Expression of TRPM8 in human reactive lymphoid tissues and mature B-cell neoplasms

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Abstract. Transient receptor potential melastatin 8 (TRPM8) is a member of the transient receptor potential superfamily of Ca^{2+} channels. The aim of the present study was to clarify TRPM8 expression in reactive lymphoid tissues and mature B-cell neoplasms. Reactive and neoplastic lymphoid tissues were used to evaluate TRPM8 expression by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). TRPM8+ cells were frequently detected in the follicular light zone and marginal zone of reactive lymphoid tissues. Double immunostaining revealed that TRPM8+ cells co-expressed cluster of differentiation (CD) 38, CD79a, CD138, interferon regulatory factor 4/melanoma associated antigen (mutated) 1, B cell CLL/lymphoma 6 and transmembrane activator and CAML interactor. TRPM8+ neoplastic cells were frequently detected in plasma cell myeloma. The positive band of TRPM8 mRNA was confirmed by RT-PCR in cases of myeloma. The present study is, to the best of our knowledge, the first to demonstrate the expression of TRPM8 in reactive lymphoid tissues and mature B-cell neoplasms, revealing that TRPM8 is frequently expressed in pre-plasmablasts, plasmablasts, plasma cells and mature B-cell lymphomas that are likely to differentiate into plasma cells.

Introduction

When naive B cells are activated by antigens, they differentiate into activated B cells (CD20+CD38±CD138-), pre-plasmablasts (CD20±CD38+CD138-), and plasmablasts

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(CD20-CD38+++CD138-) in lymphoid follicles, followed by early plasma cells (PCs; CD20-CD38+++CD138++) and then late PCs (CD19+CD20-CD38+++CD138+++) in extrafollicular areas, including bone marrow (1-3). PCs are mainly distributed in the lymphoepithelial symbiosis (SYM) /marginal zone (MZ) of the tonsils, the MZ and medullary cord of the lymph nodes (LN), and the dome of Peyer's patches.

Mature B-cell neoplasms correspond to the clonal proliferation of mature B cells derived from naive B cells to PCs in the normal B-cell differentiation pathway. Since the nature of neoplastic B cells may mimic that of normal B cells at each differentiation stage, the nomenclature and classification of these neoplasms are based on their differentiation stages (4). The clonal expansion of terminally differentiated B cells is generally called plasma cell neoplasm (PCN) (5). Although a number of antibodies that recognize PCN cells have been reported in the literature, only a few antibodies, including CD38, CD56, CD79a, CD117, CD138, and interferon-regulating factor 4/multiple myeloma oncogene 1 (IRF4/MUM-1), are valuable for and applicable to formalin-fixed and paraffin-embedded tissue sections (3,6-8).

Transient receptor potential melastatin 8 (TRPM8) is a member of the transient receptor potential (TRP) superfamily of Ca^{2+}-permeable non-selective cation channels (9-11). This channel was identified by Tsavaler *et al* (12) as a novel prostate-specific protein that is up-regulated in prostate cancer (13,14). This protein was also identified in the sensory nerves of spinal dorsal root ganglia, and because it activates the cellular influx of Ca^{2+} across the plasma membrane with cold stimuli (8-28°C), including cooling agents such as menthol, this channel protein is regarded as a cold thermoreceptor (15-20).

TRPM8 has been found in approximately 10 types of malignant tumors including carcinomas derived from the prostate gland, mammary gland, oral cavity, colon, lung, skin, pancreas, and urinary bladder (12-14,21-26). Furthermore, murine lymphocytes express several subtypes of the TRP superfamily (27-29); however, it currently remains unclear whether TRPM8 localizes in human reactive lymphoid tissues and malignant lymphomas.

The aim of the present study was to clarify the expression of TRPM8 in reactive lymphoid tissues and mature B-cell

neoplasms. We herein demonstrated for the first time that pre-plasmablasts and plasmablasts in germinal centers (GC), and PCs in the extrafollicular areas of reactive lymphoid tissues as well as some mature B-cell neoplasms express TRPM8.

Patients and methods

Patients and specimens. Tissue specimens resected from patients with chronic tonsillitis (n=5), reactive lymphadenitis (n=5), and acute appendicitis (n=5) were used as reactive lymphoid tissues. Tissue specimens were collected from LNs, bone marrow, gastrointestinal tract and kidney which were pathologically diagnosed as malignant B-cell neoplasms. Mature B-cell neoplasms included 3 cases of small lymphocytic lymphoma (SLL) [2 males, 1 female; median age, 46.7 years (range, 31-68)], 4 cases of lymphoplasmacytic lymphoma (LPL) [3 males, 1 female; median age, 65.0 years (range, 47-80)], 16 cases of PCN including 3 cases of monoclonal gammopathy of undetermined significance (MGUS) [3 females; median age, 62.0 years (range, 60-75)], 12 cases of plasma cell myeloma (PCM) including 6 cases of IgG type [3 males, 3 females; median age, 63.5 years (range, 40-83)], 6 cases of IgA type [4 males, 2 females; median age, 65.2 years (range, 19-81)], 1 case of extraosseous plasmacytoma [1 male; age, 73 years], 5 cases of extranodal MZ lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) [2 males, 3 females; median age, 70.0 (range, 53-80)], 5 cases of follicular lymphoma (FL) [only cases of a follicular pattern of >75% were selected, 1 male, 4 females; median age, 63.5 (range, 59-58)], 3 cases of mantle cell lymphoma (MCL) [except variants, 2 males, 1 female; median age, 69.0 (range, 62-73)], and 18 cases of the centroblastic and immunoblastic subtypes of common morphological variants of diffuse large B-cell lymphoma (DLBCL) including 7 cases of GC B-cell like (GCB) [3 males, 4 females; median age, 55.43 years (range, 8-81)] and 11 cases of non-GCB [5 males, 6 females; median age, 70.64 (range, 53-78)] as immunohistochemical subgroups (30). These tissue specimens were fixed in buffered 10% formalin at room temperature for 6-12 h, and then embedded in paraffin and used for immunohistochemistry. Formalin-fixed and paraffin-embedded tissue specimens resected from 4 patients with well-differentiated prostatic carcinoma were used as controls for immunohistochemistry and from one patient for the reverse transcription polymerase chain reaction (RT-PCR). Fresh-frozen tissue specimens obtained from patients with PCM (n=2) were used for RT-PCR.

Written informed consent was obtained from each patient. The use of tissues and clinical information study was approved by the Ethics Committee of Yamagata University School of Medicine (no. 23).

Immunohistochemistry. Immunohistochemistry was performed using a rabbit polyclonal anti-human TRPM8 antibody [Alomone, Jerusalem, Israel; diluted titer of 1/200; recognizes the peptide SDVD GTTYDFAHC corresponding to amino acid residues 917-929 in the 3rd extracellular loop of human TRPM8 (Accession Q7Z2W7)] (31) as a primary antibody. Regarding the specificity of TRPM8 immunostaining, the antibody was pre-incubated for 2 h with decreasing concentrations of the peptide to TRPM8 before incubating with the tissue section (antigen-preabsorption). In detail about antigen-preabsorption, the provided antigen of TRPM8 reacted with the antibodies in the used primary antibody. And then, the slides were incubated with this mixture and performed the continued steps of IHC. No positive reaction was confirmed in this IHC with antigen-preabsorption (data not shown). After that, other slides from same specimen had been performed by same immunohistochemistry (TRPM8) without antigen-preabsorption. The IHC of tissue sections without antigen-preabsorption showed positive reaction. It could be suggested that anti-TRPM-8 rabbit polyclonal antibody, used in this study, had high specificity on its own antigen (TRPM8). However, western blot by using cells lines or other specimens should be performed to verify the specificity of TRPM8. In this study, we could not perform western blot analysis to rule out non-specific staining. Furthermore, non-immune rabbit serum (Dako, Glostrup, Denmark) was used as a negative control.

Briefly, after the blocking of endogenous peroxidase activity, antigen retrieval was conducted in 0.01 M citrate buffer, pH 6.0 at 120°C for 20 min in an autoclave. After rinsing in 0.01 M phosphate-buffered saline (PBS), slides were incubated at 4°C for 6-8 h with the primary antibody. The streptavidin-biotin-peroxidase method was performed using an EnVision™/horseradish peroxidase-labeled anti-rabbit immunoglobulin antibody (Dako), and slides were incubated at room temperature for 1 h. A positive reaction was detected as brown coloration with 3,3'-diaminobenzidine (DAB; Dojin Chemicals, Kumamoto, Japan). Sections were counterstained with hematoxylin.

TRPM8$^+$ cells in reactive lymphoid tissues were estimated in different areas, such as the SYM/MZ, mantle zone, outer zone, light zone, dark zone, and interfollicular area (T-cell zone) in the tonsils; the MZ, mantle zone, light zone, dark zone, paracortical/interfollicular area, and medullary cords in the LN; and the dome, mantle zone, light zone, dark zone, and interfollicular area in the appendix. The identification of each zone in secondary lymphoid follicles was performed on serial sections for immunostaining using anti-TRPM8 and CD23 (1B12; mouse IgG1κ, Novocastra, Newcastle upon Tyne, UK) antibodies as described previously (Fig. 1A) (32).

In the estimation of the immunoreaction of TRPM8 in reactive lymphoid tissues, cells showing cell membrane and cytoplasmic staining equal to or greater than those of well-differentiated prostatic carcinoma cells and/or GC plasmablast-like cells in terms of intensity were judged as positive. At least 500 cells in each area of reactive lymphoid tissues were observed in 1-3 high-power-view fields (x400) and the percentage of TRPM8$^+$ cells among all cells in each area was calculated. Similarly, 500-1,000 neoplastic cells in mature B-cell neoplasms were observed in 1-3 high-power-view fields, and the frequency of TRPM8$^+$ cells was estimated as score 0, <1% of positive cells; score 1, 1-25%; score 2, 26-50%; score 3, 51-75%; and score 4, 76-100%. The frequencies of positive cells in reactive lymphoid tissues and B-cell neoplasms were evaluated by two independent observers (M.Y. and A.H.), and the mean values of their measurements were regarded as the results.

Double immunostaining in the tonsils. In the first step, tonsillar tissue sections were immunostained with 15 different types
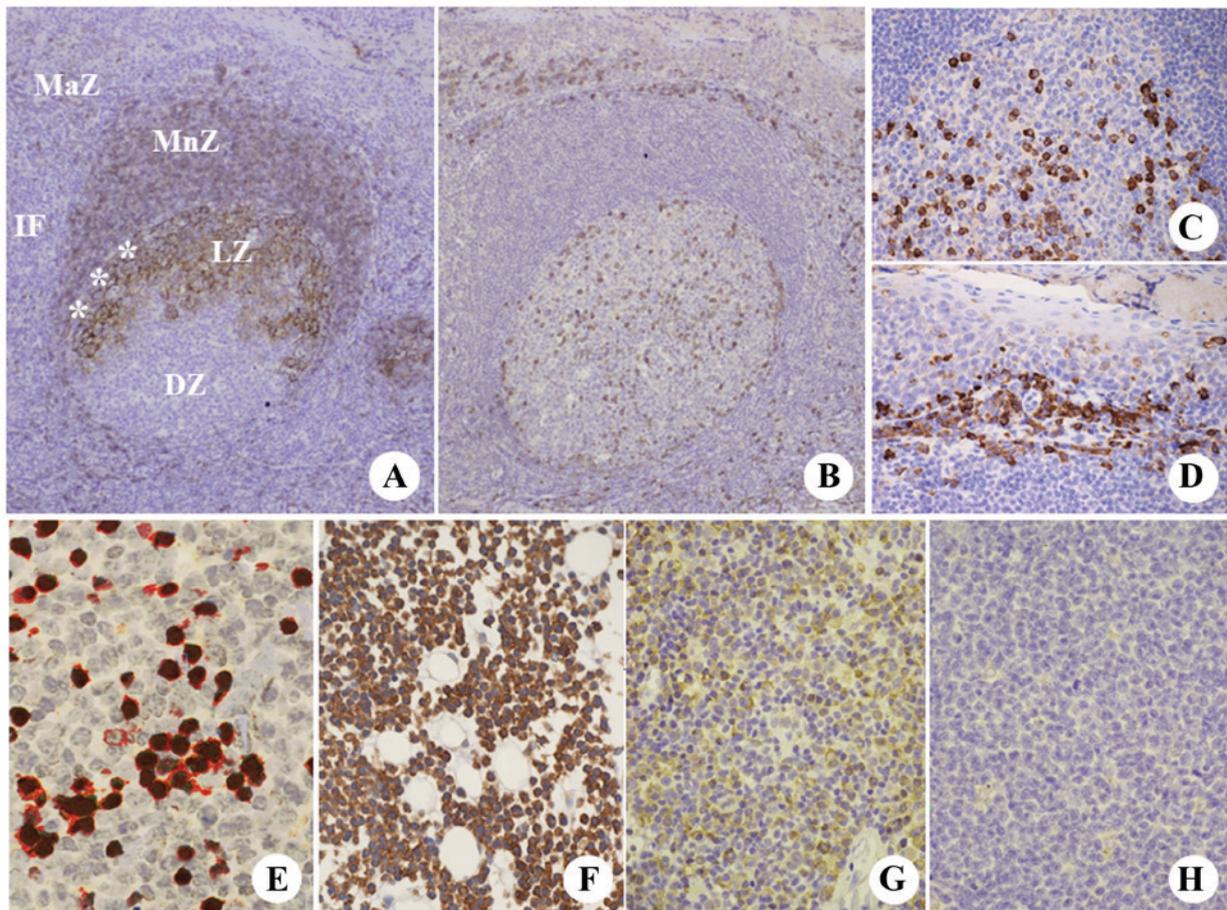
Figure 1. Immunohistochemistry of TRPM8 and lymphocyte markers in tonsils and mature B-cell neoplasms. (A) The CD23 immunostain recognizes four different zones of a tonsillar lymphoid follicle, namely, the MaZ, outer zone (*), LZ, and DZ. MZ: *SYM/MZ*; IF: Interfollicular area (x40). (B) The TRPM8 immunostain in the same lymphoid follicle as (A). There are many TRPM8^{+} cells in the light zone and SYM/MZ (x40). (C) TRPM8^{+} cells in the light zone of a tonsillar lymphoid follicle (x200). (D) TRPM8^{+} cells in SYM/MZ (x400). (E) Double immunostain of IRF4/MUM-1 and TRPM8 in the tonsillar germinal center using digital photomicrographs. The nuclei of IRF4/MUM-1^{+} cells are shown as blue in a digital photo. The same tissue section was immunostained with TRPM8 and IRF4/MUM-1^{+} TRPM8^{+} cells, shown as red in the photo. The first photo was overlaid with the second photo. IRF4/MUM-1^{+} TRPM8^{+} cells are recognized as cells having a black-colored nucleus and red-colored cytoplasm (x200). (F) TRPM8^{+} neoplastic cells in plasma cell myeloma; immunostaining score=4 (x200). (G) TRPM8^{+} cells in lymphoplasmacytic lymphoma; immunostaining score=2 (x200). (H) TRPM8^{+} cells in mantle cell lymphoma; immunostaining score=0 (x200). All sections were counterstained with hematoxylin. SYM, lymphoepithelial symbiosis; MZ, marginal zone; TRPM8, transient receptor potential melastatin 8; DZ, dark zonel LZ, light zone; MaZ, mantle zone; IF: Interfollicular area.

of lymphocyte markers to recognize B cells, T cells, natural killer cells, and plasma cells, such as antibodies against CD3 (PS1; mouse IgG2a), CD10 (56C6; mouse IgG1; both Nichirei, Tokyo, Japan), CD20 (L26; mouse IgG2κ; Dako), CD23 (1B12; mouse IgG1κ), CD38 (SPC32; mouse IgG1; both Novocastra), CD56 (1B6; mouse IgG1), CD79a (JCB117; mouse IgG1κ), CD117 (Rabbit polyclonal; all Nichirei) CD138 (MI15; mouse IgG1κ), IRF4/MUM-1 (MUM-1p; mouse IgG1κ; both Dako), BCL6 (rabbit polyclonal; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), paired box protein 5 (PAX5; 8F9; mouse IgG1κ, Abgent, San Diego, CA, USA), programmed cell death 1 (PD1)/CD279 (NAT; mouse IgG1; Abcam, Cambridge, UK), BLIMP1 (3H2-E8; mouse IgG1; Thermo, Rockford, IL, USA), and transmembrane activator and calcium-signal modulating cyclophilin ligand interactor (TACI; 4976; mouse IgG, Santa Cruz Biotechnology, Inc.). A positive reaction was detected with the UltraTech HRP Streptavidin-Biotin Detection System (Beckman Coulter, Marseille, France) and DAB as a brown color. Digital photomicrographs were taken of high-power-view fields in the SYM/MZ and light zone, which contained a large

number of TRPM8^{+} cells. In the second step, TRPM8 immunostaining was performed on the same tissue sections using the same method, and digital photomicrographs of the same fields as those in the first step were taken. Photos, bilayered by TRPM8 single immunostaining and TRPM8-lymphocyte marker double immunostaining, were created using image processing software (Adobe Photoshop Elements® 7.0, Adobe Systems, San Jose, CA, USA) (33). TRPM8 single-positive cells and double-positive cells in each of the 5 imaging photos of the SYM/MZ and light zone were counted and the ratio of double-positive cells to TRPM8^{+} cells was calculated.

RT-PCR. Frozen tissue specimens were obtained from 2 patients with PCM, stored at -80°C in a deep freezer, and a formalin-fixed paraffin-embedded tissue specimen with well-differentiated prostatic carcinoma was used as a positive control. In addition, formalin-fixed paraffin-embedded tissue specimens from PCM (n=5), FL (n=3) and MCL (n=2) were used. Undamaged mRNA was extracted from defrosted frozen cells by TRIzol® Reagent (Invitrogen; Thermo Fisher

Table I. Expression of transient receptor potential melastatin 8 in reactive lymphoid tissues.

Tissues/areas	Positive cells/total cells in each area (%) (mean ± standard deviation)[a]
Tonsil	
SYM/marginal zone	8.73±2.05[e]
Mantle zone	0.20±0.13[b,e]
Outer zone	2.82±0.90
Light zone	17.49±3.23
Dark zone	1.05±0.50
Interfollicular area	0.76±0.27[f]
Lymph node	
Marginal zone	2.70±0.83
Mantle zone	0.46±0.19[c,g]
Light zone	12.92±1.71[g]
Dark zone	1.08±0.48
Paracortex/interfollicular area	1.00±0.50
Medullary cord	37.93±11.23[c]
Appendix	
Dome	5.75±2.15[h]
Mantle zone	0.37±0.19[d,h]
Light zone	9.34±2.18[d]
Dark zone	0.99±0.23
Interfollicular area	0.99±0.41

SYM, lymphoepithelial symbiosis. [a]P<0.01 by Kruskal-Wallis test, [b]P<0.01 mantle zone vs. light zone in tonsil, [c]P<0.01 medullary cord vs. mantle zone in lymph node, [d]P<0.01 mantle zone vs. light zone in appendix, [e]P<0.05 SYM/marginal zone vs. mantle zone in tonsil, [f]P<0.05 light zone vs. interfollicular area in tonsil, [g]P<0.05 mantle zone vs. light zone in lymph node, [h]P<0.05 dome vs. mantle zone in appendix, respectively by Scheffé's multiple comparison test.

Table II. Frequency of the co-expression of lymphocyte markers on TRPM8-positive cells in the reactive tonsillar germinal center and SYM/marginal zone by double immunostaining.

Antibodies	Frequency of co-expression of lymphocyte markers on TRPM8^{+} cells (%) (mean ± standard deviation)	
	Germinal center	SYM/marginal zone
CD3	0.00±0.00	0.00±0.00
CD10	11.14±0.05	5.87±0.12
CD20	17.82±3.70	8.52±4.95
CD23	0.00±0.00	0.00±0.00
CD38	72.60±15.46	74.87±10.71
CD56	0.00±0.00	0.00±0.00
CD79a	59.57±2.76	60.41±20.15
CD117	0.00±0.00	0.00±0.00
CD138	32.29±5.61	52.28±18.51
IRF4/MUM-1	69.12±23.42	58.77±12.95
BCL6	69.75±18.35	47.73±10.45
PAX5	0.74±0.65	4.09±3.55
PD1	0.00±0.00	0.00±0.00
BLIMP1	0.00±0.00	0.00±0.00
TACI	62.54±1.44	71.21±25.85

IRF4/MUM-1, interferon-regulating factor 4/multiple myeloma oncogene 1; BCL6, B-cell CLL/lymphoma 6; PAX5, paired box protein 5; PD1, programmed cell death 1; BLIMP1, B lymphocyte maturation protein-1; TACI, transmembrane activator and calcium-signal modulating cyclophilin ligand interactor; TRPM8, transient receptor potential melastatin 8; SYM, lymphoepithelial symbiosis; CD, cluster of differentiation.

Scientific, Inc., Waltham, MA, USA). We used WaxFreeTM RNA (TrimGen, Sparks Glencoe, MD, USA) by which a sufficient volume of undamaged mRNA was successfully extracted from formalin-fixed paraffin-embedded prostatic carcinoma tissue specimens (34).

Extracted mRNA refined with water saturation phenol/chloroform was used for RT-PCR with the PrimeScript II 1st strand cDNA Synthesis kit (Takara Bio, Otsu, Japan). We used primer pairs designed by OriGene Technologies (Rockville, MD, USA) and produced by Fasmac (Atsugi, Japan); primer sequences were as follows: Sense, 5'-CTGGTTGCGAACTTCCGAAGAG-3' and antisense, 5'-GGTGCCGAGTAATAGGAGACAC-3'. The primary sequences for β-actin were: Sense, 5'-CAGAGCAAGAGAGGCATCCT-3' and antisense, 5'-ACGTACATGGCTGGGGTG-3', and the primary sequences for GAPDH were sense, 5'-GCACCGTCAAGGCTGAGAAC-3' and antisense, 5'-TGGTGAAGACGCCAGTGGA-3'. A mixture of 2 μl of primer pairs, 20 μl of dH$_2$O, 3 μl of synthesized cDNA, and 25 μl of the EmeraldAmp™ PCR Master Mix (Takara Bio) was added to a 0.2-ml microtube. The mixture was heat-denatured at 94˚C for 4 min on a thermal cycler (Veriti™; Applied Biosystems; Thermo Fisher Scientific, Inc.), at 94˚C for 2 min (50 cycles), at 57˚C for 2 min, at 72˚C for 2 min, and finally at 72˚C for 6 min. PCR products were stored at 4˚C until electrophoresis. We used a 4.0% agarose gel (NuSieve® 3-1 Agarose, Lonza, Rockland, ME, USA), 1xTAE buffer, and ethidium bromide for electrophoresis. β-actin (227 bp) and GAPDH (138 bp) primers were used as housekeeping mRNA for internal control (normalization of RT-PCR) and distilled water was used as a negative control instead of reverse transcriptase.

Statistical analysis. Dr. SPSS II software for Windows was used in statistical analyses. Spearman's correlation coefficient was used to test the interobserver correlation of the first and second observers. We performed the Kruskal-Wallis test to analyze the TRPM8-positive ratio in reactive lymphoid tissues and the scores of the TRPM8-positive ratio of mature B-cell neoplasms, and when cases were considered to be significant, we performed Scheffé's multiple comparison tests. Differences with P<0.05 were considered to be significant in each case.

Results

Localization of TRPM8^{+} cells in reactive lymphoid tissues. There were significantly more TRPM8^{+} cells in the SYM/MZ

Table III. Expression of TRPM8 in mature B-cell neoplasms.

Types of lymphomas (number of cases)	TRPM8 scores (mean ± standard deviation)[a]
Small lymphocytic lymphoma (n=3)	0.00±0.00[c]
Lymphoplasmacytic lymphoma (n=4)	2.25±0.50
Plasma cell neoplasm (n=16)	4.00±0.00[b,d,e]
Extranodal marginal zone lymphoma (n=5)	2.20±0.45
Follicular lymphoma (n=5)	0.00±0.00[b]
Mantle cell lymphoma (n=3)	0.00±0.00[d]
Diffuse large B-cell lymphoma (n=18)	0.72±0.83[e]
Germinal center B-cell-like (n=7)	0.71±0.95
Non-germinal center B-cell-like (n=11)	0.73±0.79

[a]Scores 0, 1, 2, 3 and 4 are 0%, 1-25%, 26-50%, 51-75% and 76-100% of all lymphoma cells, respectively. $P<0.01$ by the Kruskal-Wallis test, [b]$P<0.01$ plasma cell neoplasm vs. follicular lymphoma, [c]$P<0.05$ plasma cell neoplasm vs. small lymphocytic lymphoma, [d]$P<0.05$ plasma cell neoplasm vs. mantle cell lymphoma, [e]$P<0.01$ plasma cell neoplasm vs. diffuse large B-cell lymphoma, by Scheffé's multiple comparison test. TRPM8, transient receptor potential melastatin 8.
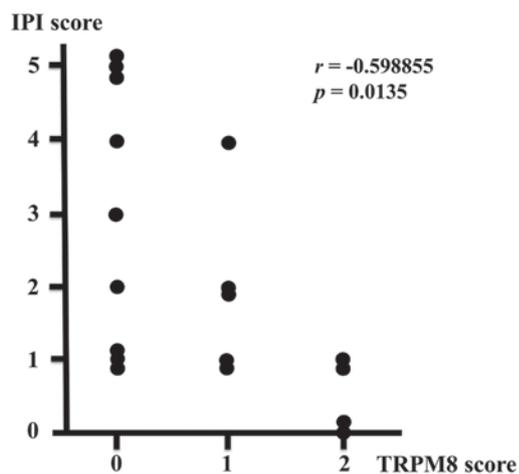
Figure 2. Relationship between IPI and TRPM8 scores. A close relationship was found between both scores (r=-0.598855, P=0.0135). TRPM8, transient receptor potential melastatin 8; IPI, international prognostic index.

and light zone (Fig. 1B-D) than in the mantle zone and interfollicular area of the tonsils (Table I). In the LN, more TRPM8+ cells were observed in the light zone and medullary cord in contrast to the mantle zone. Similarly, more TRPM8+ cells were found in the light zone and dome in contrast to the mantle zone in the appendix. Positive cells in the GC had morphological features similar to those of plasmablasts, while those in the SYM/MZ, medullary cord, and dome were morphologically similar to plasma cells.

In addition, we confirmed TRPM8+ cancer cells in prostatic carcinoma tissues as positive controls and no positive cells in negative controls using non-immune rabbit serum instead of the primary antibody.

Double immunohistochemistry of the tonsils. TRPM8+ cells frequently co-expressed CD38, CD79a, IRF4/MUM-1, BCL6 (Fig. 1E), and TACI in the GC, and CD38, CD79a, CD138, IRF4/MUM-1, BCL6, and TACI in the SYM/MZ (Table II). On the other hand, TRPM8+ cells less frequently expressed

CD10, CD20, and PAX5, and did not express CD3, CD23, CD56, CD117, PD1, or BLIMP1.

Expression of TRPM8 in mature B-cell neoplasms. In all types of PCN, such as MGUS, PCM, and extraosseous plasmacytoma, the immunostaining score was 4 (4.0±0.00), revealing that most neoplastic cells were TRPM8+ (Fig. 1F; Table III). No significant differences were observed in immunostaining scores between the IgG and IgA types of the PCM. The proportion of TRPM8+ cells/neoplastic cells of LPL (Fig. 1G) and MALT lymphoma was approximately 26-50%. The immunostaining score of DLBCL was low (<25%). Furthermore, no significant difference was observed in immunostaining scores between the GCB subtype and non-GCB subtype of DLBCL. Neither SLL, FL, nor MCL contained TRPM8+ neoplastic cells (Fig. 1H). In all lymphomas, TRPM8+ cells had an abundant cytoplasm and eccentric nucleus, resembling plasmablasts and PCs.

In the judgment of TRPM8+ cells in reactive lymphoid tissues and mature B-cell neoplasms, a good correlation was found between two observers; the interobserver correlations of the first and second observers were r=0.952 (P<0.001) in the tonsils (n=35), r=0.989 (P<0.001) in the LN (n=35), r=0.938 (P<0.001) in the appendix (n=36), and r=0.974 (P<0.001) in mature B-cell neoplasms (n=54).

Spearman's rank correlation coefficient in our DLBCL patients revealed a close relationship between TRPM8 and international prognostic index (IPI) scores (P=0.0135, r=-0.599), revealing that DLBCL patients with lower TRPM8 scores have higher IPI scores; however, the number of cases was small (Fig. 2).

RT-PCR. We recognized a positive band identical to TRPM8 mRNA at a length of 100 bp in a case of well-differentiated prostatic carcinoma (positive control), as well as in cases of PCM, but not in the negative control (Fig. 3A). In addition, positive band of TRPM8 was recognized in a case of PCM (1 out of 5 cases), but not in FL (n=3) and MCL (n=2) using FFPE specimens (Fig. 3B).
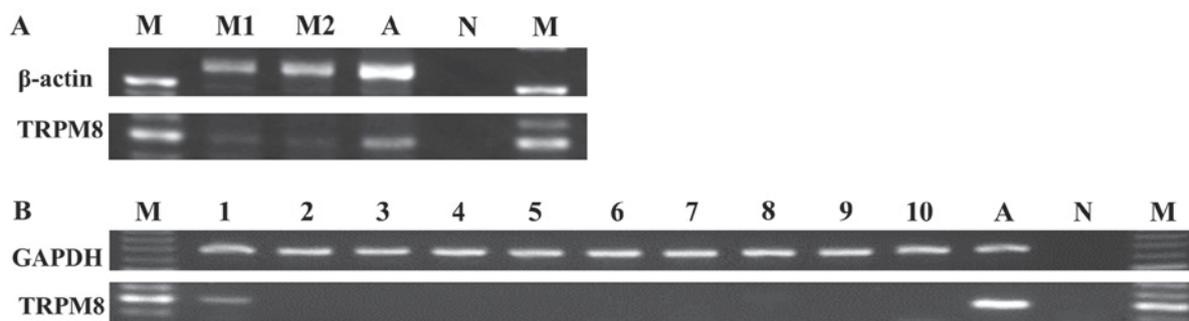
Figure 3. RT-PCR of TRPM8 mRNA in plasma cell myeloma, follicular lymphoma and mantle cell lymphoma. (A) A positive band of 100 bp in length for TRPM8 mRNA was found in two cases of plasma cell myeloma (*M1 & M2*) using frozen tissue specimens. (B) A positive band of 100 bp in length for TRPM8 mRNA was found in one case of plasma cell myeloma (1-5; n=5), but not in follicular lymphoma (6,7 and 8; n=3) and mantle cell lymphoma (9,10; n=2) using FFPE tissue specimens. (*M*) Molecular markers, (*N*) negative control, (*A*) well-differentiated prostatic carcinoma as a positive control. TRPM8, transient receptor potential melastatin 8; RT-PCR, reverse transcription polymerase chain reaction.

Discussion

TRPM8 is a thermoreceptor that is sensitive to cold stimuli below 28˚C and chemicals, such as menthol and icilin, that induce the sensation of cold (35,36). It localizes on the cell surface membrane and endoplasmic reticulum (ER). It is expressed in the male urogenital apparatus, such as the prostate gland, testis, and urinary bladder, as well as the liver and nervous system among normal organs, and is found in some malignant tumors, including androgen-dependent prostate cancer, pancreatic cancer, breast cancer, and melanoma (13,14,21-26). TRPM8 regulates the proliferation and apoptosis of prostatic epithelial cells, and cell death is induced by an inflow of Ca^{2+} into cells via TRPM8 (13,14). A previous study reported that murine B cells express TRPC1, C2, C6, V2, V4, M2, M4, M5, and M7 among the TRP superfamily (27).

To the best of our knowledge, this is the first study to demonstrate the expression of TRPM8 in reactive lymphoid tissues and mature B-cell neoplasms. In contrast to the mantle zone, the light zone had many TRPM8⁺ cells in all reactive lymphoid tissues (the tonsils, LN, and appendix). In addition, there were many TRPM8⁺ cells in the tonsillar SYM/MZ, LN medullary cord, and appendiceal dome. The morphology of positive cells in the light zone was similar to that of plasmablasts, and those in the SYM/MZ, medullary cord, and dome were carried out with PCs. In order to confirm these results, we performed double immunostaining and revealed that TRPM8⁺ cells in the follicular light zone and SYM/MZ often co-express CD38 (72 and 74%), CD79a (59 and 60%), IRF4/MUM-1 (69 and 58%), BCL6 (69 and 47%), TACI (62 and 71%), and sometimes CD138 (32 and 52%).

Jego *et al* (1) reported that the immunophenotypes of activated B cells, pre-plasmablasts, plasmablasts, and early PCs are CD20⁺CD38±CD138⁻, CD20±CD38⁺CD138⁻, CD20⁻CD38⁺⁺⁺CD138⁻, and CD20⁻CD38⁺⁺⁺CD138⁺⁺, respectively. Harada *et al* (2) demonstrated that normal PCs are CD38⁺⁺CD56⁻CD19⁺ by two-color flow cytometry. Thus, late PCs may be CD19⁺CD20⁻CD38⁺⁺⁺CD138⁺⁺⁺. CD79a is widely expressed from GC-B cells to PCs (4), IRF4/MUM-1 is found on CD20⁺CD38⁺ B cells (the intermediate of B-cell lymphoma 6 protein (BCL6)⁺ B cells and CD138⁺ B cells) in the follicular light zone (37), TACI is more likely to be expressed on late-stage B cells, such as plasmablasts and

PCs (34,38), and PAX5 is a transcription factor indispensable for B-cell differentiation, but disappears from PCs (4). In the present study, TRPM8⁺ cells were negative for CD56 and CD117 appearing on PCN (32), and for PD1 appearing on follicular helper T-cells (39). Taken together, the vast majority of TRPM8⁺ cells in the follicular light zone may be compatible with pre-plasmablasts and/or plasmablasts and in the SYM/MZ with early and/or late PCs, indicating that TRPM8 may be expressed from pre-plasmablasts to late PCs Fig. 4), available as a potential supplementary plasma cell marker. CD38 expression levels significantly decrease in 80% of all myeloma cases, plasma cells expressing low levels of CD138 have frequently been reported, and CD138⁻ plasma cells are present in the peripheral blood (3). As shown in Fig. 4, TRPM8 detected CD138⁻ plasmablasts and CD38⁻ or CD138⁻ pre-plasmablasts.

Based on the WHO classification, B-cell neoplasms have been further classified into precursor B-cell neoplasms, pre-GC neoplasms, GC neoplasms, and post-GC neoplasms (4). We examined TRPM8 immunostaining in MCL as pre-GC neoplasms, FL and DLBCL as GC neoplasms, and SLL, MALT lymphoma, LPL, DLBCL, and PCN as post-GC neoplasms. As a result, all PCN (n=16) were score 4 (76%), most LPL and MALT lymphomas score 2, and DLBCL score 0-1. The RT-PCR analysis conducted in the present study also confirmed TRPM8 mRNA expression in PCM tissues, not in FL and MCL. RT-PCR analysis on FFPE specimens of PCN (bone marrow aspirate) showed positive reaction in only one case. It may be because mass number of red blood cells in bone marrow may inhibit the RT-PCR analysis (40,41). Siebolts *et al* examined microRNAs on various tissue by quantitative PCR and showed little microRNA in bone marrow comparing with other tissues (42).

On the other hand, there were no TRPM8⁺ neoplastic cells in SLL, FL, or MCL. Therefore, TRPM8⁺ cells appear to localize in some GC neoplasms and most post-GC neoplasms, but not in pre-GC neoplasms and most GC neoplasms, further indicating that TRPM8 has potential as a marker to identify reactive plasmablasts/PCs and mature B-cell neoplasms that are likely to differentiate into PCs in a practical differential diagnosis using formalin-fixed and paraffin-embedded tissue sections.
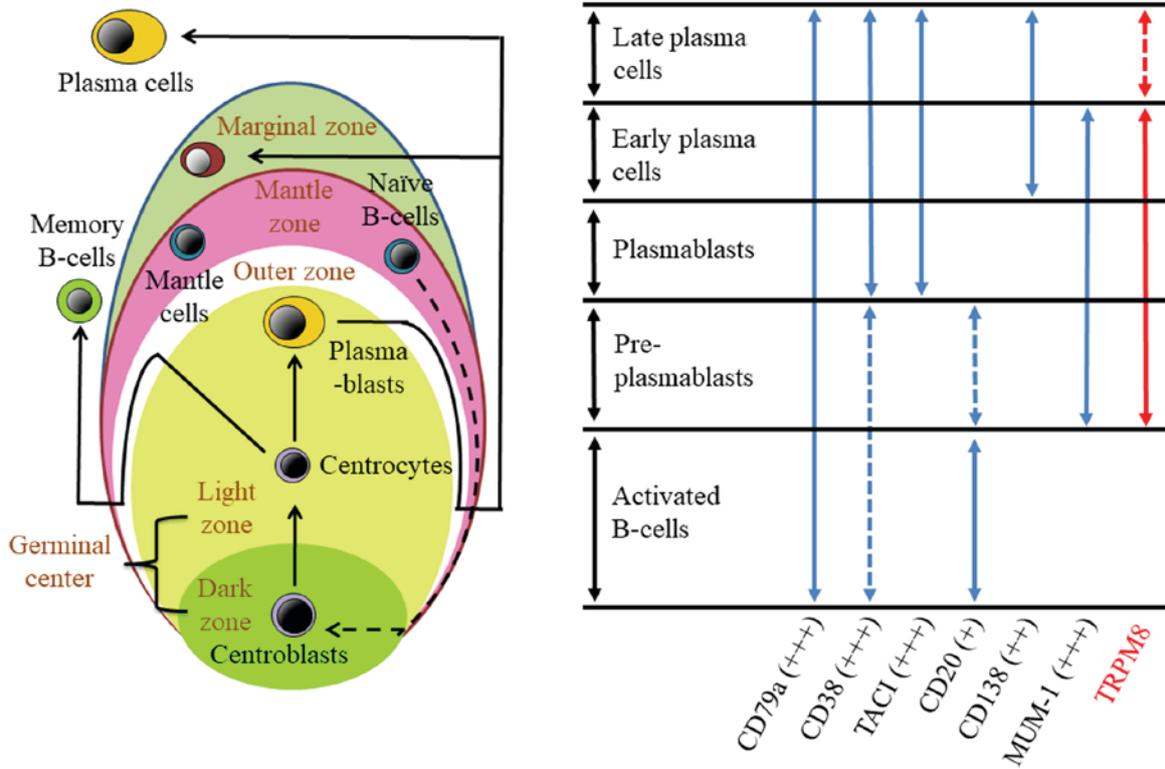
Figure 4. Scheme showing the relationship between normal B-cell differentiation (*left*) and markers including TRPM8 (*right*). Activated B cells, pre-plasmablasts, and plasmablasts in the germinal center are CD20+CD38+CD79a+, CD20+CD38+CD79a+IRF4/MUM-1+, and CD38+CD79a+TACI+IRF4/MUM-1+, respectively. Further differentiated early and late plasma cells in the extrafollicular area are CD38+CD79a+CD138+TACI+IRF4/MUM-1+ and CD38+CD79a+CD138+TACI+, respectively. The present study demonstrated that TRPM8 is expressed on cells differentiating from pre-plasmablasts to late plasma cells. TACI (+): 10-30%, (++); 30-50%, (+++); >50% of the co-expression ratio of lymphocyte markers on TRPM8+ cells. Dotted arrows: Often positive. TRPM8, transient receptor potential melastatin 8; CD, cluster of differentiation; MUM, multiple myeloma oncogene; TACI, transmembrane activator and calcium-signal modulating cyclophilin ligand interactor.

TRPM8 is up-regulated in androgen-dependent prostate cancer, and affects the growth inhibition and dedifferentiation of neoplastic cells (13,14,21-26). TRPM8 was defined as an effective diagnostic and prognostic marker in prostate cancer (13,14,21). The present study also revealed a close relationship between TRPM8 and the IPI scores of DLBCL (P=0.0135, *r*=-0.599), demonstrating that DLBCL patients with lower TRPM8 scores have a worse prognosis; however, the number of cases tested was small.

Previous studies reported that TRPM8 facilitates the inflow of Ca^{2+} and Na^+ across the plasma membrane and may mediate the release of Ca^{2+} from the ER because some TRPM8 is expressed in the ER as well as the plasma membrane (43,44). Tartakoff and Vassalli (45) showed that Na^+ and Ca^{2+} homeostasis influences immunoglobulin secretion in plasma cells. Thus, TRPM8 may have a link with immunoglobulin secretion in plasma cells. Furthermore, the extracellular domain GLMKYIGEV region of TRPM8 may activate cytotoxic T-lymphocytes, which play a role in cell destruction, suggesting that TRPM8 has potential as an immunotherapeutic target (46). However, the precise function of TRPM8 expressed on pre-plasmablasts, plasmablasts, PCs, and neoplastic cells, such as PCN, currently remains unclear and, thus, future studies including cell culture and/or mouse study to detect the influencing effect of TRPM8 on plasma cell to produce antibodies in inflammation and in cancer surrounding environment, and influencing factors of TRPM8 on worst prognosis of malignancy, are needed.

The present study is the first to demonstrate the expression of TRPM8 in reactive lymphoid tissues and mature B-cell neoplasms, revealing that TRPM8 is frequently expressed on pre-plasmablasts, plasmablasts, PCs, and mature B-cell lymphomas that are likely to differentiate into PCs. TRPM8, as one of the plasmacytic differentiation markers, may serve as a useful supplementary tool in the differential diagnosis of mature B-cell neoplasms.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AH was involved in study conception, study design, experimentation, data analysis and manuscript writing. NYA was involved in study conception, study design, experimentation, data analysis and manuscript revision. RO and HM were involved in experimentation and data analysis. AN and TK were involved in study conception and experimentation. KI and JF were involved in study conception and reviewed the manuscript. MY was involved in study conception, study design, data analysis, manuscript writing and manuscript revision. All authors read and approved the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient. The use of tissues and clinical information study was approved by the Ethics Committee of Yamagata University School of Medicine.

Patient consent for publication

Written informed consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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