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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Long Noncoding RNA Expression Profiling in Normal B-Cell Subsets and Hodgkin Lymphoma Reveals Hodgkin and Reed-Sternberg Cell–Specific Long Noncoding RNAs

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Hodgkin lymphoma (HL) is a malignancy of germinal center (GC) B-cell origin. To explore the role of long noncoding RNAs (lncRNAs) in HL, we studied lncRNA expression patterns in normal B-cell subsets, HL cell lines, and tissues. Naïve and memory B cells showed a highly similar lncRNA expression pattern, distinct from GC-B cells. Significant differential expression between HL and normal GC-B cells was observed for 475 lncRNA loci. For two validated lncRNAs, an enhanced expression was observed in HL, diffuse large B-cell lymphoma, and lymphoblastoid cell lines. For a third lncRNA, increased expression levels were observed in HL and part of Burkitt lymphoma cell lines. RNA fluorescence *in situ* hybridization on primary HL tissues revealed a tumor cell–specific expression pattern for all three lncRNAs. A potential *cis*-regulatory role was observed for 107 differentially expressed lncRNA-mRNA pairs localizing within a 60-kb region. Consistent with a *cis*-acting role, we showed a preferential nuclear localization for two selected candidates. Thus, we showed dynamic lncRNA expression changes during the transit of normal B cells through the GC reaction and widely deregulated lncRNA expression patterns in HL. Three lncRNAs showed a tumor cell–specific expression pattern in HL tissues and might therefore be of value as a biomarker. (*Am J Pathol* 2016, 186: 2462–2472; <http://dx.doi.org/10.1016/j.ajpath.2016.05.011>)

Hodgkin lymphoma (HL) is a B-cell neoplasm characterized by a minority of neoplastic cells within an extensive inflammatory background. The incidence of HL is approximately three per 100,000 per year in Western countries, and it is most common in adolescents and young adults.¹ HL has been categorized into two disease entities, classic Hodgkin lymphoma (cHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). cHL accounts for approximately 95% of all cases, whereas NLPHL is less common. The neoplastic cells of cHL [ie, Hodgkin and Reed-Sternberg (HRS) cells] originate from germinal center (GC) B cells. The HRS cells show a loss of B-cell phenotype with no or strongly reduced expression levels of the B-cell receptor, common B-cell markers, and transcription factors.^{2,3} In contrast, the neoplastic cells of NLPHL (ie, the lymphocyte predominant cells) have retained

their B-cell phenotype.⁴ HRS cells can actively influence their microenvironment through the production of various cytokines and a variety of cell surface receptors. Among the factors and pathogens that contribute to cHL pathogenesis are NF- κ B and Epstein-Barr virus.⁵ NF- κ B signaling efficiently activates transcription of a variety of genes, which are involved in survival, proliferation, and inflammation.⁶

The impact of noncoding (nc) RNAs on hematological malignancies, including HL, has been well described for miRNAs.⁷ Next to miRNAs, another class of ncRNAs [ie, the

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long (l) ncRNAs] have recently received a lot of attention as important regulators of gene expression.⁸ lncRNAs are defined as transcripts >200 nucleotides in length that lack protein-coding potential. They are mostly categorized on the basis of their genomic location and orientation compared to protein-coding genes (eg, sense, antisense, intronic, or intergenic).⁹ Previously, a subset of lncRNAs has been shown to either positively¹⁰ or negatively¹¹ regulate neighboring protein-coding genes (*cis*-acting lncRNAs).¹² For instance, some nuclear lncRNAs were shown to function as transcriptional regulators of protein-coding genes in *cis*. This mechanism involves three-dimensional folding of chromatin juxtaposing regulatory sequences located several kilobases apart into close spatial proximity.¹² There is increasing evidence that alterations in the expression levels of lncRNAs are linked to tumorigenesis.^{13,14} It is currently unknown to what extent lncRNAs are regulated in B cells during the GC reaction and possibly deregulated in HL. In this study, we generated lncRNA expression profiles of normal mature B-cell subsets and HL cell lines. RNA fluorescence *in situ* hybridization (FISH) was used to confirm expression in tumor cells of primary cHL tissues. Finally, we identified putative *cis*-regulatory lncRNA bases on differential expression patterns of nearby protein-coding genes.

Materials and Methods

Tissue Samples and Cell Lines

Five formalin-fixed, paraffin-embedded cHL tissue samples were randomly selected from the Pathology files of the University Medical Center Groningen tissue bank. All five cHL cases were young adults (aged 20 to 30 years), four were of the nodular sclerosis subtype and one was of the mixed cellularity subtype according to World Health Organization classification.¹⁵ Epstein–Barr encoding region *in situ* hybridization revealed that one of the NS cases was Epstein–Barr virus positive. Normal B-cell subsets were sorted by fluorescence-activated cell sorter from three different tonsil samples as described previously.¹⁶ The procedures were according to the guidelines of the medical ethics board of the University Medical Center Groningen. Written informed consent was obtained for the use of the tonsil samples from the parents of the children. The HL cell lines were cultured at 37°C under an atmosphere containing 5% CO₂ in RPMI-1640 medium (Cambrex Biosciences, Walkersville, MD) supplemented with 2 mmol/L ultraglutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin (Cambrex Biosciences), and 20% (DEV, L540, SUP-HD1), 10% (L1236, KM-H2), or 5% (L428) fetal calf serum (Cambrex Biosciences). We routinely confirmed the identity of our cell lines using the PowerPlex 16 HS System (Promega, Leiden, the Netherlands).

RNA Isolation from Cell Lines, B-Cell Subsets, and Nuclear and Cytoplasmic Fractions

RNA was isolated from cell lines and B-cell subsets as described earlier.¹⁷ RNA concentration was measured with a

NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and integrity was assessed by analysis of the 18S/28S bands on a 1% agarose gel. Nuclear and cytoplasmic fractions were isolated from the cell lines L1236, L428, and L540 as described previously.¹⁷

Microarray Analysis

Design of the custom lncRNA microarray, labeling and hybridization procedures, data analysis, and generation of heat maps were performed as described previously.¹⁷ Naive, memory, GC-B, and HL cell lines were Cy3 labeled using 50 to 100 ng total RNA. Probes consistently flagged as present and expressed in the 10 to 100th percentile in at least one of the three conditions for the comparison of naive, GC-B, and memory B cells were included in the statistical analyses. For the comparison of the GC-B and HL samples, probes that consistently expressed one of two conditions were included. We detected 10,469 lncRNA and 17,885 mRNA probes with consistent signals above the background for the three B-cell subsets and 9955 lncRNA and 17,551 mRNA probes for the HL versus GC-B cell comparison. Significant differences in transcript abundance were determined by one-way analysis of variance (normal B-cell subsets) or unpaired *t*-test (HL versus GC-B cells). Benjamini-Hochberg multiple testing correction was applied. Microarray data used for this publication were deposited at the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE81086).

Quantitative RT-PCR

First-strand cDNA was synthesized with 500 ng RNA in a total reaction volume of 20 µL using the following reagents: random hexamer primers, 10 mmol/L dNTP mix, 5× first-strand buffer, 0.1 mol/L dithiothreitol solution, 1 µL RNaseOUT Recombinant Ribonuclease Inhibitor, and 1 µL Superscript II Reverse Transcriptase (Life Technologies). PCRs were performed in triplicate on a Lightcycler 480 system (Roche, Penzberg, Germany). Each quantitative PCR (qPCR) contained 1 ng cDNA, SYBR Green Master Mix (Applied Biosystems), and 3 µmol/L primers in a reaction volume of 10 µL. U6 served as endogenous control for normalization. Relative expression levels are calculated as 2^{-ΔCt}. For analysis of the subcellular localization, expression was normalized to 18S when the cytoplasmic fraction was compared to the total fraction and to U3 small nucleolar RNA when the nuclear fraction was compared to the total fraction. Table 1 contains the sequences of all primers used in this study.

Processing of The Cancer Genome Atlas RNA-Seq Data

Fastq files for 20 randomly selected samples per cancer type were mapped to the human genome (build hg19) using tophat. Gene expression values were quantified using

Table 1 Sequences of the Primers Used in This Study

Transcripts	Sequence
FLJ42351 (TCONS_00002672)	F: 5'-TTGTGGCTCATGCCATATGAA-3' R: 5'-CAAAGATCCTGTGGGCAGTCA-3'
LINC00116 (TCONS_00003368)	F: 5'-CATGGCCGGCTCTTCT-3' R: 5'-TCATAAAGTGCAAGAAGAAGTCATTTC-3'
LINC00461	F: 5'-CTTAAGCGCGCAAGTATCC-3' R: 5'-GCCAGACTCCAGGTCCTGATC-3'
TCONS_l2_00015489	F: 5'-GGCTGCAGATGGCAGGATT-3' R: 5'-TGCTGTACAGATACACCACGGAAT-3'
TCONS_00002977	F: 5'-AAGCAGATGCTGTGCCTGATAC-3' R: 5'-TTCTCGACCCAGAAGCTCAAG-3'
TCONS_00021708	F: 5'-TGGATTTTACAGGCCCTCTTCA-3' R: 5'-GCTCCTGCCTCTGTTTGTCT-3'
TCONS_00025860	F: 5'-CGCGATTTCAGGATTCC-3' R: 5'-CAAATGTGGGCACTTAAAGCA-3'
TCONS_00007500	F: 5'-TGGGTAAACACTGCTTTTATGAGTTG-3' R: 5'-GCTGGCTCAGGAGTGAAGCT-3'
TCONS_l2_00030240	F: 5'-CACACTCCAAGGAAACGCA-3' R: 5'-GGGATGACTGACCTCCTCTACC-3'
TCONS_00029175	F: 5'-AGTTCATTCACCTGGTGATCTTAAGC-3' R: 5'-GCTGAGTCTACCTGGAGTCCATTATT-3'
TCONS_l2_00009699	F: 5'-GCTATTTTAAAGGGTGTCCAG-3' R: 5'-CTGTACTAAGCCTCCCCAGACT-3'
TCONS_l2_00012111	F: 5'-CTAAACCTCCTGCAAAAGTGGAA-3' R: 5'-TGTTTGCACCTTTTGTCTGAAGAT-3'
U6	F: 5'-TGGAACGATACAGAGAAGATTAGCA-3' R: 5'-AAAATATGGAACGCTTCACGAATT-3'
RPPH1	F: 5'-AGCTTGAACAGACTCACGG-3' R: 5'-AATGGGCGGAGGAGTAGT-3'
DANCR	F: 5'-CGTCTCTTACGTCTGCGGAA-3' R: 5'-TGGCTTGTGCCTGTAGTTGT-3'
MT-TK	F: 5'-CGGCTAGCTCAGTCGGTAGA-3' R: 5'-CCAACGTGGGGCTCGAAC-3'
MIAT	F: 5'-TGGAGGCATCTGTCCACCCATGT-3' R: 5'-CCCTGTGATGCCGACGGGGT-3'
ANRIL	F: 5'-AAGCCGCTCCGCTCCTCTTCT-3' R: 5'-GCCGTGTCCAGATGTCGCGT-3'
XIST	F: 5'-GTCCTTTCTTTTGTACCCAGAA-3' R: 5'-GAGCCTGGCACTTTTCTTCC-3'
18S	F: 5'-CGGCTACCACATCCAAGGA-3' R: 5'-CCAATTACAGGGCCTCGAAA-3'
U3	F: 5'-AACCCGAGGAAAGAGAGGTA-3' R: 5'-CACTCCCAATACGGAGAGA-3'
MEF2C	F: 5'-CAAATGCAGGGCCCTT-3' R: 5'-GATATGCACTTACTGAATTCCA-3'
SLC20A1	F: 5'-CTGGCTCCGGTCCAAGAA-3' R: 5'-TGTGACAAACCAGGCCATAAAA-3'

F, forward; R, reverse.

Cufflinks software version 2.1.1 (<http://cole-trapnell-lab.github.io/cufflinks/releases/v2.1.1>) on the basis of the Ensembl reference transcriptome annotation (version 75).

Immunohistochemistry and RNA-FISH

An immunohistochemical staining for chemokine ligand (CCL) 17 was performed as described previously¹⁸ to confirm that the tumor cells were positive for CCL17. FISH probe sets and reagents (QuantiGene ViewRNA ISH Tissue 2-Plex Assay kit) were purchased from Affymetrix (Santa Clara, CA). RNA-FISH was performed according to the instructions provided by the manufacturer using formalin-fixed,

paraffin-embedded tissue sections (5 µm thick). A probe set for CCL17 was used as a positive control and to identify the HRS tumor cells in combination with one of the three probe sets for the selected lncRNAs. CCL17 was visualized with a type 6 probe set (Fast Blue) and the lncRNAs were visualized with type 1 probe sets (Fast Red). Custom probe sets designed by the manufacturer covered the exons that are common to all isoforms. Slides were imaged on a Leica SP8 confocal microscope.

Identification of Differentially Expressed lncRNA-mRNA Pairs Mapping within a 60-kb Region

To identify putative *cis*-acting lncRNAs, we determined for each differentially expressed lncRNA whether there was a differentially expressed protein-coding gene in close vicinity (directly adjacent) using Galaxy (<https://usegalaxy.org>; last accessed May 13, 2016).^{19,20} The genomic coordinates of all differentially expressed probes were uploaded using the get data tool. We next defined the neighboring differentially expressed mRNAs using operate on genomic intervals tools, fetch closest nonoverlapping feature for every interval and joint data sets. The resulting data set was subsequently filtered for lncRNA-mRNA pairs within 60 kb of each other.

Results

Highly Dynamic lncRNA Expression Changes during the Germinal Center Reaction

Statistical analysis of the gene expression profiles generated for naive, GC, and memory B cells revealed 401 significantly differentially expressed lncRNA probes (251 lncRNA loci) (analysis of variance, false discovery rate <0.05) with a fold-change of at least 2 in expression levels. The naive and memory B-cell subsets showed a highly similar expression pattern with a significant differential expression for only two probes. In contrast, GC-B cells showed a distinct lncRNA expression pattern with 377 differentially expressed probes compared to naive and 376 compared to memory B cells. Unsupervised hierarchical clustering using the 401 lncRNA probes revealed two distinct clusters, one cluster with the GC-B cell samples and a second cluster with the naive and memory B-cell subsets (Figure 1A). Analysis of the differentially expressed mRNAs (2908 probes, 2505 loci) revealed a clustering pattern similar to that of lncRNAs (Supplemental Figure S1A).

Next, we clustered the six HL cell lines together with the B-cell subsets using the 401 lncRNA probes that were significantly different between the three normal B-cell subsets. This revealed a pairwise clustering of the GC-B cells and HL cell lines in one tree and the naive and memory B-cells in a separate tree (Figure 1B). The NLPHL cell line DEV clustered in the same branch as the GC-B cells, whereas the five cHL cell lines clustered in a separate

Figure 1 Unsupervised hierarchical clustering of differentially expressed lncRNAs. **A:** Heat map of the 401 lncRNA probes (251 lncRNA loci) differentially expressed between germinal center (GC), naïve, and memory (Mem) B cells. **B:** Heat map of the same 401 human lncRNA probes, including the cHL cell lines L540, KM-H2, L1236, L428, and SUP-HD1 and the NLPHL cell line DEV. **C:** Unsupervised clustering of the 639 lncRNA probes (475 lncRNA loci) differentially expressed between cHL and GC-B cells. The positions of some known and well-annotated lncRNAs are indicated in the heat maps. In bold are the three lncRNA candidates that were further studied by RNA fluorescence *in situ* hybridization.

branch next to the GC-B cells. This is consistent with the GC-B cell origin of the tumor cells of HL, with a loss-of-B-cell phenotype in cHL but not in NLPHL. A similar result was observed using the 2908 mRNA probes that were differentially expressed between the normal B-cell subsets (Supplemental Figure S1B).

lncRNA Expression Is Widely Deregulated in cHL

Comparison of the expression profiles of cHL (excluding the NLPHL cell line DEV) to GC-B cells revealed a significantly differential expression for 639 lncRNA probes (475 lncRNA loci) (Supplemental Table S1 and Figure 1C).²¹ Comparison of the mRNA expression profiles revealed 2402 differentially expressed probes (2023 loci) (Supplemental Figure S1C). lncRNAs were predominantly down-regulated in HL (74% down-regulated versus 26% up-regulated), whereas mRNAs showed a more equal

distribution between up-regulated and down-regulated transcripts (56% down-regulated versus 44% up-regulated). Comparison of the lncRNAs and mRNAs differentially expressed between the normal B-cell subsets and between cHL cell lines and GC-B cells revealed a limited overlap of 70 lncRNA and 581 mRNA probes.

To validate the differential expression patterns, we designed RT-qPCR primer sets for nine up-regulated and five down-regulated lncRNAs randomly selected from the set of lncRNAs differentially expressed between cHL and GC-B cells. All nine up-regulated lncRNAs showed a higher expression in cHL consistent with the microarray data, with significant differences for six of them (Supplemental Figure S2). For the down-regulated lncRNAs, we could confirm significant changes for three of five lncRNAs. For the remaining two lncRNAs, we were not able to design primer sets that could amplify these lncRNA transcripts.

lncRNA Expression in B Cells, Lymphoma Cell Lines, and Primary cHL Tissues

We selected three of the validated up-regulated lncRNAs (ie, FLJ42351, LINC00116, and LINC00461) (Supplemental Figure S3) for further expression analysis in normal B-cell subsets and a panel of lymphoma cell lines. This revealed high levels in HL, diffuse large B-cell lymphoma (DLBCL), and lymphoblastoid cell lines (LCL) for FLJ42351 and LINC00461 (Figure 2, A and C). For LINC00116, high levels were mainly observed in the HL cell lines and in part of the Burkitt lymphoma cell lines (Figure 2B). Reanalysis of The Cancer Genome Atlas (TCGA) RNA-seq data across 24 different cancer types

revealed relative low expression levels for FLJ42351 and LINC00116 without clear differences between different cancer tissues (Figure 2). For LINC00461, higher levels were observed only for lower-grade glioma.

Next, we used RNA-FISH to study the expression of the three in cHL cell lines up-regulated lncRNAs in tissue sections of primary cHL cases. The five randomly selected cHL cases showed positive staining for CCL17 by immunohistochemistry (data not shown). RNA-FISH using the CCL17 probe set as a positive control revealed staining of the HRS cells in four of the five cases. RNA-FISH using the lncRNA probe sets revealed a tumor cell-specific staining in the four remaining cHL cases for all three lncRNAs (Figure 3). The probe sets for FLJ42351 and LINC00461

Figure 2 lncRNA expression levels in normal B cells, lymphoma, and other cancer tissues. Quantitative RT-PCR analysis and reanalysis of The Cancer Genome Atlas RNA-seq data for FLJ42531 (A), LINC00116 (B), and LINC00461 (C). Groups consisted of: Naive, CD19⁺IgD⁺CD38⁻ cells (3); GCB, CD19⁺IgD⁺CD38⁺ (3); memory, CD19⁺IgD⁺CD38⁻ (3); HL, L428, L540, L1236, KM-H2, L591, SUP-HD1, and NLPHL, DEV (open circle); PMBL, K1106P, and MEDB1; DLBCL, Karpas 422, SU-DHL-4, SU-DHL-5, SU-DHL-6, SU-DHL-10, OCILy-3, U-2932, D0HH2; BL, ST486, DG75, RAMOS, CA46, BL65, RAJI, Jiyoye, and Nawalma; LCL, LCL6A, LCL39, LCL89; T-cell, Jurkat, HUT-78, KARPAS 299, SR678. ACC, adrenocortical carcinoma; BL, Burkitt lymphoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; COAD, colon adenocarcinoma; DLBCL, diffuse large B-cell lymphoma; GC, germinal center B cells; HL, Hodgkin lymphoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LCL, lymphoblastoid cell line; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PMBL, primary mediastinal B-cell lymphoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; T-cell, T-cell leukemia and lymphoma cell lines; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinoma.

Figure 3 Expression of FLJ42351, LINC00116, and LINC00461 is restricted to the Hodgkin and Reed-Sternberg (HRS) cells of cHL cases. Dual-color RNA fluorescence *in situ* hybridization was performed using a probe set for chemokine ligand (CCL) 17 (tumor cell specifically expressed) in combination with probe sets for FLJ42351 (A), LINC00116 (B), or LINC00146 (C). Shown are representative images of the DAPI, CCL17 (in red, fast blue), and lncRNA (in green, Cy3/fast red) signals and the overlay of the three. **Arrows** in the **top row panels** indicate the HRS tumor cell that was used for the magnified images that are shown in the **bottom row panels**. Scale bar = 10 μ m.

showed positive signals in three cases and weak positive signals in one case, the probe set for LINC00116 showed positive signals in all four cases.

lncRNAs and Neighboring Protein-Coding Genes

To identify lncRNAs that potentially affect the expression of nearby protein-coding genes, we determined which of the differentially expressed lncRNAs was close to a differentially expressed mRNA. Within the 251 lncRNAs differentially expressed between the normal B-cell subsets, we identified 51 putative *cis*-regulatory lncRNAs with a probe-to-probe distance of up to 60 kb to the differentially expressed mRNA (Supplemental Table S2). Of the 475 lncRNAs differentially expressed between cHL and GC-B cells, 59 were in close vicinity of a differentially expressed mRNA (Table 2).

Three of the putative *cis*-regulated lncRNA-mRNA pairs were detected in both the normal B-cell subsets and the HL versus GC-B analysis. For all three pairs, a positive correlation between the lncRNA and mRNA expression was observed in both analyses.

To support a putative *cis*-regulatory role for two (LINC00461 and FLJ42351) of the 59 lncRNAs, for which we already designed qPCR primers, we determined if they were preferentially localized in the nucleus. Fractionation procedures of L428, L1236, and L540 cell lines were validated by qPCR for six transcripts with a known subcellular localization. This revealed the expected enrichment of

RPPH1, DANCER, and MT-TK transcripts in the cytoplasmic fractions and of MIAT1, ANRIL, and XIST transcripts in the nuclear fractions (Figure 4, A and B). Consistent with a *cis*-regulatory role, LINC00461 and FLJ42351 showed enrichment in the nuclear fractions and depletion in the cytoplasmic fractions. Analysis of the putative *cis*-regulated protein-coding genes by RT-qPCR confirmed the inverse expression pattern between LINC00461 and the nearby upstream protein-coding gene, MEF2C (Supplemental Figure S2C). For FLJ42351, we could confirm the comparable differential expression patterns for this lncRNA and the neighboring protein-coding gene SLC20A1.

Discussion

In this study, we showed a dynamic regulation of lncRNA expression during the transition of B cells through the GC (ie, from naive B cell to GC-B cell, resulting in memory B cells). Furthermore, the comparison of cHL cell lines with GC-B cells revealed a significant differential expression pattern for a considerable number of lncRNAs. This indicates that lncRNAs play a role in normal B-cell maturation and that deregulated lncRNA expression is a prominent feature of HL.

We showed that the levels of 401 probes corresponding to 251 lncRNA loci change during the transition from naive to GC-B cells and reverse to levels comparable to naive B cells once the cells differentiate to memory B cells. Recently,

Table 2 Table of Putative *Cis*-Regulated lncRNA-mRNA Pairs Identified in the Transcriptome Comparisons of the cHL Cell Lines and GC-B Cells

lncRNA			mRNA		lncRNA to mRNA		
Chr	Probe name	Transcript(s)	Probe name	Gene symbol	Distance*	Orientation	Direction†
1	CUST_4772_PI427622066	TCONS_l2_00001761, -2, -5	A_23_P34915	ATF3	-44	Tail-to-tail	Up/up
1	CUST_4757_PI427622066	TCONS_00002580/ TCONS_00001808~11	A_23_P34915	ATF3	-11	Tail-to-tail	Up/up
1	CUST_4772_PI427622066	TCONS_l2_00001761, -2, -5	A_23_P160720	BATF3	22	Sense	Up/up
1	CUST_4751_PI427622066	TCONS_00001808~11	A_23_P160720	BATF3	54	Sense	Up/up
1	A_33_P3411328	NR_034151.1/LINC01136	A_23_P62901	BTG2	8	Head-to-head	Down/down
1	CUST_1604_PI427622066	TCONS_00001440	A_23_P35082	SESN2	34	Head-to-head	Down/up
2	CUST_9360_PI427622066	TCONS_00002672/ TCONS_00005231/ FLJ42351	A_23_P165657	SLC20A1	19	Head-to-head	Up/up
2	CUST_10333_PI427622066	TCONS_00003045	A_23_P56559	DHRS9	-5	Sense	Down/down
2	CUST_8107_PI427622066	TCONS_00003301	A_33_P3316928	PELI1	6	Sense	Down/down
2	CUST_8408_PI427622066	TCONS_l2_00014653	A_23_P68072	WDR54	12	Head-to-head	Down/up
2	CUST_6868_PI427622066	TCONS_00004118/ TCONS_00003184	A_33_P3401826	CMPK2	18	Sense	Down/down
3	CUST_11860_PI427622066	TCONS_00006507	A_33_P3288023	NCKIPSD	-51	Sense	Up/up
3	A_33_P3368771	NR_026991.1/H1FX-AS1	A_23_P251377	C3orf37	-15	Sense	Down/down
4	CUST_19108_PI427622066	TCONS_00009180~2	A_24_P337334	DCTD	13	Sense	Up/up
4	CUST_12908_PI427622068	TCONS_00008872	A_24_P103264	UGT8	-6	Sense	Down/down
5	A_32_P127350	NR_024384/LINC00461	A_23_P320739	MEF2C	56	Sense	Up/down
5	CUST_22952_PI427622066	TCONS_00011070	A_24_P97825	CCDC69	6	Sense	Down/down
6	CUST_20144_PI427622068	TCONS_l2_00024170-2	A_32_P351968	HLA-DMB	32	Tail-to-tail	Down/down
8	CUST_25512_PI427622068	TCONS_00014599, -600/ TCONS_00015212	A_24_P201064	PPP1R3B	-15	Head-to-head	Up/up
9	A_33_P3574055	TCONS_l2_00028716, -18~22	A_33_P3348001	LOC100130458	-49	Head-to-head	Down/down
9	CUST_34071_PI427622066	TCONS_00015807	A_23_P124855	ZCCHC7	-26	Tail-to-tail	Down/down
9	A_23_P216935	NR_026677.1/MIR600HG	A_24_P252575	RABGAP1	-5	Tail-to-tail	Down/down
9	A_23_P216935	NR_026677.1/MIR600HG	A_23_P252155	STRBP	15	Sense	Down/down
10	CUST_40617_PI427622066	TCONS_00017839	A_23_P161481	KIAA1274	-42	Sense	Down/down
11	CUST_42984_PI427622066	TCONS_00019374	A_23_P24444	DHCR7	-17	Head-to-head	Up/up
11	A_23_P127367	NR_046412/POLD4	A_23_P323227	PPP1CA	47	Sense	Down/down
12	CUST_44640_PI427622066	TCONS_00020398	A_33_P3215647	FAM113B	-13	Sense	Down/down
12	CUST_45746_PI427622066	TCONS_00020260/ NR_110375.1 / THRIL	A_32_P831181	BRI3BP	-14	Tail-to-tail	Down/down
12	CUST_44512_PI427622066	TCONS_00021299	A_23_P98930	C12orf35	33	Tail-to-tail	Down/down
13	CUST_38642_PI427622068	TCONS_00021766	A_24_P10137	C13orf15	-36	Sense	Down/down
14	A_23_P25746	NR_027263/ARHGAP5-AS1	A_33_P3386459	ARHGAP5	41	Head-to-head	Up/up
14	CUST_40389_PI427622068	TCONS_00022548	A_24_P273742	TTC9	-7	Sense	Down/down
14	CUST_39858_PI427622068	TCONS_00022357	A_23_P357717	TCL1A	-5	Head-to-head	Down/down
15	CUST_41903_PI427622068	TCONS_l2_00008581, -583	A_33_P3260614	PLCB2	-36	Head-to-head	Up/down
15	CUST_52013_PI427622066	TCONS_l2_00008800	A_33_P3229276	ZSCAN2	13	Sense	Down/up
15	CUST_50180_PI427622066	TCONS_l2_00009507	A_33_P3262799	ZNF280D	25	Sense	Down/down
16	A_24_P865226	NR_015396.1/CDIPT-AS1	A_33_P3294608	MVP	-17	Tail-to-tail	Up/up
16	A_24_P865226	NR_024370.1/CDIPT-AS1	A_23_P10194	SEZ6L2	6	Head-to-head	Up/up
16	CUST_54455_PI427622066	TCONS_l2_00010376~80	A_24_P193582	DEF8	44	Head-to-head	Up/down
17	CUST_55786_PI427622066	TCONS_00025420, -926	A_23_P141429	ABI3	-16	Sense	Down/down
17	CUST_46171_PI427622068	TCONS_l2_00010942, -3	A_33_P3344648	LOC440461	-6	Sense	Down/down
17	A_32_P19000	NR_040071.1/TNRC6C-AS1	A_33_P3302428	TNRC6C	-5	Antisense	Down/down
17	A_32_P19000	NR_040071.1/TNRC6C-AS1	A_23_P101013	TMC6	4	Sense	Down/down
17	CUST_46165_PI427622068	TCONS_00025471	A_33_P3344648	LOC440461	11	Sense	Down/down
17	CUST_56085_PI427622066	TCONS_l2_00011403, -406~409, -412~416, -685~687	A_24_P941441	GNA13	37	Sense	Down/down
18	A_32_P150876	NR_015389/LINC00667	A_24_P74064	ZFP161	44	Tail-to-tail	Down/down

(table continues)

Table 2 (continued)

lncRNA			mRNA		lncRNA to mRNA		
Chr	Probe name	Transcript(s)	Probe name	Gene symbol	Distance*	Orientation	Direction†
19	A_33_P3685216	NR_049780/ZNF837	A_33_P3411427	ZNF837	20	Sense	Up/down
19	CUST_48657_PI427622068	TCONS_l2_00012685~7	A_24_P310224	ZNF347	-56	Head-to-head	Down/down
19	CUST_59058_PI427622066	TCONS_00027367, -368, -759/TCONS_00026816	A_23_P90333	ZNF404	-28	Sense	Down/down
19	CUST_48777_PI427622068	TCONS_00026798, -99/TCONS_00027118~26	A_33_P3418731	ZNF542	-14	Sense	Down/down
19	A_24_P941487	NR_003148/TPM3P9	A_33_P3216433	LOC147804	-13	Sense	Down/down
19	CUST_48637_PI427622068	TCONS_l2_00012669	A_23_P130444	ZNF701	-7	Sense	Down/down
19	A_33_P3646051	NR_040027.1/ZNF790-AS1	A_33_P3423401	ZNF790	13	Antisense	Down/down
19	CUST_59260_PI427622066	TCONS_l2_00013339/TCONS_l2_00012668	A_33_P3256868	ZNF83	13	Tail-to-tail	Down/down
20	CUST_50118_PI427622068	TCONS_00027871	A_24_P91566	BMP7	-54	Head-to-head	Down/down
20	CUST_61077_PI427622066	TCONS_00028073	A_24_P413126	PMEPA1	48	Sense	Down/down
22	CUST_63826_PI427622066	TCONS_00029585, -795	A_23_P132277	MCM5	-30	Sense	Down/down
X	CUST_30264_PI427622068	TCONS_00016919	A_23_P319617	CHST7	52	Sense	Up/up
X	CUST_30840_PI427622068	TCONS_00017014	A_23_P309865	ZNF449	-34	Sense	Down/up

The lncRNA-mRNA pairs that overlap with the putative *cis*-regulated lncRNA-mRNA pairs identified in the transcriptome comparisons of the normal B-cell subsets (Supplemental Table S2) are in bold.

*Probe to probe distance in kb, positive distance means the lncRNA probe is upstream of the protein-coding gene, negative distance indicates the reverse.

†The direction of the lncRNA is indicated first, the direction of the mRNA is indicated second. Direction is based on cHL cell lines compared with GC-B cells.

four studies analyzed lncRNA expression in B-cell populations.^{22–25} Only one of the four studies also analyzed naive, memory, and germinal center B-cell populations isolated from tonsil. Consistent with our study, they showed that the lncRNA expression profiles of memory and naive B cells are similar and more distinct from GC-B cells.²² However, none of the studies provided overviews of the lncRNAs differentially expressed between similar B-cell subsets as used in our study, preventing a direct comparison to our results. At the mRNA level, we and others observed a similar pattern as we observed for lncRNAs (ie, similar

profiles for naive and memory B cells and significantly different profiles for GC-B cells).²⁶ The NLP cell line DEV clustered with normal GC-B cells, and the cHL cell lines cluster in a sub-branch next to the GC-B cells for both the lncRNAs as well as the mRNA probes. This indicates that the expression profile of lymphocyte predominant cells of NLPHL more closely resembles that of GC-B cells than that of the HRS cells of cHL. These results are consistent with previous gene expression studies showing a loss-of-B-cell phenotype in HRS cells²⁷ and intermediate expression patterns in lymphocyte predominant cells.²⁸

A direct comparison of cHL cell lines with GC-B cells revealed significant differences for 639 probes (475 lncRNA loci). Almost 75% of these lncRNAs were down-regulated in the cHL cell lines, whereas the percentage of up-regulated and down-regulated mRNAs was similar. This suggests that lncRNAs might, similar to miRNAs,²⁹ show a global down-regulation in cancer. Previous studies comparing the expression profiles of microdissected primary HRS cells and cHL cell lines to GC-B cells showed a considerable overlap between the differentially expressed genes in primary HRS cells and cHL cell lines.^{27,30} However, a subset of the genes differentially expressed in primary HRS cells, including chemotaxis and surface receptor signaling related genes, were not differentially expressed in the cell lines.^{27,30} This indicates that it is likely that the lncRNA expression profile of the HL cell lines will also not fully reflect the lncRNA profile of primary HRS cells.

Three of the up-regulated lncRNAs (ie, LINC00116, LINC00461, and FLJ42351) were studied in more detail,

Figure 4 LINC00461 and FLJ42351 are nuclear enriched lncRNAs. As a validation for the isolation of cytoplasmic (A) and nuclear (B) fractions, the enrichment of three cytoplasmic control RNAs (RPPH1, DANCER, and MT-TK) in the cytoplasmic fraction and three nuclear control (MIAT, ANRIL, and XIST) RNAs in the nuclear fraction were confirmed by quantitative RT-PCR. Analysis of LINC00461 and FLJ42351 shows a clear enrichment in the nuclear fraction and depletion in the cytoplasmic fraction. $\Delta\Delta C_t$ values are shown with the average of the total fractions of L428, L1236, and L540 HL cell lines set to 1 (dashed line). 18S (cytoplasmic) and U3 (nuclear) were used for normalization of cytoplasmic and nuclear fractions, respectively.

including expression analysis in primary cHL tissues. RNA-FISH for LINC00116 revealed a remarkable specific expression pattern, with fluorescent signals being restricted to the HRS cells. The RT-qPCR across normal B-cell subsets and lymphoma cell lines showed a similar pattern with a preferential expression in HL cells. TCGA data did not show a preferential expression pattern across multiple other types of cancer. LINC00116 has three known transcript variants, two short variants and one long variant. The longer transcript (LINC00116-001) might encode for a 138 amino acid uncharacterized protein ([http://www.uniprot.org/uniprot; accession number Q8NCU8](http://www.uniprot.org/uniprot;accession%20number%20Q8NCU8)), whereas the shorter variants represent true lncRNA transcripts without protein-coding potential. The expression pattern of the probes present on our array pointed to a predominant expression of shorter isoforms (LINC00116-002 and LINC00116-003) (Supplemental Figure S3). Moreover, the probes for RNA-FISH and the primers used for the qPCR were also designed on the basis of these short noncoding transcript isoforms. Thus, our data support an HRS cell-specific expression pattern of one or both of the shorter LINC00116 transcript variants.

LINC00461 showed a highly specific expression pattern in the HRS cells by RNA-FISH. We also noticed enhanced expression of LINC00461 in HL, DLBCL, and LCL cell lines. Analysis of the TCGA data revealed expression only in low-grade gliomas and not in DLBCL tissues. The difference between the qPCR and TCGA results for DLBCL may be related to differences that sometimes can be observed between cell lines and primary tissues. The LINC00461 locus contains several isoforms of which the array probe, the RT-qPCR primer set, and the FISH probe set all detect both the 3.6-kb (NR_024384) and the 3.4-kb (NR_024383) isoforms (Supplemental Figure S3). The last exon of both lncRNA isoforms contains the pre-miR-9-2 sequence. Whether this lncRNA, besides serving as the primary transcript of miR-9, also has other functions has yet to be determined. It has been reported that miR-9 expression is increased in HL and glioma and that inhibition of miR-9 inhibits growth of HL cell lines in a xenograft mouse model.^{31–34} The expression pattern of the nuclear enriched LINC00461 showed an inverse correlation with the expression of the nearby upstream protein-coding gene, *MEF2C*. *MEF2C* is essential for B-cell proliferation and survival in response to BCR stimulation *in vitro*.³⁵ *In vivo*, *MEF2C* loss results in reduced antibody responses to T-cell-dependent antigens and impaired GC formation.^{35,36} Studies in rats have shown that *MEF2C* can stimulate expression of the miR-9-2 locus by binding to a highly conserved site in the promoter of the miR-9-2 host gene.³⁷ On the other hand, *MEF2C* is a predicted miR-9 target via a broadly conserved 8-mer site in the 3' untranslated region. How LINC00461 and *MEF2C* regulate each other's expression and to what extent this is relevant for cHL remains to be investigated.

FLJ42351, the third lncRNA studied in more detail, also showed a remarkable HRS cell-specific expression in primary HL tissues. qPCR confirmed expression in HL cell lines and showed in addition expression in DLBCL and LCL cell

lines. TCGA RNA-seq data did not show substantial expression in other types of cancer tissues. The expression pattern of FLJ42351 is similar to that of the immediately downstream phosphate transporter *SLC20A1*. Consistent with a *cis*-regulatory role of FLJ42351 we observed a preferential nuclear localization in the cHL cell lines. *SLC20A1* knockdown in HeLa and hepatic cell lines reduced cell proliferation,³⁸ and *SLC20A1* knockout mice showed increased apoptosis rates in erythroid cells.³⁹ We previously identified FLJ42351 as a MYC-induced transcript in P493-6 cells.¹⁷ In support of a putative *cis*-regulatory role of this lncRNA, we also identified *SLC20A1* as a positively correlating neighboring gene of FLJ42351 in our MYC-regulated lncRNA study. Thus, in summary, FLJ42351 is a HRS cell-expressed lncRNA whose expression pattern is similar to that of the neighboring protein-coding gene *SLC20A1*, which is known to have important functions in cell growth.

Despite several attempts, we were not able to efficiently knock down LINC00461 and FLJ42351 with siRNA- and lentiviral shRNA-based approaches. This precluded a further analysis to provide more definitive proof that these two lncRNAs can regulate the expression of the neighboring genes. Interestingly, both lncRNAs do have a DNase I hypersensitivity site, which is indicative of regulatory sequences (ie, approximately 15 kb upstream of the transcriptional start site of *LINC00461* in both L1236 and L428 cell lines⁴⁰ and approximately 45 kb downstream of the *FLJ42351* transcriptional start site in L1236).

In summary, our comprehensive expression analysis shows dynamic regulation of lncRNA expression during the GC transition of B cells and a widely deregulated expression in HL. These lncRNAs, together with the putative *cis*-acting data, provide a valuable source for further studies aiming at understanding the role of lncRNAs in normal B-cell biology and in the pathogenesis of HL.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.05.011>.

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