Differential gene expression profiling linked to tumor progression of splenic marginal zone lymphoma

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The genetic events that lead to aggressive transformation of cases of splenic marginal zone lymphoma (SMZL) after the chronic clinical stage have not been well understood. We aimed to find candidate genes associated with aggressive features of SMZL. We have successfully established two SMZL cell lines, designated SL-15 and SL-22, derived from the same patient's tumor clone in chronic and aggressive phases, respectively. Microarray analysis identified cell cycle-associated genes—specifically PLK1—as the most significantly upregulated in primary aggressive SMZL cells compared with cells from chronic phase. EPHA4 and MS4A1 (CD20) were found to be downregulated dramatically. These gene expression patterns were reproduced in both cell lines. Genetic knockdown of PLK1 resulted in inhibition of cell proliferation and induction of apoptosis in SL-22 cells, which expressed higher levels of PLK1 than SL-15 cells. SL-22 cells needed higher concentrations of chemical PLK1 inhibitors to achieve greater effects. In addition, we found homozygous deletion of the MS4A1 gene as a newly identified molecular mechanism of CD20-negative conversion. Our findings are expected to stimulate further studies on whether PLK1 could be a potential therapeutic target for this tumor. Furthermore, cases with CD20-negatively converted lymphomas should be screened for the genomic loss of MS4A1.

Splenic marginal zone lymphoma (SMZL), also called splenic lymphoma with villous lymphocytes, is a rare B-cell neoplasm involving the spleen, bone marrow, and usually peripheral blood. Most patients with SMZL show a chronic course with a median survival of around 10 years, whereas in a subset of patients the disease transforms to a more aggressive course with rapidly progressive and treatment-resistant form with increased mortality. In the last few years, molecular genetic studies have identified a plethora of somatic mutations in cases of SMZL. The most frequently mutated genes are KLF2 and NOTCH2, with a prevalence of 20–40% and ~10–25%, respectively. Inactivation of KLF2 and upregulation of NOTCH2 are involved in the physiological differentiation and proliferation of splenic marginal zone B cells, which might contribute to lymphomagenesis. However, the genetic changes underlying the transformation of SMZL into a high-grade aggressive malignancy remain unknown. Although recognition of the sequential gene expression profiles during progression from chronic to aggressive phases of SMZL is helpful in revealing markers for tumor progression, the rarity of the disease, coupled with a lack of suitable in vitro study systems, might have hindered the biologic and genetic investigation of the aggressive transformation of SMZL. This study aimed to identify candidate genes associated with aggressive features of SMZL.

One approach to understand malignant transformation is by comparing gene expression of tumor cells derived from a chronic phase to their evolved malignant counterparts. Cell lines represent invaluable tools for research on rare diseases such as SMZL. Our previous study described an SMZL cell line, SL-15, established form a tumor in a chronic phase. The case had a prolonged chronic clinical course with a good therapeutic response to monotherapy using the anti-CD20 monoclonal antibody rituximab, but later transformed into an aggressive disease. We have again successfully established another cell line, designated SL-22, from the transformed and

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aggressive tumor in the same patient. Comparison of the primary lymphoma cells as well as their evolved cell lines derived from a single patient with SMZL in two different phases of the disease has provided an opportunity to study sequential gene expression profiles during such transformation. In this study, microarray analysis showed a differential gene expression profile between SMZL cells derived from the chronic and aggressive clinical phases. We raised several therapeutic potential targets especially linked to cell cycle regulation, most notably PKLI, for further investigation of the genetic basis of SMZL transformation.

Rituximab-based treatment is a valid therapy for SMZL and is associated with a high overall response rate (~90%), with complete remission in more than half of these responding cases. Although consecutive treatment with rituximab further improves the complete remission rate in patients with SMZL, acquired resistance to this drug has become a considerable problem. Studies have suggested that loss of CD20 expression is a major mechanism in such resistance. Epigenetic mechanisms, in part, might contribute to the downregulation of CD20 expression, but the molecular mechanisms are still unclear. One of the major limitations in defining the mechanism of CD20-negative conversion from CD20-positive lymphomas after rituximab use is the lack of a laboratory model by which unlimited supplies of CD20-negative clones derived from CD20-positive cells can be studied repeatedly and extensively. So far, a few CD20-negative cell lines have been established from patients with CD20-positive lymphomas treated with rituximab. However, paired CD20-positive and -negative cell lines derived from the same clones before and after rituximab use, respectively, have been lacking. In this context, our two lymphoma cell lines, SL-15 and SL-22, are valuable for studying the negative conversion of CD20. By utilizing these cell lines, we show here that genomic deletion of the MS4A1 (CD20) gene is another molecular mechanism in the loss of CD20 expression.

Results
Comparative characterization of the cell lines. The Epstein–Barr virus (EBV)-immortalized SL-15 and SL-22 cell lines were established from a single patient with SMZL. The cell surface marker profile of SL-22 cells was similar to that of SL-15 cells, except for being negative for CD20 expression. The karyotype of SL-22 cells was identical to that of the primary SMZL cells at an aggressive phase (Fig. 1A), confirming that these cells were derived from the clone of the patient’s tumor cells. The two cell lines possessed common chromosome aberrations, including a unique t(9;14) chromosomal translocation involving 9q13 and 14q32, where PAX-5 and the immunoglobulin (Ig) heavy-chain gene are located, respectively, indicating that the SL-15 and SL-22 lines had evolved from the same clone. Southern blot analysis of DNA showed that SL-22 cells exhibited a rearrangement of the Ig heavy-chain gene bands identical to those of SL-15 cells (Fig. 1B), also signifying that the two cell lines were clonally identical. Clearly SL-15 and SL-22 cells are paired SMZL cell lines derived from the same clone.

Differential gene expression profiles between different clinical phases of SMZL. We compared gene expression profiles of the paired primary SMZL cells derived from the chronic (designated PB-15 cells) and aggressive (PB-22 cells) clinical phases using microarray analysis. A list of the differentially expressed genes was formed under criteria of 2.54-fold upregulation (Z-score > 2) and downregulation (Z-score < -2) in PB-22 cells compared with PB-15 cells (Table 1). A total of 1161 upregulated genes and 1112 downregulated genes were identified and further subjected to gene ontology (GO) analysis using the DAVID analysis. In this, the expression of genes associated with cell cycle regulation was involved in the aggressive transformation of the disease in our patient.

GO analysis was also performed on the downregulated genes. Annotation cluster 1 with the highest enrichment score of 28.63 included genes assigned to GO terms involved in cadherin and cell adhesion (Table 2). The EPHA4 (erythropoietin-producing hepatocellular receptor A4) gene, which was the most strongly downregulated gene (Table 1), belonged to the category of cell adhesion in this cluster. Annotation cluster 2 with an enrichment score of 13.91 contained genes linked to cell membrane functions, including membrane-spanning 4A (MS4A) family genes such as MS4A1 (CD20) and MS4A7. KEGG pathway analysis also identified the MS4A family of genes in the inclusive hematopoietic cell lineage pathway as being significantly downregulated (Table 2).

Changes in the expression levels of KLF2 and NOTCH2 were also investigated. Our microarray analysis showed that KLF2 expression was significantly downregulated in PB-22 cells (18.5-fold lower; Table 1). A higher expression of NOTCH2 (1.5-fold higher) was observed, although this was not significant.

Validation of microarray gene expression profiles. The differentially upregulated genes in the cell cycle pathway were validated by real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) on primary lymphoma cells (PB-22 versus PB-15 cells) and their evolved cell lines (SL-22 versus SL-15 cells) in three separate experiments. The cell cycle pathway included genes regulating cell proliferation and mitosis. The selected genes were PLK1, E2F2, MAD2L1, AURKB, CDC5, CCNA2, CCNB1, CCNB2, CDK1, CDK2, PTTG1, and UBEC. The differential expression patterns were confirmed for all genes in both the primary tumors and cell lines (Fig. 2A). Among the cell cycle related-genes upregulated, PLK1 showed the greatest difference in expression, as demonstrated by both microarray analysis and RT-qPCR. Immunoblot analysis also validated differential expression patterns of the protein polo-like kinase 1 (PLK1; Fig. 2B).

We also validated the expression levels of EPHA4 by RT-qPCR, because microarray analysis identified this as the most downregulated gene (Table 1). The EPHA4 expression levels were dramatically suppressed in PB-22 (P < 0.001) and SL-22 cells (P = 0.020) compared with those in PB-15 and SL-15 cells, respectively (Fig. 2A). The reduced expression of EphA4 in PB-22 and SL-22 cells was also confirmed at the protein level (Fig. 2B).
The RT–qPCR results showed that expression of \textit{KLF2} and \textit{NOTCH2} was significantly downregulated and upregulated, respectively, in PB-22 cells (\textit{KLF2}: \( P = 0.010 \); \textit{NOTCH2}: \( P = 0.002 \)) and SL-22 cells (\textit{KLF2}: \( P = 0.016 \); \textit{NOTCH2}: \( P = 0.018 \); Fig. 2A). Given these results, we next tested the \textit{KLF2} and \textit{NOTCH2} genes for the presence of somatic mutations. We screened the entire coding regions of \textit{KLF2} (exons 1–3) and \textit{NOTCH2} exon 34 in which somatic mutations are frequently reported\(^7,9,10\). Somatic mutations were not found in PB-15 and PB-22 cells and cell lines SL-15 and SL-22, except for a silent mutation, c.528C > A, in \textit{KLF2} exon 1.

\textbf{Antiproliferative effect of PLK1 inhibition evaluated using PLK1-specific small hairpin RNA (shRNA) and small interfering RNA (siRNA).} PLK1, belonging to the family of serine/threonine protein kinases, plays a key role in centrosome maturation, bipolar spindle formation, and cytokinesis during mitosis\(^{19}\).
It is likely to be one of the key molecular candidates associated with malignant transformation of SMZL, and thus could be a potential therapeutic target. In this context, we assessed the effects of PLK1 inhibition through applying PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). Likewise, transduction of siRNA targeting different sequence of PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). Likewise, transduction of siRNA targeting different sequence of PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). Likewise, transduction of siRNA targeting different sequence of PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). Likewise, transduction of siRNA targeting different sequence of PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). Likewise, transduction of siRNA targeting different sequence of PLK1-specific shRNA on the proliferation and apoptosis of SL-22 cells. Transduction of this shRNA into the cells resulted in significant decreases in PLK1 gene expression at the RNA and protein levels compared with transduction of control shRNA (Fig. 3A,B). Genetic knockdown of PLK1 caused a significant decrease in cell proliferation and an increase in apoptosis in SL-22 cells (Fig. 3C,D). Next, we conducted experiments to determine whether the antiproliferative effects on PLK1 inhibition would be mediated through cell cycle inhibition. Inhibition of PLK1 expression caused a significant increase in the proportion of the cell population at the G2/M phase of the cell cycle (Fig. 3E). 

### Differential sensitivity to chemical inhibition of PLK1

Next, we performed experiments to determine whether there would be a difference in the antiproliferative effect of chemical inhibition of PLK1 between SL-15 and SL-22 cell lines. Both cell lines showed no difference in cell growth in RPMI 1640 medium supplemented with 10% fetal calf serum without PLK1 inhibitors (Supplementary Fig. S2). The cells were treated with various concentration of volasertib, a selective PLK1 inhibitor, for 48 h. This resulted in efficient growth inhibition of both cell types at higher concentrations, but the two cell lines had differing sensivities to this drug (Fig. 4A). The 50% growth inhibition (EC50) value of SL-22 (25 nM) was 1.9-fold higher than SL-15 (13 nM). The EC50 values (42 nM for SL-22 and 17 nM for SL-15) distinguished the drug sensitivity more clearly, with a 2.5-fold difference. These differences in drug sensitivity were apparent when volasertib was used at concentrations ranging from 20 to 40 nM. Similar results were obtained when another PLK1 inhibitor, BI 2536, was used (data not shown).

Exposure of SL-15 and SL-22 cells to volasertib for 24 h induced apoptosis in both lines. However, consistent with the results of the cell proliferation assay, SL-15 cells were more susceptible to apoptosis than SL-22 cells at 50 nM (P < 0.01; Fig. 4B). We next compared the effects of volasertib on the cell cycle between SL-15 and SL-22

| Gene symbol | Description | Gene symbol | Description |
|-------------|-------------|-------------|-------------|
| TLR5        | toll-like receptor 5 | EPHA4   | EPH receptor A4 |
| SYT1        | synaptotagmin XVII | MAPT    | microtubule-associated protein tau |
| RGS7        | regulator of G-protein signaling 7 | GLYCTK | glyceraldehyde kinase |
| FGFRL1      | fibroblast growth factor receptor-like 1 | RBP7    | retinol binding protein 7, cellular |
| BTN1L9      | butyrophilin-like 9 | ADAMTS9 | ADAM metalloprotease with thrombospondin type 1 motif 9 |
| NPA54       | neuronal PAS domain protein 4 | MS4A1   | membrane-spanning 4-domains, subfamily A, member 1 |
| GLRA3       | glycine receptor, alpha 3 | NRN1L   | neurtin 1-like |
| PLK         | polo-like kinase 1 | RGS4    | regulator of G-protein signaling 4 |
| DMXL1       | Dmxd-like 1 | TSPAN15 | tetraspanin 15 |
| LIFR        | leukemia inhibitory factor receptor alpha | SOX5    | SRY (sex determining region Y)-box 5 |
| HMBOX1      | homeobox containing 1 | TNFRSF8 | tumor necrosis factor receptor superfamily, member 8 |
| LHPPL2      | lipoma HMGC fusion partner-like 2 | MS4A7   | membrane-spanning 4-domains, subfamily A, member 7 |
| ZNF90       | zinc finger protein 90 | RAB31   | RAB31, member RAS oncogene family |
| CRYM        | crystallin, mu | DENR    | density-regulated protein |
| NXT1        | NTF2-like export factor 1 | DHR54   | dehydrogenase/reductase (SDFR family) member 4 |
| ZNF407      | zinc finger protein 407 | NOMO1   | NODAL modulator 1 |
| CHI3L2      | chitinase 3-like 2 | MEF2C   | myocyte enhancer factor 2 C |
| ACACB       | acetyl-CoA carboxylase beta | SORL1   | sortilin-related receptor, L(DLR class) A repeats-containing |
| TTC39B      | tetratricopeptide repeat domain 39B | MACROD2 | MACRO domain containing 2 |
| CDCP1       | CUB domain containing protein 1 | EPDR1   | ependymin related protein 1 (zebrafish) |
| NBL         | nebulette | SDK1    | sidekick homolog 1, cell adhesion molecule (chicken) |
| PLN         | phospholamban | TFEC    | transcription factor EC |
| MYRPC2      | myosin binding protein C, fast type | HEATR1  | HEAT repeat containing 1 |
| TTC3        | tetratricopeptide repeat domain 3 | MYOM1   | myomesin 1 |
| FETUB       | fetuin B | OSBP10  | oxysterol binding protein-like 10 |
| CLDN11      | claudin 11 | DDX60L  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like |
| CSor93      | chromosome 9 open reading frame 93 | PTPRU   | protein tyrosine phosphatase, receptor type, U |
| HBD         | hemoglobin, delta | ARHGAP18 | Rho GTPase activating protein 18 |
| FLJ37543    | hypothetical protein FLJ37543 | KLF2    | Kruppel-like factor 2 |
| SPAG1       | sperm associated antigen 1 | PLEKHA2 | pleckstrin homology domain containing, family A (phosphonosidase binding specific) member 2 |

**Table 1.** Top 30 genes of 1161 upregulated and 1112 downregulated genes in PB-22 cells identified as >2.54-fold (Z-score: >2 or <−2) compared with PB-15 cells. *The underlined genes were investigated further in this study.*
| Category               | Term                                                                 | Count | P-value   | Benjamini |
|------------------------|----------------------------------------------------------------------|-------|-----------|-----------|
| UP_KEYWORDS            | Cell cycle^1                                                          | 90    | 5.97E-17  | 5.01E-14  |
| UP_KEYWORDS            | Cell division^1                                                       | 59    | 6.10E-13  | 9.16E-11  |
| UP_KEYWORDS            | Mitosis^1                                                            | 46    | 1.99E-12  | 2.24E-10  |
| GOTERM_BP_DIRECT       | Cell division (GO:0051301)                                           | 48    | 2.95E-08  | 1.00E-04  |
| GOTERM_BP_DIRECT       | Mitotic nuclear division^1 (GO:0007067)                               | 36    | 5.26E-07  | 4.47E-04  |

Enriched KEGG pathways for the upregulated genes

| Category               | Term                                                                 | Count | P-value   | Benjamini |
|------------------------|----------------------------------------------------------------------|-------|-----------|-----------|
| KEGG_PATHWAY           | Cell cycle^1 (hsa04110)                                              | 25    | 1.16E-07  | 3.01E-05  |
| KEGG_PATHWAY           | p53 signaling pathway (hsa04115)                                     | 13    | 3.85E-04  | 4.87E-02  |
| KEGG_PATHWAY           | Progesterone-mediated oocyte maturation (hsa04914)                   | 14    | 1.34E-03  | 1.09E-01  |
| KEGG_PATHWAY           | Oocyte meiosis (hsa04114)                                            | 16    | 1.39E-03  | 8.64E-02  |
| KEGG_PATHWAY           | Transcriptional misregulation in cancer (hsa05202)                   | 20    | 3.61E-03  | 1.71E-01  |
| KEGG_PATHWAY           | Malaria (hsa05144)                                                   | 9     | 6.44E-03  | 2.43E-01  |
| KEGG_PATHWAY           | Jak-STAT signaling pathway (hsa04630)                                | 17    | 9.17E-03  | 2.89E-01  |
| KEGG_PATHWAY           | Biosynthesis of amino acids (hsa01230)                               | 11    | 9.59E-03  | 2.68E-01  |

Enriched gene ontology functions for the downregulated genes

| Annotation Cluster 1  | Enrichment Score: 28.63     | Count | P-value   | Benjamini |
|------------------------|-------------------------------|-------|-----------|-----------|
| INTERPRO               | Cadherin, N-terminal (IPR013164) | 42    | 1.05E-35  | 1.61E-32  |
| UP_SEQ_FEATURE         | domain:Cadherin 6            | 46    | 1.54E-35  | 4.9E-32   |
| UP_SEQ_FEATURE         | domain:Cadherin 5            | 49    | 2.59E-33  | 4.11E-30  |
| UP_SEQ_FEATURE         | domain:Cadherin 2            | 50    | 3.39E-32  | 3.59E-29  |
| UP_SEQ_FEATURE         | domain:Cadherin 1            | 50    | 3.39E-32  | 3.59E-29  |
| UP_SEQ_FEATURE         | domain:Cadherin 4            | 49    | 1.05E-31  | 8.32E-29  |
| UP_SEQ_FEATURE         | domain:Cadherin 3            | 49    | 1.05E-31  | 8.32E-29  |
| INTERPRO               | Cadherin (IPR002126)         | 49    | 7.89E-30  | 6.03E-27  |
| INTERPRO               | Cadherin conserved site (IPR020894)                                 | 48    | 9.35E-30  | 4.77E-27  |
| INTERPRO               | Cadherin-like (IPR015919)    | 49    | 1.98E-29  | 7.56E-27  |
| SMART                  | Cadherin repeats (SM00112)   | 49    | 6.76E-28  | 2.18E-25  |
| UP_KEYWORDS            | Cell adhesion^1             | 92    | 1.86E-27  | 8.65E-25  |
| GOTERM_BP_DIRECT       | Homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156) | 53    | 3.02E-26  | 1.06E-22  |
| UP_KEYWORDS            | Calcium                     | 121   | 1.12E-22  | 1.74E-20  |
| GOTERM_MF_DIRECT       | Calcium ion binding (GO:0005509)                                     | 95    | 1.07E-14  | 1.21E-11  |

Enriched KEGG pathways for the downregulated genes

| Category               | Term                                                                 | Count | P-value   | Benjamini |
|------------------------|----------------------------------------------------------------------|-------|-----------|-----------|
| UP_KEYWORDS            | Cell membrane^1                                                       | 294   | 1.20E-24  | 2.79E-22  |
| GOTERM_CC_DIRECT       | plasma membrane (GO:0005886)                                         | 351   | 5.24E-20  | 2.89E-17  |
| UP_SEQ_FEATURE         | glycosylation site-N-linked (GlcNAc)                                 | 342   | 4.47E-18  | 2.84E-15  |
| UP_KEYWORDS            | Glycoprotein                                                          | 358   | 4.52E-18  | 5.26E-16  |
| UP_KEYWORDS            | Membrane                                                              | 520   | 5.24E-16  | 5.17E-14  |
| UP_SEQ_FEATURE         | topological domain:Extracellular                                     | 238   | 2.10E-14  | 9.52E-12  |
| GOTERM_CC_DIRECT       | integral component of plasma membrane (GO:0005887)                   | 146   | 1.27E-13  | 3.50E-11  |
| UP_SEQ_FEATURE         | topological domain:Cytoplasm                                         | 273   | 1.59E-12  | 5.60E-10  |
| UP_KEYWORDS            | Transmembrane                                                         | 396   | 1.05E-11  | 8.15E-10  |
| UP_KEYWORDS            | Transmembrane helix                                                  | 394   | 1.64E-11  | 1.09E-09  |
| UP_SEQ_FEATURE         | transmembrane region                                                 | 362   | 5.12E-11  | 1.63E-08  |
| GOTERM_CC_DIRECT       | integral component of membrane (GO:0016021)                          | 334   | 4.06E-04  | 3.66E-02  |

Enriched KEGG pathways for the downregulated genes

| Category               | Term                                                                 | Count | P-value   | Benjamini |
|------------------------|----------------------------------------------------------------------|-------|-----------|-----------|

Continued
cells. Although chemical inhibition of PLK1 caused an increase in the proportions of cells at the G2/M phase in a dose-dependent manner in both cells, this was significantly greater in SL-15 than in SL-22 cells at 50 nM (P < 0.01; Fig. 4C). Collectively, these results suggest that, compared with SL-15 cells, SL-22 cells harboring a higher expression of PLK1 needed higher levels of volasertib to be killed.

**Genomic deletion of the MS4A1 (CD20) gene.** PB-22 cells sampled when the tumor was refractory to rituximab-based treatment showed a CD20-negative phenotype. RT–qPCR showed that MS4A1 mRNA was detected in PB-15 and SL-15 cells, whereas the transcript was not expressed in PB-22 or SL-22 cells. As expected, microarray analysis demonstrated over a 200-fold downregulation of MS4A1 expression in PB-22 cells compared with PB-15 cells (Table 1). The MS4A1 gene, located on chromosome 11q12, belongs to the MS4A gene family with at least 18 subgroups (MS4A1–MS4A18)20. Karyotypes of PB-22 and SL-22 cells had no chromosomal translocations and deletions involving the 11q12 region, so we suspected a partial genomic deletion around MS4A1 in these cells. Confirming this, we identify a homozygous MS4A1 deletion (Fig. 5). We explored the deleted span of the genomic DNA on chromosome 11q12. A 487-kilobase (kb) region from MS4A1 to MS4A13 of the genomic DNA on chromosome 11q12. A 487-kilobase (kb) region from

| Enriched gene ontology functions for the upregulated genes | Enrichment Score: 10.79 | Count | P-value | Benjamini |
|---|---|---|---|---|
| KEGG_PATHWAY | Natural killer cell mediated cytotoxicity (hsa04650) | 19 | 1.76E-04 | 4.55E-02 |
| KEGG_PATHWAY | cAMP signaling pathway (hsa04024) | 24 | 8.63E-04 | 1.08E-01 |
| KEGG_PATHWAY | cGMP-PKG signaling pathway (hsa04022) | 20 | 2.82E-03 | 2.21E-01 |
| KEGG_PATHWAY | Hematopoietic cell lineage (hsa04640) | 13 | 3.04E-03 | 1.83E-01 |
| KEGG_PATHWAY | Amphetamine addiction (hsa05031) | 11 | 3.95E-03 | 1.89E-01 |
| KEGG_PATHWAY | Tuberculosis (hsa05152) | 20 | 5.74E-03 | 2.24E-01 |
| KEGG_PATHWAY | Cocaine addiction (hsa05030) | 9 | 6.07E-03 | 2.06E-01 |
| KEGG_PATHWAY | Neuroactive ligand-receptor interaction (hsa04080) | 27 | 8.29E-03 | 2.41E-01 |
| KEGG_PATHWAY | Insulin secretion (hsa04911) | 12 | 8.65E-03 | 2.26E-01 |
| KEGG_PATHWAY | Adrenergic signaling in cardiomyocytes (hsa04261) | 17 | 8.91E-03 | 2.11E-01 |

Table 2. Enriched gene ontology (GO) functions and KEGG pathways for the upregulated and downregulated genes. 1The underlined terms include PLK1. 2The underlined term includes EPHA4. 3The underlined terms include MS4A1 (CD20) and MS4A7.
Figure 2. Expression analysis of the target genes in primary SMZL cells (PB-15 and PB-22 cells) and their evolved cell lines (SL-15 and SL-22 cells). (A) Analysis of mRNA expression. Differential expressions of genes related to the cell cycle, which showed the highest enrichment score by microarray analysis, were validated by RT–qPCR. Expression levels of EPHA4, the most downregulated gene shown by microarray analysis, KLF2 and NOTCH2 were also analyzed. Ratios of the expression levels in PB-15 versus PB-22 cells (left panel) and ratios of SL-15 versus SL-22 cells (right panel) are plotted. Data are shown as the mean ± standard deviation (SD) of three independent experiments. (B) Analysis of protein expression. Immunoblotting analysis showed upregulation of PLK1 and downregulation of EphA4 in PB-22 (left panel) and SL-22 cells (right panel) compared with PB-15 and SL-15 cells, respectively. Intensities of the bands obtained by immunoblotting were quantified and normalized to the levels of β-actin. The relative amounts of PLK1 and EphA4 in PB-22 cells and SL-22 cells were also normalized to the level (value = 1) for PB-15 cells and SL-15 cells. Data are shown as the mean ± SEM of the three separate experiments. Significant expression differences are shown as *P < 0.05; **P < 0.01. The full-length blots are presented in Supplementary Fig. S3.
suggesting that higher PLK1 expression appears to be associated with a more severe grade of malignancy. Therefore, the role of PLK1 on cell proliferation and apoptosis was investigated further using the SMZL cell lines. Genetic knockdown of PLK1 through shRNA and siRNA-mediated RNA interference caused a reduction in cell proliferation through cell cycle inhibition and an increase in apoptosis. Although the selective PLK1 inhibitor volasertib also showed antiproliferative effects in both SL-15 and SL-22 cells, there were clear associations between the levels of PLK1 expression and the sensitivity of cells to volasertib. SL-22 cells, which expressed higher levels of PLK1 than SL-15 cells, needed higher concentrations of volasertib to achieve more efficient inhibition of cell proliferation and induction of apoptosis. The EC50 value of SL-22 shown in this study was similar to that of multiple cell lines derived from various cancer tissues, including carcinomas of the colon (HCT 116, EC50 = 23 nM) and lung (NCI-H460, EC50 = 21 nM), as shown in previous studies. Thus, these data suggest that volasertib could serve as a potential therapeutic agent against PLK1-expressing SMZL tumors, as shown in many forms of solid cancer. Indeed, PLK1 overexpression has been found in a variety of cancers in advanced stages, and several PLK1 inhibitors are currently in various stages of clinical trials. In certain cancer types, such as invasive breast cancers.
and renal cell carcinomas, *PLK1* has significantly higher expression levels in late than in early stages, which is in line with the results of the present study. Among hematological malignancies, *PKL1* is often overexpressed in acute myeloid leukemia. A few studies have suggested that high-grade non-Hodgkin's lymphomas show a trend toward higher expression levels of *PLK1* than low-grade forms. Our findings suggest that upregulation

Figure 4. Effects of volasertib on cell proliferation, apoptosis and cell cycle. SL-15 and SL-22 cells were cultured in the presence of various concentrations of volasertib. (A) Cell proliferation assay. Exposure of the cells to volasertib for 48 h reduced the viable cells, but the two cell lines had diverse sensitivity to treatment with the drug. (B) Apoptosis assay. Cells were treated with volasertib at the indicated concentrations for 24 h. Susceptibility to apoptosis was different between the two cell lines at 50 nM. (C) Cell cycle analysis. After treatment of the cells with volasertib at the indicated concentrations for 24 h, cell cycle stage distributions are determined. Percentages of the cell population in each stage of the cell cycle are presented outside the graph. All experiments were independently repeated three times and data are expressed as the mean ± SEM. Significant expression differences are shown as *P* < 0.05; **P** < 0.01.
of PLK1 might be involved in the biological aggressiveness of SMZL and promote its progression. In this context, although high levels of PLK1 expression should be confirmed in more patients with advanced SMZL, clinical management with combination chemotherapy including PLK1 inhibitors is worth studying in the future.

It is notable that CD20 expression was lost in the late-stage tumor cells in our patient. Several studies have suggested that epigenetic mechanisms are linked to the loss of CD20 expression after rituximab treatment, and that the expression can be restored by DNA methyltransferase inhibitors and histone deacetylase inhibitors14–17. Our study revealed a genomic deletion of the entire MS4A1 gene along with its neighboring genes on chromosome 11q12. Based on the literature to date, Nakamaki et al.11 reported a case in which the MS4A1 gene was deleted after rituximab-containing chemotherapy for the treatment of diffuse large B-cell lymphoma (DLBCL). Their report showed that genomic loss was observed intensively around a region including MS4A1 and MS4A5, and spanned a 700-kb region involving some genes of the MS4A family. Consistent with that report, here we showed the genomic loss of a 600-kb segment around MS4A1. Loss of CD20 expression leads to the potential loss of a therapeutic target during relapse and/or disease progression of CD20-positive B-cell malignancies, and is often associated with poor prognosis for the patients at that point6,12. Accordingly, clinicians and researchers should note that genomic losses around MS4A1 are missed by conventional karyotyping, so it is necessary to recognize this genomic MS4A1 deletion as a new molecular mechanism for CD20-negative conversion.

Our microarray identified EPHA4 as the most downregulated gene. GO analysis revealed that EPHA4 belonged to the category of ‘cell adhesion’ in annotation cluster 1 with the highest enrichment score. For these reasons, we payed attention to EPHA4 as a representative downregulated gene. EPHA4 downexpression was verified at both the RNA and protein levels by RT–qPCR and immunoblotting, respectively. Epha comprises the largest family of receptor tyrosine kinases, being composed of nine EphA and five EphB33. Recent evidence indicates that the Eph receptors have both tumor-promoting and suppressing activities, depending on their expression pattern in different tumor types; thus, some of the EPH genes are oncogenic and are upregulated in various cancers33. On the other hand, the EPH genes can act as tumor suppressors, and loss of their expression is evident in some tumors, for example, EphB4 in colorectal and breast cancers34,35. Likewise, Epha4 has also been found to have a multifaceted function as a tumor suppressor and promoter in some solid cancers33,36–38. However, its role in the pathogenesis of hematological malignancies has not been fully determined. DNA methylation of EPHA4 has been observed in cases of acute lymphoblastic leukemia39, and Epha4 expression has been shown to inhibit lymphocyte proliferation40. Thus, a potential role of EphA4 as a tumor suppressor in lymphoid malignancies is currently receiving increasing attention. We hypothesize that signaling pathways involved in EphA4 might be associated with the aggressive transformation of SMZL, and this should be clarified by further studies. Of note, Koivula et al.41 showed that a low level of EPHA4 expression was associated with poor overall survival in patients with DLBCL. Interestingly, EPHA4 was found to be one of the most important genes associated with the responsiveness to rituximab in cases of B-cell lymphoma41. In this context, it is plausible that the dramatic downregulation of EPHA4 might be caused, in part, in association with CD20 downregulation, thereby directly or indirectly contributing to the poor prognosis in our patient.

In summary, we have presented a differential gene expression profile associated with tumor progression of SMZL, and have identified specific genes for further studies to identify the molecules involved in the transformation process of this disease. Some of the gene expression changes reported here, specifically PLK1, might be involved in the biological aggressiveness of SMZL and could serve as potential therapeutic targets. Although a limitation of the current study was that only one case of transformed SMZL and a pair of cell lines was analyzed,
future studies promise to elucidate the important issues. Another limitation of our study is the use of EBV immortalization to create the SMZL cell lines. Although their differential gene expression profile was confirmed in the primary SMZL cells, the experimental results should be interpreted with caution. If our findings are confirmed, we hope that PKL1 inhibitors will prove efficacious in improving the outcome of patients with advanced SMZL who have limited therapeutic options. Furthermore, we have demonstrated a homogenous MS4A1 deletion as a unique molecular mechanism of CD20-negative relapse in a patient with B-cell lymphomas. This finding suggests that cases of B-cell lymphomas with loss of CD20 should be screened for the genomic loss of MS4A1. Such screening will help identify patients who need early intensive treatments including stem cell transplants to overcome a CD20-negative relapse of B-cell lymphomas, because genomic deletion of MS4A1 appears to be an irreversible event that leads to the permanent loss of the immunotherapeutic target, and ultimately to reduced survival of the patient.

Materials and Methods

Cell lines. The EBV-immortalized SL-15 cell line was established from a 53-year-old man with SMZL in a chronic phase. The detailed characteristics have been reported11. The SL-15 line was demonstrated to be derived from the clone of the patient’s primary lymphoma.

Complete remission was achieved in this patient after rituximab-based treatment, but the disease relapsed three times. After 3 years and 4 months of repeated rituximab monotherapy for each relapsed disease, the patient developed bilateral pleural effusions and ascites infiltrated with lymphoma cells. The patient died of the progressive disease with resistance to rituximab-inclusive combination chemotherapy when his white blood cell count was 60.0 × 10^9/l with 70% lymphoma cells. The immunophenotype of these cells was similar to that of the lymphoma cells at diagnosis, with the exception that CD20 expression became negative. The karyotype of the lymphoma cells was 47, XY, add(3)(p13), add(3)(p13), t(9;14)(p13;q32), add(10)(q24), add(11)(q21), +add(11), der(11;13)(q10;q10), +12, and add(16)(p11.2), showing a close resemblance to that of the lymphoma cells at diagnosis11. These findings indicated that the lymphoma cells from the pre- and post-rituximab therapy were of the same clonal origin. Following informed consent from the patient, peripheral blood was obtained 2 weeks before his death, and mononuclear cells were separated by Ficoll–Hypaque density gradient centrifugation. The cells were cultured under the same conditions used for the establishment of SL-1511. The cells began to proliferate after a week from initiation of the culture and could then be regularly passaged. The cell line was designated SL-22 and characterized as described11. This study was approved by the Ethics Committee of Kochi Medical School, Kochi University, Japan. All experiments were performed in accordance with the relevant guidelines and regulations.

Oligonucleotide microarray. The CodeLink Human Whole Genome Bioarray (Applied Microarrays, Tempe, AZ, USA) was used to define and compare gene-expression profiles between primary SMZL cells derived from the chronic and aggressive clinical phases. The array targets most of the known and predicted genes of the human genome, and is composed of approximately 55,000 probes designed to blind to conserved exons. Labeling of complementary DNA targets, hybridization, and scanning of the arrays were carried out following the manufacturer’s instructions. Raw intensity measurements of all probe sets were background-corrected, normalized, and converted into expression measurements using the MicroArray Data Analysis Tool Version 3.2 (Filgen, Nagoya, Japan). All microarray data were submitted to Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE94318. Differentially expressed genes were identified using a cutoff fold change of >2.54. GO analysis and pathway analysis (KEGG_PATHWAY) were performed using the DAVID Bioinformatics Resource 6.7 online software (https://david.ncifcrf.gov/).

Real-time quantitative reverse-transcription polymerase chain reaction (RT–qPCR). Real-time RT–qPCR was used to validate selected data from microarray experiments in both primary lymphoma cells and their corresponding cell lines. Total RNA was extracted using High Pure RNA Tissue kits (Roche Diagnostics, Tokyo, Japan). The total RNA was treated with DNase to avoid any amplification of genomic DNA and reverse-transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies, Tokyo, Japan). An aliquot of cDNA was subjected to qPCR analysis. The reaction was conducted in triplicate on a StepOnePlus thermocycler (Life Technologies) with SYBER green PCR master mix containing 0.4 μM of each primer. The primer sequences used to determine the gene expression are listed in Supplementary Table S1. The β-globin (HBB) gene was amplified to confirm the presence of PCR-amplifiable cDNA. The PCR conditions were 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative gene expression levels in PB-22 and SL-22 cells were calculated using the 2^−ΔΔCT method43, with the β-actin (ACTB) gene used as a housekeeping control, and the value was expressed as an n-fold change relative to that in PB-15 and SL-15 cells, respectively. Statistical analysis was performed at the ΔΔCT stage using unpaired two-tailed Student’s t-tests. A statistically significant difference was defined as P < 0.05.

Immunoblot analysis. Immunoblotting was performed as described43. The following antibodies were used: rabbit monoclonal anti-PLK1 (clone 208G4; Cell Signaling Technology, Danvers, MA, USA); mouse monoclonal anti-EPHA4 (clone 4C8H5; Thermo Fisher Scientific, Waltham, MA, USA); mouse monoclonal anti-β-actin (clone AC-74; Merck KGaA, Darmstadt, Germany); IRDye 680RD goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE, USA); and IRDye 800CW goat anti-mouse IgG (LI-COR Biosciences). Bands were visualized using ODYSSEY CLx (LI-COR Biosciences). Signal intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA; https://imagej.nih.gov/ij/). Levels of proteins were normalized to that of β-actin.
DNA sequencing analysis. Nested PCR was performed using Platinum SuperFi DNA polymerase (Thermo Fisher Scientific). To amplify exon 1–2 and exon 3 of KLF2 and exon 34 of NOTCH2, the PCR conditions were 10 s at 98 °C (30 s for the first cycle), followed by 20 cycles of 30 s at 58 °C and 1.5 min at 72 °C (5 min for the last cycle) for the first round of PCR and 30 cycles for the second round of PCR. Amplification products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Presence of somatic mutations in the KLF2 (exon 1–3) and in the NOTCH2 (exon 34) were investigated by Sanger sequencing as described9, 10. The primer sequences used for nested PCR and sequencing analysis are listed in Supplementary Table S2.

Plasmid construction and production of PLK1-specific shRNA and siRNA. The doxycycline-regulated and RNA polymerase II-inducible shRNA-expression plasmid vector, designated as pTRE3G1, was generated for this study. The pTRE3G1 plasmid vector contains the following constructs: reverse tetracycline activator coding region derived from pCMV-TET3G (Takara Bio Inc., Shiga, Japan); an improved variant of the copedoped Pontellina plumata green fluorescent protein (max GFP) coding region derived from pmaxGFP (Lonza, Basel, Switzerland); the GAPDH promoter from nucleotides –376 to +183 relative to the transcription start site; a tetracycline-response element promoter derived from pTRE3G (Takara Bio); 5′ and 3′ miR-155 flanking region derived from pcDNA 6.2-GW/EmGFP-miR (Thermo Fisher Scientific); and a Luciferase shRNA coding region derived from pSingle-tTS-Anti-Luc (Takara Bio Inc.) for control shRNA. The DNA fragments were synthesized using the GeneArt Strings system (Thermo Fisher Scientific) and cloned into Sall–PciI restriction enzyme sites of pcDNA3.1 (Thermo Fisher Scientific) using the In-Fusion HD cloning kit (Takara Bio Inc.), according to the manufacturer’s instructions.

For constructing the PLK1 shRNA-expression plasmid vector, the DNA fragment containing a PLK1 shRNA coding region was cloned into BamHI/SpeI sites of pTRE3G1. The PLK1 shRNA sequence was designed using siDirect version 2.0 software (http://sidirect2.rnai.jp/). The hairpin targeting sequence was 5′−GGAUCAAGAAGAAUGAAUA−3′ in the PLK1-coding region (National Center for Biotechnology Information accession number NM_005030.5). Fluorescent-labeled siRNAs targeting the PLK1 sequence 5′−CACCCUGCAGUAUGAGCGUGAU−3′ and control siRNA were obtained from Nippon Gene (Toyama, Japan).

PLK1 inhibition. For genetic inhibition of PLK1, cells were transfected with PLK1 shRNA plasmid vector, PLK1 siRNA, or their controls on Nucleofector (Lonza) using C solution and the D-23 program. The cells transfected with shRNA plasmid vectors were isolated 2 days after transfection by sorting maxGFP-expressing cells on a FACSARia II flow cytometer (Becton Dickinson, Mountain View, CA, USA). The isolated cells were treated with 1 μg/ml of doxycycline for 48–96 h to induce the expression of shRNA before further experiments. The transfection efficiency of siRNAs was more than 85%, as determined using fluorescent siRNA. For chemical inhibition of PLK1, cells were treated with the PLK1 inhibitor volasertib (BI 6727) (ChemScene, Monmouth Junction, NJ, USA) or BI 2536 (ChemScene) at concentrations of 5–50 ng/ml.

Cell proliferation, apoptosis and cell cycle analyses. For cell proliferation assays, cells were seeded in 96-well plates (8 × 103 cells/well) and viable cells were counted after 48 h or every 24 h on a FACSCalibur flow cytometer (Becton Dickinson) by gating out cells stained with propidium iodide as described11. For apoptosis assays, cells were stained with annexin V–phycoerythrin and 7-amino-actinomycin D according to the manufacturer’s instructions. For cell cycle analysis, cells were fixed in cold 70% ethanol, treated with RNase, and stained with propidium iodide. Cells were analyzed using a FACSCalibur as above, and all flow cytometry data were analyzed using CellQuest Pro software (Becton Dickinson). All experiments were performed in triplicate.

Real-time qPCR for detecting genomic DNA of the MS4A gene cluster. Real-time qPCR was used to detect and quantify genomic DNA of the MS4A gene cluster and their neighboring genes. Genomic DNA was extracted using the phenol–chloroform method. The reaction was conducted in duplicate with 200 ng of extracted DNA and SYBR green PCR master mix containing 0.4 μM of each primer. The primer sequences used to determine the gene levels are listed in Supplementary Table S3. The PCR conditions were 10 min at 95 °C, followed by 30 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative gene loads in SL-22 cells were calculated using the 2−ΔΔCt method, with the β-globin (HBB) gene used as housekeeping control, and the value was expressed as an n-fold change relative to that in SL-15 cells. The PCR products were separated electrophoretically on 2% agarose gels, visualized with ethidium bromide staining, and photographed.

Data Availability. All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

References
1. Isaacson, P. G. et al. Splenic B-cell marginal zone lymphoma in WHO classification of tumours of haematopoietic and lymphoid tissues (eds. Swerdlow, S. H. et al.) 185–187 (IARC Press, 2008).
2. Arcaini, L., Rossi, D. & Paulli, M. Splenic marginal zone lymphoma: from genetics to management. Blood 127, 2072–2081 (2016).
3. Olzewska, A. J. & Castillo, J. J. Survival of patients with marginal zone lymphoma: analysis of the Surveillance, Epidemiology, and End Results database. Cancer 119, 629–638 (2013).
4. Arcaini, L. et al. Splenic marginal zone lymphoma: a prognostic model for clinical use. Blood 107, 4643–4649 (2006).
5. Martinez, N. et al. Whole-exome sequencing in splenic marginal zone lymphoma reveals mutations in genes involved in marginal zone differentiation. Leukemia 28, 1334–1340 (2014).
6. Parry, M. et al. Whole exome sequencing identifies novel recurrently mutated genes in patients with splenic marginal zone lymphoma. PLoS One 8, e83244 (2013).

SCIENTIFIC REPORTS | 7: 11026 | DOI:10.1038/s41598-017-11389-5
7. Clipson, A. et al. KLF2 mutation is the most frequent somatic change in splenic marginal zone lymphoma and identifies a subset with distinct genotype. *Leukemia* 29, 1177–1185 (2015).
8. Piva, R. et al. The Krüppel-like factor 2 transcription factor gene is recurrently mutated in splenic marginal zone lymphoma. *Leukemia* 29, 503–507 (2015).
9. Parry, M. et al. Genetics and Prognostication in Splenic Marginal Zone Lymphoma: Revelations from Deep Sequencing. *Clin. Cancer Res.* 21, 4174–4183 (2015).
10. Rossi, D. et al. The coding genome of splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating marginal zone development. *J. Exp. Med.* 209, 1537–1551 (2012).
11. Daibata, M. et al. In vitro Epstein-Barr virus immortalized lymphoma cell line carrying t(9;14)(p13;q32) chromosome abnormality, derived from splenic lymphoma with villous lymphocytes. *Int. J. Cancer* 118, 513–517 (2006).
12. Kalpadakis, C. et al. Treatment of splenic marginal zone lymphoma with rituximab monotherapy: progress report and comparison with splenectomy. *Oncologist* 18, 190–197 (2013).
13. Czuczman, M. S. et al. Acquisition of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. *Clin. Cancer Res.* 14, 1561–1570 (2008).
14. Hiraga, J. et al. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. *Blood* 113, 4885–4893 (2009).
15. Sugiimoto, T. et al. Escape mechanisms from antibody therapy to lymphoma cells: downregulation of CD20 mRNA by recruitment of the HDAC complex and not by DNA methylation. *Biochem. Biophys. Res. Commun.* 390, 48–53 (2009).
16. Shimizu, R. et al. HDAC inhibitors augment cytotoxic activity of rituximab by upregulating CD20 expression on lymphoma cells. *Leukemia* 24, 1760–1768 (2010).
17. Tomita, A. et al. Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy. *Int. J. Hematol.* 86, 49–57 (2007).
18. Sonoki, T. et al. Establishment of a novel CD20 negative mature B-cell line, WILL2, from a CD20 positive diffuse large B-cell lymphoma patient treated with rituximab. *Int. J. Hematol.* 89, 400–402 (2009).
19. Kumar, S., Sharma, A. R., Sharma, G., Chakraborty, C. & Kim, J. PLK1-1: Angel or devil for cell cycle progression. *Biochem. Biophys. Acta*. 1865, 190–203 (2016).
20. Eon Kuek, L., Leffler, M., Mackay, G. A. & Hulett, M. D. The MS4A family: counting past 1, 2 and 3.
21. Inokuchi, K. et al. Establishment and characterization of a villous lymphoma cell line from splenic B-cell lymphoma. *Leuk. Res.* 19, 817–822 (1995).
22. Martinez-Climent, J. A. et al. Genomic abnormalities acquired in the blast transformation of splenic marginal zone B-cell lymphoma. *Leuk. Lymphoma* 44, 459–464 (2003).
23. Matsushahi, Y. et al. Establishment and characterization of the new splenic marginal zone lymphoma-derived cell line UCH1 carrying a complex rearrangement involving t(8;14) and chromosome 3. *Leuk. Lymphoma* 48, 767–773 (2007).
24. Rudolph, D. et al. BL6272, a Polo-like kinase inhibitor with improved pharmacokinetic profile and broad antitumor activity. *Clin. Cancer Res.* 15, 3094–3102 (2009).
25. Gitteridge, B. E., Ndiaye, M. A., Liu, X. & Ahmad, N. PLK1 inhibitors in cancer therapy: from laboratory to clinics. *Mol. Cancer Ther.* 15, 1427–1435 (2016).
26. Liu, Z., Sun, Q. & Wang, X. PLK1, A potential target for cancer therapy. *Transl. Oncol.* 10, 22–32 (2016).
27. Renner, A. G. et al. Polo-like kinase 1 is overexpressed in acute myeloid leukemia and its inhibition preferentially targets the proliferation of leukemic cells. *Blood* 114, 659–662 (2009).
28. Berezoe, T. et al. A novel treatment strategy targeting polo-like kinase 1 in hematological malignancies. *Leukemia* 23, 1564–76 (2009).
29. Mito, K. et al. Expression of Polo-Like Kinase (PLK1) in non-Hodgkin’s lymphomas. *Leuk. Lymphoma* 46, 225–231 (2005).
30. Liu, L., Zhang, M. & Zhou, P. Expression of PLK1 and survivin in non-Hodgkin’s lymphoma treated with CHOP. *Acta Pharmacol. Sin.* 29, 371–373 (2008).
31. Nakamaki, T. et al. CD20 gene deletion causes a CD20-negative relapse in diffuse large B-cell lymphoma. *Eur. J. Haematol.* 89, 350–355 (2012).
32. Johnson, N. A. et al. Diffuse large B-cell lymphoma: reduced CD20 expression is associated with an inferior survival. *Blood* 113, 3773–3780 (2009).
33. Surawska, H., Ma, P. C. & Salgia, R. The role of ephrins and Eph receptors in cancer. *Cytokine Growth Factor Rev.* 15, 419–433 (2004).
34. Davalos, V. et al. EphB4 and survival of colorectal cancer patients. *Cancer Res.* 66, 8943–8948 (2006).
35. Noren, N. K., Foos, G., Hauser, C. A. & Pasquale, E. B. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway. *Nat. Cell Biol.* 8, 815–25 (2006).
36. Oshima, T. et al. Overexpression of EphA4 gene and reduced expression of EphB2 gene correlates with liver metastasis in colorectal cancer. *Int. J. Oncol.* 33, 573–577 (2008).
37. Saintjnguy, P. et al. Global evaluation of Eph receptors and ephrins in lung adenocarcinomas identifies EphA4 as an inhibitor of cell migration and invasion. *Mol. Cancer Ther.* 11, 2021–2032 (2012).
38. Sun, Y., Qian, J., Lu, M. & Xu, H. Lower and reduced expression of EphA4 is associated with advanced TNM stage, lymph node metastasis, and poor survival in breast carcinoma. *Pathol. Int.* 66, 506–10 (2016).
39. Kuang, S. Q. et al. Aberrant DNA methylation and epigenetic inactivation of Eph receptor tyrosine kinases and ephrin ligands in acute lymphoblastic leukemia. *Blood* 115, 2412–2419 (2010).
40. Huang, Y. C. et al. Regulation of EBV LMP1-triggered EphA4 downregulation in EBV-associated B lymphoma and its impact on patients’ survival. *Blood* 128, 1578–1589 (2016).
41. Koivula, S., Valo, E., Raunio, A., Hautaniemi, S. & Leppä, S. Rituximab regulates signaling pathways and alters gene expression associated with cell death and survival in diffuse large B-cell lymphoma. *Oncol. Rep.* 25, 1183–1190 (2011).
42. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt Method. *Methods* 25, 402–408 (2001).
43. Higuchi, T., Nakayama, T., Arao, T., Nishio, K. & Yoshie, O. SOX4 is a direct target gene of IRA-2 and induces expression of HDAC8 in adult T-cell leukemia/lymphoma. *Blood* 121, 3640–3649 (2013).

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**Author Contributions**

T.H., Y.H., M.K., and M.D. performed the experiments. A.T. collected the clinical samples. T.H., Y.H., and M.D. wrote the manuscript. M.D. designed the study and contributed the acquisition of funding. All authors read and approved the final manuscript.
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