or relapse-free survival, which was measured from the date of diagnosis to disease-specific death or first relapse, or otherwise censored at the time of the last follow-up visit or at non-disease-related death. Time to first relapse or disease-specific death was plotted as Kaplan–Meier survival curves. Cox proportional hazards regression was used for univariate analysis of the prognostic impact of Fat1 expression. For statistical analysis, SPSS (Version 15.0.1; SPSS Inc., Chicago, IL, USA) software was used.

Isolation of HSC from bone marrow and peripheral blood

Peripheral blood (PB) hematopoietic stem cells (HSCs) were isolated using fluorescence-activated cell sorting from samples obtained from therapeutic donors with their informed consent (for example, polycythemic patients) using CD34 and CD133 cell surface markers (detailed method in Supplementary Information). HSCs from commercial normal bone marrow (BM) mononuclear cells (no. 2M-25D; Lonza, Mt Waverley, VIC, Australia) were prepared using positive immunomagnetic enrichment. Briefly, anti-CD34 (no. CBL496; Chemicon International, Boronia, VIC, Australia) or CD133 (no. 130-090-422, Miltenyi Biotec, North Ryde, NSW, Australia) monoclonal antibodies (mAbs) were used to prepare bispecific tetrameric antibody complexes and antigen-positive cells from BM isolated according to manufacturer's instructions (EasySep, Stem Cell Technologies, Tullamarine, VIC, Australia). To validate the successful isolation of functional HSCs in the isolated populations, cells were inoculated into methylcellulose media (no. 130-091-280; Miltenyi Biotec) to confirm their *in vitro* clonogenic capacity.

Real-time quantitative PCR analysis

Total RNA was isolated and reverse transcribed to complementary DNA using the Illustra RNAspin Mini Isolation Kit (GE Healthcare, Rydalmere, NSW, Australia) and Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Castle Hill, NSW, Australia), respectively. The Applied Biosystems 7500 Real-Time PCR System (Mulgrave, VIC, Australia) was used to compare the expression level of Fat1 with that of GusB and ABL housekeeping controls. For patient BM samples, quantitative PCR (qPCR) was carried out as described previously[21] (primer sequences listed within Supplementary Table S1).

BM samples from patients with acute lymphoblastic and AML leukemia

BM samples from ALL and AML patients were sourced from the Tumour Bank at The Children's Hospital at Westmead, with BM collected at the time of diagnosis. Informed consent was obtained from the parents of all patients according to the regulations of The Children's Hospital at Westmead Ethics Committee.

RESULTS

Fat1 expression is found in leukemia cell lines but not in PB cells or enriched hematopoietic progenitor cells

The Fat1 cadherin is a type I transmembrane protein with 34 cadherin repeats, 5 epidermal growth factor-like repeats and 1 laminin G motif on the extracellular side of the cell, followed by a transmembrane region and cytoplasmic domain (Figure 1a). Initial analysis of Fat1 expression focused on a leukemic cell line panel and normal PB cells from four healthy donors using western blotting and qPCR. In the western blot, the preB- and B-ALL cell lines LK63 and Nalm-6, AML cell line THP-1 and T-ALL cell lines Jurkat, JM and MOLT-4 all showed an immunoreactive band for Fat1 resolving at $\sim$550 kDa. The remaining cell lines and PB mononuclear cells from four healthy donors showed no immunoreactivity in this region indicative of either no Fat1 expression or a level of expression below detection limit (Figure 1b). The levels of the protein largely reconciled with the level of Fat1 mRNA transcript as measured by qPCR, with the exception of HPB-ALL and HL-60, both of which had significant Fat1 mRNA signal but no discernable full-length Fat1 protein expression as detected by western blot (Figure 1c). The expression of vertebrate Fat1 cadherin in mouse and rat has previously been shown to be high during development,[14,15] and therefore we determined whether Fat1 may be enriched in hematopoietic progenitors. To this end, $CD34^+/CD133^{dim}$ and $CD34^+/CD133^{bright}$ cell were sorted and validated from the circulating PB of non-leukemic patients using flow cytometry (Figure 2a), and then analyzed by qPCR to determine the levels of Fat1 mRNA within these two defined populations. The qPCR results show that there was no significant expression of Fat1 mRNA in either of the enriched $CD34^+/CD133^{dim}$ and $CD34^+/CD133^{bright}$ cell populations (Figure 2b). Utilizing an independent method, CD34+ and CD133+ cells were enriched from the BM of non-leukemic patients using magnetic beads and analyzed by qPCR for Fat1 mRNA expression. This analysis also demonstrated that there was no enrichment of Fat1 associated with hematopoietic progenitor populations, as there was less Fat1 transcript in the enriched population compared with presorted control population of cells (Figure 2c).

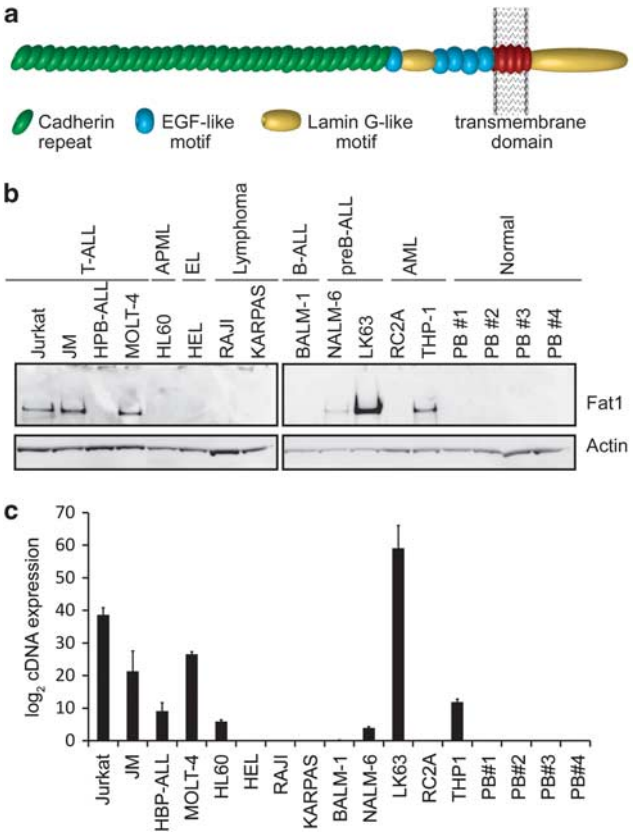

Figure 1. Fat1 cadherin protein expression in leukemia cell line panel. (**a**) Schematic diagram of the full-length Fat1 cadherin protein, which is 4588 amino acids long and has a predicted molecular weight of ~550 kDa. It is a type I transmembrane protein with 34 extracellular cadherin domains, 5 epidermal growth factor-like motifs and 1 laminin G-like domain. (**b**) The expression of Fat1 protein in a leukemia cell line panel reveals an immunoreactive band at the predicted molecular weight for Fat1 at 550 kDa in three of the four T-cell ALLs (Jurkat, JM and MOLT-4), both preB-ALLs (Nalm-6 and LK63) and one of the two AMLs (THP-1) examined. There was no visible expression of Fat1 in the acute promyelocytic leukemia (HL60), erythroleukemia (HEL), B-cell ALL (Balm-1) or lymphoma (RAJI and KARPAS) cell lines. Similarly, normal PB cells from four separate healthy donors had no detectable Fat1 protein expression. (**c**) qPCR analysis for Fat1 mRNA of the same cell lines and normal PB cells from healthy donors generally reconciled with protein levels, except for both HL-60 and HPB-ALL, which had significant Fat1 mRNA signal but no equivalent full-length Fat1 protein present as measured by western blot.

To extend these studies and determine whether Fat1 was expressed at any significant level during normal hematopoiesis, the level of Fat1 transcript was analyzed within the context of the recently published data by Novershtern *et al*.,[22] in which gene expression profiling was performed in multiple stages of hematopoietic differentiation (GSE24759). In this study, 38 subpopulations representing different lineages and maturation states were obtained using multiparameter fluorescence-activated cell sorting and profiled using HG_U133AAofAv2 microarrays (Affymetrix). We examined Fat1 transcript levels *in silico* using the deposited $\log_2$-transformed normalized data. Consistent with our fluorescence-activated cell sorting data, there was no significant Fat1 transcript ($P<0.05$) in their HSC1 (Lin^-, $CD133^+$ and $CD34^{dim}$) or HSC2 (Lin^-, $CD38^-$ and $CD34^+$) population (Figure 2d). We then used the same data set[22] to determine the extent and level of Fat1 expression across six separate lineage signatures (Figure 3). From this analysis, significant but relatively low Fat1 transcript expression occurred only within the erythroid lineage and, in particular, in the defined early and late erythroid signatures. Here, Fat1 in the early erythroid signature ranked 131/1228 (P-value $= 1.55249 \times 10^{-26}$), and within the late erythroid signature ranked 149/1270 (P-value $= 5.34305 \times 10^{-43}$). To determine whether red blood cells in the PB express Fat1 protein, we carried out immunoprecipitation, but were unable to detect Fat1 in circulating red blood cells (data not shown).

In silico analysis of clinical microarray data reveals that Fat1 mRNA transcript is present in AML, B-ALL and T-ALL but not in normal blood cells and their progenitors

Publicly available microarray data deposited within the GEO or similar databases using Affymetrix-based platforms were mined to determine the number of cases where Fat1 transcript is present within cohorts of clinically relevant leukemia samples, including AML, B-ALL, T-ALL and normal PB and BM cells. The MAS5.0 algorithm used (see Materials and methods) generates three distinct calls (present, marginal or absent) for each probe present on the array (Affymetrix ID), with empirical threshold probability values of $P<0.06$, including both marginal and present calls, and a P-value >0.06 classified as absent. For this study, we aimed to determine the extent of Fat1 expression (significance set for both present and marginal) in B-ALL, T-ALL and AML, and thereby examine the applicability of Fat1 as a unique leukemia target compared with normal blood cells and their progenitors. The significant detection of Fat1 transcript in eight separate AML array studies is presented in Table 1, with the sum of all cases resulting in Fat1 transcript present in 11% of cases. In eight separate B-ALL array studies, the sum of all cases shows that Fat1 transcript is present in 29% of cases (Table 2); and for eight T-ALL array studies the sum of all cases shows that Fat1 transcript is present in 63.5% (Table 3). A total of six separate analysis incorporating different subsets of normal blood cells was then analyzed (Table 4). In the study looking at early hematopoietic cell progenitors isolated from either umbilical cord blood or the BM,[23] only one out of four cases of BM cells enriched for $CD34^+CD38^-CD33^-Rho^{high}$ had a significant signal for Fat1 transcript. In a second study analyzing total PB,[24] only one case out of five had a significant signal for Fat1 transcript. These data showing the lack of significant Fat transcript signals in the majority of healthy normal blood cells and their progenitors reconcile with our findings of low expression levels of Fat1 transcript from $CD34^+$- and $CD133^+$-isolated HSCs (Figures 2 and 3).

Fat1 transcript can be detected by qPCR in clinically relevant leukemia BM aspirate samples

To extend the *in silico* analysis and directly verify the presence of Fat1 transcript in clinically relevant pediatric leukemia samples, a cohort of 18 preB-ALL, 19 T-ALL and 7 AML pediatric BM samples (patient characteristics and phenotypes listed in Supplementary Table S2) were assessed for Fat1 using qPCR. The clinical samples were also directly compared in the same assays with representative cell lines expressing high levels of Fat1 (Jurkat T-ALL and LK63 preB-ALL), low levels of Fat1 (Nalm-6 preB-ALL) and Fat1-negative cells (Raji lymphoma). The qPCR analyses of clinical samples (Figure 4) were normalized to LK63 (high Fat1; set as 1) and with an arbitrary cutoff equivalent to Nalm-6 (low Fat1; Fat1 qPCR signal $2^{-Ct} > 0.06$; Supplementary Table S2 and S3). This criterion was then used to assess the number of Fat1-positive cases' results with 10/18 Fat1 positive for B-ALL, 18/19 Fat1 positive for T-ALL and Fat1 1/7 positive for AML. Although this clinical cohort was small, the overall trend for Fat1-positive clones across the different leukemia phenotypes is consistent with the *in silico* analysis of clinical microarray data (T-ALL > B-ALL > AML). This finding corresponds well with data obtained from the *in silico* gene expression

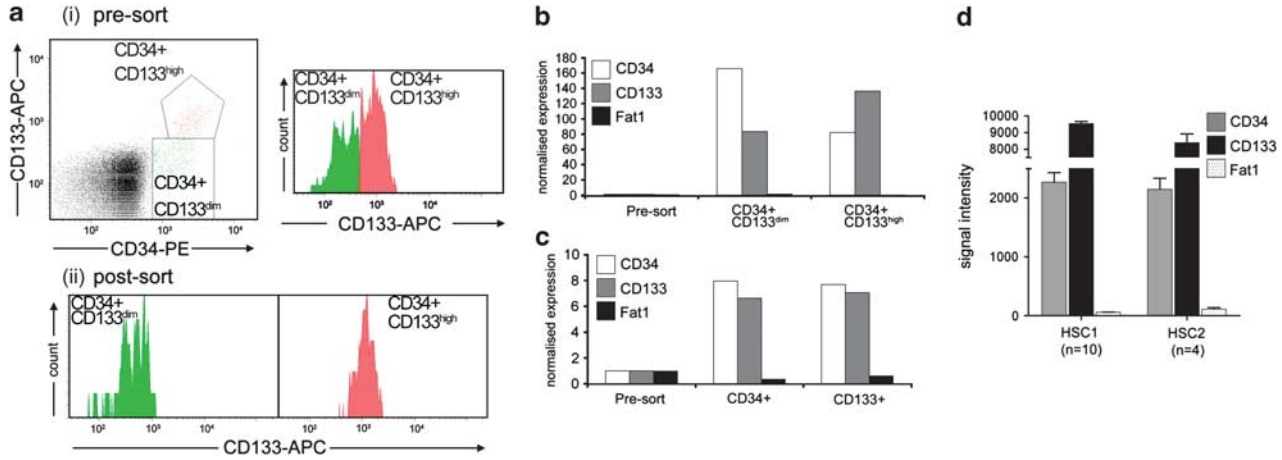

Figure 2. Fat1 cadherin expression is negligible in HSCs isolated from PB or BM. (**a**; i) Illustration of the simplified gating strategy used for fluorescence-activated cell sorting of circulating HSCs from PB. After defining CD34+ HSCs by ISHAGE gating, the small CD34+ population (0.1%) was divided into $CD133^{dim}$ and $CD133^{high}$ subpopulations, with sorted gates indicated in green and red, respectively. (ii) Post-sort analysis to validate the purity of each population. (**b**) Total RNA from the sorted PB populations was isolated, and the expression of Fat1, CD34 and CD133 measured by qPCR. The relative mRNA expression levels were calculated after normalizing against the *GusB* and *ABL* housekeeping genes, resulting in no detectable Fat1 transcript. (**c**) qPCR analysis of Fat1, CD34 and CD133 mRNA expression in CD34+ and CD133+ cells enriched from BM using magnetic-bead-based sorting, showing that Fat1 transcript is lower in enriched progenitors compared with presorted population. All results are representative of at least two independent experiments. (**d**) *In silico* analysis of Fat1 mRNA expression in HSC1 ($CD133+$ $CD34^{dim}$) and HSC2 (CD38− CD34+) hematopoietic progenitor populations[22] shows no significant Fat1 expression.

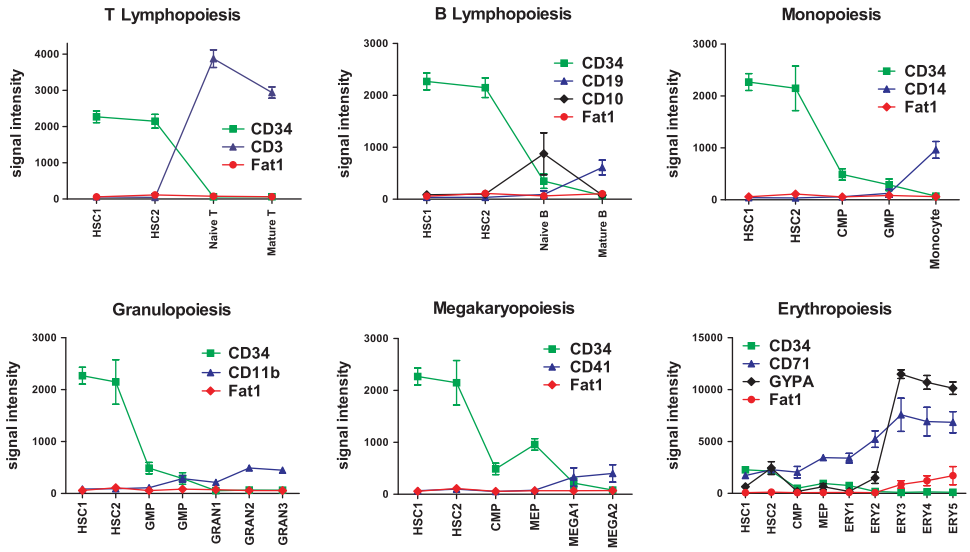

Figure 3. Fat1 mRNA expression tracked during differentiation of the major hematopoietic lineages. Analysis of the data set GSE24759 [ref. 22] shows Fat1 mRNA expression from early hematopoietic progenitors (HSC1-$CD133+/CD34^{dim}$ and HSC2-$CD38^{-}/CD34^{+}$), intermediate populations and differentiated cells across six different lineage signatures relative to differentiation markers used in the fluorescence-activated cell sorting. In each lineage signature, the expression of the primitive marker CD34 decreased concordant with increases in positive expression of lineage-specific differentiation markers. The only significant ($P<0.05$) level of Fat1 mRNA expression occurs during the final stages of erythropoiesis.

arrays, although the proportion of cases deemed Fat1 positive in the arrays is less overall. Resolution of these discrepancies may await the availability of suitable anti-Fat1 antibodies for flow cytometry, but collectively these assays provide clear evidence for Fat1 expression in pediatric clinical samples.

Fat1 expression is prognostic for disease relapse and overall survival in pediatric preB-ALL in paired diagnosis-relapse samples

For relevant microarray data sets in which patient outcome data were available, the level of Fat1 expression was assessed as a predictor of patient outcome for ALL subsets. For preB-ALL, two recently published array sets[25,26] with matched pediatric diagnosis-relapse patients were analyzed. The use of matched diagnosis-relapse samples affords several advantages over a conventional cohort; it not only lends itself to the identification of genetic pathways and molecular mechanisms involved in relapse but also provides insights into the origins of the relapsed clone. Both are important aspects for consideration in identifying putative targets for future therapies. In the study by Bhojwani *et al.*,[25] pediatric BM samples from 35 (32 preB-ALL and 3 T-ALL) patients comprising of matched samples from initial diagnosis and first marrow relapse were profiled using the Affymetrix U133A microarray platform and deposited in the GEO (accession number

GSE3912). For the matched preB-ALL patients, the primary end points for the survival analyses was either disease-specific survival or relapse-free survival, which was measured from the date of diagnosis to disease-specific death or first relapse, or otherwise censored at the time of the last follow-up visit or at non-disease-related death. For the 32 preB-ALL patients, the Fat1 signal intensity assessed by the MAS5.0 algorithm ranged from 58 to 1683 (mean = 304) at the time of diagnosis, and ranged from 21 to 3630 (mean = 363) at the time of relapse. High Fat1 expression at the time of diagnosis (upper quartile cutoff) had significantly increased risk of relapse compared with lower Fat1 levels of expression (remaining 75%; hazard ratio = 5.09; 95% confidence interval 1.80–14.41; $P = 0.002$; Figure 5), with a median relapse-free survival of 15.3 months compared with 30.1 months, respectively.

In the second study, Staal *et al.*[26] also carried out genome-wide expression analysis to unravel the biological mechanisms of relapsed ALL in paired diagnosis–relapse samples for 41 pediatric patients, including 27 preB-ALL and 14 T-ALL patients. In concordance with the Bhojwani study above, univariate analysis

Table 1. *In silico* analysis of AML samples for the presence of the Fat1 transcript

GEO/source	*AML subtype/sample*	*Cases (Fat1 transcript present; P < 0.06)*	*Percentage (%)*
GSE14468		52/525	10
GSE1159		40/285	14
GSE14471		8/111	7
GSE15434	Normal karyotype	30/251	12
GSE12417	Normal karyotype	23/163	14
GSE12326	CD34+ paired (BM and PB)	0/10	0
GSE9476	BM and PB	3/26	12
GSE17061	M0	4/35	11
Sum expression		160/1406	11.4

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; GEO, gene expression omnibus; PB, peripheral blood.

Table 2. *In silico* analysis of B-cell ALL samples for the presence of the Fat1 transcript

GEO/source	*Cases (Fat1 transcript present; P < 0.06)*	*Percentage (%)*
GSE3912	32/105	30
GSE18497	13/54	24
GSE4698	15/51	29
GSE13425	34/154	22
http://www.stjuderesearch.org/data/ALL1	93/286	33
GSE7440	27/99	27
GSE635	35/145	24
GSE11877	74/220	34
Sum expression	323/1114	29

Abbreviations: ALL, acute lymphoblastic leukemia; GEO, gene expression omnibus.

Table 3. *In silico* analysis of T-cell ALL samples for the presence of the Fat1 transcript

GEO/source	*T-ALL subtype/sample*	*Cases (Fat1 transcript present; P < 0.06)*	*Percentage (%)*
GSE3912		5/10	50
GSE18497		23/28	82
GSE4698	PreT-ALL	1/6, 1/2	17, 50
GSE13425		23/36	64
GSE635		18/28	64
GSE8879	Atypical	35/55	64
http://www.stjuderesearch.org/data/ALL1		29/45	64
GSE11877		20/34	59
Sum expression		155/244	63.5

Abbreviations: ALL, acute lymphoblastic leukemia; GEO, gene expression omnibus; preT, precursor T cell.

Table 4. *In silico* analysis of normal PB and BM samples for the presence of the Fat1 transcript

GEO/source	*Normal PB/BM subtype/sample*	*Cases (Fat1 transcript present; P < 0.06)*	*Percentage (%)*
GSE1493	Lin+CD34+ (PB or BM)	0/2	0
	Lin−CD34+ (PB or BM)	0/2	0
	Lin−CD34− (PB or BM)	0/2	0
GSE2666	CD34+CD38−CD33−Rho(lo)c-kit+ (BM)	0/4	0
	CD34+CD38−CD33−Rho(hi) (BM)	1/4	25
	CD34+CD38−CD33−Rho(lo)c-kit+ (UC)	0/5	0
	CD34+CD38−CD33−Rho(hi) (UC)	0/5	0
GSE1159	Total (PB or BM)	1/5	20
	CD34+ purified from three patients	0/3	0
GSE10438	CD34+CD38−Lin− (UC)	0/3	0
	CD34+CD38−CD36− (UC)	0/3	0
	CD34+CD38+ (UC)	0/3	0
	Whole blood	0/3	0
GSE 14924	T-cell CD4+	0/10	0
	T-cell CD8+	0/10	0
GSE9476	CD34+ (BM or PB)	0/8	0
	Unselected BM or PB	0/10	0

Abbreviations: BM, bone marrow; GEO, gene expression omnibus; PB, peripheral blood.

shows high Fat1 expression at diagnosis (upper quartile cutoff) compared with lower Fat1 levels of expression (remaining 75%), which had significantly shortened relapse-free (median = 13 versus 23.5) and overall survival (median = 14.5 versus 32) (Figure 5, Table 5). Multivariate analysis against other criteria of risk assessment, including age, white blood cell count and sex,[27,28] shows that Fat1 is an independent prognostic marker for relapse-free survival and overall survival in preB-ALL (Table 5). The small number of T-ALL cases in both the Bhojwani and Staal data sets precluded any significant analysis to determine whether Fat1 expression is prognostic.

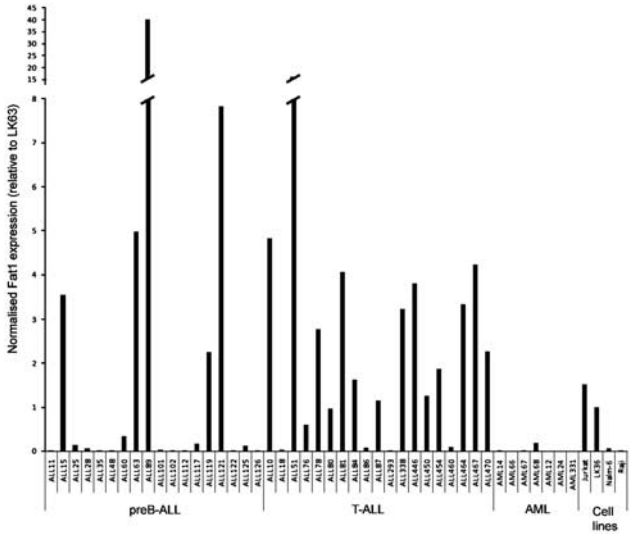

Figure 4. Fat1 is expressed in clinically relevant BM samples of leukemia. qPCR analysis of preB-ALL, T-ALL and AML in concert with cell lines Jurkat, LK63, Nalm-6 and Raji were analyzed for Fat1 expression relative to β-actin and then normalized to LK63 (set to 1). The level of Fat1 mRNA signal is varied, and using the raw Fat1 qPCR signal associated with Nalm-6 ($2^{\Delta Ct} > 0.06$; Supplementary Table S2, S3), there are 10/18 Fat1 positive for B-ALL, 18/19 Fat1 positive for T-ALL and Fat1 1/7 positive for AML.

DISCUSSION

Aberrant expression of the Fat1 cadherin has previously been described for a small number of solid tumors, and in the current study this work is extended for the first time to hematological malignancies. Using an *in vitro* cell line panel we found Fat1 to be expressed at the protein level in three out of the four T-ALLs (Jurkat, JM and Molt-4), both preB-ALL cell lines (LK63 and Nalm-6) and one AML cell line (THP-1). Consistent with this analysis, examination of microarray data from clinical specimens confirmed the significant presence of Fat1 transcript in 63.5% of T-ALL, 29% of B-ALL and 11% of AML cases, thereby demonstrating that Fat1 expression in leukemia was not an artifact of cell culture. Significantly, during revision of this manuscript and concordant with our results presented here, Coustan-Smith *et al*.[29] reported that Fat1 was overexpressed in 34.4% of B-ALL cases using genome-wide gene expression analysis of 270 patients with B-lineage ALL compared with normal $CD19^+CD10^+$ cells. Furthermore, we found no detectable Fat1 expression in PB-mononuclear cell either at the transcript level by qPCR or at the protein level by western blotting, and was confirmed *in silico* with no Fat1 expression in the analysis of 38 hematopoietic cell line precursors, with the exception of early and late erythroid lineage signatures.[22] Thus, the expression of Fat1 on a significant proportion of leukemic but not normal hematopoietic cells highlights it as a potential leukemic marker. The reasons for the high levels of Fat1 observed in some leukemic cells are unclear, but they do not appear to result from significant copy number abnormalities as these have not been reported in AML, B-ALL or T-ALL across the Fat1 locus.[30–32]

We next undertook to examine whether Fat1 expression provides any significance for patient outcomes. We showed in two independent genome-wide array data sets from matched pediatric preB-ALL diagnosis–relapse samples that Fat1 expression was an independent prognostic marker, whereby high Fat1 expression at diagnosis predicted poor outcome. The use of matched pairs has a number of distinct advantages, especially the fact that each patient acts as its own control. Moreover, these cohorts provide important information on the genetic changes and biological mechanisms that occur in preB-ALL relapse. One of the earliest studies to investigate the genetic changes in matched preB-ALL diagnosis–relapse samples used seven patients and glass-slide microarray technology.[33] Two subsequent studies using

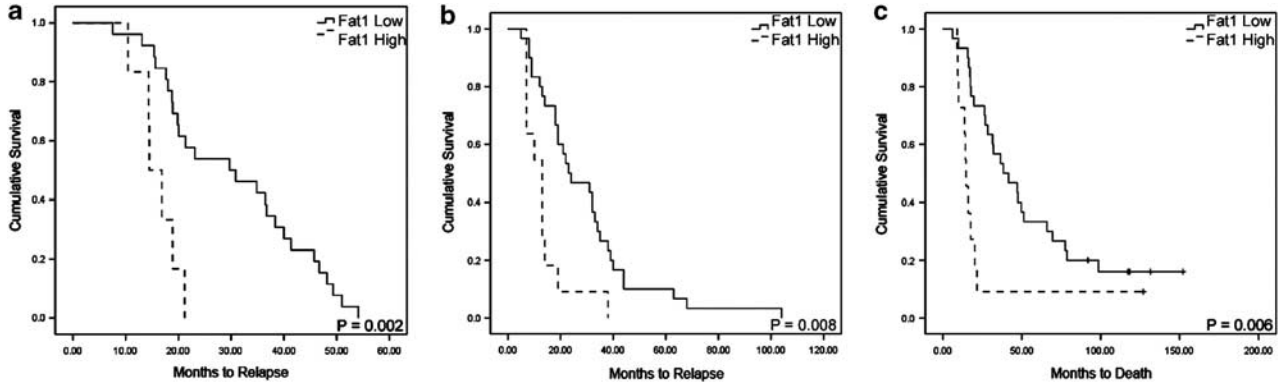

Figure 5. Fat1 expression is associated with poor prognosis in paired diagnosis–relapse samples of preB ALL. (**a**) Kaplan–Meier plot of relapse-free survival in 32 patients with preB-ALL from GEO data set GSE3912. Those patients expressing high Fat1 (solid line, upper quartile) versus medium/low levels of Fat1 (dotted line, remaining 75%) have significantly poorer outcome (hazard ratio (HR) = 5.1, $P = 0.002$). (**b**) Kaplan–Meier plots for 27 preB-ALL patients in GEO data set GSE18497. Patients with high Fat1 (solid line, upper quartile) had a significantly higher incidence of relapse compared with those expressing lower Fat1 (dotted line, remaining 75%; HR = 3.0, $P = 0.008$). (**c**) Similarly, patients with high Fat1 in GSE18497 (solid line, upper quartile) had a significantly poorer overall survival compared with those expressing lower Fat1 (dotted line, remaining 75%; HR = 2.9, $P = 0.006$).

Table 5. Univariate and multivariate Cox proportional hazards regression analysis of age, gender, WBC count and Fat1 expression (at diagnosis) for RFS and overall survival in GEO data set GSE18497. High Fat1 expression was significantly prognostic with respect to both RFS and overall survival, and was independent of other clinical variables

	RFS			*Overall survival*		
	HR	*P-value*	*95% CI*	*HR*	*P-value*	*95% CI*
Univariate						
Clinical variable						
Fat1 (upper quartile vs rest)	3.0	0.008	1.4–6.3	2.9	0.006	1.4–6.2
Age (10 vs <10)	1.5	0.291	0.7–3.0	1.7	0.177	0.8–3.6
Gender (female vs male)	0.8	0.471	0.4–1.6	0.8	0.631	0.4–1.8
WBC ($<50\times10^9$/l vs 50×10^9/l)	2.1	0.028	1.1–4.1	1.4	0.292	0.7–2.8
Multivariate						
Fat1 (upper quartile vs rest)	2.8	0.007	1.3–5.9	2.9	0.007	1.3–6.2
Age (10 vs <10)	*	*	*	*	*	*
Gender (female vs male)	*	*	*	*	*	*
WBC ($<50\times10^9$/l vs 50×10^9/l)	*	*	*	*	*	*

Abbreviations: CI, confidence interval; GEO, gene expression omnibus; HR, hazard ratio; RFS, relapse-free survival; WBC, white blood cell.
*Not significant in final multivariate model.

matched diagnosis–relapse samples[25,26] (also analyzed in this current report) increased the numbers of patient samples and utilized Affymetrix-based arrays. In the first study, Bhojwani *et al*.[25] found that a number of genes implicated in malignant transformation and/or drug resistance and that many relapse blast expressed genes involved in cell proliferation. In the second study, Staal *et al*.[26] identified four distinct gene clusters corresponding to pathways associated with cell cycle regulation, DNA replication and repair, molecular transport, cellular assembly, protein trafficking and B-cell signaling. This second study also concluded that in some cases relapse appears to result from therapy-induced selection of minor clones present at diagnosis, rather than from genetic adaptation of the original tumor cells. There was overall little overlap of significant gene changes between the Staal study[26] and that of Bhojwani,[25] except for the level of Fat1 expression that is prognostic at time of diagnosis for both sets of matched preB-ALL diagnosis–relapse samples as reported here.

The emergence of resistance and prognostic value of any factor is directly associated with the type of treatment delivered. In the present study, high Fat1 expression is associated with earlier relapse and appears not to be associated with treatment, as different treatment regimes 'contemporary cooperative treatment protocol'[25] versus 'Dutch Childhood Oncology Group protocol'[26] were used for each study analyzed. Therefore, the supposition can be made that expression of Fat1 by preB-ALL may have a more general role in the mechanism and emergence of relapse, with perhaps Fat1 expression selected in a 'Darwinian' manner in line with the proposed model of clonal evolution after treatment.[34] Furthermore, while concluding the current report, a study was published analyzing the genetic basis for relapse in 207 uniformly treated children with high-risk preB-ALL.[35] This study revealed eight unique genetic clusters within high-risk ALL patients, of which the expression of *Fat1* was highly ranked (59th) within cluster R2, a cluster also associated with the t(1;19)(*E2A-PBX1*) translocation; however, we found that Fat1 within this cohort was not prognostic on its own for overall survival (data not shown). This does not contradict the work presented in the current manuscript where Fat1 expression was found to be an independent prognostic marker in paired diagnosis-relapse patients, but yields important information behind the biology of relapse, information that continues to be needed to successfully cure the patients whom relapse. Further, by verifying that Fat1 is abnormally expressed in leukemia cells compared with normal blood cells, we provide evidence for Fat1 as a bona fide target that may help overcome some of the mechanisms by which resistant lymphoblasts evade cytotoxicity.

But how might ectopic Fat1 expression contribute to leukemia onset, progression or relapse? As this is the first report examining Fat1 expression in the context of a hematological malignancy, this is as yet unclear but we anticipate that the biological effects of Fat1 would be multifaceted. Preliminary data from our laboratory in which Fat1 expression is knocked down by small interfering RNA in preB-ALL cell lines did not result in any significant changes to cell proliferation (data not shown), and therefore it is speculated that biological effects of Fat1 *in vivo* are not simply due to an overt growth phenotype. One potential mechanism may involve disturbances in cell polarity and asymmetric cell division, since these are important for the self-renewal of somatic stem cells and proposed to be awry in leukemia.[36] Recently, Fat1 in zebrafish has been shown to form a physical and functional association with scribble, one of the key proteins that helps establish cell polarity as part of a larger polarity complex with discs large and large giant larvae.[37] Hence, ectopic Fat1 expression may perturb cell polarity and asymmetric cell division aiding leukemogenesis.

Functional studies have shown a role for Fat1 in both the Hippo and Wnt signaling pathways,[38,39] as well as the ability to alter actin filament organization, cell–cell adhesion and cell migration.[40] The Wnt/β-catenin pathways have been shown to be essential in the development of all hematopoietic cells, and mutations in this pathway lead to leukemia.[41] In addition, the t(1;19)(*E2A-PBX1*) translocation has been shown to induce high expression of Wnt16 and to regulate leukemia–stroma interactions, with β-catenin expression highly expressed and localized at the cell membrane.[42] Our current study has corroborated previous studies that Fat1 transcript is enriched in those cases harboring the t(1;19)(*E2A-PBX1*) translocation[43–46] and illustrated in Supplementary Figure S1 (ref. 45) and Supplementary Figure S2.[46] Although this translocation is no longer associated with poor prognosis in pediatric patients due to new and intensified chemotherapy,[47,48] they do suffer from increased risk of central nervous system relapse.[49,50] Previous reports have established high Fat1 expression within the central nervous system and its importance for central nervous system development,[14,51,52] and we therefore speculate that high Fat1 expression associated with the t(1;19)(*E2A-PBX1*) translocation, together with the ability of the cytoplasmic tail of Fat1 to directly bind β-catenin,[53] may provide another potential mechanism whereby ectopic Fat1 expression can either aid central nervous system infiltration or stabilize

leukemia–stroma interactions with its unknown extracellular ligand. Finally, our laboratory has recently shown that the Fat1 protein is expressed as both an unprocessed full-length protein and furin-processed heterodimer on the surface of melanoma cells.[19] We suggested that these dual forms of Fat1 could perform different signaling functions, and notably both are also evident in representative preB- and T-ALL cell lines (data not shown). This observation highlights that changes in both transcriptional and/or post-translational mechanisms may contribute to any functional role for Fat1 in leukemia onset, progression or relapse.

To date, there remains a great need to identify new therapeutic targets for ALL, and large-scale global gene expression profiling is uncovering a range of new candidates for drug development. Currently, the use of mAbs–whether simply humanized or further modified by coupling to toxins or radioactive isotopes–are emerging as an important therapeutic tool in leukemia, and mAbs to a range of CD antigens are now in clinical use or clinical trials. Those mAbs used clinically in ALL include CD20 (rituximab), CD22 (epratuzumab), CD52 (alemtuzumab) and CD19. However, in none of these cases can the mAb be considered as specific in targeting malignant cells, as all are directed against relatively common leukocyte antigens. In the present study, we show that Fat1 is overexpressed in a B-ALL, a T-ALL and to a lesser extent in an AML, and has been independently confirmed for B-ALL by Coustan-Smith *et al*.,[29] showing 34.4% abnormally overexpressed Fat1 compared with normal CD19 + CD10 + cells. Importantly, from a therapeutic perspective, there was no significant Fat1 mRNA expression in early hematopoietic precursors, except for early- and late-stage erythroid cells,[22] although we could not confirm Fat1 expression on differentiated red blood cells. This leads us to hypothesize that given the frequency of Fat1 cadherin expression in a range of phenotypically diverse leukemias concomitant with little or no expression on normal PB cells and their early hematopoietic progenitors, Fat1 would provide an ideal target for the generation of a novel antibody-based therapeutic. Indeed, one of the limitations of the current study is the lack of an anti-Fat1 antibody precluding a detailed multicolor flow cytometric analysis of Fat1 expression performed in concert with B-cell and T-cell antibody differentiation markers. In this regard, we are currently generating new anti-Fat1 antibodies raised against a natively folded Fat1 protein antigen that would allow us to undertake this work, while simultaneously paving the way for a potential novel targeted therapeutic agent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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