

Original Article

Low expression of HS1 protein in plasma cells and H/RS cells

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Abstract: Hematopoietic cell-specific Lyn substrate 1 (HS1) is an intracellular protein that mainly over-expressed in cells of lymphohematopoietic origin. However, little knowing about HS1 located at the stage of B lymphocytes. To demonstrate differentially expressing of HS1 in B lymphopoiesis, we screened a panel of B lymphocytes representing various developmental stages selected by Fluorescence activated Cell Sorting (FACS) according to corresponding surface markers. We also examined HS1 protein in normal lymphoid tissue, B lymphoid tumor and classical Hodgkin lymphoma (CHL) tissues by using immunohistochemistry, examined HS1 protein and mRNA level in lymphoid tumor cell lines by using Western blot and quantitative real-time PCR. We found that HS1 expression was mainly located at the stage of B cell from pre-B to mature B cells and fewer in plasma cells, but not in H/RS cells which derive from crippled pre-apoptotic germinal centre B cells. This result indicates that HS1 maybe as a useful diagnostic marker for CHL to different diagnose from T cell/histiocyte-rich large B-cell lymphoma (THRLBCL). To certify this hypothesis, we further examined 21 CHL and 12 THRLBCL for immunoreactivity to HS1 as compared to commonly used markers such as CD30, CD15 and CD20. Finally, our findings show that HS1 maybe as a useful diagnostic marker of CHL to different diagnose from THRLBCL, helping to improve diagnostic accuracy for this malignancy tumor.

Keywords: HS1, expression, B lymphocytes, diagnosis

Introduction

B cell lymphoma means that B cell neoplasms are clonal tumors of mature and immature B cells at various stages of differentiation. B cell in many respects appear to recapitulate stages of normal B cell differentiation, so that they can be to some extent classified according to the corresponding normal stage [1]. Hodgkin lymphoma is a tumor composed with the H/RS cells, and the cells are mainly derived from crippled pre-apoptotic germinal center B cells [2].

HS1 is a 75 kDa intracellular protein that has been shown to be expressed mainly in hematopoietic cell lineages [3, 4], and it was involved in the signal transduction pathways that initiate at the antigen receptors of both B [5-7], T [8-12] lymphocytes, natural killer cells [13] and neutrophils [14]. According to some literatures, HS1 also performs a seminal role in apoptosis [15-18] and impact on prognoses of lymphoid

neoplasm [19, 20], and it is critical for erythropoietin-induced differentiation of erythroid cells [21].

Literatures have been reported that HS1 is over expressed in hematopoietic cell lineages, but HS1 protein really over express in all cells of lymphohematopoietic origin? In our study, we find that not all cells of lymphohematopoietic origin are over-expressed HS1 protein, and find HS1 protein expression mainly located at the stage of B cells from pre-B to mature B, fewer in plasma cells, and not expresses at the stage of crippled pre-apoptotic germinal centre B cells, and this expression pattern will do a good for diagnose CHL in clinic.

Material and methods

Cells and specimens

The cell lines used for western blot and Quantitative real-time PCR are, B lymphoblastic line,

Nalm6; diffuse large B cell lymphoma (DLBCL), OCI-ly1, OCI-ly3, OCI-ly8 and OCI-ly10; Burkitt lymphoma lines, Raji, Daudi, Ramos and BJAB; multiple myeloma lines, RPMI-8226 and IM9; CHL line, L428 and L428-99 [22].

The specimens used for Fluorescence activated Cell Sorting fresh tonsil tissue of patient with chronic tonsillitis, used for immunohistochemistry are 2 tonsil with chronic tonsillitis, 15 lymph node with reactive hyperplasia (RH) and a total of 214 cases of lymphoid neoplasm from archived paraffin-embedded formalin-fixed tissue blocks from the Department of Pathology at the Nan fang Hospital affiliated to Southern Medical University and Guizhou Provincial People's Hospital, from the period of 1997 to 2014. Ethics approval for this study was granted by the Local Ethics Committees.

These 214 cases included 35 B lymphoblastic lymphomas (BLL), 13 mantle cell lymphoma (MCL), 27 Burkitt lymphoma (BL), 14 follicular lymphoma (FL), 53 diffuse large B cell lymphoma, not otherwise specified (DLBCL, NOS), 11 nodal marginal zone lymphoma (NMZL), 28 plasma cell myeloma (PCM), 21 CHL and 12 THRLBCL, diagnosed and classified according to the criteria of the World Health Organization classification of lymphoid neoplasm [1].

Antibody

For Fluorescence activated Cell Sorting, immunohistochemistry and Western blots, a large panel of antibodies were used (Table S1).

Fluorescence activated cell sorting (FACS)

B cell subpopulations were purified from tonsils by using FACS according to the corresponding surface markers [23]: B cells CD19+, naïve B cells CD19+IgD+CD38-, centroblasts CD19+IgD-CD38+CD77+, centrocytes CD19+IgD-CD38+CD77-, memory B cells CD19+IgD-CD38- and plasma cells CD19-CD38+CD138+.

Quantitative real-time PCR

For qRT-PCR, we isolated RNA using Trizol reagent (TaKaRa Biotechnology, Dalian, Co. Ltd, China), amplified cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, Co. Ltd, China) and measured mRNA expression using the Bestar® SYBR Green qPCR mastermix (DBI-2043, DBI

Bioscience, Germany) according to per manufacturer's protocol. The sequences of the primers used are listed in Table S2. The reaction conditions consisted of an initial activation step (2 min at 95°C), a cycling step (denaturation for 10 s at 95°C, annealing for 34 s at 60°C and elongation for 30 s at 72°C for 40 cycles), and melting curve step (1 min at 95°C, 1 min at 55°C, 55°C-98°C (10 s per cycle, 0.5°C per cycle) for 86 cycles. All reactions were carried out with three biological replicates, and each analysis consisted of three technical replicates. A melting curve analyses was carried out after each run. The results were standardized against the reference GAPDH gene, and the relative expression levels were quantified by the $2^{-\Delta\Delta C_t}$ method, with the relative fold changes normalized to the control values, where $\Delta\Delta C_t$ is $(C_{T_{sample}} - \text{average } C_{T_{GAPDH}}) - \text{average } (C_{T_{control}} - \text{average } C_{T_{GAPDH}})$.

Western blot

We obtained whole cell lysates through lysis of a defined number of cells in RIPA lysis buffer followed by brief sonication. We separated proteins by 10% SDS-PAGE, and the proteins were transferred from gels onto nitrocellulose membrane in a standard procedure used in our lab. The membranes were incubated with HS1 monoclonal antibody or α -tubulin monoclonal antibody at dilutions of 1:1000 overnight at 4°C in Primary Antibody Diluent, after blocking them in 5% non-fatty blocking grade milk (Bio-Rad) in TBST (Tris Buffered Saline with Tween 20) for an hour. Peroxidase-conjugated Affinipure Goat anti-rabbit or anti-mouse IgG (H+L) (FD-GAR007 and FD-GAM007 respectively, USA) secondary antibody at 1:10000 dilution was used in blocking solution. Membranes were washed after each incubation. The protein bands were visualized under imaging machine.

Immunohistochemistry (IHC)

Immunohistochemistry of paraffin-embedded tissue sections was carried out using a panel of primary antibodies are presented in Table S1, biotinylated Second antibody kit (SP kit, Zhongshan Co., China), and DAB substrate kit (Zhongshan Co., China). In brief, the tissue sections were routinely deparaffinized and rehydrated, then subjected to antigen retrieval and blocked endogenous peroxidase activity.

HS1 low-expressed in plasma cells and H/RS cells

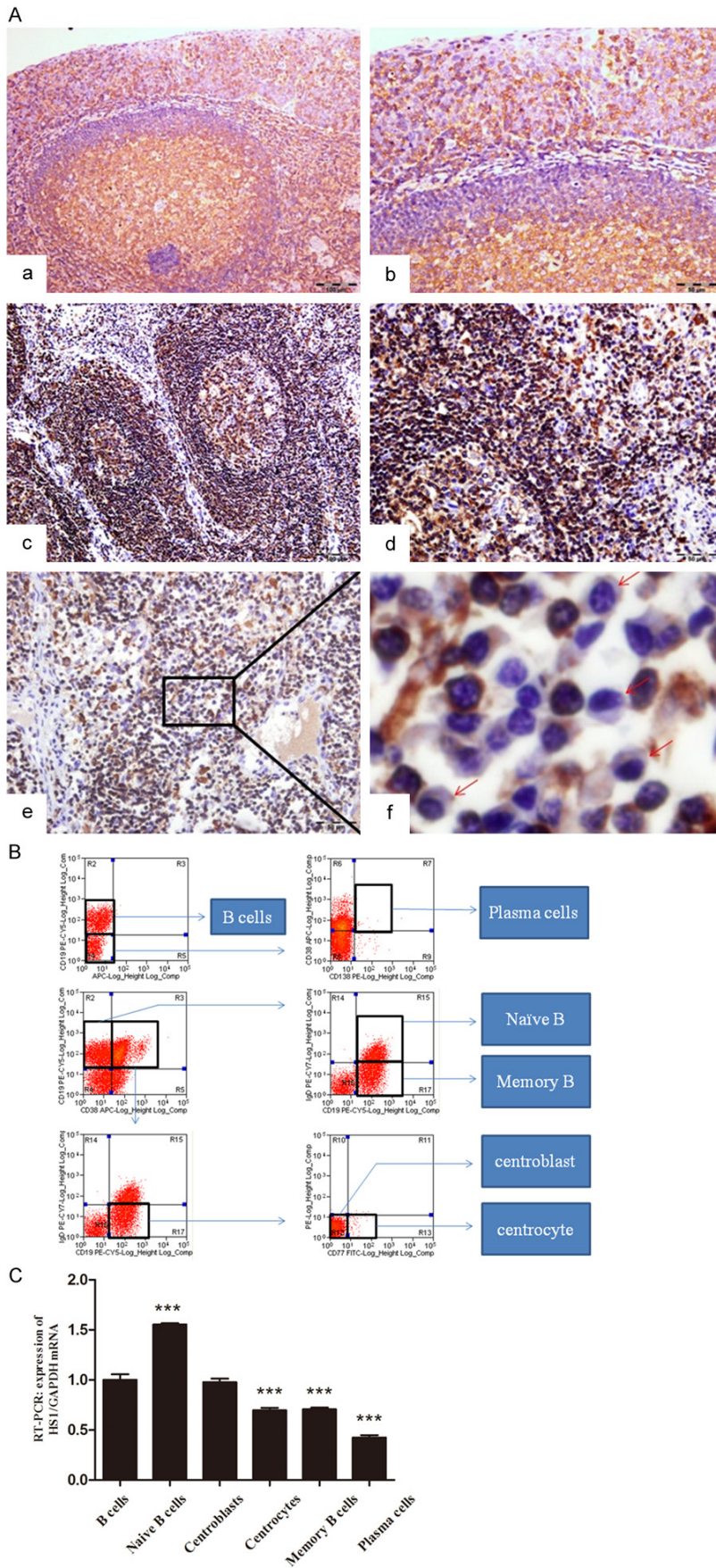


Figure 1. (A) HS1 IHC in normal lymphoid tissue. Normal tonsil with strong staining (a and b) ($\times 200$, $\times 400$, respectively) and reactive hyperplasia lymph node with moderate staining (c-e) ($\times 200$, $\times 400$, $\times 400$, respectively). Note that HS1 is ubiquitously expressed in follicular, perifollicular area, interfollicular area, marginal zones, and even in epithelia of tonsil. (f) is focal zoom in from (e) ($\times 400$) to show that HS1 expression in plasma cells is negative (red arrows). (B) FACS was used to purify B cells (CD19+) and plasma cells (CD19-CD38+CD138+) from tonsil tissue. B cells (CD19+CD38-) were further sorted into Naïve B (IgD+) and Memory B (IgD-) cells, and B cells (CD19+CD38+IgD-) were further sorted into centrocyte (CD77+) and centroblast (CD77-) cells. (C) Relative HS1 mRNA level in different B cells and plasma cells. Note that HS1 mRNA is differentially expressed at various stages of B cell differentiation, highest in naïve B cells ($P=0.000$), moderate in centroblasts, lower in centrocytes and memory B cells, lowest in plasma cells ($P=0.000$), and the mRNA level was slide down follow with the B cell from immature to mature. ($P<0.001^{***}$).

Sections were then immunostained with HS1 (or other primary antibodies) and biotinylated Second antibodies in brown with the DAB substrate and then counterstained with hematoxylin.

Two pathologists (T.Z. and J.W.) working independently and blinded to the clinical data carried out semi-quantitative evaluation of immunohistochemical staining on the unidentified samples using a 4-tiered system. Staining was scored as positive if

Table 1. HS1 expression in different subtypes of lymphoid neoplasm (n=214) and normal lymphoid tissues (n=17)

Subtype	Total case (n)	1+	Positive (n) 2+	3+	Positive (n/%)	Negative (n/%)
Tonsillitis	2	0	0	2	2 (100%)	0 (0%)
RH	15	0	9	6	15 (100%)	0 (0%)
BLL	35	10	4	7	21 (60.00%)	14 (40.00%)
MCL	13	9	4	0	13 (100%)	0 (0%)
BL	27	6	6	2	14 (51.85%)	13 (48.15%)
FL	14	2	8	4	14 (100%)	0 (0%)
DLBCL, NOS	53	8	5	40	53 (100%)	0 (0%)
NMZL	11	8	3	0	11 (100%)	0 (0%)
PCM	28	3	5	1	9 (32.14%)	19 (67.86%)
THRLBCL	12	1	3	7	12 (100%)	0(0%)
CHL	21	1	0	0	1 (4.76%)	20 (95.24%)

Abbreviation: RH, reactive hyperplasia; BLL, B lymphoblastic leukaemia/lymphoma; MCL, Mantle cell lymphoma; BL, Burkitt lymphoma; FL, Follicular lymphoma; DLBCL, NOS, Diffuse large B cell lymphoma, not otherwise specified; NMZL, Nodal marginal zone lymphoma; PCM, Plasma cell myeloma; THRLBCL, T cell/histiocyte-rich large B-cell lymphoma; CHL, Classical Hodgkin lymphoma.

at least 10% of the tumor cells or background cells were immunoreactive, and then scored as weak (1+), moderate (2+), or strong (3+) according to staining intensity.

Statistical analysis

Data were represented as the mean \pm Standard Error of Mean (S.E.M) and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test.

Result

HS1 protein expression in normal lymphoid tissue

To identify HS1 protein expression in normal lymphoid tissue, we carried out IHC experiments on tonsil and lymph node tissues, and found that both tissues were strong staining of HS1. This phenomenon noted that HS1 was ubiquitously expressed in follicular, perifollicular area, interfollicular area, marginal zones, and even in epithelia of tonsil (**Figure 1A**). From image A and C (**Figure 1A**), we found that HS1 expression in germinal center B cells were bright staining than marginal and mantle zones. Somewhat surprisingly, image F (**Figure 1A**) showed that HS1 protein expression in plasma cell was negative.

Isolation of the B cell sub-population

To further investigate HS1 mRNA expression in B cell subpopulation, firstly, we got the purified B cells subpopulation from tonsil tissues. For this purpose, we carried out FACS analysis to isolation B cell subpopulation according to the B cell surface markers were differ from different B cell subsets by the use of mAbs of B cell surface marker. In our study, B cells (CD19+) and plasma cells (CD19-CD38+CD138+) were purified from other sorts of cells, B cells (CD19+CD38-) were further sorted into

Naïve B (IgD+) and Memory B (IgD-) cells, and B cells (CD19+CD38+IgD-) were further sorted into centrocyte (CD77+) and centroblast (CD77) cells (**Figure 1B**).

Relative HS1 mRNA level in B cell subpopulation

Up sorted B cells subpopulation mRNA level of HS1 were tested, and shown that HS1 mRNA was different expressed at various stages of B cell differentiation, highest in naïve B cells, moderate in GCs and mature B cells, lowest in plasma cells. We find that HS1 mRNA level is slide down follow with the B cell from immature to mature (P=0.000) (**Figure 1C**).

HS1 protein expression in B cell neoplasm tissues

In order to found whether B cell neoplasm had the same trend with HS1 expression, we examined HS1 expression in 214 cases of lymphoid neoplasm, and found that the same trend was in those tumors (P=0.000) (**Table 1**). All of the DLBCL, THRLBCL and FL specimens demonstrated the highest percentage of positive cases, was 53/53 (100%), 12/12 (100%) and 14/14 (100%), respectively, and the tumor cells are often with bright staining of HS1. Meanwhile, MCL and NMZL also have the highest percentage of positive cases 100% (13/13 and 11/11, respectively) with weak to moderate staining

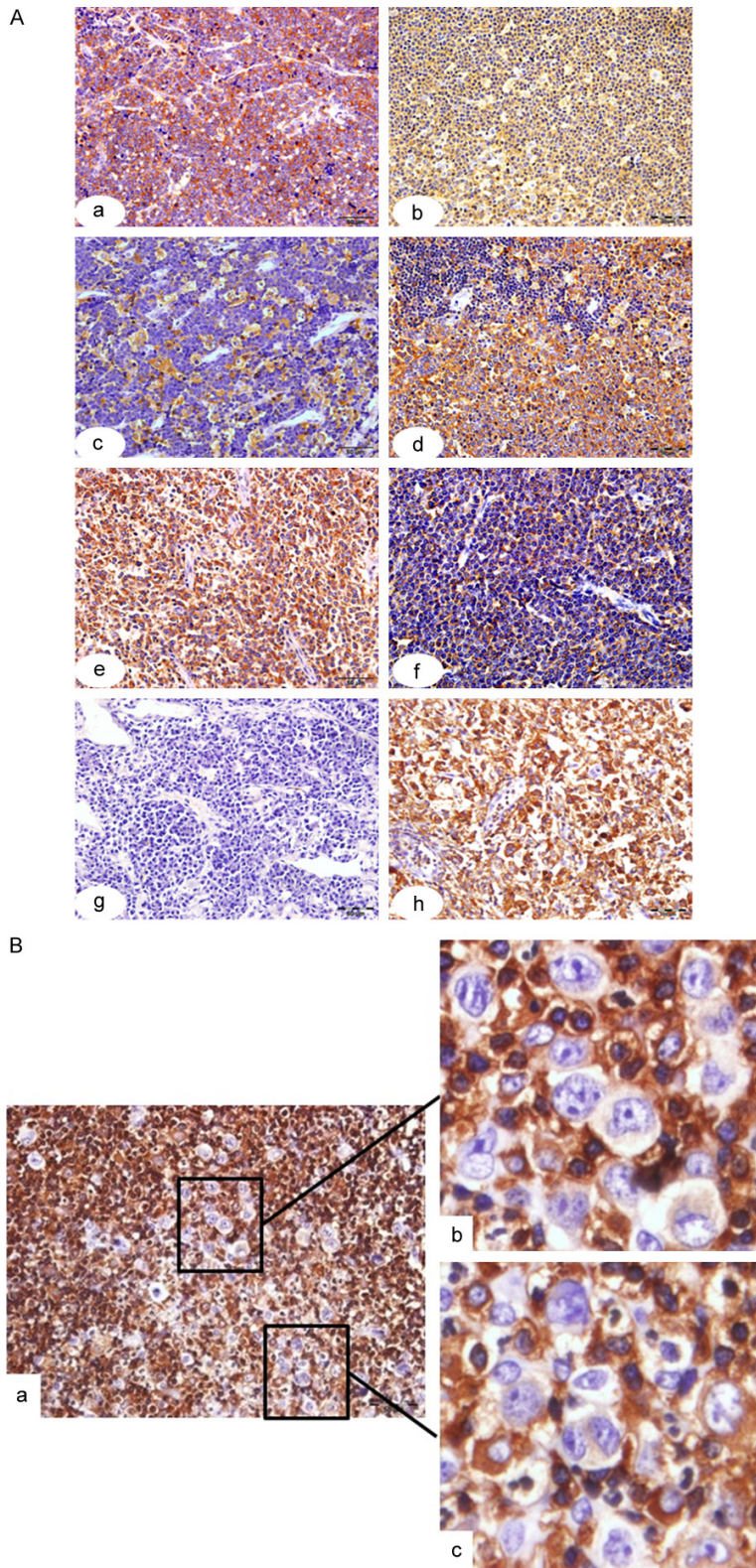


Figure 2. (A) HS1 IHC in non-Hodgkin lymphoma. (a) (B lymphoblastic leukaemia/lymphoma, BLL), (b) (Mantle cell lymphoma, MCL), (c) (Burkitt lymphoma, BL), (d) (Follicular lymphoma, FL), (e) (Diffuse large B cell lymphoma, DLBCL) (f) (Nodal marginal zone lymphoma, NMZL), (g) (Plasma cell myeloma, PCM), (h) (T cell/histiocyte-rich large B-cell lymphoma, THRLBCL) ($\times 400$,

respectively), all these images represent that HS1 expression in the majority of each type of neoplasm. (B) HS1 IHC in classic Hodgkin lymphoma. a ($\times 400$) note that HS1 was negative in H/RS cells, and strong staining in the background cells, which include (b), T lymphocyte and other inflammation cells. (b and c) was focal zoom in from (a) to show that HS1 expression in classic Hodgkin lymphoma tumor cells is negative.

for HS1. But in other B lymphoma subtypes, HS1 positive staining is differ from the former subtypes, 21/35 (60%) in B lymphoblastic lymphomas, 14/27 (51.85%) in Burkitt lymphoma and 9/28 (32.14%) in plasma cell myeloma. And the percent of HS1 positive-staining in plasma cell myeloma is the lowest in all type of non-Hodgkin lymphoma. Image a, b, c, d, e, f, g and h represents staining intensity of majority of cases of neoplasm, respectively (**Figure 2A**).

Classical Hodgkin lymphoma is a unique lymphoid neoplasm in HS1 staining of IHC. And our data showed that nearly all (95.24%, 20/21) of the Hodgkin lymphoma specimens were negative for HS1 staining in H/RS cells, and only one cases (4.76%, 1/21) were shown weakly staining intensity (**Table 1**). Image a, b and c (**Figure 2B**) shows that Hodgkin and Reed-Sternberg (H/RS) cells of classical Hodgkin lymphoma IHC staining were negative.

HS1 protein and mRNA expression in lymphoid tumor cell lines

Figure 3 showed that HS1 over-expression of protein and mRNA were existed in

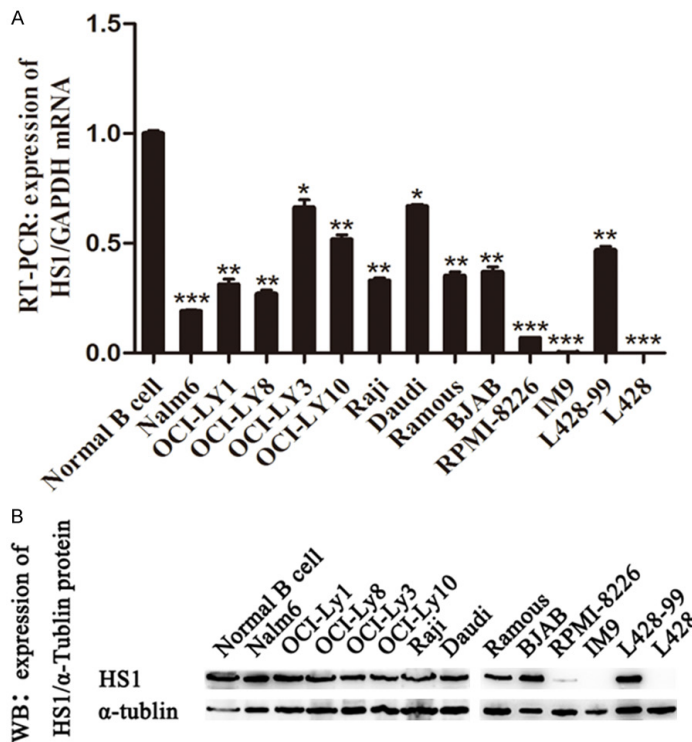


Figure 3. HS1 mRNA and protein expression in normal B cell and 13 lymphoid neoplasm cell lines. A. HS1 mRNA was examined in normal B lymphocytes and lymphoid neoplasm cell lines, but the level was lower than normal B lymphocytes, and lowest expressed in RPMI-8226 (plasma cell myeloma) and negative in IM9 (plasma cell myeloma) and L428 (Hodgkin lymphoma) ($P < 0.001^{***}$, $P < 0.01^{**}$, $P < 0.05^{*}$). B. HS1 protein was examined in normal B lymphocytes and lymphoid neoplasm cell lines, but lower expressed in RPMI-8226 (plasma cell myeloma) and negative in IM9 (plasma cell myeloma) and L428 (Hodgkin lymphoma).

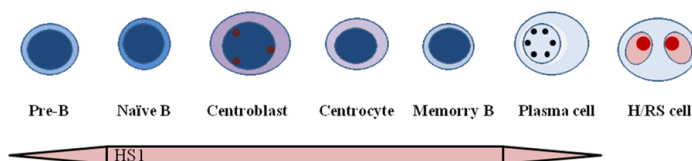


Figure 4. Expression model of HS1 gene at the stage of B cell differentiation.

lymphoma cell lines (Nalm6, OCI-ly1, OCI-ly3, OCI-ly8, OCI-ly10, Raji, Daudi, Ramos, BJAB, RPMI-8226, L428-99), but not in cell lines (IM9 and L428), in contrast with the normal B cells. **Figure 3A** showed that HS1 mRNA level decreased as the B cells matured into plasma cells. HS1 protein levels also fell, in contrast with the normal B cells (**Figure 3B**) ($P < 0.05$). These data indicated that HS1-negative was associated with mature B cell convert to plasma cell.

The gene expression of HS1 located at the stage of B cell differentiation

According to our research results, an expression model of HS1 gene was drawn in B cell differentiation. **Figure 4** showed that the gene expression of HS1 located at the stage of B cell is from pre-B to mature B cells, fewer in plasma cells, and not in H/RS cells which derive from crippled pre-apoptotic germinal centre B cells.

Comparison of HS1 protein expression to CD30, CD15 and CD20 expression in classical Hodgkin lymphoma and T cell/histiocyte-rich large B-cell lymphoma

In **Table 2**, the majority of CHL specimens were positive for CD30 (17/21, 80.95%) and CD15 (13/21, 61.9%), but parts of the CHL specimens were positive for CD20 (2/21, 9.52%) in the H/RS cells. In all THRLBCL specimens, 100% (12/12) were positive staining for CD20, one case was positive for CD30 (8.33%, 1/12), no specimen (0%, 0/12) was detected positive staining for CD15. In fact, there existed a part of specimens, which CD30-negative (4/21, 19.05%) or CD20-positive (2/21, 9.52%) cases of CHL and CD30-positive (8.33%, 1/12) case of THRLBCL. These overlap of immunophenotype led to confusion to diagnose THRLBCL or CHL when the haematoxylin and eosin (HE) images didn't typical. HS1 protein expressed in the tumor cells of THRLBCL, not in H/RS cells, was compared to commonly diagnostic markers, we were

enthralled to find that HS1 expressions may be complementary for pathological diagnosis of CHL from THRLBCL. For example, one THRLBCL specimen show double-positivity for HS1 and CD30 ($n=1$), but CHL specimens of CD20-positive ($n=2$) or CD30-negative ($n=4$) did not show positivity for HS1 ($n=6$). Therefore, when immunostaining was performed for HS1, all 12 THRLBCL cases were detected. For the THRLBCL and CHL specimens, HS1 immunos-

HS1 low-expressed in plasma cells and H/RS cells

Table 2. Different markers expression in CHL (n=21) and THRLBCL (n=12)

Diagnosis	CD30 (n/%)		CD15 (n/%)		CD20 (n/%)	
	Positive	Negative	Positive	Negative	Positive	Negative
CHL (n=21)	17 80.95%	4 19.05%	13 61.90%	8 38.10%	2 9.52%	19 90.48%
THRLBCL (n=12)	1 8.33%	11 91.67%	0 0%	12 100%	12 100%	0 0%

taining may be useful as a complement diagnostic marker.

Discussion

In this study, we also have demonstrated that HS1 is over-expressed in normal B lymphocytes, lymphoid tissues, and non-Hodgkin lymphoma tissues and cell lines. As these samples contain cells of lymphoid origin, the result is consistent with the data reported previously [3]. Notably, plasma cells and H/RS cells of classic Hodgkin lymphoma are all derived from lymphoid origin show no detectable of HS1 expression in protein and mRNA levels, and plasma cell myeloma is low expression of HS1 in protein and mRNA levels. Finally, our results illuminate that HS1 protein expression located at the stage of pre-B cells to mature B cells, low expressed at the terminal stage of B cells (namely, plasma cells). The H/RS cells arise from crippled pre-apoptotic germinal center B cells [2] are not express HS1 protein, and this maybe do good for CHL to different diagnose from THRLBCL.

HS1 is an intracellular protein that is expressed mainly in hematopoietic and lymphoid cells [3]. Our experiments confirm that HS1 is over-expressed in normal tonsil and lymph node, and the expression of HS1 in germinal center B cells were bright staining than in marginal and mantle zones. To our surprised, HS1 expression in plasma cell is negative. And plasma cell myeloma is low expression of HS1 in protein and mRNA levels. Next, we used FACS to sorting various stages of B cells, detected HS1 mRNA level in these cells, and found that HS1 level was slide down follow with the B cell differentiation from immature to mature. To further confirm this idea, we examined numerous samples of lymphoid neoplasm and cell lines and found the same trend. Finally, an expression model of HS1 gene was drawn in B cell differentiation, which expression located at the stage of B cell is from pre-B to mature B cells, fewer in plasma cells, and not in H/RS cells.

Lyn and HS1 interact in erythroid cells and this association is critical for Epo-induced erythroid differentiation [21, 24]. HS1 was differentially expressed in murine B lymphoid tumor cell lines, including pre-B lymphoma and B lymphoma cell lines and plasmacytoma cell lines [25]. But our data show that, in human, HS1 is differentially expressed in B lymphocyte and B lymphoid tumor, not expressed in normal plasma cell, lower in plasma cell myeloma. Thus, we hypothesize that low expression of HS1 is associated with the terminal differentiation of normal B lymphocytes to plasma cells, and this hypothesis need to be further certified in the future.

Accurate diagnosis of classical Hodgkin lymphoma [26] and T cell/histiocyte-rich large B-cell lymphoma [27] is essential for initiating a timely and appropriate treatment strategy, thereby improving patient prognosis and survival. While the morphologic and immunohistochemical features of tumor cells are the current standard of diagnosis, no single biomarker has yet been identified for use as a specific target of clinical tests. The current panel of markers, including CD30, CD15, CD20, CD79 α , CD3, Mum-1 and so on, help to identify tumor cells, but some limitations exist and interpretation of the results remains somewhat subjective. For example, differential diagnosis is confounded by the fact that THRLBCL also partly expresses CD30 and such large B cells with Hodgkin features, as well as CD30-negative (4/21, 19.05%) and CD20-positive (2/21, 9.52%) cases of CHL present in our study. Thus, there is a need for identifying more biomarkers that may help to improve the immunohistochemical diagnosis of CHL and THRLBCL. Based on HS1 expression is negative in H/RS cells, is positive in tumor cells of THRLBCL and which imply HS1 might as a negative marker to auxiliary differential diagnosis CHL from THRLBCL.

In addition, we accidentally found that epithelial cells of the tonsil also express HS1 protein. These results further imply that the gene

expression of HS1 is not restricted to cells of hematopoietic origin [4].

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Disclosure of conflict of interest

None.

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HS1 low-expressed in plasma cells and H/RS cells

Table S1. Antibodies used in IHC, WB and FACS

Antibody specificity	Source	Species	Clone	Application
HS1	Cell Signaling Technology	Rabbit	D83A8	IHC, WB
CD20	Abcam	Rabbit	EP459Y	IHC
CD30	Abcam	Mouse	CON6D/B5	IHC
CD15	Santa Cruz	Mouse	TG-1	IHC
α -tublin	Proteintech	Mouse	66031-1-Ig	WB
HU-CD19-PE-CY5	BD Pharmingen	Mouse	HIB19	FACS
HU-CD38-APC	BD Pharmingen	Mouse	HIT2	FACS
HU-CD138-PE	BD Pharmingen	Mouse	DL-101	FACS
HU-IgD-PE-CY7	BD Pharmingen	Mouse	IA6-2	FACS
HU-CD77-FITC	BD Pharmingen	Mouse	5B5	FACS

Table S2. Primers used in qRT-PCR

Gene	Sequence
HS1	Forward 5' GGGGCAAGTACGGAGTTGAG 3'
	Reward 5' CCCTTGTAGTCATATCCCAGAGC 3'
GAPDH	Forward 5' GGAGCGAGATCCCTCCAAAAT 3'
	Reward 5' GGCTGTTGTCATACTTCTCATGG 3'