STIMULUS REPORT

blood advances

Clinical and functional impact of recurrent *S1PR1* mutations in mantle cell lymphoma

Agata M. Wasik,[1,*] Chenglin Wu,[2,*] Larry Mansouri,[3,4] Richard Rosenquist,[3,4] Qiang Pan-Hammarström,[2,†] and Birgitta Sander[1,†]

[1]Division of Pathology and [2]Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; [3]Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden; and [4]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

Key Points

- *S1PR1* mutations are present in 7.8% of patients with MCL and are significantly more frequent at relapse.
- *S1PR1* mutations reduce expression of the S1PR1 receptor, which mediates migration towards the tissue-to-blood egress factor S1P in MCL.

Introduction

Mantle cell lymphoma (MCL) is currently an incurable disease with a median survival of 3 to 5 years.[1] The initiating oncogenic event is the translocation t(11;14) resulting in high constitutive levels of cyclin D1, while secondary oncogenic events include mutations/deletions affecting key cellular processes (eg, DNA damage response and the B-cell receptor/NF-κB pathway).[2-5] Although MCL cells depend on signals from stromal cells within the residing tissue for survival and proliferation, these stromal cells can also protect MCL cells from chemotherapy and hence maintain residual tumor cells that may give rise to subsequent relapse(s).[6,7] Several receptors responsible for MCL cell localization within the tissue microenvironment have been reported including integrins and homing receptors, such as CXCR4, CXCR5,[8] CCR7,[9] and sphingosine-1-phosphate receptor 1 (S1PR1).[10,11] The importance of the microenvironment was recently reinforced by drugs targeting B-cell receptor signaling (eg, the Bruton tyrosine kinase inhibitor ibrutinib, which was shown to be effective in MCL).[12]

Sphingosine-1-phosphate (S1P) is a sphingolipid that regulates migration of lymphocytes through a concentration gradient with 10 000× higher levels in blood and lymph compared with tissue. S1P signals through 5 cognate G-protein coupled receptors (S1PR1-S1PR5). In diffuse large B-cell lymphoma (DLBCL), the expression of S1PR2 is disturbed either because of mutations (in germinal center B-cell subtype DLBCL)[13,14] or suppression by the transcription factor FOXP1 (in activated B-cell subtype DLBCL)[15] and contributes to the pathogenesis of DLBCL.

S1PR1 controls egress of lymphocytes to the blood and lymph[16] and is expressed in normal mantle zone B cells but also in MCL,[10,11] suggesting that this receptor might have a specific function for mantle zone B cells and their malignant counterparts. Although few mutations have been hitherto identified in homing/egress receptors, we recently reported *S1PR1* as 1 of 25 recurrent mutations by whole exome sequencing (WES) and Sanger sequencing of primary and relapsed tumors from 13 MCLs.[17] In the current study, we aimed to investigate the frequency and the possible clinical and functional impact of *S1PR1* mutations in MCL. We thus extended the screening of the coding region of *S1PR1* in 200 MCL samples from 179 patients and provide novel insight into functional consequences of these mutations.

Methods

Patient material and Sanger sequencing

The coding region of *S1PR1* (exon 2) was analyzed in 200 (diagnostic and/or relapse) samples from 179 MCL patients (including 6 samples that have been sequenced by WES and described by Wu et al[17]) diagnosed between 1997 and 2013. Paired normal DNA from 4 *S1PR1* mutated samples that were sequenced by WES did not show mutations in the S1PR1 germ line. For the remaining cases that were Sanger sequenced, paired normal DNA was not available, and thus we cannot ascertain the somatic origin of *S1PR1* mutations in these cases. However, in-depth analyses were made to subtract all previously known somatic variants as described by Wu et al.[17] Information on age, sex, stage, and survival status

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*A.M.W. and C.W. are joint first authors.

†Q.P.-H. and B.S. are joint senior authors.

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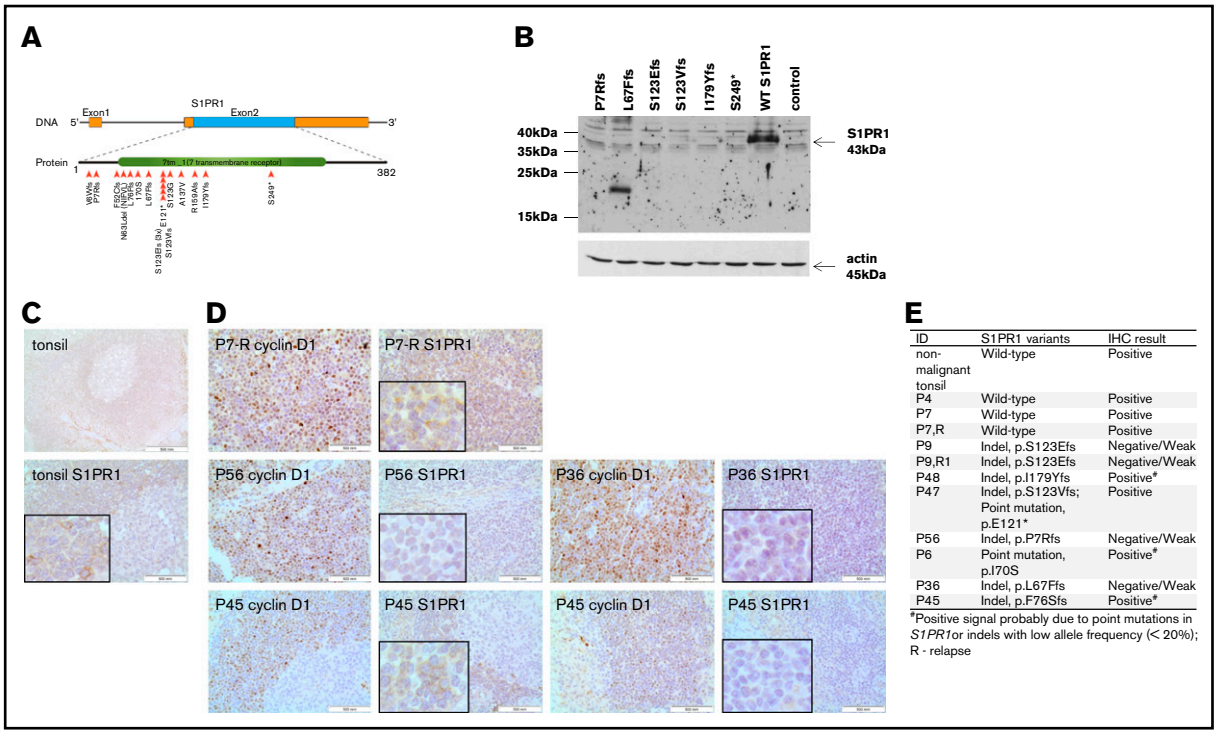

ID	S1PR1 variants	IHC result
non-malignant tonsil	Wild-type	Positive
P4	Wild-type	Positive
P7	Wild-type	Positive
P7,R	Wild-type	Positive
P9	Indel, p.S123Efs	Negative/Weak
P9,R1	Indel, p.S123Efs	Negative/Weak
P48	Indel, p.I179Yfs	Positive#
P47	Indel, p.S123Vfs; Point mutation, p.E121*	Positive
P56	Indel, p.P7Rfs	Negative/Weak
P6	Point mutation, p.I70S	Positive#
P36	Indel, p.L67Ffs	Negative/Weak
P45	Indel, p.F76Sfs	Positive#

#Positive signal probably due to point mutations in *S1PR1*or indels with low allele frequency (< 20%); R - relapse

Figure 1. Deleterious variants identified in *S1PR1* gene resulted in reduced expression of S1PR1. (A) The distribution of *S1PR1* variants identified in the discovery and extended cohort of MCL patients. Altogether, 200 Swedish MCL samples from 179 patients were screened by WES and/or Sanger sequencing. Most of the variants identified in *S1PR1* were frameshift insertion or deletions (indels). In P9, an identical mutation (p.S123Efs) was detected in all 3 samples. In P47, 2 mutations were detected. (B) S1PR1 protein expression in a fibroblast cell line (WB detection) transfected with wild-type (WT) or mutant S1PR1 constructs; β-actin was used as a loading control. In lane 2, a band between 15 and 20 kDa is detected, which is probably nonspecific because the mutations would lead to a truncated protein of ~35 kDa. IHC staining of S1PR1 in control tonsil samples (C; note S1PR1 expression in the mantle zone of germinal centers) and in representative MCL cases (D) carrying WT (P7) or mutated S1PR1 (P56, P36, P45; note that in P45 different tumor areas vary in S1PR1 expression); magnification ×200. (E) The full summary on the S1PR1 expression by IHC in MCL cases.

were collected from patients' records. Genomic DNA was extracted from peripheral blood, frozen tumor samples, or viability frozen cells using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Taufkirchen, Germany), following the manufacturers' instructions. Polymerase chain reaction (PCR) and sequencing primers (available upon request) were designed with National Center for Biotechnology Information/Primer-BLAST.[18] The resulting PCR products were purified by using the Qiaquick PCR purification kit (Qiagen) and sequenced at Macrogen Inc. (Seoul, Korea) or Eurofins MWG Operon (Ebersberg, Germany). The institutional review boards at Karolinska Institutet and Uppsala University approved the study. The study was completed in accordance with the Declaration of Helsinki.

S1PR1 expression constructs

Wild type or 6 mutants (P7Rfs, L67Ffs, S123Efs, S123Vfs, I179Yfs, and S249*) of *S1PR1* were amplified and cloned into the *Hin*dIII and *Bam*HI sites of the pcDNA3.1 plasmid (Invitrogen, Gaithersburg, MD). Transient transfection of *S1PR1* wild type or mutants into a human dermal fibroblast cell line with Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's recommendations.

Protein expression

S1PR1 detection was performed using western blot (WB) or immunohistochemistry (IHC). In both methods, primary antibody anti-S1PR1 antibody sc-25489 (Santa Cruz, CA) was used.

Migration assay

MCL cell lines Granta519 and JVM2 (DSMZ, Germany) with transiently downregulated *S1PR1* using gene-specific short interfering RNA (siRNA) (cat. 4390824, Invitrogen) or negative control siRNA (cat. 4390844, Invitrogen) were stained with calcein-AM (ThermoFisher Scientific), and migration toward S1P (Cayman Chemicals) in fatty-acid–free bovine serum albumin (Sigma) was measured using Fluoroblok inserts (8-μm pore size, 96-well plate format; Corning). The migration is presented as percent of migration toward conditioned media (control).

Results and discussion

Using Sanger sequencing, we screened the coding region of *S1PR1* in lymphoma tissue from 179 MCL patients. In total, 200 biopsies were available, and out of these, 152 were taken at diagnosis and 48 at relapse. Among those, paired samples from 17 patients were analyzed (either diagnostic biopsy and relapse biopsy/biopsies or multiple relapse biopsies). *S1PR1* was found to be mutated in 8.0% (16/200) of the tumor samples and in 7.8% (14/179) of the patients. In 1 case (P47), 2 mutations in the S1PR1 gene were found. Notably, most (10/15) of the mutations identified were frameshift insertions/deletions (indels), located in the N-terminal region of the protein (Figure 1A). The remaining alterations consisted of 2 stop-gain and 3 nonsynonymous missense single-nucleotide variations that were predicted to be damaging by Combined Annotation Dependent Depletion (CADD) scores, SIFT, and PolyPhen-2 (Table 1). Thus, the majority of *S1PR1* mutations are likely to

Table 1. Summary of mutations discovered in the *S1PR1* gene in our MCL cohort

Gene	Nucleotide change	AA change	Found in WES	Found in Sanger	Mutated tumor	Novel or not	CADD scores	SIFT	PolyPhen-2
S1PR1	c.14_21delGCGTCCC	p.V6Wfs		Yes	1 (P170,R)	Novel	24	N.A.	N.A.
	c.19_20 insGGGA	p.P7Rfs		Yes	1 (P56)	Novel	23.6	N.A.	N.A.
	c.154_155 insGT	p.F52Cfs		Yes	1 (P18,R)	Novel	28.8	N.A.	N.A.
	c.186_201delGAACATCTTTGTCTT	p.N63Ldel (NIFVL)		Yes	1 (P166)	Novel	20.3	N.A.	N.A.
	c.201_202 insT	p.L67Ffs		Yes	1 (P36)	Novel	32	N.A.	N.A.
	c.T209G	p.I70S	Yes	Yes	1 (P6)	Novel	27.2	Damaging	Damaging
	c.226_227 insGACACCTGTTGGCAGGAGTAGCC-TACACAGCTAACCTGCTCTTG	p.F76Sfs		Yes	1 (P45)	Novel	27.7	N.A.	N.A.
	c.361 G>T	p.E121*		Yes	1 (P47)	Novel	36	N.A.	N.A.
	c.362_363 delA	p.S123Vfs		Yes	1 (P47)	Novel	27.5	N.A.	N.A.
	c.366_367insG	p.S123Efs	Yes	Yes	3 (P9; P9,R1; P9,R2)	Novel	26.8	N.A.	N.A.
	c.367 A>G	p.S123G		Yes	1 (P236,R)	Novel	8.766	Damaging	Damaging
	c.410 C>A	p.A137V		Yes	1 (P235,R)	Novel	28.9	Damaging	Damaging
	c.475_476delC	p.R159Afs		Yes	1 (P172,R)	Novel	23.7	N.A.	N.A.
	c.535_536 insT	p.I179Yfs		Yes	1 (P48)	Novel	28.8	N.A.	N.A.
	c.743_744 insT	p.S249*		Yes	1 (P242)	Novel	34	N.A.	N.A.

Two hundred samples in total, including cases characterized by WES.[17]
AA, amino acid; N.A., not applicable; R, relapse.

be pathogenic and affect the expression/function of the protein. For samples that underwent WES,[17] we analyzed copy number alterations.[19] We found no gains or losses at 1p21 in the diagnostic biopsies, but we found loss at 1p21 in relapse samples (supplemental Figure 1). In order to investigate whether other genetic alterations coexist with *S1PR1* mutations, we analyzed WES results (n = 27 from 13 patients) and found that *S1PR1* mutated cases carry mutations in *MEF2B* but not *ATM*.[17] For the 179 samples from 171 patients, we had full information on *CARD11* mutations, and among cases with *S1PR1* mutations, only 1 case (P9R2) had mutations in both genes.

To gain insight into the functional consequences of *S1PR1* mutations, we introduced wild-type or mutant (P7Rfs, L67Ffs, S123Efs, S123Vfs, I179Yfs, and S249*) constructs to fibroblast cells and analyzed protein expression by WB analysis. Although expression of S1PR1 was detected in cells transfected with wild-type *S1PR1*, it was absent in cells transfected with the different mutants (Figure 1B). We then examined the S1PR1 protein expression by IHC in 3 primary MCL cases carrying wild-type *S1PR1* and 8 mutated MCL samples; nonmalignant tonsil was used as a control. In tonsil, S1PR1 expression was detected in cells within the mantle zone (Figure 1C). All wild-type MCL cases showed immunopositivity for S1PR1 (Figure 1D, showing P7-R case). In 4 samples carrying *S1PR1* indels, staining was weak/negative, exemplified by cases P56 and P36 in Figure 1D, whereas the remaining 4 samples with either missense mutations in *S1PR1* or indels in a smaller proportion of the tumor clone (<20%) were partly immunoreactive for S1PR1. Interestingly, in P45 S1PR1 was highly expressed in areas with mantle zone growth pattern but not in areas with diffuse growth (Figure 1D, P45). Thus, our results indicate that most *S1PR1* frameshift mutations identified in this study would diminish S1PR1 protein expression.

To investigate functional consequences of reduced S1PR1 protein levels, we transiently downregulated *S1PR1* by siRNA in MCL cell lines (Granta519 and JVM2). Our results demonstrated that migration toward S1P was reduced in cells with downregulated S1PR1 (supplemental Figure 2). Thus, S1PR1 in MCL cells is functional and, similar to nonmalignant B and T cells,[20-22] regulates migration toward S1P. Mutations diminishing S1PR1 expression may therefore influence how MCL cells respond to the S1P gradient and as a consequence disturb homing/egress of MCL cells, by preventing them from recirculation and contributing to their retention in the supportive microenvironment.

S1PR1 mutations were further correlated with selected clinical parameters (supplemental Table 1). Stage 4 disease was more prevalent among cases with mutated *S1PR1* ($P = .02$, 2-sample test for variance). The median survival of the MCL cases carrying mutations in *S1PR1* was longer, and these patients were also younger at diagnosis; however, none of these differences were statistically significant.

Interestingly, mutations in *S1PR1* were observed in 5.9% (9/152) of biopsies taken at diagnosis and in 14,6% (7/48) of biopsies taken at relapse ($P = .0002$), which is consistent with the copy number variation analysis where specific loss of 1p21, where *S1PR1* is located, was observed in relapse samples.[17]

Taken together, it is possible that dysfunctional/diminished S1PR1 contributes to the retention of malignant cells in the surrounding tissue, constituting a minimal residual disease reservoir that later may give rise to a relapse. In DLBCL, mutations of *S1PR1* have not yet been reported, but lack of membrane expression of S1PR1 in DLBCL at early stages (Ann Arbor I+II) correlates to superior outcome.[23,24] MCL patients treated with ibrutinib release lymphoma cells from the tumor site into the bloodstream.[12] However, ~40% of MCL patients develop resistance to ibrutinib, and adhesion of MCL cells to the stroma contributes to this resistance.[25] Patrussi et al reported that ibrutinib elevates expression of S1PR1 (simultaneously decreasing CCR7) in chronic lymphocytic leukemia.[26] However, further studies are needed to determine if *S1PR1* mutations impact the outcome of ibrutinib therapy in MCL.

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Authorship

Contribution: A.M.W. and C.W. were responsible for experimental work; A.M.W., C.W., Q.P.-H., and B.S. performed data analysis and interpretation; L.M., R.R., and B.S. were responsible for clinical data; R.R., L.M., and B.S. provided tissue samples; and all authors contributed to writing the manuscript and study design.

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Correspondence: Birgitta Sander, Division of Pathology, Department of Laboratory Medicine, F46, Karolinska Institutet, Flemingsberg campus, SE 141 86 Stockholm, Sweden; e-mail: birgitta.sander@ki.se.

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