Identification of a *miR-146b*-Fas ligand axis in the development of neutropenia in T large granular lymphocyte leukemia

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 doi:10.3324/haematol.2019.225060

Received: May 3, 2019.

Accepted: August 23, 2019.

Pre-published: August 29, 2019.

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SUPPLEMENTARY MATERIALS AND METHODS

Flow cytometry analysis

T-LGL phenotype was assessed by flow cytometry. Briefly, cells were stained with the following antibodies: anti-CD3-PE (SK7), anti-CD3-APC (SK7), anti-CD4-FITC (SK3), anti-CD8-APC (RPA-T8), anti-CD16-PECy7 (B73.1), anti-CD56-PE (B159), anti-CD57-FITC (NK-1), and analysed using a FACSCanto analyser and the BD FACSDiva software (everything from BD Bioscience). TCR Vβ repertoire analysis was performed using the IOTest Beta Mark TCR-Vβ Repertoire kit (Beckman Coulter).

STAT3 and STAT5b mutations analysis

For the screening of STAT3 and STAT5b mutations we used the set of primers reported by Koskela et al[1] and Rajala et al[2], respectively, to amplify the hot spot regions for STAT3 and STAT5b mutations (exons 19-24 of STAT3 and exons 16-18 of STAT5b). DNA was extracted from purified T-LGL and remaining autologous PBMCs using the Puregene Cell Kit Plus (Qiagen) and sequenced on the ABI 3130 sequencer (Applied Biosystem). The presence of D661Y and Y640F STAT3 mutations undetectable by direct sequencing, was also analysed by a DNA tetraprimer amplification refractory mutation system assay (ARMS-PCR), as previously reported[3].

miRNA and Gene-Expression Analysis

High-throughput and single-miRNA expression analyses were performed by TaqMan as previously described[4]. Briefly, the small RNA fraction was purified by using the miRCURY RNA Isolation Kit (Exiqon) according to the manufacturer's instructions. High throughput and single miRNA analysis were carried out by using the TaqMan® Human microRNA Array (Card Set v3.0, Applied Biosystems) and the TaqMan® microRNA Human Assays (Applied Biosystems), respectively. Array data were analysed by Gene Expression Suite software (Applied Biosystems). miRNA expression values both from arrays and from single assays were calculated according to the comparative threshold cycle method using U6 as endogenous control. Relative miRNA expressions have been reported as fold change (FC). miRNAs samples with a FC>2 or FC<0.5 and P<0.05 were considered as differentially expressed.

mRNA and primary transcript data were calculated with LinReg PCR 7.0 (http:/LinRegPCR.nl) and Q-Gene software (http://www.gene-quantification.de/download.html) and then expressed as mean normalized expression (MNE) units after GAPDH or RPL32 normalization, unless otherwise indicated.

Methylated DNA Immunoprecipitation (meDIP) assay

Genomic DNA was firstly treated with 20 μg/mL RNAse A (Sigma Aldrich) for 30 min at 37°C and subsequently shared to generate fragments between 300 bp and 1000 bp using the Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Bandelin). Fragmented DNA (4 μg) was immunoprecipitated with 5 μg anti-5-methylcytosine mouse mAb (clone 33D3, Millipore). Immunopreciptated DNA was purified using the QIAquik PCR Purification Kit (Qiagen) according to the manufacturer's instructions. qPCR data were expressed as percentage over input.

Western blot

The blots were incubated with antibodies anti-STAT3 (79D7, Cell Signaling), anti-phospho-STAT3 (Tyr705) (STAT3-YP; D3A7, Cell Signaling), anti-GAPDH (clone 6C5, Millipore), anti-HuR (19F12, Thermo Scientific) and anti-β-actin (Sigma Aldrich) Abs. Detection was carried out with an horseradish peroxidase–conjugated anti–rabbit and anti–mouse immunoglobulins (Amersham International Biotechnology) and an enhanced chemiluminescent detection system (Thermo Scientific) for STAT3 and STAT3-YP. Goat anti-Mouse IgG antibody DyLight™ 800 conjugated (Rockland antibodies & assay) and AlexaFluor® 680 goat anti-Rabbit IgG (Molecular Probes, Thermo Scientific) and were used for HuR, GAPDH and β-actin detection, respectively. Detection was performed using the ImageQuant LAS 500 and quantified by ImageQuant TL v8.1 software (GE Healthcare) for STAT3 and STAT3-YP, and using the Odyssey infrared imaging system (LI-COR Biosciences) for HuR, GAPDH and β-actin.

Oligonucleotide used in cell transfection

miR-146b mimic (PM10105), pre-miR Negative Control#2, ELAVL1 Silencer Select (si-HuR, s4610) and Silencer Select negative control #2 (all from Ambion, Thermo Scientific).

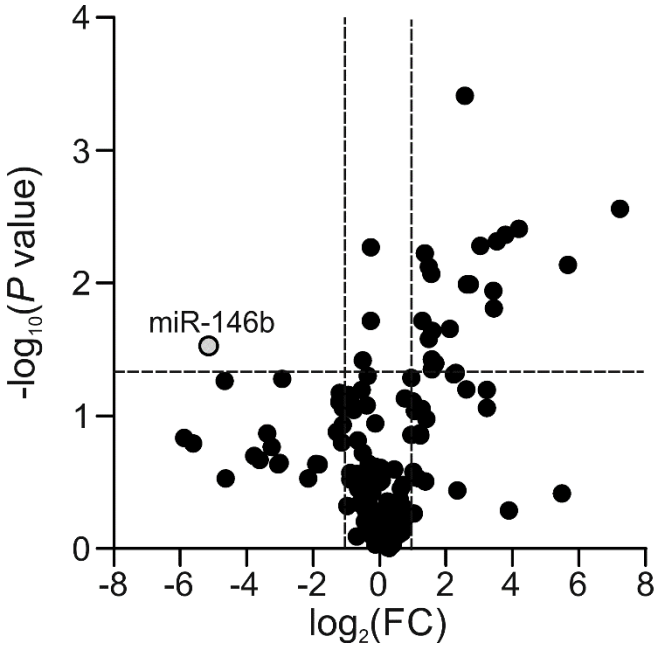
Enzyme-linked immunosorbent assay (ELISA)

Absorbance was immediately read at 450 nm using the Victor Multilabel plate (PerkinElmer).

Statistics

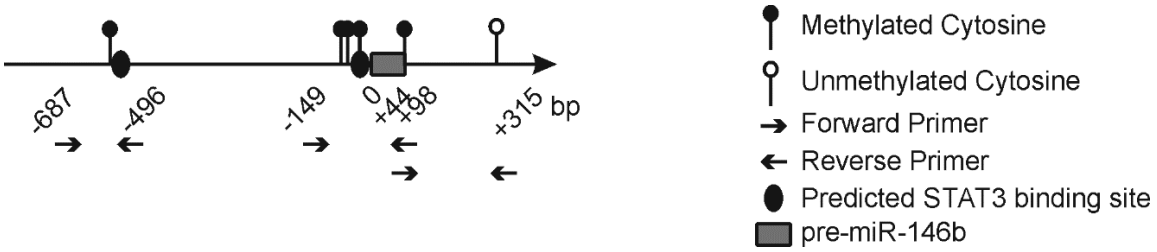
Hierarchical Clustering Analysis (HCA) of TaqMan Human microRNA Array data was performed by using the Multi Experiment Viewer (MEV, mev.tm4.org/) software with the following parameters: *Pearson Correlation and complete linkage*. For all the statistical analysis $P<0.05$ was considered significant.

SUPPLEMENTARY FIGURES



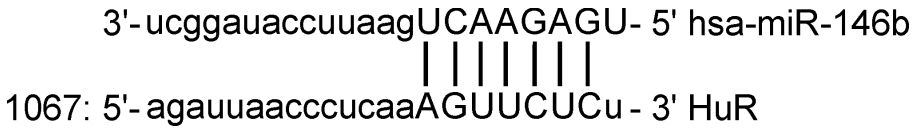
Supplementary Figure S1. miRNAs differentially expressed in CD8 as compared to CD4 T-LGLL.

Volcano plot showing miRNAs differentially expressed in CD8 as compared to CD4 T-LGLs. For each miRNA the –log10 (P value) is plotted against the average log2 (Fold Change). Vertical dashed lines represent the 0.5 and 2 boundary values for the Fold Change (FC); horizontal dashed line represents the 0.05 P value boundary.



Supplementary Figure S2. Schematic representation of the human miR-146b promoter.

Arrows represent the three primer pairs used in the MeDIP analysis. Primer position in the miR-146b locus is reported as bp upstream (-) or downstream (+) the miR-146b transcriptional start site arbitrarily indicated as 0. Positions of methylated and unmethylated cytosine as well as predicted STAT3 binding site are also reported.



Supplementary Figure S3: Base pairing comparison between mature miR-146b and HuR 3′ UTR.

Base pairing comparison between mature miR-146b and HuR 3′ UTR putative target site is shown according to microRNA.org (http://34.236.212.39/microrna/home.do).

SUPPLEMENTARY TABLES

Supplementary Table S1. Laboratory and clinical features of thirty patients affected by T-LGLL.

Patient ID	T-LGLL	T-LGL Phenotype	Sex/Age, y	Ly, %	LGLs, % on Ly	ANC, $\times 10^9$/L	Hb, g/L	Plts, $\times 10^9$/L	STAT3 mutation	STAT5 mutation	TCR gene analysis	Relevant Vβ expression	Associated neoplasia
LGL 1	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/57	72	74	0.95	151	282	MUT (Y640F)	WT	C	NF	no
LGL 3	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/79	66	52	1.43	142	338	WT	WT	C	NF	no
LGL 5	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/73	74	86	1.10	121	199	MUT (Y640F)	WT	C	5.2	no
LGL 9	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/53	90	86	0.80	144	135	MUT (Y640F)	WT	C	NF	no
LGL 10	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	F/82	53	51	5.70	139	198	WT	WT	C	13.1	yes
LGL 17	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/85	60	78	2.29	150	183	WT	WT	C	13.6	no
LGL 18	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/48	48	70	4.30	159	204	WT	WT	C	5.1	no
LGL 19	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/51	56	55	2.40	151	183	WT	WT	C	NF	no
LGL 20	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/72	43	50	3.18	149	252	WT	MUT (N642H)	C	17	yes
LGL 22	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/76	73	74	1.50	126	160	MUT (Y640F)	WT	C	21.3	no
LGL 23	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/79	69	59	0.50	125	66	MUT (N647I)	WT	C	17	no
LGL 24	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	F/62	59	53	2.86	132	238	WT	WT	C	NF	no
LGL 26	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/39	92	88	0.83	152	175	WT	WT	C	NF	no
LGL 31	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/69	57	48	3.28	163	175	WT	WT	C	8	yes
LGL 32	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/66	73	85	0.71	134	204	MUT (D661Y)	WT	C	2	no
LGL 33	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/68	86	66	0.46	128	104	MUT (D661Y)	WT	C	13.6	no
LGL 34	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/85	37	49	0.65	129	233	MUT (Y640F)	WT	C	3 and 20	no
LGL 36	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/54	64	91	0.70	155	111	WT	WT	C	8	no
LGL 37	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/71	73	83	1.82	154	218	WT	MUT (Y665F)	C	13.1	no
LGL 38	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/73	63	63	3.72	135	263	WT	WT	C	NF	no
LGL 39	CD4+	$CD3^+CD4^+CD8^-CD16\text{-}CD56^+CD57^+$	M/63	56	55	3.20	143	237	WT	WT	C	9	no

LGL 40	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/57	89	87	0.79	124	239	WT	WT	C	17	no
LGL 41	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/56	72	57	0.94	130	193	MUT (D661V)	WT	C	2	no
LGL 42	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/49	83	50	0.51	147	126	MUT (Y640F)	WT	C	7	no
LGL 43	CD4+	$CD3^+CD4^+CD8^-$CD16-$CD56^+$CD57	M/55	57	50	3.00	153	192	WT	WT	C	13.1	no
LGL 44	CD4+	$CD3^+CD4^+CD8^-$CD16-$CD56^+CD57^+$	F/45	48	50	4.90	121	334	WT	WT	C	NF	yes
LGL 45	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/69	56	45	1.08	145	186	MUT (D661Y)	WT	C	3	no
LGL 46	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	M/69	45	40	0.41	146	159	WT	WT	C	17	no
LGL 47	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/56	65	88	0.61	113	194	MUT (D566N)	WT	C	22	no
LGL 48	CD8+	$CD3^+CD8^+CD16^+CD56^-CD57^+$	F/58	78	54	1.4	154	219	MUT (D661Y)	WT	C	9	no

ANC indicates absolute neutrophil count, C indicates clonal rearrangement of TCR, Hb indicates haemoglobin, LGLs indicates large granular lymphocytes, Ly indicates lymphocytes and Plts indicates platelets.

Supplementary Table S2. Primers used in RT-qPCR analysis.

	Sequence	
RT-qPCR target	**Forward primer**	**Reverse primer**
DNMT1	CCAAAGCCCGAGAGAGTGCCTCAG	CCTTAGCAGCTTCCTCCTCCTT
FasL	CCACCCCCTGAAAAAAAGGAG	ATAGGTGTCTTCCCATTCCAG
FasL-PT	GCTGCCACCCCTGAAGAAG	CCCTCCATCCCCTTATGCC
HuR	TTTGGGCGGATCATCAACTC	ATGGGCTCAGAGGAACCTG
pri-miR-146b	GAACGGGAGACGATTCACAG	CCTTGGCATTGATGTTGTAGC
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
meDIP-qPCR target	**Forward primer**	**Reverse primer**
miR-146b +44bp +315bp	ATGCCCTGTGGACTCAGTTC	AGGATGCGATGGAAGATCAG
miR-146b –149bp +98bp	TCAGACCCTCCCTGGAATAG	CCTTGGCATTGATGTTGTAGC
miR-146b –687bp -496bp	CCTCTCTCTGGATTCAAGC	GAAGTACCTGGGATAGTGG

Supplementary Table S3. miRNAs differentially expressed in CD8 and CD4 T-LGLs.

	FC	*P*
hsa-miR-146b	0.028	0.030
hsa-miR-501	150.621	0.030
hsa-miR-1249	50.970	0.007
hsa-miR-1303	18.288	0.004
hsa-miR-1227	13.830	0.004
hsa-miR-571	11.546	0.005
hsa-miR-335#	10.839	0.015
hsa-miR-566	10.807	0.011
hsa-miR-1247	8.215	0.005
hsa-miR-1285	6.586	0.010
hsa-miR-33a#	6.194	0.010
hsa-miR-591	5.927	0.000
hsa-miR-623	4.937	0.047
hsa-miR-636	4.719	0.049
hsa-miR-625#	4.350	0.022
hsa-miR-1267	3.198	0.040
hsa-miR-516-3p	3.015	0.039
hsa-miR-1253	3.002	0.023
hsa-miR-197	2.993	0.038
hsa-miR-331-5p	2.970	0.045
hsa-miR-1233	2.946	0.008
hsa-miR-1825	2.808	0.026
hsa-miR-484	2.796	0.008
hsa-miR-630	2.572	0.006
hsa-miR-939	2.445	0.019

FC indicates Fold Change.

Supplementary Table S4. Correlation analysis of miRNA expression, Absolute Neutrophil Count and STAT3-YP

	ANC		STAT3-YP	
	p	***P***	***p***	***P***
hsa-miR-630	**-0.866**	**0.030**	**1.000**	**0.003**
hsa-miR-146b	**0.866**	**0.030**	**-0.866**	**0.033**
hsa-miR-939	-0.829	0.058	**0.943**	**0.017**
hsa-miR-33a#	-0.829	0.058	**0.943**	**0.017**
hsa-miR-636	-0.829	0.080	**0.943**	**0.017**
hsa-miR-1285	-0.657	0.173	0.829	0.058
hsa-miR-1303	-0.600	0.242	0.829	0.058
hsa-miR-623	-0.600	0.242	0.829	0.058
hsa-miR-516-3p	-0.543	0.297	0.771	0.103
hsa-miR-566	-0.543	0.297	0.771	0.103
hsa-miR-1233	-0.543	0.297	0.771	0.103
hsa-miR-1247	-0.829	0.059	0.657	0.175
hsa-miR-1267	-0.600	0.240	0.657	0.175
hsa-miR-591	-0.543	0.297	0.657	0.175
hsa-miR-1249	-0.600	0.242	0.600	0.242
hsa-miR-484	-0.429	0.419	0.600	0.242
hsa-miR-331-5p	-0.429	0.419	0.600	0.242
hsa-miR-197	-0.429	0.419	0.600	0.242
hsa-miR-335#	-0.486	0.356	0.543	0.297
hsa-miR-571	-0.429	0.419	0.543	0.297
hsa-miR-625#	-0.486	0.356	0.486	0.356
hsa-miR-1253	-0.486	0.356	0.486	0.356
hsa-miR-1825	-0.371	0.497	0.486	0.356
hsa-miR-501	-0.600	0.242	0.429	0.419
hsa-miR-1227	-0.429	0.419	0.429	0.419

ANC indicates absolute neutrophil count; *p* indicates the Spearman correlation coefficient.

Supplementary Table S5. Patients used in each analysis.

	Samples used																		
Figure ID	**CD8**									**CD4**									
1C	LGL 1	LGL 3	LGL 5	LGL 9						LGL 10	LGL 17								
2A	LGL 1	LGL 3	LGL 5	LGL 9	LGL 22	LGL 23	LGL 26	LGL 32	LGL 33	LGL 10	LGL 17	LGL 18	LGL 19	LGL 20	LGL 31				
2B - LEFT	LGL 1	LGL 3	LGL 5	LGL 9	LGL 22	LGL 23	LGL 26			LGL 10	LGL 17	LGL 18	LGL 20	LGL 31					
2B - CENTRE	LGL 1	LGL 3	LGL 5	LGL 9	LGL 22	LGL 23	LGL 26	LGL 32	LGL 33	LGL 10	LGL 17	LGL 18	LGL 19	LGL 20	LGL 31	LGL 39	LGL 43	LGL 44	
2C - RIGHT	LGL 1	LGL 3	LGL 5	LGL 9	LGL 22	LGL 23	LGL 26			LGL 10	LGL 17	LGL 18	LGL 20	LGL 31					
3A	LGL 40	LGL 41	LGL 42							LGL 18	LGL 39	LGL 44							
3B - LEFT	LGL 32	LGL 36	LGL 40	LGL 45	LGL 47	LGL 48													
3B - RIGHT	LGL 23	LGL 32	LGL 40	LGL 42	LGL 45	LGL 46	LGL 48												
4A	LGL 1	LGL 3	LGL 9	LGL 26						LGL 10	LGL 17	LGL 20	LGL 31						
4B	LGL 1	LGL 3	LGL 9	LGL 22	LGL 26	LGL 32	LGL 33			LGL 10	LGL 17	LGL 19	LGL 20	LGL 31					
4C	LGL 1	LGL 3	LGL 9	LGL 22	LGL 26	LGL 32	LGL 33			LGL 10	LGL 17	LGL 19	LGL 20	LGL 31					
4D	LGL 1	LGL 9	LGL 23	LGL 32						LGL 19	LGL 39	LGL 43	LGL 44						
4E	LGL 1	LGL 3	LGL 9	LGL 22	LGL 26	LGL 32	LGL 33			LGL 10	LGL 17	LGL 19	LGL 20	LGL 31					
6A	LGL 32	LGL 33																	

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