Long non-coding RNA *LINC00926* is a biomarker for naïve B-cells with prognostic value in advanced stage classic Hodgkin lymphoma

In classic Hodgkin lymphoma (HL), neoplastic Hodgkin and Reed-Sternberg cells (HRSC) account for less than 5% of the tumor mass. The remaining 95% of the tumor is composed of various cell types of the immune system. Among the tumor infiltrating lymphocytes, a high B-cell content in the tumor microenvironment (TME) of HL has been associated with favorable outcome. This phenomenon was demonstrated by gene expression profiling of the bulk tissue as well as whole slide image analysis.[1-3] Given the clinical relevance of B-cell content in HL, we and others have studied the composition of B-cell subtypes in the TME of HL patients and found that the number of naïve B cells correlates with B-cell content.[3,4] Recently, single-cell RNA sequencing (scRNA-Seq) showed that up to 30% of the cells in HL TME belong to B-cell clusters, with 70% of these cells being defined as naïve B cells.[5,6] The data also show that HL TME contains ten times less germinal center (GC) derived B cells relative to a reactive lymph node.[6] It is, therefore, reasonable to speculate that the prognostic value of B-cell content is associated with content of naïve B cells, more than with other B-cell subtypes.

Evaluation of tissue infiltrating naïve B cells requires detection of multiple surface markers,[4] which can be technically challenging. A single biomarker specific for infiltrating naïve B cells is not yet available. Given that the expression of long non-coding RNA (lncRNA) is known to be highly cell type specific relative to protein coding genes,[7] we sought to identify lncRNA transcripts that could be used as markers for the detection of naïve B cells in tissue sections. A similar analysis of lncRNA expression in normal B-cell subsets and in HL cell lines has been previously performed for the identification of HL-specific lncRNA transcripts.[8]

First, we explored publicly available gene expression data sets to search for B-cell specific transcripts.[9,10] The lncRNA *LINC00926* was selected for further analysis due to its expression pattern in purified subsets of peripheral blood mononuclear cells (PBMC) that was restricted to the B-cell lineage, similar to the B-cell marker *CD20* (Figure 1A, B). To confirm an expression pattern limited to B cells, we also interrogated other expression datasets available at the Genotype-Tissue Expression (GTEx) Portal, the Expression Atlas of the European Molecular Biology Labora-

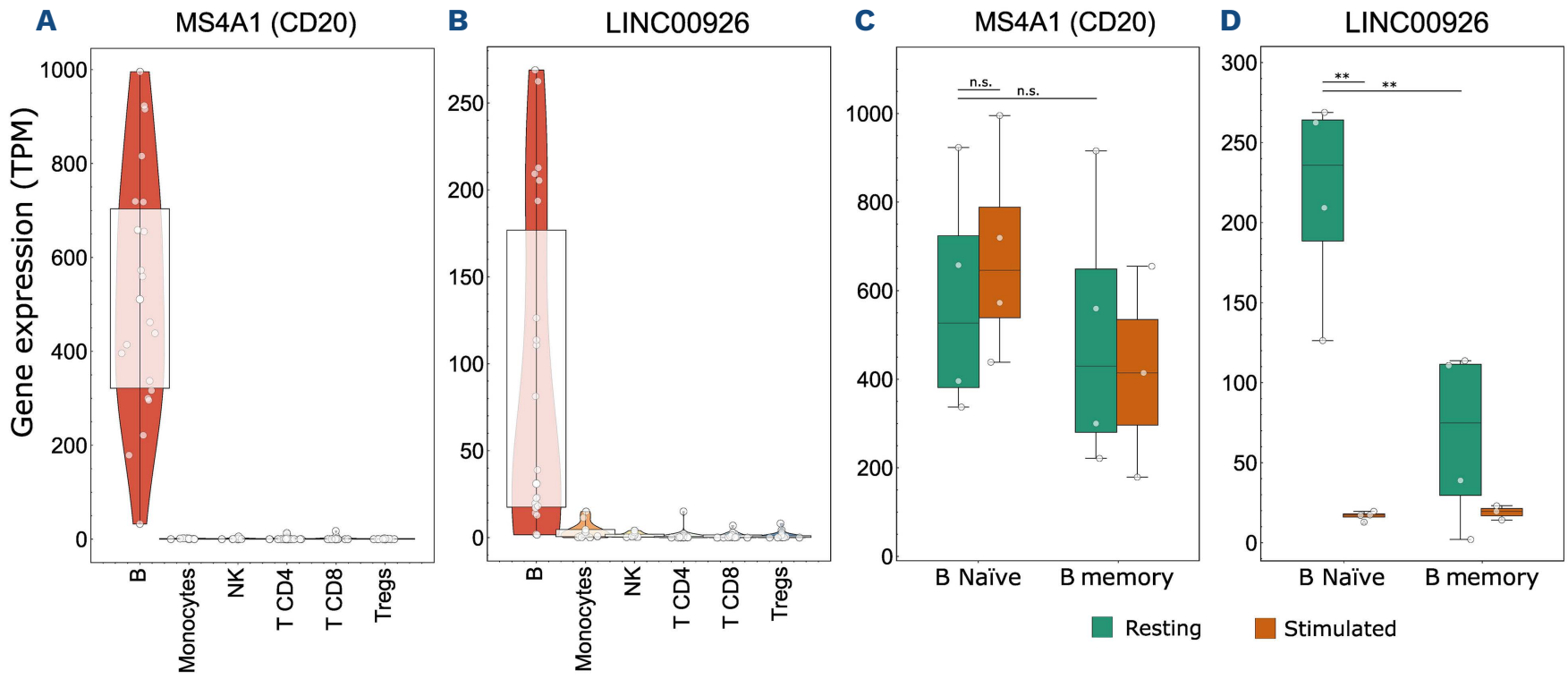


Figure 1. The lncRNA LINC00926 is specifically expressed in resting naïve B cells. RNA-Seq data from Calderon *et al.*[9] was analyzed for the expression of the *CD20* gene (*MS4A1*) and the lncRNA *LINC00926* in different peripheral blood mononuclear cells (PBMC) cellular components (A and B) and in naïve and memory B cells under resting condition or after stimulation with anti-IgG/IgM + IL-4 (C and D). The difference in expression between naïve resting and memory resting B cells, as well as between naïve resting and naïve stimulated B cells, was significant for *LINC00926* (**P<0.05) and not significant (n.s.) for *MS4A1*. NK: natural killer cells; TPM: transcripts per million; Tregs: regulatory T cells.

tory's European Bioinformatics Institute and the Cancer Cell Line Encyclopedia of the Broad Institute. Data from all these databases confirmed that *LINC00926* expression is restricted either to the blood or to lymphocyte rich organs (spleen, small intestine) or to cancer cells with B lymphocytic origin. Interestingly, in the Calderon dataset,[9] the transcript *LINC00926* was strongly down-regulated following B-cell activation in both naïve and memory B cells (Figure 1D). In the same dataset, expression of *CD20* was not differentially expressed comparing either naïve and memory B cells or stimulated and unstimulated cells (Figure 1C). These data indicate that *LINC00926* is a good marker for naïve B cells in PBMC.

Next, we asked if *LINC00926* could be considered a marker for naïve B cells also in HL tissue biopsies. This study was approved by the ethics commission of the Kiel University (D 562/24). To address this issue, we designed a nanoString custom panel covering several known cell type-specific protein coding genes together with a number of lncRNAs transcripts, including *LINC00926* (*Online Supplementary Table S1*) and analyzed 23 HL specimens of the NIVHAL trial.[11] Analysis of the expression data from the HL samples showed a strong correlation of expression between *LINC00926* and known B-cell marker genes like *CD19, CD20* (*MS4A1*) and *TCL1A* (Figure 2A). Furthermore, analysis of the 23 HL samples using the CIBERSORTx tool, that provides an estimation of the abundances of cell types in a mixed cell population, showed a strong correlation between the expression of *LINC00926* and the naïve B-cell content score of the same samples (Pearson = 0.796), but not with the memory B-cell content score (Pearson = 0.147). To confirm naïve B-cell restricted expression of *LINC00926*, we analyzed single cell RNA sequencing (scRNA-Seq) data published by the group of Steidl[5,6] (European Genome-phenome Archive, EGAD00001008270). The scRNA-Seq data confirmed that *LINC00926* expression was restricted to the B-cell lineage (*Online Supplementary Figure S1*). By focusing on the B-cell compartment, we found that cells with highest *LINC00926* expression level belonged to two B-naïve clusters: C1 and C3. A low percentage of memory B cells also expressed *LINC00926* but at a significantly lower level (*P*<10e-20). On the contrary, the gene coding for the pan B-cell marker *CD20* (*MS4A1*) was expressed at high level in all B-cell clusters and the *TCL1A* gene was strongly expressed in GC B cells. *CD19* showed high expression in naïve B cells but was also found in a high percentage of GC B and memory B cells (Figure 2B, C). As expected, *LINC00926* and other B-cell genes displayed highest expression in the lymphocyte rich HL subtype relative to the other subtypes and to reactive lymph nodes (Figure 2D).

To localize *LINC00926* expression *in situ*, we used RNAscope *in situ* hybridization (ISH). *LINC00926* expression was first analyzed in formalin-fixed paraffin-embedded (FFPE) sections of a reactive lymph node. The expression of *LINC00926* is mostly limited to cells with a lymphocyte morphology present in the mantle zone of GC and in the interfollicular space, areas know to harbor naïve B cells (*Online Supplementary Figure S2A*). Next, we analyzed FFPE sections of HL (nodular sclerosis type). In the HL TME, *LINC00926*-positive cells were observed sparsely distributed but distant to CD30-positive HRSC (*Online Supplementary Figure S2C*). Moreover, up to 80% of the *LINC00926*-positive cells co-expressed IgM, a typical feature of naïve B cells (*Online Supplementary Figure S2D*). Notably, HRSC were negative for *LINC00926* expression. In summary, the *LINC00926* can be considered a good marker for naïve B cells not only in PBMC but also in tissue samples from HL patients.

Given the existing correlation between B-cell content and clinical outcome in HL, we next asked if the expression of *LINC00926* can be used as marker for overall survival (OS) and/or progression-free survival (PFS) in HL. To answer this question, we used real time quantitative PCR (RT-qPCR) to measure the expression of *LINC00926* in RNA extracted from diagnostic biopsies of 99 patients with advanced stage HL treated within the HD15 and 78 biopsies of patients treated within the HD12 trials of the German Hodgkin Study Group.[12,13] Initially, we used the HD15 cohort as a training cohort to define the best cut-off value of *LINC00926* expression dividing good from bad responders. For this purpose, we evaluated all potential thresholds, performed a log rank test between the high and low-score groups for OS and PFS, and selected the threshold with the lowest *P* value corresponding to highest significance. We, therefore, used the identified cut-offs (0.111 for OS and 0.090 for PFS) to validate the association of *LINC00926* expression with OS and PFS in patients belonging to the HD12 cohort, respectively. Interestingly, this analysis showed a strongly significant (*P*=0.0045) difference in OS comparing patients with *LINC00926* expression below *versus* above the 0.111 cut-off (Figure 3). Differences in PFS, although showing the same trend, were not significant (*P*=0.31). Using Cox regression analysis, including the parameter *LINC00926* (below *vs*. above the threshold of 0.111), and the binarized international prognostic score (IPS>2) for Hodgkin lymphoma, *LINC00926* expression retained prognostic information for OS (N=78, 17 events, *P*=0.016). The corresponding analysis for PFS missed the level of significance (*P*=0.279). Finally, a significant association with OS was observed also testing for *LINC00926* as a continuous variable together with IPS (*P*=0.0143).

Interestingly, a correlation with *LINC00926* expression and a better prognosis in HL was suggested by a previous observation showing a higher *LINC00926* expression in HL biopsies of patients with late relapses in comparison with early relapse.[14]

In summary, given the predominance of cells of the naïve subtype among the B cells infiltrating HL tissue, we asked if the know prognostic value of B-cell content in HL was

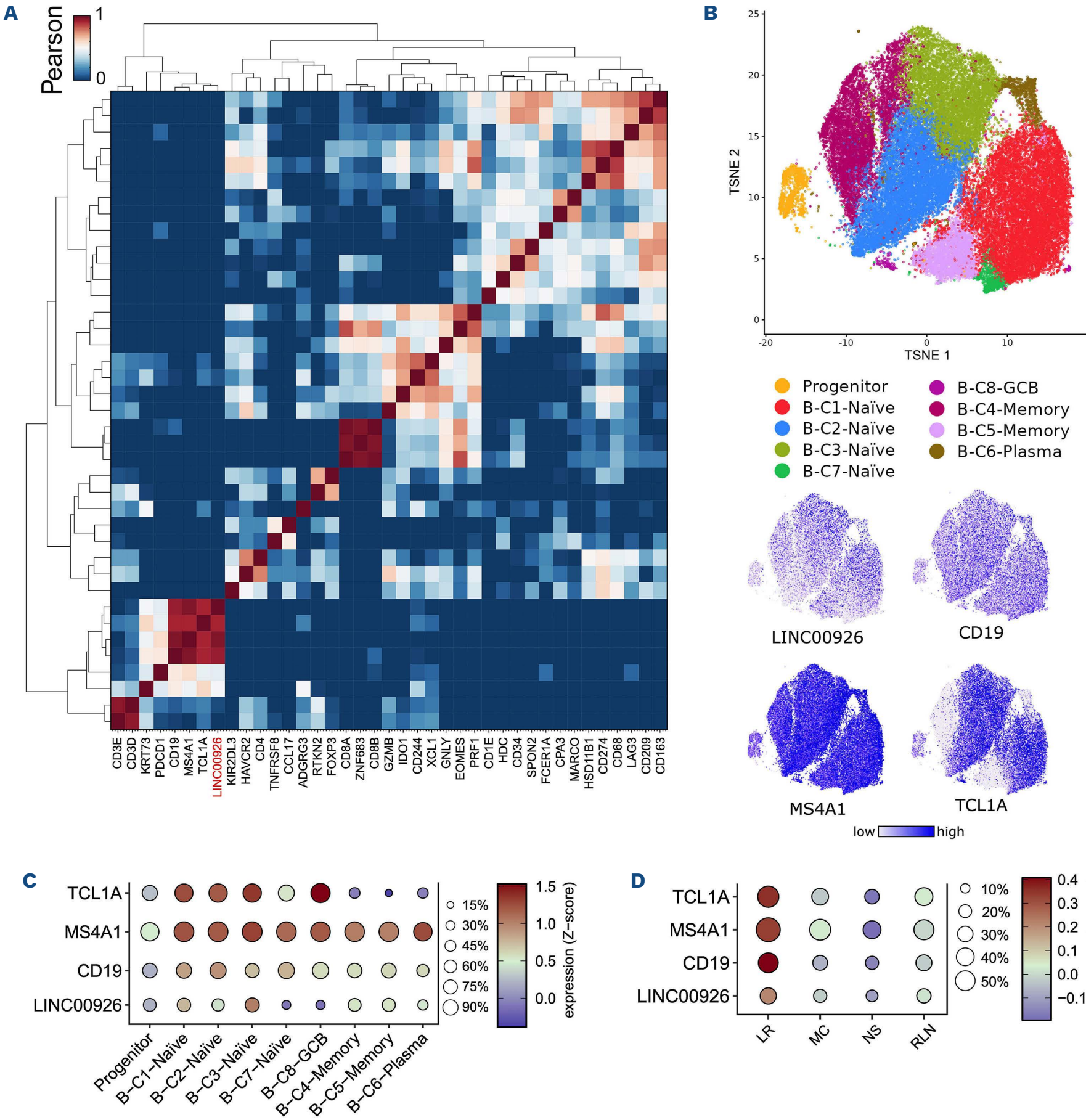
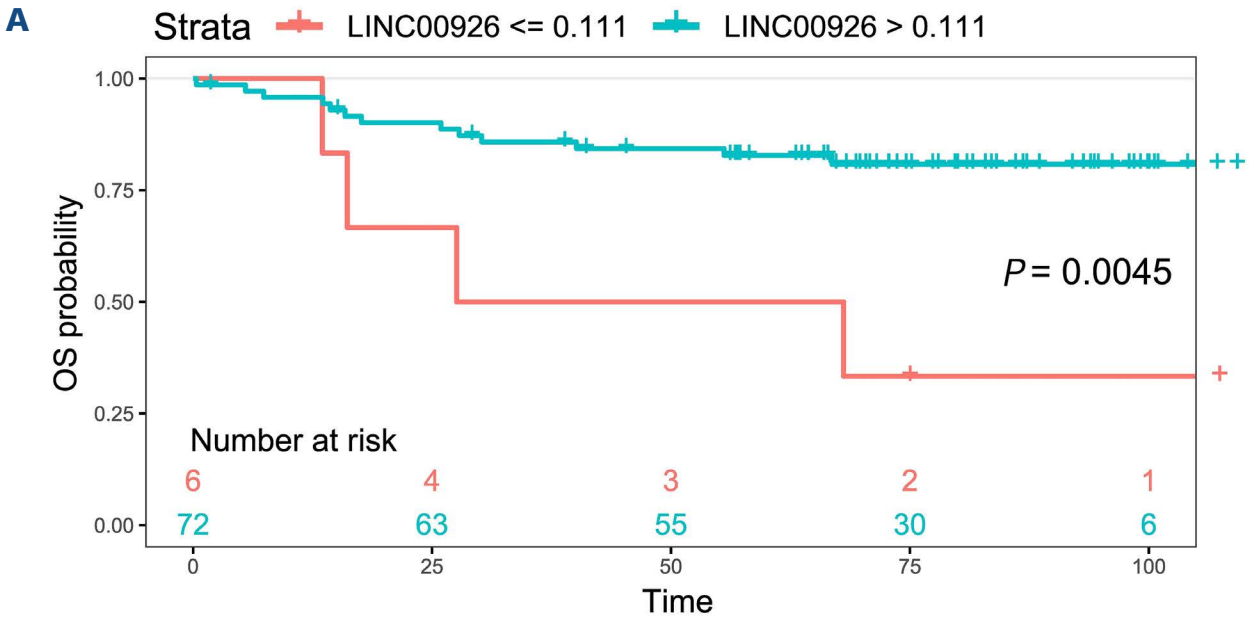


Figure 2. The lncRNA LINC00926 is expressed in naïve B cells in classic Hodgkin lymphoma samples. (A) Correlation matrix of nanoString expression data in 23 classical Hodgkin lymphoma (cHL) samples. The expression of *LINC00926* (in red) has a strong expression correlation with the B-cell marker genes *CD19, MS4A1* and *TCL1A*. Gene expression using the nanoString technology was performed using a custom panel targeting 95 genes of which: 24 corresponded to protein coding genes with a cell type expression pattern defined from nanoString annotations and from the literature; 15 protein coding genes predicted to have cell type restricted expression pattern by our own *in silico* analysis (*data not shown*); 43 lncRNA transcripts predicted to have a cell type restricted expression pattern; 13 housekeeping genes for expression normalization. A list of the selected genes and their predicted cell-type expression pattern is presented in the *Online Supplementary Table S1*. Expression data was processed using the nSolver software, as recommended by the manufacturer. (B) Single-cell expression of B cells from 28 cHL in tSNE space (first two dimensions), as in data from Aoki *et al.*[5,6] Cells are colored according to PhenoGraph cluster. (C) Bubble plot showing the expression level of the genes shown on the y axis, as well as the % of expressing cells in the clusters defined on the x axis. In the Bubble plot the diameter of the bubble is related to the % of cells expressing the analyzed gene and the color of the bubble to the intensity of expression. (D) Bubble plot showing the expression level of the genes shown on the y axis, as well as the % of expressing cells in the cHL subtypes defined on the x axis. The tSNE plots were generated using the scatter (1.32.1) R package. GCB: germinal center-derived B cells ; LR: lymphocyte rich; MC: mixed cellularity; NS: nodular sclerosis; RLN: reactive lymph node, as in Aoki *et al.*[5,6]



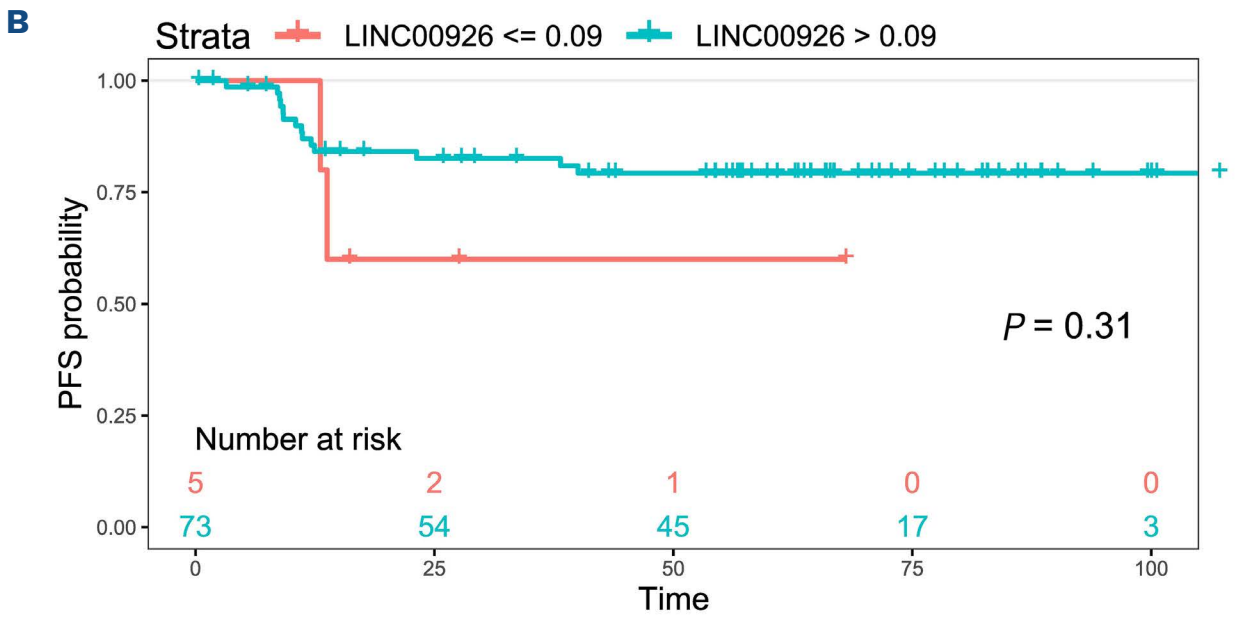


Figure 3. Overall survival and progression-free survival according to *LINC00926* expression. (A) Kaplan-Meier plots of overall survival (OS) and (B) progression-free survival (PFS) according to *LINC00926* expression below or above the cut-offs of 0.111 and 0.09, respectively. The expression of *LINC00926* and *GUSB* were analyzed by quantitative PCR using the TaqPath™ 1-Step Multiplex Master Mix (Thermo Fischer Scientific) and the TaqMan assay Hs03680805_m1 for *LINC00926*, and Hs00939627_m1 for *GUSB*. Reactions were performed in 20 µL containing 200ng RNA, 1 µL (1X) of each TaqMan Assay and 4 µL of TaqPath™ 1-Step Multiplex Master Mix. Amplifications were performed using a LightCycler 480 (Roche) with the following program: UNG Incubation: 25°C, 2 minutes (min); Reverse Transcription: 53°C, 15 min; Polymerase Activation: 95°C, 2 min; Amplification: 95°C, 3 seconds (sec) - 60°C, 30 sec; 45 cycles.

associated with naïve B cells. With the aim of identifying single biomarkers specific for infiltrating naïve B cells, we found that the lncRNA *LINC00926* can be used to quantify naïve B cells in HL tissue specimen and may serve as a prognostic biomarker for HL that is accessible by a single, widely available assay.

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Contributions

II and WK conceived the study; II performed analyses and analyzed data; TB analyzed scRNA-Seq data; SR performed image analyses and IHC; PB and BvT provided samples and data; MA performed bioinformatic analyses; II and WK wrote the manuscript.

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Data-sharing statement

Original data produced in the context of this study will be provided to other investigators upon request.

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