Sphingosine-1-phosphate receptor 1 as a prognostic biomarker and therapeutic target for patients with primary testicular diffuse large B-cell lymphoma

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Running title: S1PR1 as a biomarker and therapeutic target in DLBCL

Summary

Sphingosine-1-phosphate (S1P) is a potent lipid mediator that is produced during the metabolism of sphingolipid by sphingosine kinase. S1P has been implicated in the migration and trafficking of lymphocytes and several lymphoid malignancies through S1P receptors. Moreover, the overexpression of **sphingosine-1-phosphate receptor 1 (S1PR1)** has been correlated with the constitutive activation of signal transducer and activator of transcription (STAT)3 and poor prognosis of diffuse large B-cell lymphoma (DLBCL). Thus, in this study, we examined the expression of **S1PR1** in 198 DLBCL samples collected from nodal and various extranodal sites and sub-classified formalin-fixed paraffin-embedded tissue samples into germinal centre B-cell-like (GCB) and non-GCB subgroups using immunohistochemistry. These analyses showed S1PR1 overexpression in 15.7% of all cases with DLBCL and in 54.2% of 24 cases with primary testicular (PT)-DLBCL, and S1PR1 expression correlated with ***S1PR1*** mRNA expression and STAT3 phosphorylation in fresh samples. Analyses of data from a single institution suggested that S1PR1 overexpression was an independent negative prognostic marker in 68 patients with DLBCL of clinical stages I and II. The present high prevalence of S1PR1 overexpression warrants the consideration of PT-DLBCL as a distinct disease subtype and suggests the potential of the S1P/S1PR1 axis as a therapeutic target.

Key words: sphingosine-1-phosphate receptor 1, diffuse large B-cell lymphoma, STAT3, primary testicular lymphoma, immunohistochemistry

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a clinically and genetically heterogeneous disease and is the most common subtype of non-Hodgkin lymphoma, accounting for 30% in Western countries (Swerdlow *et al*, 2008) and 40% of Japanese cases (Lymphoma Study Group of Japanese Pathologists, 2000). The International Prognostic Index (IPI) is considered the most important prognostic factor for survival in patients with non-Hodgkin lymphoma. However, the IPI does not provide any indication of responsiveness to therapies, warranting investigations of additional biomarkers that predict outcomes and form the basis of novel treatment strategies for patients with DLBCL. Gene expression profiling analyses identified two distinct subgroups of DLBCL, including germinal centre B-cell-like (GCB) and activated B-cell-like (ABC) subgroups (Alizadeh *et al*, 2000). Moreover, immunohistochemical (IHC) analyses of formalin-fixed paraffin-embedded (FFPE) sections classified DLBCL into GCB and non-GCB types based on the expression of BCL6, CD10 and MUM-1 (Hans *et al*, 2004). Numerous biological markers and related novel therapies for DLBCL have selective activity in GCB or non-GCB DLBCL groups (de Jong *et al*, 2007; Carbone *et al*, 2014). In particular, nuclear factor kappa B (NFκB; Compagno *et al*, 2009) and signal transducer and activator of transcription (STAT)3 pathways (Ding *et al*, 2008; Huang *et al*, 2013) are constitutively activated in non-GCB DLBCL and concomitantly contribute to poor prognosis in affected patients (Lam *et al*, 2008).

Sphingosine-1-phosphate (S1P) is a potent lipid mediator that is produced during the metabolism of sphingolipid by sphingosine kinase (SPHK; Spiegel & Milstien *et al*, 2003; Kihara *et al*, 2007). In addition to intracellular roles in NFκB activation and epigenetic regulation of gene expression (Alvarez *et al*, 2010), extracellular S1P transduces intracellular signals by activating the cell surface sphingosine-1-phosphate receptor (S1PR)1, S1PR2, S1PR3, S1PR4 and S1PR5 and seven truncated plasma membrane receptors that are ubiquitously expressed in various tissues (Blaho & Hla, 2014). Accordingly,

the S1P/S1PR axis has reported effects on various inflammatory and neoplastic diseases (Pyne & Pyne, 2010; Maceyka *et al*, 2012). In particular, STAT3 is activated by S1Pl/S1PR1 signalling (Lee *et al*, 2010; Liang *et al*, 2013) and reciprocally enhances the transcription of target genes such as *S1PR1* in cancer cells. Liu *et al* (2012) showed that blockade of S1PR1/STAT signalling decreased ABC-DLBCL tumour cell proliferation and apoptosis *in vitro*. They also validated the effects of S1PR1/STAT3 inhibition on tumour progression using a xenograft model of human DLBCL.

In our previous studies, *in situ* localisation of S1PR1 in adult human tissues, including normal lymphoid tissues and malignant lymphomas, was observed after immunostaining FFPE sections with a well-defined commercially available anti-S1PR1 antibody (Akiyama *et al*, 2008; Nishimura *et al*, 2010 a, b). Subsequently, strong membrane expression of S1PR1 was observed in normal mantle cells in all cyclin D1-positive mantle cell lymphoma cells, in 10–20% of DLBCL and in some cases of chronic lymphocytic leukaemia, Hodgkin lymphoma and peripheral T-cell lymphoma (Nishimura *et al*, 2010b). These findings were corroborated by others using the same antibody (Kluk *et al*, 2013; Sic *et al*, 2014), and in a recent study, Paik *et al* (2014) showed that IHC expression of S1PR1 in lymphoma cells in FFPE tissues was correlated with inferior overall survival (OS) among 103 patients with DLBCL treated with rituximab (R). However, these investigators demonstrated S1PR1-positive cytoplasmic and nuclear staining of lymphoma cells. Hence, the clinicopathological significance of S1PR1 overexpression in IHC analyses of DLBCL specimens remains ambiguous.

In the present study, we initially examined expression of S1PR1 in 198 patients with nodal or extranodal DLBCL that was diagnosed using World Health Organization (WHO) criteria (Swerdlow *et al*, 2008) and assessed the prognostic value of membrane S1PR1 expression. We demonstrated that IHC analyses of S1PR1 reliably predicted outcomes in patients with early-stage DLBCL. In addition, S1PR1 overexpression was more prevalent in primary testicular (PT)-DLBCL

specimens than in DLBCL samples from other sites.

Materials and Methods

The study design (1568-1, 1901) was approved by the research ethics committee of Kawasaki Medical School and Hospital.

Patients and tissue samples

A total of 186 biopsies from 170 patients with primary DLBCL, not otherwise specified (NOS) and 16 patients with specific subtypes of DLBCL (9 primary DLBCL of the central nervous system (CNS), 1 primary leg type cutaneous DLBCL and 6 EBV-positive DLBCLs in elderly patients) were diagnosed in Kawasaki Medical School Hospital during 1990–2014 according to WHO criteria (Swerdlow *et al*, 2008). Diagnoses of lymphoma cases were confirmed using histology, flow cytometry immunophenotyping, IHC analysis, fluorescence *in situ* hybridisation, Southern blotting and polymerase chain reaction (PCR). Patients with follicular lymphoma or other types of indolent lymphoma that transformed into DLBCL were excluded. Subsequently, 182 patients received chemotherapy, 158 received R-based chemotherapy, and of these, 141 received **R-CHOP** (cyclophosphamide, doxorubicin, vincristine and prednisolone) chemotherapy. Three patients received CHOP chemotherapy and 21 patients received other chemotherapeutic agents. Only one patient received local radiation therapy (RT), and three patients were untreated.

Additional samples were included from 12 patients with PT-DLBCL from Teikyo University, Chiba Medical Center.

Immunohistochemistry

Biopsy tissues were fixed overnight in 4% buffered formalin. Paraffin-embedded tissues were cut into 3-μm sections and were deparaffinised. Subsequently, sections were incubated in antigen retrieval CC1 solution for 60 min at 98°C followed by staining with the Ventana XT system discovery using an avidin–biotin detection system. Primary antibodies included the well-defined rabbit polyclonal anti-S1PR1/EDG-1 (Santa

Cruz Biotechnology, Santa Cruz, CA, USA; 1:20 dilution as described previously; Akiyama *et al*, 2008; Nishimura *et al*, 2010b), rabbit monoclonal antibodies against STAT3 (1:60 dilution; Abcam, Tokyo, Japan), phospho-STAT3 (tyrosine705, p-STAT3^{Tyr705}; 1:20 dilution; Abcam), NFκB/p65 (1:80 dilution; Abcam), phospho NFκB/p65 (serine 536; p-NFκB/p65^{Ser536}; 1:30 dilution; Abcam) and mouse monoclonal antibody against anti-Ki-67 (1:50 dilution; DAKO Japan, Tokyo, Japan). Sections were incubated with primary antibodies against S1PR1, STAT3, NFκB/p65 and for 60 min or with antibodies against anti-p-STAT3^{Tyr705} or p-NFκB/p65^{Ser536} for 9 h. A diaminobenzidine hydrochloride solution with hydrogen peroxide was used as the chromogen, and slides were counterstained with hematoxylin.

All DLBCL samples were assessed by two independent observers, and consensus decisions were made according to the following criteria: In positive cases, >20% of tumour cells had membrane S1PR1 staining with or without cytoplasmic staining and with an intensity similar to or slightly weaker than intrinsic endothelial cell controls. Nuclear immunostaining of p-STAT3^{Tyr705} and p-NFκB/p65^{Ser536} in lymphoma cells indicated activation of STAT3 and NFκB/p65 proteins, and nuclear p-STAT3^{Tyr705} immunostaining of vascular endothelial cells was used as an endogenous positive control. Analyses of phosphorylation state-specific antibody immunoreactivity were limited to the outer 5 mm of surgical specimens as described previously (Mandell, 2003).

Western blot

Prior to western blot (WB) analyses, proteins from frozen tissue were removed from storage at −80°C and were directly resuspended and boiled in lysis buffer containing 1% sodium dodecyl sulfate, 1.0 mM sodium orthovanadate and 10 mM Tris (pH 7.4). Lysates were then homogenised, boiled for 5 min and passed through a 26G needle 5–10 times and centrifuged. Protein concentrations of cell extracts were determined using a NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Proteins (20 μg) were loaded onto NuPAGE 4%–12%

Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) with molecular weight marker (Novex Sharp Pre-Stained Protein Standard) and MagicMark XP Western Protein Standard (Life Technologies). After electrophoretic separation, proteins were transferred to polyvinylidene difluoride membranes using iBlot (Life Technologies). Membranes were treated with a blocking reagent (Roche Diagnostics; Basel, Switzerland) at room temperature for 1 h and were then incubated with rabbit polyclonal anti-S1PR1 (1:500 dilution) at 4°C overnight as described previously (Nishimura *et al*, 2010b), with rabbit monoclonal antibodies against STAT3 (1:1000 dilution), **p-STAT3^{Tyr705}** (1:1000 dilution), NFκB/p65 (1:1000 dilution; Abcam), or **p-NFκB/p65^{Ser536}** (1:1000 dilution) at room temperature for 1 h, or with mouse anti–β–actin monoclonal antibody (1:5000 dilution; Sigma-Aldrich; St Louis, MO, USA) at room temperature for 1 h. Membranes were then washed with Tris-buffered saline (TBS) for 30 min, were incubated at room temperature for 1 h with a horseradish peroxidase-conjugated anti-mouse/rabbit IgG antibody (Roche Diagnostics), were washed with TBS for 30 min and were finally treated with BM chemiluminescence Western blotting kit (Roche Diagnostics). Specific bands were visualised using LAS-1000UVmini (GE Healthcare, Tokyo, Japan) and were analysed for density using Image Studio, ver. 4.0 (Techonosystems, Osaka, Japan).

Quantitative real-time RT-PCR

Total mRNA was extracted using a RiboPure kit (Life Technologies) and was quantified using a NanoDrop 1000, and cDNA was synthesised from extracts containing 1 μg of mRNA using a QuantiTect Reverse Transcription kit (Qiagen; Hilden, Germany). Real-time PCR primers were purchased from Qiagen (QuantiTect Primer Assay) for human ***S1PR1*** **(QT00208733),** ***S1PR2*** **(QT00230846),** ***SPHK1*** **(QT01011927),** ***SPHK2*** **(QT00085386),** ***STAT3*** **(QT00068754)** and RPS18 (QT00248682). Gene expression levels were analysed in triplicate using an Applied Biosystems StepOne Plus PCR System (Life Technologies) with a QuantiFast SYBR Green

PCR kit (Qiagen). PCR amplification was performed for 5 min at 95°C to activate the HotStarTaq DNA polymerase, followed 40 cycles of 95°C for 10 s and a combined annealing/extension step at 60°C for 30 s. Because the PCR efficiency of the reaction was comparable between target and endogenous reference (RPS18) genes, normalised ***S1PR1, S1PR2, SPHK1, SPHK2*** **and** ***STAT3*** expression was calculated using StepOne software, version 2.2.2 (Life Technologies) and 2-ΔΔCt analysis.

Statistical analysis

Correlations between S1PR1 expression and clinicopathological parameters were compared using chi-square **test** or Mann–Whitney *U* test. Data from qRT-PCR and WB analyses of S1PR1-positive and -negative groups were analysed using Mann–Whitney *U* test. Survival parameters were estimated using the Kaplan–Meier method, and differences between groups were compared using the log-rank test. OS was measured from the date of first diagnosis to the date of last follow-up or death from any cause. Recurrence-free survival (RFS) of patients was calculated from the date of first diagnosis until relapse or death. Univariate and **multivariate** analyses were performed using Cox proportional hazard models. Differences and correlations were considered significant when $P < 0.05$. Statistical analyses were performed at the **Statistical Analysis Section, Kureha Special Laboratory Co., Ltd.** (Tokyo, Japan) using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Correlations between parameters were identified using Pearson's correlation coefficients with StatMate (StatMate Discovery Software, version 5.01, ATMS, Tokyo, Japan).

Results

Clinicopathological context of 186 cases with DLBCL with S1PR1 expression in a single institution (Kawasaki Medical School)

Among 186 patients with DLBCL treated at our institution, 91 (48.9%) and 95 patients (51.1%) carried GCB and non-GCB types, respectively, according to Hans IHC algorithm (Hans *et al*, 2004). Based on our IHC criteria, 25 of 186 patient samples (13.4%) were positive for S1PR1, including 2 of 170 cases with DLBCL, NOS, 2 of 9 cases with primary DLBCL of the CNS and 1 case of primary cutaneous leg type DLBCL. Clinicopathological features of these S1PR1-positive cases are summarised in Table I. Most S1PR1-positive DLBCL was morphologically classified as the centroblastic type (Fig. 1), and all differences in clinicopathological variables between S1PR1-positive and -negative cases are shown in **Table II.** Among 25 S1PR1-positive cases, 5 carried GCB-type and 20 carried non-GCB type DLBCL and the ensuing correlation was significant (P = 0.004). Moreover, patients with Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≥ 2 tended to have S1PR1-negative DLBCL (P = 0.061). No significant relationships were observed between the expression of S1PR1 and the clinical variables age, gender, B symptoms, disease stage, elevated lactate dehydrogenase (LD; ≥ 240 IU/L), bone marrow involvement, extranodal involvement or IPI risk.

In subsequent analyses, correlations of S1PR1 expression with primary biopsy site were examined (Table III), and whereas 13.0% (10 of 77) of cases were positive for S1PR1 in lymph nodes, 14.0% (15 of 109) were positive for S1PR1 in extranodal sites. However, extranodal S1PR1 expression was frequently observed in patients with PT-DLBCL (7 of 12 cases, 58.3%). Because individual tissues were too few to analyse statistically, the prevalence of S1PR1 expression was compared between primary biopsies from lymph nodes (10 of 77), testis (7 of 12) and other sites **(primary biopsy sites, excluding testis and lymph nodes)** (8 of 97) and was most prevalent among cases of PT-DLBCL **(Table II)**.

High prevalence of S1PR1 expression in PT-DLBCL

The present IHC analyses of FFPE sections showed relatively high prevalence of S1PR1 expression in patients with PT-DLBCL from our hospital. Therefore, we added 12 cases from Teikyo University, Chiba Medical Center and performed analyses of a total of 24 cases (**Table IV**). Among these cases, five (20.8%) had GCB and 19 (79.2%) had non-GCB type PT-DLBCL. Moreover, S1PR1 expression was observed in 13 cases (54.2%), including 3 (23.1%) with GCB and 10 (76.9%) with non-GCB-type disease. We also investigated the relationship between S1PR1 expression, Ki-67 indices and STAT3 phosphorylation in IHC analyses of FFPE sections from patients with PT-DLBCL. However, no significant correlations were observed between S1PR1 expression and Ki-67 indices (P = 0.685) or STAT3 phosphorylation (P = 0.807).

IHC expression of S1PR1 protein is correlated with S1PR1 but not STAT3 mRNA expression

In analyses of correlations between IHC S1PR1 expression in FFEP sections and mRNA expression of ***S1PR1***, ***S1PR2***, ***SPHK1***, ***SPHK2*** **or** ***STAT3*** in frozen tissues **(Supplementary Table I)**, relative ***S1PR1*** mRNA expression was significantly higher in S1PR1-positive lymphoma specimens than in the S1PR1-negative lymphoma group **(0.79 ± 0.47 *vs* 0.23 ± 0.14, P = 0.023).** However, no correlations between IHC S1PR1 protein expression and relative mRNA expression of ***S1PR2*** **(0.56 ± 0.42 *vs* 0.61 ± 0.46, P = 0.894)**, ***SPHK1*** **(1.33 ± 0.59 *vs* 1.37 ± 0.90, P = 0.790)**, ***SPHK2*** **(0.67 ± 0.48 *vs* 0.32 ± 0.19, P = 0. 182)** **or** ***STAT3*** **(0.60 ± 0.39 *vs* 0.46 ± 0.45, P = 0.286) were identified in cases with DLBCL.**

IHC expression of S1PR1 protein is correlated with constitutive activation of STAT3 protein in fresh frozen tissues

WB analyses of frozen tissues from 13 patients with DLBCL (GCB type, six cases; non-GCB type, seven cases) were performed, and correlations between S1PR1 protein expression and activated STAT3 or NFκB/p65 were examined **(Fig. 2, Supplementary Table II)**. These analyses showed a significant

correlation between S1PR1 expression and STAT3 phosphorylation ($r = 0.75$, $P = 0.004$) but not between S1PR1 expression and NFκB/p65 phosphorylation ($r = -0.38$, $P = 0.206$).

Subsequently, correlations between S1PR1 expression in IHC and WB and STAT3 or NFκB phosphorylation were examined. In these analyses, IHC and WB assessments of S1PR1 protein levels were highly correlated (0.11 ± 0.05 *vs* 0.03 ± 0.02, $P = 0.002$) and were strongly correlated with STAT3 phosphorylation (0.55 ± 0.11 *vs* 0.25 ± 0.09, $P = 0.004$) and tended to be correlated with NFκB/p65 phosphorylation (0.52 ± 0.45 *vs* 1.07 ± 1.36, $P = 0.067$) but were not correlated with Akt phosphorylation (0.13 ± 0.17 *vs* 0.10 ± 0.06, $P = 0.608$).

Impacts of S1PR1 expression on the survival of patients with DLBCL

We next evaluated the impact of S1PR1 expression on the survival of patients from a single institution. First, univariate and multivariate Cox regression analyses of data from a total of 186 patients with DLBCL were performed to determine prognostic implications of the present clinicopathological variables, including age, gender, IPI risk and S1PR1. In multivariate analysis, IPI [high intermediate (Hi) and high (H)] risk was an independent poor prognostic factor for OS [Hazard Ratio (HR), 1.73; 95% confidence interval (CI), 1.01–2.95; $P = 0.045$)] and RPS (HR, 1.90; 95% CI, 1.20–3.01; $P = 0.006$), whereas age (≥60), gender (male) and S1PR1 expression were not significant for OS and RPS (Table V).

Subsequently, multivariate Cox analyses with age, gender, IPI risk and S1PR1 were performed on data from a total of 68 patients in the early stage patients subgroup with Ann Arbor stage I + II DLBCL (Table V continued). In this subgroup, S1PR1 expression was a significant, independent and poor prognostic factor of OS (HR, 4.58; 95% CI, 1.64–12.83; $P = 0.004$) and RFS (HR, 3.56; 95% CI, 1.35–9.36; $P = 0.010$).

Clinical outcomes of patients with S1PR1-positive DLBCL

Kaplan–Meier survival curves comparing S1PR1-positive and

-negative cases were generated for OS and RFS **among 68 patients with stage I or II DLBCL (Fig. 3A). In these experiments, S1PR1 expression significantly correlated with inferior OS ($P = 0.026$).** Similarly, S1PR1 expression tended to be correlated with inferior RFS among patients with stage I or II DLBCL ($P = 0.064$). However, OS ($P = 0.518$) and RFS ($P = 0.474$) did not differ between patients with GCB and non-GCB types in Kaplan–Meier survival analyses of 186 patients with DLBCL **(Fig. 3B)**.

Discussion

In this study, we confirmed that IHC expression of S1PR1 in FFPE sections represents expression levels of S1PR1 protein in lymphoma cells. Accordingly, IHC expression of S1PR1 was well correlated with corresponding mRNA and protein expression in fresh frozen samples. In agreement, antigenicity of S1PR1 protein is reportedly well preserved, and S1PR1 remains detectable even in FFPE sections of autopsy samples that lack cyclin D1 expression (Nishimura *el al*, 2010a). Moreover, analyses of S1PR1 expression in lymphoma cells from FFPE sections relative to that in vascular endothelium indicated that S1PR1 was expressed in 13.4% of all cases with DLBCL. In contrast, a previous study in **another** institution showed that 40% of DLBCL were positive for S1PR1 (Paik *et al*, 2014). However, whereas only membrane-stained lymphoma cells were considered positive in the present study, Paik *et al* (2014) included cytoplasmic S1PR1 staining in their analyses. Accordingly, the present tumour cells often showed both membrane and cytoplasmic S1PR1 immunostaining but were not considered S1PR1 positive without membrane localisation.

The present 186 cases were divided into GCB (n = 91) and non-GCB (n = 95) groups according to the Hans algorithm. However, in agreement with previous studies (Colomo *et al*, 2003; Gutierrez-Garcia *et al*, 2011), no differences in OS and RFS were identified between GCB and non-GCB subtypes. **In contrast, multivariate Cox regression analysis without a factor of non-GCB subtype significantly correlated with S1PR1 expression revealed that S1PR1 expression is an independent predictor of inferior OS and RFS in patients with stage I + II DLBCL.**

Recently, various cell signalling biomarkers of lymphoma cell trafficking have attracted attention as therapeutic targets (Roussos *et al*, 2011; Yoshie & Matsushima 2015; Moreno *et al*, 2015). In particular, the S1P/S1PR1 axis has been correlated with the regulation of motility in various cell types and has been shown to be crucial for lymphocyte egression and localisation in lymphoid organs (Rosen *et al*, 2005; Cyster & Schwab 2012). It can be assumed that lymphoma cells with

significant S1PR1 expression spread using similar mechanisms. Specifically, S1PR1 overexpressing lymphoma cells may egress toward the circulation under S1P gradients in patients with early-stage DLBCL.

Activation of S1PR1 by S1P reportedly led to phosphorylation of STAT3, which reciprocally induced transcription of S1PR1 in cancer cells (Lee *et al*, 2010). Moreover, blockade of S1PR1/STAT signalling decreased tumour cell proliferation and apoptosis of ABC-DLBCL cells (Liu *et al*, 2012). The present **Western blot (WB)** experiments **(Fig. 2 and Supplementary Table II)** confirmed the close correlation between S1PR1 overexpression and STAT3 activation in fresh samples. However, this correlation was not reflected in IHC analyses with an anti-phospho STAT3 antibody, and no correlation was identified between numbers of PT-DLBCL cells with STAT3-positive nuclei and STAT activation. This discrepancy may reflect fixation conditions of tissue samples, sizes of tissue samples and/or time-dependent penetration of the formalin fixative and, as previously suggested, indicates the inappropriateness of IHC analyses of FFPE sections for assessments of STAT3 activation using anti-phospho STAT3 antibodies (Mandell *et al*, 2003).

The unique clinical and molecular features of PT-DLBCL may warrant consideration as a distinct entity (Fonseca *et al*, 2000; Menon *et al*, 2012; Cheah *et al*, 2014). Accordingly, Menter *et al* (2014) showed that most PT-DLBCL are of non-GC origin and that the majority of cases exhibit active STAT3 and CXCR4 signalling, as indicated by expression of the respective phosphorylated proteins in IHC analyses of FFPE sections. Moreover, molecular alterations such as somatic mutations in ***CD79B*** and ***MYD88*** have been demonstrated in PT-DLBCL and may lead to induction of constitutive NFκB and/or janus kinase (JAK)/STAT3 signalling in this lymphoma type (Ngo *et al,* 2011; Kraan *et al*, 2014). In the present patients, S1PR1 overexpression was prevalent in PT-DLBCL and was specific to the tissue of origin but was not related to the ratio of GCB and non-GCB subtypes. Taken together, these data support the distinction of PT-DLBCL from other lymphomas.

Poor outcomes of PT-DLBCL reportedly follow specific anatomic manifestation in the testis, which is an immune-privileged microenvironment and leads to preferential dissemination to other immune-privileged sites such as contralateral testis and the CNS (Cheah *et al*, 2014). The standard treatment for localised PT-DLBCL (stage I to II) is R-CHOP with CNS prophylaxis and contralateral testis irradiation (Zucca *et al*, 2003; Vitolo *et al*, 2011) and was used to treat PT-DLBCL patients in our institution. Given the possible role of S1PR1 expression in site-specific dissemination of PT-DLBCL in early-stage patients, targeted treatments for PTL may have influenced the relationship between S1PR1 expression and survival in this study. However, the blockade of S1P/S1PR1 signalling may inhibit cell motility and thereby prevent the progression of S1PR1 expressing lymphomas, warranting further investigations of this treatment strategy in experimental animal models with brain metastases of S1PR1-positive DLBCL.

In conclusion, we found that IHC expression of S1PR1 in FFPE samples was correlated with OS of patients with stages I and II DLBCL with activated STAT3. Accordingly, the high prevalence of S1PR1 expression in PT-DLBCL warrants distinction as a DLBCL subtype. Although more studies are needed to elucidate the biological mechanisms behind these findings *in vivo*, the present data indicate that the blockade of the S1PR1 pathway may be an effective therapeutic strategy for patients with S1PR1-positive DLBCL.

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Disclosure/conflicts of interest

The authors declare no conflicts of interest.

Author contributions

RK performed experiments, acquired pathologic data, analysed pathologic and clinical data and wrote the manuscript; DO, HF, HN, TA, SH and KY acquired and analysed pathologic data; HW and TS acquired and analysed clinical data and YS designed research, acquired pathologic data, analysed pathologic and clinical data and wrote the manuscript. All the authors have read the manuscript and have approved this submission.

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Table I. Clinicopathological features of 25 cases of S1PR1-positive DLBCL at **Kawasaki Medical School Hospital**

Case	Gender	Age	Ann Arbor stage	IPI	LD	BM	Tissue	% p-STAT3	%Ki-67	COO	Outcome
1	F	64	IIIB	H	+	–	LN	73.6	84.5	non-GCB	CR
2	M	68	IVB	H	+	+	LN	76.5	58.4	non-GCB	CR
3	F	54	IIIA	L	–	–	LN	43.0	50.6	non-GCB	2nd CR
4	F	60	IIB	L	–	–	LN	55.3	52.3	non-GCB	Died of lymphoma
5	F	69	IIA	Li	+	–	LN	5.30	56.5	non-GCB	relapse
6	M	76	IVA	H	+	–	LN	75.8	95.0	GCB	Died of lymphoma
7	M	71	IIA	Li	+	–	LN	28.3	80.0	non-GCB	Died of lymphoma
8	M	64	IIA	L	+	–	LN	17.0	87.2	non-GCB	CR
9	M	60	IIA	L	–	–	LN	56.3	32.0	non-GCB	CR
10	M	57	IIA	L	–	–	LN	31.8	72.2	non-GCB	CR
11	M	61	IEA	L	–	–	Testis	4.00	36.6	GCB	Died of other disease
12	M	55	IIEA	L	–	–	Testis	48.9	61.3	GCB	Stopped follow-up
13	M	68	IVA	Hi	–	–	Testis	12.0	48.1	non-GCB	CR
14	M	68	IAE	L	+	–	Testis	5.20	74.5	non-GCB	Died of other disease
15	M	81	IVA	Hi	–	–	Testis	37.8	73.4	non-GCB	CR
16	M	70	IIAE	L	–	–	Testis	20.7	89.2	non-GCB	CR
17	M	54	IVA	Li	–	–	Testis	41.2	82.3	GCB	CR
18	M	70	IVA	Li	–	–	CNS	40.1	76.3	non-GCB	Died of lymphoma
19	M	78	IVB	Hi	+	–	CNS	70.3	83.0	non-GCB	CR
20	F	73	IA	Li	–	–	Nasal cavity	11.0	93.0	GCB	Died of lymphoma
21	F	76	IVA	H	+	–	Skin	52.2	92.4	non-GCB	CR
22	F	65	IVA	H	+	–	Soft tissue	45.8	52.0	non-GCB	2nd CR
23	F	30	IA	L	+	–	Breast	61.5	35.0	non-GCB	Died of lymphoma
24	M	75	IA	L	–	–	Others	44.6	42.0	non-GCB	2nd CR
25	F	56	IVB	Hi	+	+	Others	12.9	43.0	non-GCB	CR

BM, bone marrow involvement; CNS, central nervous system; COO, cell of origin; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; F, female; H, high risk; Hi, high-intermediate risk; IPI, International Prognostic Index; L, low risk; Li, low-intermediate risk; LD, elevated lactate dehydrogenase (LD) (≥240 IU/L); LN, lymph node; M, male; p-STAT3, phosphorylated STAT3 **(tyrosine705)**.

Table II. Clinicopathological features of 186 patients with S1PR1-positive and -negative diffuse large B-cell lymphoma **at Kawasaki Medical School Hospital**

Characteristics (%)		Total	S1PR1+	S1PR1−	*P*-value
Patient		186	25 (13.4)	161 (86.5)	
Age, Median		68	68	68	*P* = 0.395 (MW)
	Range	19–90	30–81	19–90	
Gender, Male		108 (58.1)	16 (64.0)	92 (57.1)	*P* = 0.668 (C)
B symptoms		41 (22.0)	5 (20.0)	36 (22.4)	*P* = 0.996 (C)
Ann Arbor stage	I	28 (15.1)	5 (20.0)	23 (14.3)	
	II	40 (21.5)	8 (32.0)	32 (19.9)	*P* = 0.204 (MW)
	III	28 (15.1)	2 (8.0)	26 (16.1)	
	IV	90 (48.4)	10 (40.0)	80 (49.7)	
ECOG PS ≥ 2		37 (19.9)	1 (4.0)	36 (22.4)	*P* = 0.061 (C)
LD elevation		104 (55.9)	12 (48.0)	92 (57.1)	*P* = 0.522 (C)
Bone marrow involvement		**30 (16.1)**	**2 (8.0)**	**28 (17.4)**	***P* = 0.370 (C)**
Extranodal involvement	0	59 (31.7)	8 (32.0)	51 (31.7)	
	1	85 (45.7)	14 (56.0)	71 (44.1)	*P* = 0.458 (MW)
	≥2	42 (22.6)	3 (12.0)	39 (24.2)	
IPI risk: L, Li		95 (51.1)	16 (64.0)	79 (49.1)	*P* = 0.240 (C)
Non-GCB type		95 (51.1)	20 (80.0)	75 (46.6)	*P* = 0.004 (C)
Primary biopsy sites					***P* = 0.001(KW)**
LN		**77 (41.4)**	**10 (13.0)**	**67 (87.0)**	
Testis		**12 (6.5)**	**7 (58.3)**	**5 (41.7)**	
Other sites		**97 (52.2)**	**8 (8.2)**	**89 (91.8)**	

C, chi-square test; ECOG PS, Eastern Cooperative Oncology Group performance status; GCB type, germinal centre B-cell type; IPI, International Prognostic Index; **KW, Kruskal–Wallis H-test;** LD level, lactate dehydrogenase level; L, low risk; Li, low-intermediate risk; **MW, Mann–Whitney *U*-test; Other sites, primary biopsy sites, excluding testis and lymph node**

Table III. Primary biopsy sites of S1PR1-positive and -negative DLBCL **at Kawasaki Medical School Hospital**

Tissue	Total (%)	S1PR1+ (%)	S1PR1– (%)	Non-GCB type (%)
Total no.	186	25 (13.4)	161 (86.6)	95 (51.1)
Lymph node	77 (41.4)	10 (13.0)	67 (87.0)	41 (53.2)
Gastrointestinal tract*	22 (11.8)	0	22 (100)	9 (40.9)
Tonsil	19 (10.2)	0	19 (100)	5 (26.3)
Testis	12 (6.5)	7 (58.3)	5 (41.7)	7 (58.3)
CNS	10 (5.4)	2 (20.0)	8 (80.0)	6 (60.0)
Pharynx	5 (2.7)	0	5 (100)	4 (80.0)
Breast	5 (2.7)	1 (20.0)	4 (80.0)	2 (40.0)
Parotid gland	4 (2.2)	0	4 (100)	2 (50.0)
Nasal cavity	4 (2.2)	1 (25.0)	3 (75.0)	2 (50.0)
Skin	3 (1.6)	1 (33.3)	2 (66.7)	3 (100)
Adrenal gland	3 (1.6)	0	3 (100)	2 (66.7)
Soft tissue	3 (1.6)	1 (33.3)	2 (66.7)	2 (66.7)
Others	19 (10.2)	2 (10.5)	17 (89.5)	10 (52.6)

*DLBCL specimens from the gastrointestinal tract included two from the oesophagus, nine from the duodenum, six from the small intestine and three from the appendix.

CNS, central nervous system; DLBCL, diffuse large B-cell lymphoma; GCB, germinal centre B-cell like; S1PR1, sphingosine-1-phosphate receptor 1

Table IV. Clinicopathological features of 24 patients with PT-DLBCL with S1PR1 overexpression from the Kawasaki Medical School Hospital and the Teikyo University, Chiba Medical Center.

Case	Age	S1PR1	p-STAT3 (%)	Ki-67 (%)	COO	Survival time (Mo)	Outcome
1	61	+	4.0	36.6	GCB	248	Died of other disease
2	55	+	48.9	61.3	GCB	155	Untraceable
3	54	+	41.2	82.3	GCB	18	2nd CR
4	63	+	ND	80.1	non-GCB	24	CR
5	65	+	ND	72.1	non-GCB	57	Died of lymphoma
6	60	+	59.0	46.4	non-GCB	46	CR
7	81	+	22.0	48.2	non-GCB	0	Under treatment
8	64	+	ND	30.9	non-GCB	22	Died of lymphoma
9	79	+	6.4	32.3	non-GCB	13	Died of lymphoma
10	68	+	12.0	48.1	non-GCB	110	Died of other disease
11	68	+	5.2	74.5	non-GCB	79	CR
12	81	+	37.8	73.4	non-GCB	53	CR
13	70	+	20.7	89.2	non-GCB	18	CR
14	44	–	ND	59.2	GCB	240	3rd CR
15	62	–	8.0	20.5	GCB	177	CR
16	72	–	ND	62.3	non-GCB	2	Non-CR
17	54	–	17.9	24.3	non-GCB	45	Untraceable
18	62	–	38.1	90.2	non-GCB	23	CR
19	68	–	ND	22.8	non-GCB	223	CR
20	71	–	ND	61.2	non-GCB	23	Died of lymphoma
21	79	–	17.6	87.2	non-GCB	1	Under treatment
22	71	–	16.0	52.3	non-GCB	128	CR
23	62	–	10.2	72.3	non-GCB	82	CR
24	77	–	39.0	49.1	non-GCB	2	Died of lymphoma

IHC prevalence of S1PR1, p-STAT3 and Ki67 expression.

COO, cell of origin; CR, complete remission; GCB, germinal centre B-cell-like; IHC, immunohistochemical; ND, not detected; PT-DLBCL, primary testicular diffuse large B-cell lymphoma; S1PR1, sphingosine-1-phosphate receptor 1; STAT, signal transducer and activator of transcription

Table V. Univariate and multivariate **Cox** regression analyses of OS and RFS among patients with DLBCL **from the Kawasaki Medical School Hospital**

(A) OS, (entire patients, n = 186)

Clinicopathologic variables	**Category**	Univariate		Multivariate	
		Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age	60 ≥ vs < 60	1.435 (0.762–2.705)	*P* = 0.263	1.244 (0.646–2.392)	*P* = 0.514
Gender	male vs female	0.884 (0.529–1.479)	*P* = 0.639	0.894 (0.533–1.499)	*P* = 0.671
IPI risk	(Hi + H) vs (Li + L)	1.802 (1.073–3.027)	*P* = 0.026	1.727 (1.011–2.950)	*P* = 0.045
S1PR1	+ vs –	1.186 (0.613–2.294)	*P* = 0.613	1.233 (0.636–2.390)	*P* = 0.535

(B) PFS, (entire patients, n = 186)

Clinicopathologic variables	**Category**	Univariate		Multivariate	
		Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age	60 ≥ vs < 60	1.160 (0.693–1.941)	*P* = 0.573	1.000 (0.588–1.702)	*P* = 0.999
Gender	male vs female	0.798 (0.516–1.235)	*P* = 0.312	0.808 (0.521–1.253)	*P* = 0.341
IPI risk	(Hi + H) vs (Li + L)	1.903 (1.218–2.972)	*P* = 0.005	1.901 (1.202–3.007)	*P* = 0.006
S1PR1	+ vs –	1.123 (0.627–2.011)	*P* = 0.696	1.176 (0.656–2.110)	*P* = 0.585

Table V. continued

(C) Ann Arbor stage I + II (n = 68), OS

Clinicopathologic variables	Category	Univariate		Multivariate	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age	60 ≥ vs < 60	6.205 (0.825-46.669)	*P* = 0.076	6.542 (0.855-50.057)	*P* = 0.070
Gender	male vs female	0.235 (0.083-0.668)	*P* = 0.007	0.160 (0.054-0.470)	*P* < 0.001
IPI risk	(Hi + H) vs (Li + L)	2.341 (0.534-10.265)	*P* = 0.259	3.251 (0.666-15.860)	*P* = 0.145
S1PR1	+ vs –	2.823 (1.095-7.281)	*P* = 0.032	4.582 (1.637-12.828)	*P* = 0.004

(D) Ann Arbor stage I + II (n = 68), RFS

Clinicopathologic variables	Category	Univariate		Multivariate	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age	60 ≥ vs < 60	2.121 (0.619-7.262)	*P* = 0.231	2.325 (0.667-8.108)	*P* = 0.185
Gender	male vs female	0.344 (0.137-0.866)	*P* = 0.023	0.249 (0.095-0.651)	*P* = 0.005
IPI risk	(Hi + H) vs (Li + L)	2.275 (0.525-9.857)	*P* = 0.272	3.064 (0.659-14.249)	*P* = 0.153
S1PR1	+ vs –	2.287 (0.926-5.646)	*P* = 0.073	3.561 (1.354-9.364)	*P* = 0.010

CI, confidence interval; DLBCL, diffuse large B-cell lymphoma; **H, high risk; Hi, high-intermediate risk; IPI, International Prognostic Index; L, low risk; Li, low-intermediate risk; OS, overall survival; RFS, recurrence-free survival; S1PR1, sphingosine-1-phosphate receptor 1**

Supplementary Table I. Comparison of ***S1PR1, STAT3, S1PR2, SPHK1*** **and** ***SPHK2*** mRNA expression and S1PR1 IHC expression

case	S1PR1 (IHC)	mRNA				
		S1PR1	*STAT3*	*S1PR2*	*SPHK1*	*SPHK2*
1	+	1.00	1.00	1.000	1.000	1.000
2	+	0.87	1.06	0.866	1.774	1.345
3	+	0.44	0.28	0.119	0.455	0.235
4	+	1.41	0.28	0.107	1.867	0.292
5	+	0.23	0.40	0.705	1.547	0.478
6	–	0.28	1.20	0.502	2.353	0.391
7	–	0.21	0.32	0.501	0.816	0.487
8	–	0.03	0.17	1.010	0.524	0.231
9	–	0.17	0.10	1.568	0.804	0.261
10	–	0.21	0.20	0.363	1.220	0.195
11	–	0.26	0.25	0.465	1.481	0.512
12	–	0.45	1.29	0.836	3.272	0.618
13	–	0.03	0.24	0.116	0.672	0.046
14	–	0.36	0.38	0.161	1.211	0.162

S1PR1 status was determined using IHC analyses. **Gene expression levels were shown by the relative amount of case 1.**

S1PR1, sphingosine-1-phosphate receptor 1; SPHK, sphingosine kinase; STAT, signal transducer and activator of transcription

Supplementary Table II. Comparison of protein expression levels between IHC and WB analyses

case	S1PR1 (IHC)	S1PR1/β-actin	p-STAT3^{Tyr705} /STAT3	p-NFκB/p65^{Ser536} /NFκB	p-Akt^{Ser473}/Akt
1	+	**0.094**	0.530	0.239	0.005
2	+	**0.100**	0.547	0.399	0.074
3	+	**0.072**	0.416	1.323	0.104
4	+	**0.072**	0.715	0.324	0.0344
5	+	**0.187**	0.543	0.339	0.417
6	–	**0.003**	0.175	4.418	0.202
7	–	**0.045**	0.353	0.535	0.0414
8	–	**0.033**	0.250	0.942	0.0541
9	–	**0.011**	0.113	0.448	0.124
10	–	**0.034**	0.391	0.605	0.057
11	–	**0.011**	0.219	0.689	0.133
12	–	**0.044**	0.277	0.461	0.127
13	–	**0.026**	0.211	0.483	0.0415

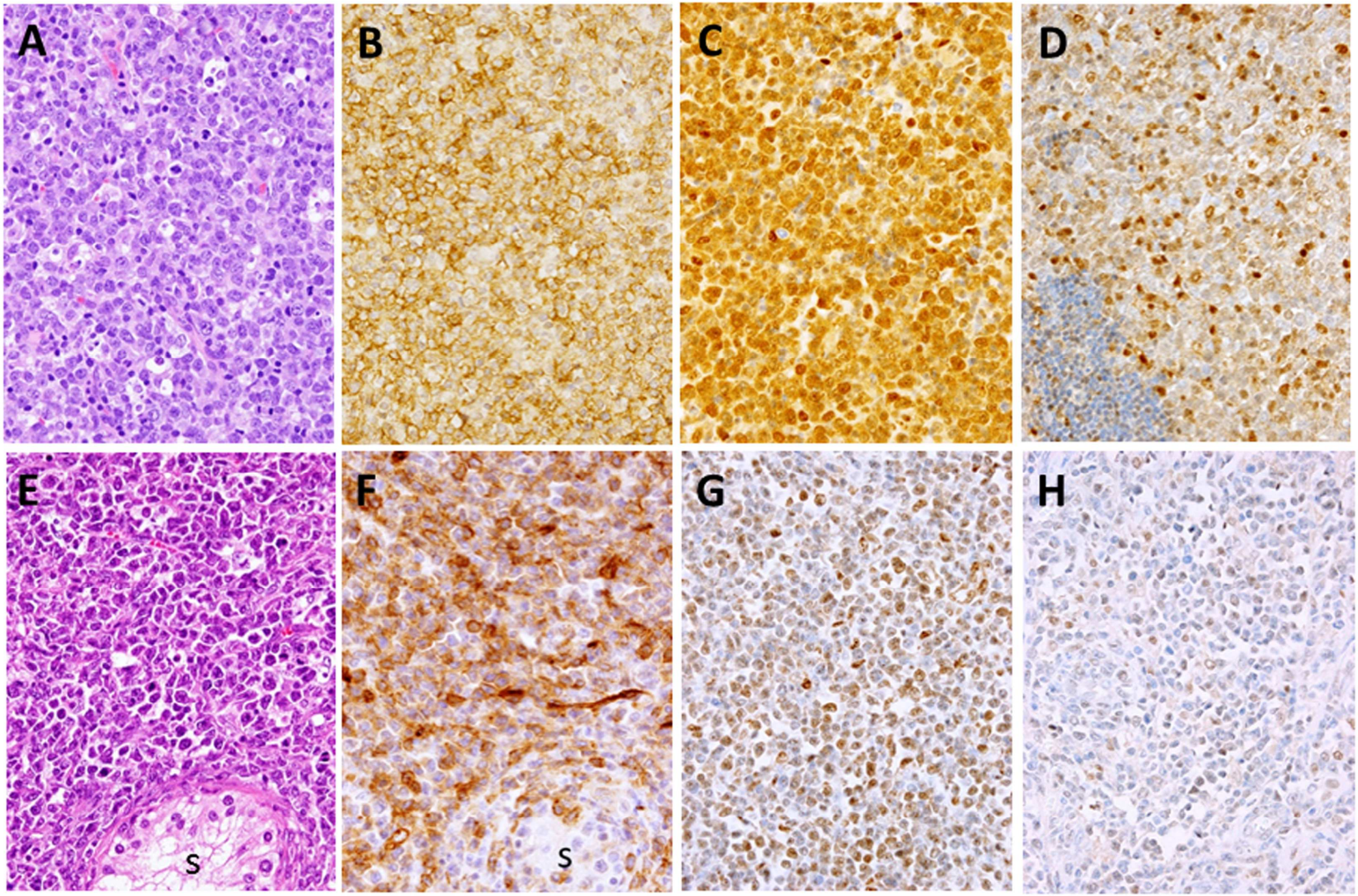
S1PR1, phosphorylated STAT3 (p-STAT3^{Tyr705}/STAT3), p-NFκB/p65^{Ser536}/NFκB, or p-Akt^{Ser473}/Akt were quantified in WB of tissue lysates from five cases of S1PR1-positive and **eight** cases of S1PR1-negative diffuse large B-cell lymphoma. S1PR1 status was determined using IHC analyses.

IHC, immunohistochemistry; NFκB, nuclear factor kappa B; p-STAT3, p-STAT3^{Tyr705}/STAT3; p-NFκB, p-NFκB/p65^{Ser536}/NFκB; p-Akt, p-Akt^{Ser473}/Akt; S1PR1, sphingosine-1-phosphate receptor 1; STAT, signal transducer and activator of transcription; WB, Western blot

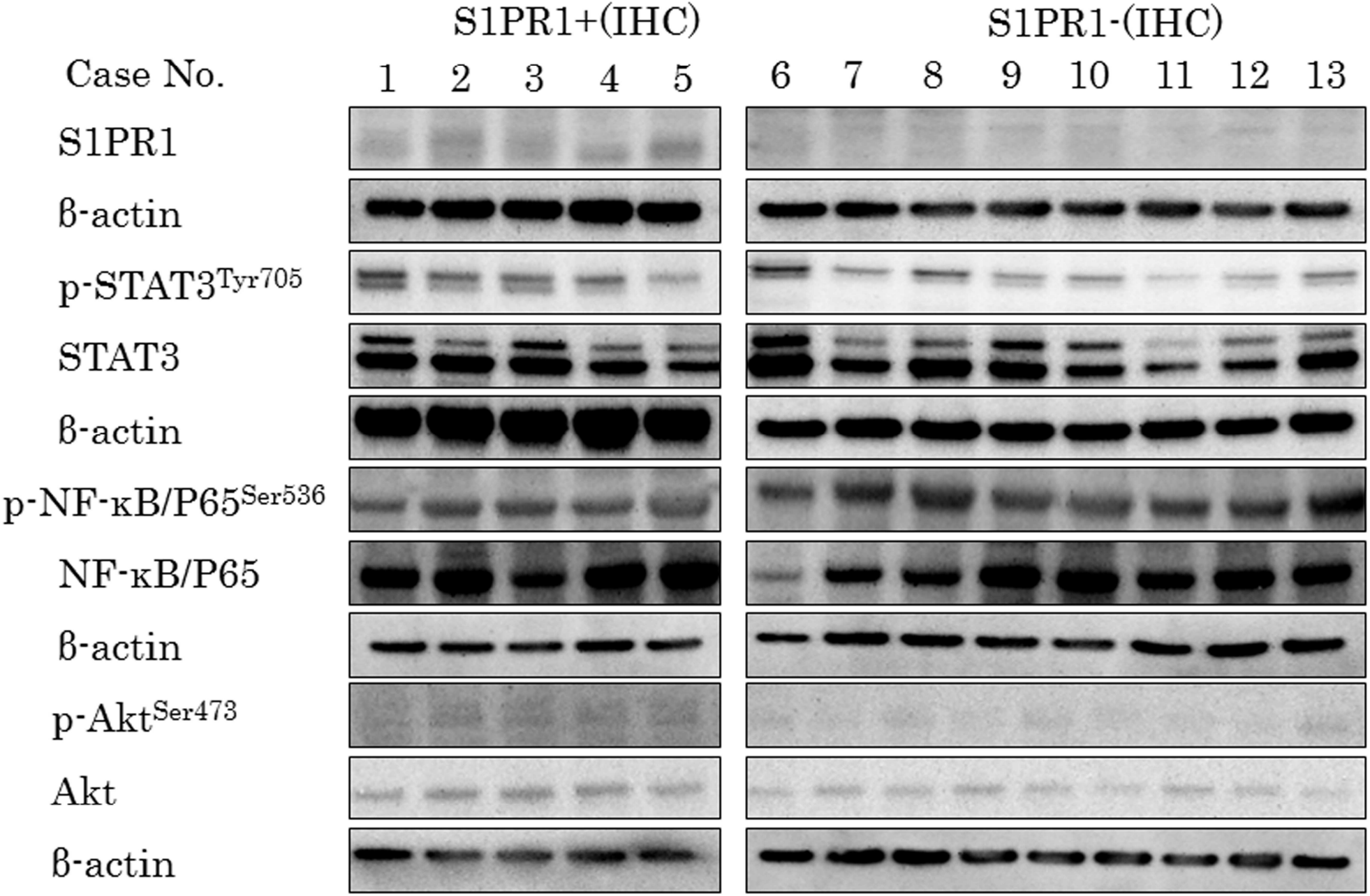
Figure 1. S1PR1-positive diffuse large B-cell lymphoma in lymph nodes (case 2 in Table I; A–D) and testis (case 17 in Table I; E–H). (**A**) Hematoxylin and eosin (HE)-stained sections; Lymphoma cells are relatively large lymphocytes with round nuclei containing one to two distinct nucleoli. (**B**) Strong membrane staining for S1PR1 was observed. (**C**) Most cells were stained for STAT3 and (**D**) over 70% of lymphoma cells were stained for **p-STAT3^{Tyr705}**. (**E**) HE-stained sections: infiltrated lymphoma cells destroying seminiferous tubules (S); (**F**) membrane staining for S1PR1 in lymphoma cells; (**G**) most cells show nuclear staining for STAT3 and (**H**) over 40% of lymphoma cells are immunopositive for **p-STAT3^{Tyr705}.** S1PR1, sphingosine-1-phosphate receptor 1; STAT, signal transducer and activator of transcription.

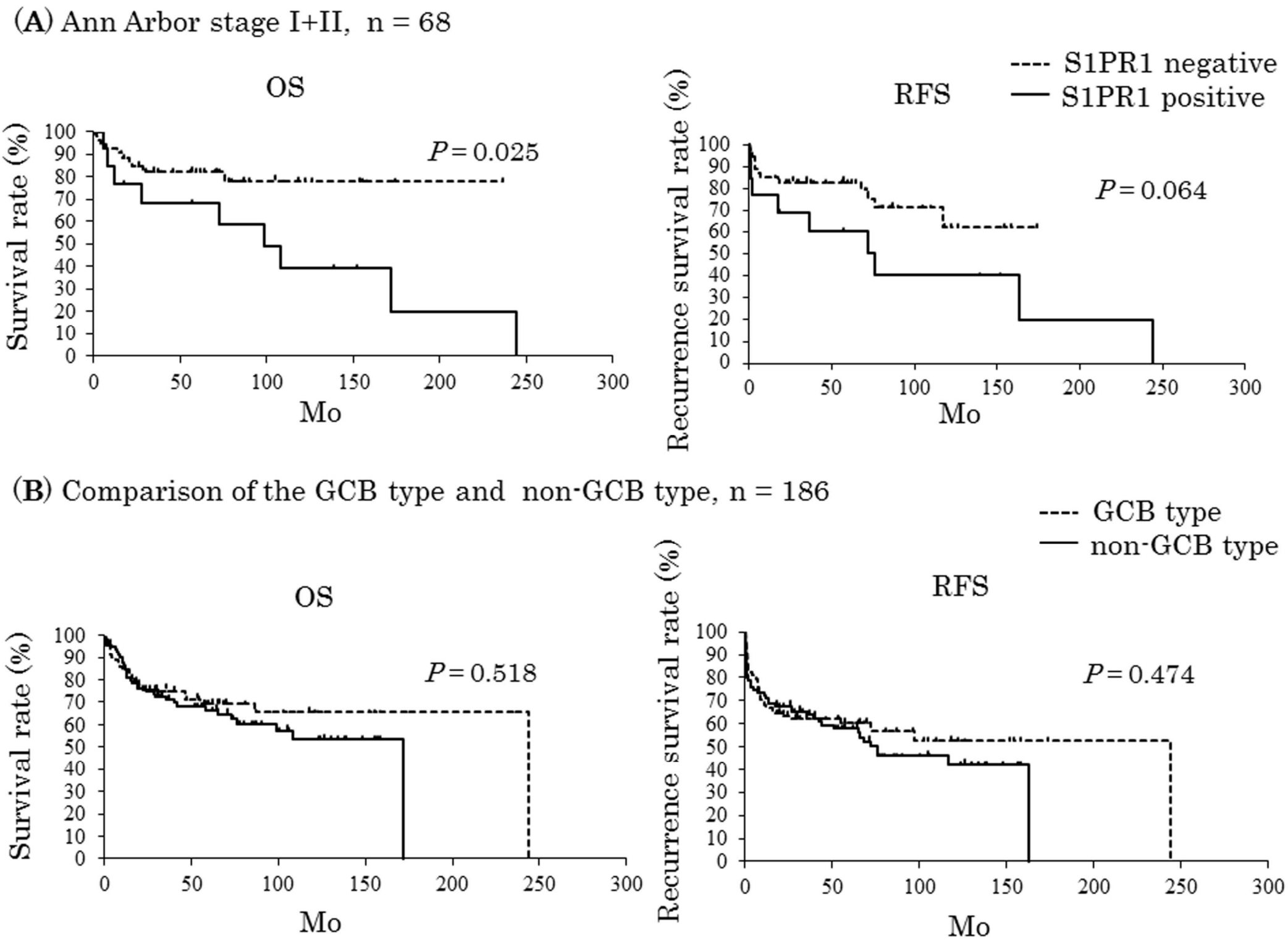
Figure 2. Western blots of S1PR1, **p-STAT3^{Tyr705}**, STAT3, **p-NFκB/P65^{Ser536}**, NFκB/p65, **p-Akt^{Ser473}**, Akt in tissues from five S1PR1-positive and **eight** S1PR1-negative cases of diffuse large B-cell lymphoma. S1PR1 status was determined using immunohistochemical analyses. Sample tissues from case numbers 1 and 2 were from testis and those from case numbers 3, 4 and 5 were from lymph nodes. All S1PR1-negative lymphoma samples (numbers 6–13) were derived from lymph nodes. S1PR1, sphingosine-1-phosphate receptor 1; STAT, signal transducer and activator of transcription.

Figure 3. Kaplan–Meier analyses of OS and RFS in S1PR1-positive and -negative 68 patients with Ann Arbor stage I + II DLBCL (**A**) and Kaplan–Meier survival analyses comparing OS and RFS **between 186 patients with DLBCL of GCB and non-GCB types** (**B**). DLBCL, diffuse large B-cell lymphoma; GCB, germinal centre B-cell like; OS, overall survival; RFS, recurrence-free survival; S1PR1, sphingosine-1-phosphate receptor 1.


A
B
C
D
E
S
F
S
G
H

(A)


S1PR1+(IHC)
S1PR1-(IHC)
Case No.
1 2 3 4 5 6 7 8 9 10 11 12 13
S1PR1
β-actin
p-STAT3Tyr705
STAT3
β-actin
p-NF-κB/P65Ser536
NF-κB/P65
β-actin
p-AktSer473
Akt
β-actin


(A) Ann Arbor stage I+II, n = 68
OS
Survival rate (%)
P = 0.025
Mo
RFS
Recurrence survival rate (%)
P = 0.064
Mo
---- S1PR1 negative
—— S1PR1 positive
(B) Comparison of the GCB type and non-GCB type, n = 186
OS
Survival rate (%)
P = 0.518
Mo
RFS
Recurrence survival rate (%)
P = 0.474
Mo
---- GCB type
—— non-GCB type

Supplementary Table I. Comparison of ***S1PR1, STAT3, S1PR2, SPHK1*** **and** ***SPHK2*** mRNA expression and S1PR1 IHC expression

case	S1PR1 (IHC)	mRNA				
		S1PR1	*STAT3*	*S1PR2*	*SPHK1*	*SPHK2*
1	+	1.000	1.000	1.000	1.000	1.000
2	+	0.870	1.060	0.866	1.774	1.345
3	+	0.440	0.280	0.119	0.455	0.235
4	+	1.410	0.280	0.107	1.867	0.292
5	+	0.230	0.400	0.705	1.547	0.478
6	–	0.280	1.200	0.502	2.353	0.391
7	–	0.210	0.320	0.501	0.816	0.487
8	–	0.030	0.170	1.010	0.524	0.231
9	–	0.170	0.100	1.568	0.804	0.261
10	–	0.210	0.200	0.363	1.220	0.195
11	–	0.260	0.250	0.465	1.481	0.512
12	–	0.450	1.290	0.836	3.272	0.618
13	–	0.030	0.240	0.116	0.672	0.046
14	–	0.360	0.380	0.161	1.211	0.162

S1PR1 status was determined using IHC analyses. **Gene expression levels were shown by the relative amount of case 1.**

S1PR1, sphingosine-1-phosphate receptor 1; SPHK, sphingosine kinase; STAT, signal transducer and activator of transcription

Supplementary Table II. Comparison of protein expression levels between IHC and WB analyses

case	S1PR1 (IHC)	S1PR1/β-actin	p-STAT3^{Tyr705} /STAT3	p-NFκB/p65^{Ser536} /NFκB	p-Akt^{Ser473}/Akt
1	+	**0.094**	0.530	0.239	0.005
2	+	**0.100**	0.547	0.399	0.074
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S1PR1, phosphorylated STAT3 (p-STAT3^{Tyr705}/STAT3), p-NFκB/p65^{Ser536}/NFκB, or p-Akt^{Ser473}/Akt were quantified in WB of tissue lysates from five cases of S1PR1-positive and **eight** cases of S1PR1-negative diffuse large B-cell lymphoma. S1PR1 status was determined using IHC analyses.

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