

Distinct gene signatures of transient and acute megakaryoblastic leukemia in Down syndrome

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Approximately 10% of newborns with Down syndrome develop Transient Leukemia (TL), a disorder that is unique to infants with constitutional trisomy 21 (or trisomy 21 mosaicism). TL blasts disappear spontaneously within the first 3 months of life in the majority of cases. Despite the resolution of TL, 20–30% of these newborns will go on to develop acute megakaryoblastic leukemia (AMKL) later in life. In this study, samples from both TL and AMKL patients were examined using cDNA microarrays to study the pathogenic progression from TL to AMKL. TL and AMKL samples partition separately by cluster analysis, and AMKL samples had substantial increases in apolipoprotein C-I, transporter 1, myosin alkali light chain 4, and spermidine/spermine N-acetyltransferase, compared to TL samples. Although these findings will require validation in an independent series of TL and AMKL samples, they indicate that TL and AMKL have distinct gene signatures, and provide a basis for studies of the different mechanisms underlying either the resolution of TL or its progression to AMKL.

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abnormal proliferation of hematopoietic stem cells (HSC).⁷ A current model for the development of leukemia suggests that at least two classes of mutations are required to transform a normal hematopoietic stem cell into a malignant leukemic cell.⁸ In this model, one class of mutation increases cell survival or proliferation, while the second causes lineage-specific blocks in differentiation. Recently, somatic mutations in *GATA1*, a zinc-finger transcription factor required for normal erythroid and megakaryocyte differentiation,⁹ were identified in AMKL blasts from individuals with Down syndrome.¹⁰ Functionally equivalent mutations in *GATA1*, likely occurring *in utero*,¹¹ are also present in the blasts of TL, indicating that *GATA1* mutations precede the onset of TL and that other as yet undetermined secondary events underlie the progression of TL to AMKL in Down syndrome.^{12–16}

To identify genes that are involved in the development of AMKL in Down syndrome, we profiled gene expression patterns in blasts of TL and AMKL using cDNA microarrays. Our results show that TL and AMKL blasts have distinct gene expression signatures with statistically significant changes in genes involved in multiple cell processes.

Introduction

Individuals with Down syndrome (constitutional trisomy 21) have a 10–20-fold increased incidence of acute leukemia¹ and a 150-fold higher incidence of acute myeloid leukemia during the first 4 years of life.² While rare in the general pediatric population, the predominant form of acute myeloid leukemia in children with Down syndrome is acute megakaryoblastic leukemia (AMKL), the subtype defined by a platelet precursor phenotype of the leukemic blasts (AML subtype M7; French–American–British Cooperative Group).^{3,4} Prior to the onset of AMKL, approximately 10% of newborns with Down syndrome initially develop transient leukemia (TL),⁵ also referred to as transient myeloproliferative disorder (TMD) or transient abnormal myelopoiesis (TAM), which is an accumulation of leukemic blasts that are indistinguishable from those in AMKL. Strikingly, the blast population of TL disappears spontaneously within the first 3 months of life in the majority of cases. Following this resolution of TL, however, approximately 20% of cases go on to develop full AMKL usually by 4 years of age.⁶

The basis of the predisposition of Down syndrome individuals to leukemia remains unknown, although it is believed to involve increased expression of genes on chromosome 21 that stimulate

Materials and methods

Cell and RNA samples

Clinical samples were obtained from Down syndrome patients after informed consent and with the approval of the institutional review board at The Hospital for Sick Children. Samples of seven TL and six AMKL patients were studied. All patients had constitutional trisomy 21 with the exception of patient A8, who is a phenotypically normal child with trisomy 21 mosaicism. The *GATA1* gene was sequenced in six of seven samples from patients with TL and two of five samples with AMKL. In all cases studied, deletions and insertions within exon 2 were found that result in the use of a downstream initiator codon and a mutant *GATA1* protein lacking the N-terminal activation domain (previously reported in Hitzler *et al*¹⁶). Cytogenetics data were available on two cases with TL and three cases with AMKL. Constitutional trisomy was the sole abnormality in both cases of TL. In the three AMKL cases, trisomy 8, +22p and 2q–, respectively, were found in addition to trisomy 21. Samples were derived from either peripheral blood or bone marrow, with blast counts ranging from 43 to 93%. RNA was extracted by standard methods from mononuclear bone marrow and peripheral blood cells prepared by Ficoll density separation. Total RNA was purified using the RNeasy minprep kit (Qiagen, Valencia, CA, USA) in combination with DNase treatment (Qiagen). RNA quality was assessed using the Agilent 2100 bioanalyzer and RNA 6000 Nano LabChip kit and (Agilent Technologies, Palo Alto, CA, USA).

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Microarrays

Human 19K microarrays containing 19200 characterized and unknown ESTs were manufactured in house at the University Health Network Microarray Centre (Toronto, Ontario, Canada). A complete list of the cDNA collection used for these arrays and protocols used for array construction can be found at the University Health Network Microarray Centre web site (<http://www.microarrays.ca>).

RNA amplification and labeling for microarrays

Owing to the limiting amounts of RNA isolated from cell samples (ranging from 0.5. to 5 μ g), total RNA was amplified before performing microarray hybridizations. RNA was amplified using the T7-based Amino Allyl MessageAmp aRNA kit (Ambion, Inc., Austin, TX, USA),¹⁷ following the manufacturer's instructions. An amount of 100 ng of total RNA from patient samples and a common reference RNA (Universal Reference Human RNA) (Stratagene, La Jolla, CA, USA) were used for each amplification. An amount of 4 μ g of amplified antisense RNA from patient and reference was labeled with Superscript II (Invitrogen, Carlsbad, CA) using Cy5-dCTP or Cy3-dCTP and cohybridized to human 19K microarrays. Dye switches were performed on each sample to minimize the effect of labeling bias. Only high-quality hybridizations were used for further microarray data analysis. Details of hybridization and labeling can be found on the UHN Microarray Centre website (www.microarrays.ca).

Data collection and analysis

Arrays were scanned using ScanArray 4000 scanner (Perkin Elmer, Boston, MA, USA). GenePix Pro 3.0 software (Axon Instruments, Union City, CA, USA) was used to quantitate spot intensities and to flag absent or poor quality spots. GeneTraffic (lobion Informatics/Stratagene) was used to generate log₂ ratios and to normalize ratios by LOWESS (subgrid). Filtering, adjustment, and hierarchical clustering of log₂ gene ratios were performed using Cluster.¹⁸ Briefly, ratios were filtered using a stringency factor of 80% present. At this filter level, 8543 genes remained for analysis. Filtered ratios were then median centered, followed by hierarchical clustering using a correlation (uncentered) similarity metric. Clustered data were visualized using Treeview (<http://rana.lbl.gov/EisenSoftware.htm>). Multi-dimensional scaling of the same data set of 8543 genes carried out done using BRB ArrayTools, which was developed by Dr Richard Simon and Amy Peng Lam (Biometric Research Branch, NCI). The learning software SAM¹⁹ was applied to spot ratios to identify genes with *statistically significant* changes in gene expression between TL and AMKL samples, using a false discovery rate (FDR) of 1% and a two-fold differential expression. Statistical significance of RT-PCR data was determined by a two-tailed Mann-Whitney *U*-test using Analyse-it add-in (Analyse-It Software, Ltd, UK) within Microsoft Excel. Gene ontology data were obtained from the SOURCE database.²⁰ Microarray data are available at www.microarrays.ca/data/Lightfoot.zip

Quantitative real time RT-PCR

Real-time RT-PCR was carried out with the ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). Total RNA (50 ng

was reverse transcribed with Superscript II, and cDNAs were used to determine gene expression levels by relative quantitation, using SYBR green and 2.5 ng of sample cDNA per reaction. PCR primers were designed with Primer Express software (Applied Biosystems) for the following genes: Apolipoprotein C-I (APOC1; Spermidine/spermine *N*-acetyl transferase (SAT); Myosin, light polypeptide 4, alkali (MYL4); Transporter 1 (TAP1). Cyclophilin A was used as an endogenous control to normalize expression levels among samples. Standard curves were generated using cDNA prepared from Universal Reference Human RNA. A detailed description of the method is available in the Applied Biosystems User Bulletin #2.

Results and discussion

We performed microarray data analysis initially by hierarchical clustering of normalized expression data from all 13 samples. As shown in Figure 1a, TL and AMKL separated into two main branches. The left branch contained five of the six AMKL samples. The right branch partitioned the TL samples to two sub-branches, one of which contained AMKL sample A6. The relatively long length of the A6 branch suggests a low degree of similarity of A6 to the TL samples. A similar grouping of samples was seen when multidimensional scaling was applied, which showed spatial relationships between the same groups of AMKL and TL samples (Figure 1b). These unsupervised forms of analysis indicate that TL and AMKL samples are sufficiently distinct to partition to separate groups based solely on gene expression. Patient A6 did not show unusual clinical or sample characteristics (Table 1), indicating that the separate clustering of this sample is due to its atypical molecular profile.

To determine statistically significant changes in gene expression between TL and AMKL, SAM¹⁹ was applied to the data set of 8543 genes. SAM employs a modified *t*-test to identify statistically significant differentially expressed genes and estimates the false discovery rate (FDR) by a permutation analysis. The expression of a total of 67 genes was found to be significantly different (2-fold or higher) between TL and AMKL (Table 2) with a 1% FDR. TL and AMKL samples separated into two groups when these genes were clustered as part of supervised analysis (Figure 2). This result lends further support for distinct molecular signatures for TL and AMKL, and strongly suggests that the SAM-derived genes are key components of these signatures.

Expression levels of four genes that were elevated in AMKL (APOC1, SAT, MYL4, TAP1) were determined by real-time RT-PCR. Genes with a fold-change ranging from 2.1 to 4.2 were chosen as a representative subset of differentially expressed genes to validate the expression changes found by microarray analysis. The RT-PCR data verified differential expression of these genes, in that TL showed substantially lower levels of transcripts for these genes compared to AMKL (Figure 3). Values of MYL4 and APOC1 were particularly high in AMKL with an over 30-fold increase in some samples. The results of the Mann-Whitney *U*-test, which tests for a difference between the medians of two sample populations within a set confidence interval,²¹ confirmed the statistical significance ($P < 0.05$) of differential expression (data not shown). Expression levels were uniform among most of the TL samples. In contrast, AMKL samples showed a wider range of transcript levels. This observation does not appear to be due to differences in blast counts among AMKL samples (Table 1), but may instead be the result of a greater degree of differentiation among AMKL blasts.

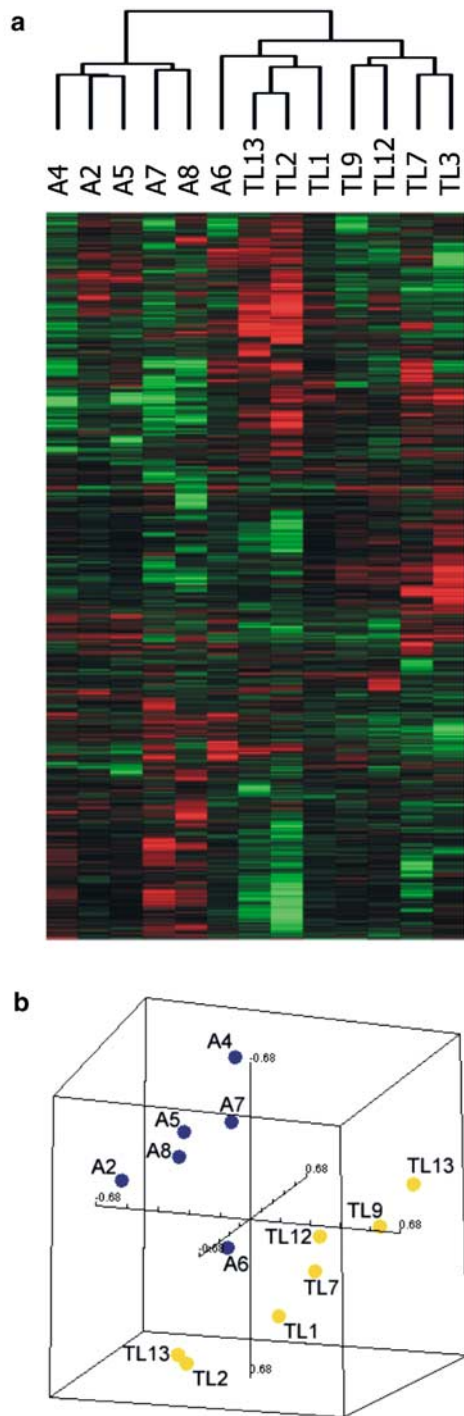


Figure 1 Unsupervised microarray analysis of AMKL and TL samples. (a) Unsupervised hierarchical clustering. Normalized expression ratios from 8543 genes of six AMKL samples and seven TL samples were clustered. Separate clustering of TL and AMKL samples is seen, with the exception of one AMKL sample (A6), which clusters more closely with TL. (b) Multidimensional scaling of normalized log ratios from 8543 genes of all TL and AMKL samples. Each sample is represented by a sphere in three-dimensional space, and samples that are close together have similar expression profiles.

The potential biological effects of the differentially expressed genes provide further support for the distinctions between TL and AMKL (Table 2). The uniqueness of TL and AMKL is such that there is no normal hematopoietic counterpart to the blasts of

Table 1 Clinical characteristics of Down syndrome patients with TL and AMKL, and qualities of samples used for analysis

Patient Identifier	Age at diagnosis ^a	Sex	Blast percentage	Sample source ^b	Developed AMKL
Transient Leukemia					
TL1	17	F	83	BL	Yes
TL2	10	M	83	BL	No
TL3	4	M	81	BL	No
TL7	11	M	86	BL	No
TL9	7	M	43	BL	No
TL12	6	?	>90	BL	No
TL13	4	F	86	BL	Yes
AMKL					
A2	20	M	93	BL	—
A4	38	M	59	BL	—
A5	27	M	78	BM	—
A6	25	F	68	BM	—
A7	7	M	64	BL	—
A8	2	F	56	BL	—

The sex of patient TL12 is unknown.

^aAge of diagnosis: days for TL; months for AMKL.

^bSample source: BL, blood; BM, bone marrow.

these disorders, either in Down syndrome or non-Down syndrome individuals. As a result of this, the gene expression of the leukemic cells of TL and AMKL were compared directly to each other. The relatively low levels of TAP1 expression seen in TL may confer a survival advantage to these cells. TAP1 is a member of the superfamily of ATP-binding cassette (ABC) transporters, and is responsible for transporting peptides to class I molecules of the major histocompatibility complex for presentation to immune cells.²² Decreased expression of TAP1 has been shown to give tumor cells a survival advantage, in that malignant cells can avoid being recognized as foreign by the immune system.²³ Owing to this, maintaining low levels of TAP1 may be important for survival and proliferation of TL blasts.

Differential expression of SAT in TL and AMKL suggests differences in polyamine catabolism in these disorders. SAT is a rate-limiting enzyme in the catabolism of the polyamine growth factors spermine and spermidine,²⁴ and induction of SAT expression is commonly seen in response to treatment of cells with polyamine analogues.^{25,26} An effect of this response is the initiation of downstream events leading to apoptosis,^{27,28} so differential expression of SAT provides the potential for differences in apoptotic response in TL and AMKL blasts. Apoptotic responses in TL and AMKL have not been reported, and the increased expression of SAT in AMKL seen here suggests that AMKL blasts are less sensitive to SAT-mediated induction of apoptosis.

Apolipoprotein C-I is expressed primarily in the liver and is associated with triglyceride-rich chylomicrons and VLDL.²⁹ Significant changes in APOC1 expression are seen in leukemia cell lines in the NCI60 cancer cell line collection³⁰ (see also <http://genome-www.stanford.edu/nci60>), although a role for APOC1 in leukemia has not been described. A similar situation exists for MYL4, which is a component of the contractile protein myosin and is found in embryonic muscle and adult atria.³¹ Elevated levels of MYL4 in AML contribute to the separate clustering of AML and ALL clinical samples examined by microarrays,³² although the biological significance of this gene in AML is not known. It seems possible, however, that APOC1 and MYL4 share a pattern of expression associated with certain subsets of AML.

Table 2 Statistically significant gene expression differences among AMKL and TL samples

Gene name	Symbol	IMAGE ID	Fold change
Overexpressed in AMKL			
Apolipoprotein C-I	APOC1	205499	Up 4.2
Thyroid peroxidase autoantibody light chain variable EST		161528 25744	3.3 3
Spermidine/spermine N1-acetyltransferase	SAT	152226	3
Immunoglobulin kappa light chain		161395	3
Transporter 1, ATP-binding cassette, sub-family B	TAP1	22629	2.4
Immunoglobulin kappa light chain VKJ region cDNA FLJ26472 fis, clone KDN04506		158155 49167	2.3 2.2
Chromosome 6 open reading frame 142	C6orf142	5762809	2.2
Major histocompatibility complex, class I, C	HLA-C	485984	2.1
Dihydroliipoamide dehydrogenase	DLD	47595	2.1
Zinc-finger CCCH type domain containing 1	ZC3HDC1	205497	2.1
Myosin, light polypeptide 4, alkali; atrial, embryonic EST	MYL4	5749628 40037	2.1 2
Hypothetical protein BC005868	LOC90233	429707	2
Underexpressed in AMKL			
Glycophorin B (includes Ss blood group)	GYPB	195256	Down 4.3
Thymosin, beta 4, X-linked EST	TMSB4X	273224 249978	3.2 3
Hypothetical protein FLJ20397		321162	2.8
Ortholog of mouse polydom	FLJ20397	290986	2.7
Pregnancy-specific beta-1-glycoprotein 1	POLYDOM	135075	2.6
Sestrin 2	PSG1	213665	2.6
EST	SESN2/Hi95	186755	2.5
Ras suppressor protein 1		111522	2.5
EST	RSU-1	5555913	2.5
EST		192327 446443	2.5 2.4
Complement component 4 binding protein, alpha	C4BPA	202665	2.4
RaP2 interacting protein 8	RPIP8	181115	2.4
Cyclin M3	CNNM3	160008	2.3
Probable cation-transporting ATPase 2	FLJ16025	188132	2.3
Anaphase-promoting complex 1		149286	2.3
Transaldolase 1	TALDO1	5812026	2.3
EST		147732	2.2
Hypothetical protein MGC20486	MGC20486	5091359	2.2
cDNA clone YZ88E12		290158	2.2
Splicing factor YT521-B	YT521	172010	2.2
Glutathione S-transferase pi	GSTP1	236069	2.2
Small EDRK-rich factor 2 EST	SERF2	5756172	2.2
Nuclear factor of activated T-cells		259940	2.2
Transcription factor AP-2 beta EST	NFATC1	208875	2.2
	TFAP2B	222083	2.2
Hypothetical protein FLJ13912		161929	2.1
Collagen, type XVIII, alpha 1	FLJ13912	154907	2.1
Rac/Cdc42 guanine nucleotide exchange factor 6	COL18A1	489524	2.1
Zinc-finger protein 35	ARHGEF6	159355	2.1
Tetraspan 2	ZNF35	163209	2.1
cDNA DKFZp686A17165	TSPAN-2	489701	2.1
EST		166867	2.1
Glycogen phosphorylase, liver form		301492	2.1
Lin-7 homolog A (<i>C. elegans</i>)	PYGL	156926	2.1
Lymphotoxin beta receptor (TNFR superfamily)	LIN7A	123251	2.1
Solute carrier family 11, member 2	LTBR	144233	2
Ankyrin repeat domain 6	SLC11A2	291059	2
Ortholog of rat vacuole membrane protein 1	ANKRD6	321714	2
Glutathione peroxidase 1	VMP1	5725681	2
	GPX1	270088	2
Similar to RIKEN cDNA 1700009P17			
Amyotrophic lateral sclerosis 2 (juvenile) candidate 3		156132	2
Aldo-keto reductase family 1, member C2	ALS2CR3	266981	2
Excision repair cross-complementing group 1	AKR1C2	209473	2
Cytoplasmic linker 2	ERCC1	220443	2
Cholesterol 25-hydroxylase	CYLN2	178581	2
Rho GDP dissociation inhibitor (GDI) alpha	CH25H	279562	2
EST	ARHGDI A	5180471	2
Crystallin, mu		167228	2
EST	CRYM	206621	2
		278098	2

Genes are represented as over- and underexpressed in AMKL relative to TL. We arbitrarily chose to describe gene expression changes as relative to TL; genes that are overexpressed in AMKL, for instance, can also be defined as underexpressed in TL. Gene symbols and names derived from the SOURCE database are shown, as well as fold changes (two-fold and above) for each gene.

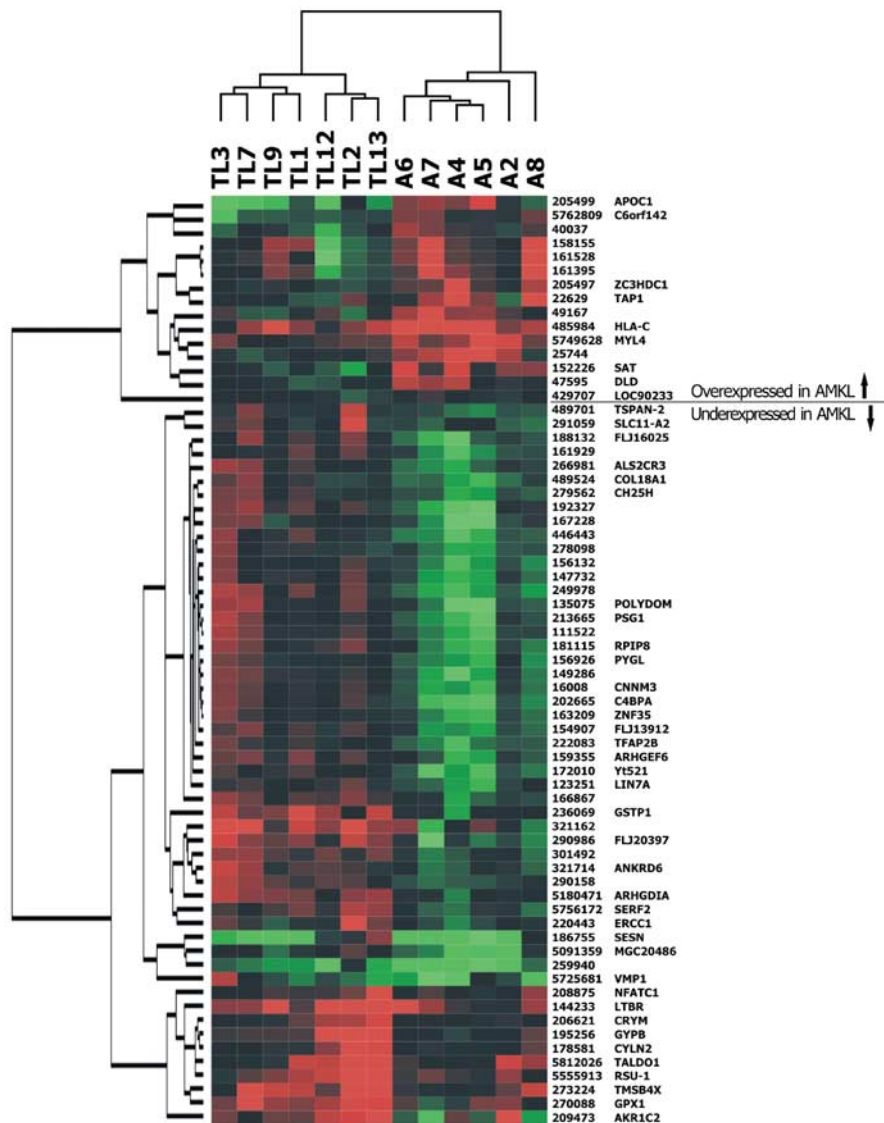


Figure 2 Clustering of AMKL and TL samples in supervised analysis. Magnified view of statistically significant changes in gene expression as derived from SAM (see Table 2). IMAGE clone IDs and corresponding UniGene symbols are indicated.

Microarray analysis revealed that AMKL cells showed decreased expression of Thymosin beta 4 (TMSBX4) and sestrin 2 (SESN), both of which influence cell proliferation and differentiation. TMSBX4 is an actin sequestering peptide that is highly expressed in CD15+ myeloid progenitor cells,³³ and is involved in the differentiation of lymphocytes, macrophages, and granulocytes.³⁴ TMSBX4 has been shown to inhibit growth of AML cell lines,³⁵ suggesting that decreased expression in AMKL cells may promote proliferation compared to TL cells. A number of DNA binding genes (ZNF35, NFATC1, TFAP2B) are decreased in AMKL, suggesting the potential for differential expression of putative transcription factors necessary for the AMKL cellular phenotype.

Conclusion

In summary, we have found that blasts of AMKL in Down syndrome and those of its precursor lesion TL can be

distinguished based on their gene expression profiles. The ontologies of the differentially expressed genes provide important clues for the design of mechanistic studies aimed at studying the progression of the reversible leukemic precursor lesion TL into the fully transformed leukemic phenotype AMKL. Although the present findings will require validation on an independent series of TL and AMKL samples, this study is a first step to understanding these two forms of megakaryoblastic leukemia in Down syndrome as a model of stepwise leukemic transformation that holds principal lessons beyond Down syndrome for the understanding acute leukemia.

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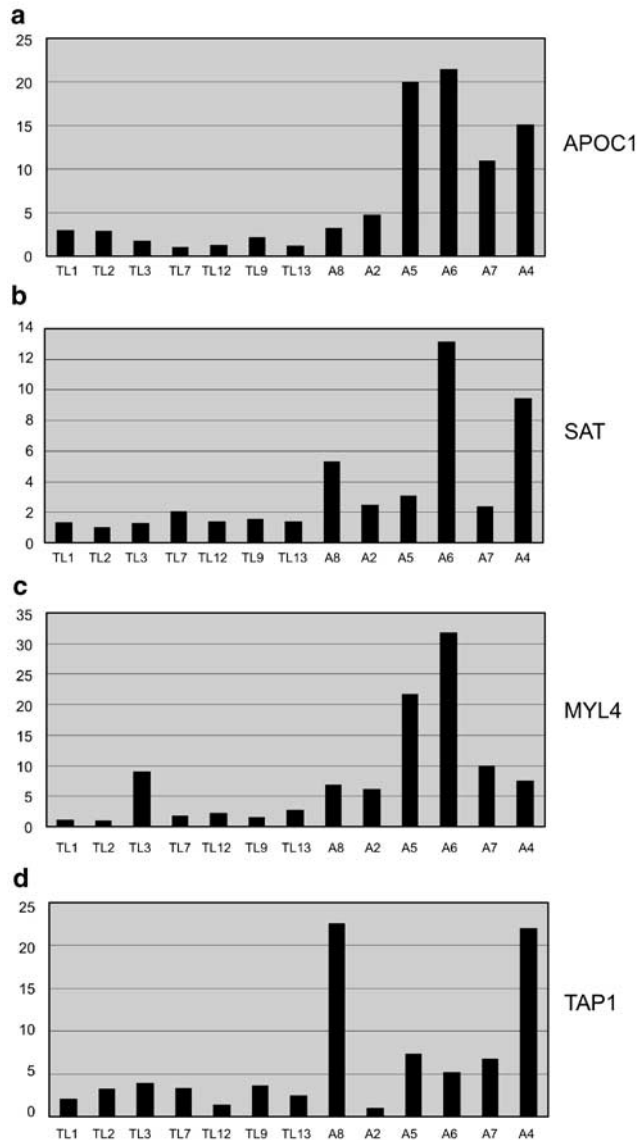


Figure 3 Quantitation of transcripts for selected genes using real time RT-PCR. Each panel represents quantitation of an individual transcript among all samples: (a) apolipoprotein C-I (APOC1), (b) spermidine/spermine *N*-acetyltransferase (SAT), (c) myosin light polypeptide 4 (MYL4), (d) transporter 1 (TAP1). Transcript levels are shown as arbitrary units. Values represent the mean of quadruplicate samples normalized to cyclophilin A and calibrated to the TL sample with the lowest mean expression value. Standard deviations for each value were below 0.1. All samples were carried out in quadruplicate and mean C_t values were used for quantitation. Mean C_t values for cyclophilin A varied less than 0.5 cycles among all samples (not shown on graph).

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