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Multiple mechanisms induce ectopic expression of LYL1 in subsets of T-ALL cell lines

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ABSTRACT

Basic helix–loop–helix (bHLH) transcription factors are essential for lymphocytic differentiation. Here, we have analyzed the complete bHLH family in T-cell acute lymphoblastic leukemia cell lines by expression profiling. Differential expression was detected for BHLHB2, HES1, HES4, HEY1, ID1, ID2, ID3, LYL1 and TAL1, highlighting dysregulation of family members with inhibitory activity. Subsequently we focused on the mechanisms responsible for aberrant expression of LYL1 in comparison to TAL1. Quantitative genomic PCR indicated microdeletions upstream of both, TAL1 and LYL1, targeting STIL/SIL and TRMT1, respectively. Additionally, one LYL1-expressing cell line exhibited amplification of TRMT1. While deletion of STIL correlated with expression of the STIL-TAL1 fusion transcript, no TRMT-LYL1 fusion transcripts were detected in parallel with genomic rearrangements thereof. Sequence analysis of the LYL1 promoter region revealed potential binding sites for transcription factors HOXA10, LMO2 and NKX2-5. Overexpression analysis, reporter gene assays and chromatin immuno-precipitation confirmed their activating impact on LYL1 expression. In conclusion, we identified multiple mechanisms which activate LYL1 in leukemic cells, including structural genomic alterations, namely microdeletion or amplification, together with the involvement of prominent oncogenic transcription factors.

1. Introduction

The differentiation of T-cells begins in the bone marrow where hematopoietic stem cells (HSC) develop into pluripotent precursors which migrate to the thymus. Here, they differentiate as thymocytes into mature T-cells, passing several developmental stages which are distinguished by cell surface markers, T-cell receptor (TCR) gene rearrangements and transcription factor expression patterns [1,2].

T-cell acute lymphoblastic leukemia (T-ALL) cells display differentiation arrests at certain stages, corresponding with particular mutations and oncogene expressions [2,3]. Prominent oncogenes in T-ALL are Lim domain only (LMO) genes (LMO1, LMO2), homeobox genes of both the NK-like family (TLX1/HOX11, TLX3/HOX11L2, NKX2-5/CSX) and the clustered type (HOXA9, HOXA10), and basic helix–loop–helix (bHLH) genes [4]. The last group is subdivided into ubiquitous expressed class-A bHLH genes, comprising TCF3/E2A and TCF12/HEB, and tissue specific class-B genes, including ID2, ID3, LYL1 and TAL1/SCL. Several proteins of the bHLH class-B share the ability to inhibit the activity of class-A proteins [5,6]. This type of regulation is important for normal lymphocyte development, notably thymocytes, since aberrant activities of inhibitory class-B bHLH proteins promote T-cell leukemogenesis [6–9].

TAL1 and LYL1 are the most prominent oncogenic members of the bHLH family which are dysregulated in leukemic thymocytes. Normally TAL1 and LYL1 are expressed in hematopoietic progenitors, including very early stages of thymocytes [10,11]. Juxtaposition with TCR genes is the classic mechanism underlying aberrant activation for both genes, TAL1 and LYL1 [12,13]. Additionally, TAL1 is targeted by a cryptic microdeletion, creating a fusion with its neighbor STIL/SIL which then drives TAL1 expression [14]. This deletion, present in about 25% of all T-ALLs, is much more frequent than TAL1 translocations at about 3% [15]. Here, we analyzed and compared the mechanisms underlying leukemic dysregulation of TAL1 and LYL1, thereby discovering mechanistic parallels linking both bHLH oncogenes.

2. Materials and methods

2.1. Cell lines

Cell lines were supplied by the DSMZ (Braunschweig, Germany). Cultivation was performed as described by Drexler [16]. For stimulation of T-ALL cell lines we used TNFalpha (R&D Systems, Wiesbaden, Germany), NFkB-Activation-Inhibitor (Calbiochem, Darmstadt, Germany) and cAMP derivate

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8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (Sigma, Taufkirchen, Germany).

2.2. Expression profiling

For quantification of gene expression via profiling we used DNA chips U133A Plus 2.0 obtained from Affimetrix (Buckinghamshire, UK). Chip-data analysis was performed as described recently [17]. Analysis of expression data was performed using online programs. For creation of heat maps we used CLUSTER version 2.11 and TREEVIEW version 1.60 (http://rana.lbl.gov/EisenSoftware.htm).

2.3. RNA, cDNA and genomic DNA

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Karlsruhe, Germany). cDNA was subsequently synthesized from 5 μg RNA by random priming, using Superscript II (Invitrogen). Preparation of genomic DNA was performed, using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany).

2.4. Polymerase chain reaction (PCR) analysis

Reverse transcriptase (RT)-PCR was performed, using taqpol (Qiagen, Hilden, Germany) and thermocycler TGradient (Biometra, Göttingen, Germany). Oligonucleotides were obtained from MWG Eurofins (Martinsried, Germany). Their sequences are listed in Table 1. Oligonucleotides used for detection of STIL-TAL1 fusion transcripts and for analysis of the TAL1 deletion type are described elsewhere [18,19]. Quantitative expression analysis was performed using the 7500 Real-time System, commercial buffer and primer sets for TAL1, LYL1, LMO2, HOXA9 and HOXA10 (Applied Biosystems, Darmstadt, Germany). For normalization of expression levels we used TBP. Copy number determination was performed using 50 ng genomic DNA per replicate. For normalization we used TAL1 or LYL1. Quantitative analyses were performed in triplicates and repeated twice. 5′RACE was performed using 5′RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen). Nested PCR products were subcloned and sequenced by MWG Eurofins.

2.5. Fluorescence in situ hybridization (FISH)

FISH analysis was performed as described recently [20]. The following RP11-clones were obtained from the (now defunct) clone facility at the Sanger Centre (Cambridge, UK): 349B5, 1061K17 (TAL1); 148D11, 245A21, 343E23 (LYL1); and supplemented with fosmid clones G248P87537C11 and G248P80397F3. Fluorescence images were captured using an Axioskop 2 plus microscope (Zeiss, Göttingen, Germany) and analyzed with Cytovision 2 software (Applied Imaging, Newcastle, UK).

2.6. Cloning procedures

cDNAs of HEX, HOXA9, HOXA10, LMO2, TLX3 cloned into expression vector pCMV6-XL5 were obtained from Origene (Rockville, MD, USA). NKX2-5 and TLX1 were cloned into CMV-driven expression vector as described previously [21]. To construct the lentiviral plasmids the respective cDNA cassettes were blunt-end cloned into the blunted BamHI site of the pHR'-SIN-SIEW-SnaBI vector, placing the cDNA fragment downstream of the SFFV promoter.

For creation of the LYL1 reporter construct, we cloned genomic PCR products (Table 1) of the LYL1 promoter and of the HOXA9 gene, comprising exon1–intron1–exon2 (reporter), into the HindIII/BamHI and EcoRI sites, respectively, of the expression vector pcDNA3 downstream of the CMV enhancer. The validity of the construct was confirmed by sequence analysis (MWG Eurofins). Taqman real-time PCR using the commercial HOXA9 assay quantifies the spliced reporter-transcript, corresponding to promoter activity.

2.7. Gene transfer

Transfection of expression constructs into cervix carcinoma cell line HELA was performed using transfection reagent SuperFect (Qiagen). Transfection experiments were performed twice at an efficiency of about 30% as determined by fluorescence microscopy.

VSV.G-pseudotyped lentiviral particles were generated by calcium phosphate cotransfection of 293T cells and viral supernatants were concentrated as previously described [22]. Lentiviral transduction of T-ALL cell lines JURKAT and MOLT-4 was performed twice with a multiplicity of infection of approximately two.

2.8. Chromatin immuno-precipitation (ChIP) analysis

ChIP analysis was performed with the ChIP Assay Kit (Millipore-Upstate, Schwalbach, Germany). Antibodies (anti-HOXA10, anti-LMO2 and anti-NKX2-5) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The subsequent PCR analysis was performed as described above, using nested oligonucleotides as listed in Table 1.

3. Results

3.1. Expression analysis of bHLH genes in T-ALL cell lines

For a comprehensive examination of the bHLH family in T-ALL cells, the transcriptional profiles of 115 informative genes were compared in T-ALL and control cell lines. The expression data were clustered and transformed into a heat map as shown in Fig. 1.

Table 1
Oligonucleotides used for PCR analysis.

Gene	Acc no.	Comment	Forward	Reverse
TAL1	NM_003189	Fusion		CGCGCCCAGTTCGATGAC
		Copy number	AAGCCAAGGCACTTCACAAG	ATATTTGAGGACAGGGACCG
STIL	NM_001048166	Fusion	TCCCGCTCCTACCCTGCAA	
		Copy number	ACGCAGATACGCCAGTATTG	GGATGTTGAGTGAACTCTGC
		RT-PCR	TTCACCAGACATCATGGAGC	TTCTCGACCAGGATCAAAGC
LYL1	NM_005583	Fusion		TCTCTGCCTTCTCAGTCATG
		Copy number	AGGCCCGCGCTGCTAGGTCC	CCCGGTTTCCTCCCTCTCAC
		RACE cDNA		CTCCACCTGC
		RACE1		TCTCTGCCTTCTCAGTCA
		RACE 1 nested		ACCTCTGCCTGTGCCTGAGG
		ChIP	AGGCCCGCGCTGCTAGGTCC	CCCGGTTTCCTCCCTCTCAC
		ChIP nested	GTCCCCGCCCGCTGGTTTCC	TTCCTCCCTCTCACCCCTGG
		ChIP control	ATGGAGCAAACCGCTTTGAG	TCTTCACTGGTCCTTCTTCG
		ChIP control nested	AAACCGCTTTG AG CCCAGAG	AGTTCGCTCCCGAACATGCG
		Promoter construct	CTAAGCTTAGGACCCTCCTTGTAGCG	CGGGATCCCGGTTTCCTCCCTCTCAC
TRMT1	NM_017722	RT-PCR	AGAAGAACGTCCACGTGAAG	AATGGAACGTAGGCCTGAAG
		Copy number	TTATAGGCATGAGCCACTGC	AGTGGCTCACGTCTGTAATC
		Fusion	CCACTATCATGTGATTCGCC	
		Fusion	CAGAGTTAACAAGTCGCAGG	
		Fusion	GTGAGAACTGCTACAGACAC	
		Fusion	CATCTCTGGGATCTCCTATC	
		Fusion	GTGAGTGCCTGATTTGATCC	
BTBD14B	NM_052876	Copy number	TTCTAGCCTTGCTGGGAAACCG	CGAGTGGCTAGCACTGTCTGG
HOXA9	NM 152739	Reporter construct	TGGCATTAAACCTGAACCGC	ACTCTTTCTCCAGTTCCAGG
HEX	NM_002729	RT-PCR/real-time	GCAAACCTCTACTCTGGAGC	TTCACTGGGCAAATCTTGCC
NKX2-5	NM_004387	RT-PCR	TCTATCCACGTGCCTACAGC	TGGACGTGAGTTTCAGCACG
CREB1	NM_134442	RT-PCR	GTGACGGAGGAGCTTGTACC	GCAATCTGTGGCTGGGCTTG