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Serum soluble VSIG4 as a surrogate marker for the diagnosis of lymphoma-associated hemophagocytic lymphohistiocytosis

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Summary

Lymphoma-associated haemophagocytic lymphohistiocytosis (L-HLH) is characterized by excessively activated macrophages and cytotoxic T lymphocytes, but few reliable markers for activated macrophages are available clinically. This study, designed to discover novel biomarkers for the diagnosis of lymphoma patients with L-HLH, was initiated between 2016 and 2018. Fifty-seven adult lymphoma patients were enrolled — 39 without HLH and 18 with HLH. The differential serum protein expression profile was first screened between lymphoma patients with and without L-HLH by a quantitative mass spectrometric approach. Soluble V-set and immunoglobulin domain-containing 4 (sVSIG4), specifically expressed by macrophages, was significantly upregulated in the L-HLH group. Subsequently, sVSIG4 concentration was confirmed by enzyme-linked immunosorbent assay to be significantly increased in lymphoma patients with L-HLH. When it was exploited for the diagnosis of lymphoma patients with L-HLH, the area under a receiver operating characteristic curve was 0·98 with an optimal cut-off point of 2195 pg/ml and the corresponding sensitivity and specificity were 94·44% and 94·87% respectively. In addition, the one-year overall survival was significantly worse in patients with a sVSIG4 concentration above 2195 pg/ml compared with those below 2195 pg/ml (5·3% vs. 72·2%, $P < 0·0001$). sVSIG4 may be a surrogate marker of activated macrophages for the diagnosis of lymphoma patients with L-HLH.

Keywords: lymphoma, haemophagocytic lymphohistiocytosis, VSIG4, macrophage, biomarker.

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Haemophagocytic lymphohistiocytosis (HLH) or haemophagocytic syndrome (HPS) in adults is a rare but life-threatening syndrome characterized by multiple organ failure and severe inflammation, usually as an acquired form resulting from excessive activation and expansion of macrophages and cytotoxic T lymphocytes (CTLs) (Filipovich, 2009; Jordan *et al.*, 2011; Riviere *et al.*, 2014; Grom *et al.*, 2016; Lorenz *et al.*, 2018). In terms of aetiology, systemic infections, malignancies and autoimmune disorders or immune system iatrogenic treatments are the main causative triggers of adult HLH, but the predominant cause differs in each country (Janka, 2012; Ramos-Casals *et al.*, 2014; Emile *et al.*, 2016; Al-Samkari & Berliner, 2018). For instance, infection is the leading cause of adult HLH in the USA, while malignancy is the leading cause in China (Ramos-Casals *et al.*, 2014). Patients with haematological malignancies, especially lymphoma, are more prone to develop HLH than those with any other type of cancer (Ramos-Casals *et al.*, 2014). The overall prognosis of adult HLH is dismal, with a very high rate of early mortality (Bigenwald *et al.*, 2018). Particularly, patients with haematological malignancy-associated HLH (hM-HLH) have a poorer prognosis than those with other underlying diseases (Riviere *et al.*, 2014; Arca *et al.*, 2015; Schram *et al.*, 2016). Therefore, improving the early diagnosis and initial therapeutic treatment of hM-HLH, is critical for improving the patient's outcome (Bigenwald *et al.*, 2018).

Many clinical criteria have been applied in the diagnosis of adult HLH (Hayden *et al.*, 2016). Most of them use the Histiocyte Society's HLH-2004 diagnostic criteria for paediatric HLH. However, both the HLH-94 and HLH-2004 criteria have the common criterion of "no evidence of malignancy", which demonstrates that the specific reference standard for the diagnosis of adult malignancy-associated HLH including lymphoma-associated HLH (L-HLH) has not been fully established. Usually, haemophagocytic macrophages in the bone marrow, peripheral blood or tissue samples are regarded as the hallmark of HLH, but these may not be present or be easily found at the early stage, leading to diagnostic difficulty (Zhang *et al.*, 2011; Larroche, 2012; Maruoka *et al.*, 2014). Particularly in the early stages of L-HLH, the clinical symptoms and laboratory results often do not formally fulfil the diagnostic HLH-2004 criteria, further leading to under- or delayed diagnosis of HLH in patients (Tamamyan *et al.*, 2016; Lin *et al.*, 2017).

Activated CTLs and macrophages are two crucial players in HLH. During the development and progression of HLH, impaired cytotoxicity leads to prolonged stimulation of CTLs and natural killer (NK) cells secreting large amounts of pro-inflammatory cytokines, including IFN-γ, which causes macrophage activation and expansion, further resulting in subsequent overproduction of other pro-inflammatory cytokines and ultimately the cytokine storm (Lehmberg & Ehl, 2013; Grom *et al.*, 2016; Al-Samkari & Berliner, 2018). The soluble interleukin-2 receptor alpha (sIL-2Rα; also termed sCD25) is a truncated protein shed from the surface of activated CTLs. Thus, in the widely accepted HLH-2004 criteria, prolonged high sIL-2Rα level has been regarded as a surrogate serum marker of activated CTLs under enduring stimulus by pro-inflammtory cytokines for the diagnosis of HLH (Groen-Hakan *et al.*, 2017; Hayden *et al.*, 2017). In contrast, a reliable marker related to activated macrophages remains elusive devoid in clinical practice (Schulert & Grom, 2015; Al-Samkari & Berliner, 2018).

In the current study, we focused on lymphoma patients with L-HLH and sought to identify novel serum biomarkers specifically for activated macrophages. We used a quantitative mass spectrometric method to compare the serum proteome of lymphoma patients with L-HLH and those without HLH (non-HLH). By this method, soluble V-set and immunoglobulin domain-containing protein-4 (sVSIG4), also referred to as complement receptor of the immunoglobulin superfamily (CRIg) or Z39Ig, existing exclusively on the surface of human and mouse macrophages (Munawara *et al.*, 2017; van Lookeren Campagne & Verschoor, 2018), was identified as being highly expressed in the serum of lymphoma patients with L-HLH. We further demonstrated that sVSIG4 may be regarded as an alternative biomarker to discriminate L-HLH from non-HLH lymphoma patients and refine the prognosis of these patients.

Materials and methods

Study population and design

Initially, the present study was a single-institution, retrospective study designed to discover novel serum biomarkers for the diagnosis of patients with L-HLH. All aspects of the current study were performed under the approval of the 307 Hospital Ethics Committees. Informed written consent was obtained from each cancer patient for storage and use of remaining blood samples. Collection and use of remnant serum from healthy adult controls after routine diagnostic procedures followed the National Regulations on the Use of Clinical Samples in China. Sample collection took place at the 307 Hospital between August 2016 and September 2017. Each serum sample was stored at −80°C. Clinical and follow-up data were obtained by review of patient medical records. Subsequently, further HLH samples were obtained through a collaboration with Professor Jiangfeng Zhou's group in Wuhan. All of their specimens were plasma samples, stored at −80°C and mailed to our laboratory on dry ice.

Protein identification and quantification

Eight-plex isobaric tags for relative and absolute quantitation (iTRAQ) analysis was performed in the State Key Laboratory of Proteomics (Beijing Proteome Research Center, National Center for Protein Sciences Beijing). Sample preparation, mass spectrometry, peptide and protein identification were

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performed as previously described (Wang *et al*., 2015; Sun *et al*., 2016; Zhang *et al*., 2017). All reported data were recorded using Excel for evaluation of candidate proteins based on proteomic fold-change and false discovery rate (FDR).

Soluble VSIG4 measurement by ELISA

Serum soluble VSIG4 (sVSIG4) was measured at least in triplicate by enzyme-linked immunosorbent assay (ELISA) according to the protocol provided by the manufacturer (Sino Biological Inc., Beijing, China). All serum samples were diluted 1:10 with phosphate-buffered saline (PBS) and assayed consecutively within the same batch of reagents. All plasma samples were diluted 1:1·5 with PBS and detected in the same way.

Clinical data

All clinical parameters of specimens from Professor Song's group were measured in the Department of Laboratory Medicine, The Fifth Medical Center, Chinese PLA General Hospital (Former 307th hospital of the PLA). Serum sIL-2Rα levels were measured by the automated immunoassay system (Siemens IMMULITE 1000 Immunoassay Platform; Chinese normal adult reference range, 0–710 U/ml; Siemens Healthcare Diagnostics Inc. Flanders, USA). The measurable upper limit of sIL-2Rα is 7500 U/ml (Rothkrantz-Kos *et al*., 2004). If the sIL-2Rα level was >7500 U/ml, the corresponding frozen serum sample was diluted 1:2–1:8 with dilution buffer in order to calculate the exact value. For ferritin and triglyceride levels, the most frequently missing data, some of the cases were measured using the frozen serum if enough was available before final analysis.

Statistical analysis

Continuous variables are presented as medians and categorical variables as numbers and percentages (%). Variables were evaluated for an association with the diagnosis with the use of Fisher's exact test for categorical data and the Mann–Whitney test or *t*-test for numerical data. The relation between sIL-2Rα and sVSIG4 was assessed with the use of Pearson's *r*-test. To evaluate the diagnostic value of sIL-2Rα and sVSIG4 in the serum, receiver operating characteristic (ROC) curves were constructed and the area under the ROC curve (AUC) was calculated. On the basis of the sensitivities and specificities produced by ROC curves, the Youden index (= sensitivity + specificity − 1) was used to determine the optimal cut-off points for sVSIG4 and sIL-2Rα respectively. Analysis of multiple paired data was performed by one-way anova with Tukey's multiple comparison test. Overall survival was defined as the date from initial diagnosis to death. The probabilities of overall survival were estimated by the Kaplan–Meier method and compared with the log-rank test. $P < 0{\cdot}05$ was considered statistically significant. Most of the statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, Inc.). The difference in AUCs between sIL-2Rα and sVSIG4 was analyzed using the DeLong method performed with MedCalc software version 11.4.2.0.

Results

Characteristics of patients

From August 2016 to September 2017, we sequentially collected 80 serum specimens from patients with HLH and/or lymphoma at the time of diagnosis. Assessment of the NK cell function was not available in our hospital and was not included in the current study. Patients were diagnosed with L-HLH if they fulfilled at least five of the eight HLH-2004 criteria. After excluding duplications, non-adult and non-lymphoma-associated HLH, the remaining 57 patients with lymphoma were enrolled for further analysis, comprising 39 non-HLH and 18 L-HLH cases. Like another centre's report (Riviere *et al*., 2014), ferritin and triglyceride levels were the most frequently missing data in the current study. Ferritin levels were missing in five of the 39 non-HLH patients, and triglyceride levels were missing in 15 of the 39 non-HLH patients and two of the 18 L-HLH patients. There were missing data on fibrinogen levels in four non-HLH patients. Clinical characteristics of the 57 patients are shown in Table I. Most of them were male, 21 (53·8%) in the non-HLH and 15 (83·3%) in the HLH groups respectively ($P = 0{\cdot}04$). The median age was 50 years (range, 23–83 years) and 55 years (range, 26–84 years) in the non-HLH and the L-HLH groups respectively ($P = 0{\cdot}7867$). Fever was present in seven patients (18%) and 18 patients (100%) and splenomegaly occurred in five patients (13%) and 14 patients (78%) in the non-HLH and L-HLH groups respectively (both $P < 0{\cdot}0001$). The difference was significant between the non-HLH and L-HLH groups with respect to white blood cell and red blood cell counts, and levels of haemoglobin, platelet, triglyceride, fibrinogen, ferritin and sIL-2Rα. However, the difference in ongoing infection with Epstein–Barr virus (EBV) or bacteria at the time of sample collection was not significant between the non-HLH group and the L-HLH group. There was no statistically significant difference in lymphoma subtypes between the non-HLH and the L-HLH groups (B-cell lymphoma: 54% vs. 44%, $P = 0{\cdot}5765$; T/NK-cell lymphoma: 28% vs. 50%, $P = 0{\cdot}2378$; non-HLH vs. L-HLH) either. The median follow up was shorter and correspondingly the mortality was higher in the L-HLH group compared with the non-HLH group (34·5 days vs. 332 days, 94% vs. 28%, respectively, both $P < 0{\cdot}0001$).

Identification of soluble VSIG4 in the serum using iTRAQ

To identify the protein expression profile in L-HLH, eight-plex iTRAQ analysis was performed on the serum of four

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diffuse large B-cell lymphoma (DLBCL) patients with HLH (L-HLH group) and four DLBCL patients without HLH (non-HLH group) from the enrolled 57 patients (Table SI). After running this experiment, 533 proteins were identified, 418 of which were quantified. Proteins with ratios of L-HLH/non-HLH above 1·5 or below 0·67 (1/1·5) and FDR below 5% were considered to be significantly differentially expressed in the L-HLH group. Overall, 89 upregulated and 37 downregulated proteins were identified in L-HLH group compared with the non-HLH group (Table II). Among these soluble proteins upregulated in the serum, CD14, CD163, β2-microglobulin, and C-reactive protein (CRP) had been revealed to be present at higher levels in patients with HLH in previous studies (Bleesing *et al*., 2007; Nanno *et al*., 2016; Tamamyan *et al*., 2016; Koh *et al*., 2017; Machowicz *et al*., 2017). In addition to CD14 and CD163, other transmembrane proteins expressed on the macrophage's surface were also shown to be significantly upregulated in the serum of L-HLH, such as Fc gamma receptor III-A (FcγRIIIA), scavenger receptor MARCO, CSF1 receptor (CSF1R) and VSIG4. Notably, of all detected receptor proteins in the serum originally found to be expressed on the surface of macrophages, sVSIG4 had the highest ratio of being upregulated in L-HLH versus non-HLH patients. Hence, we proceeded to examine the level of confidence of serum sVSIG4 in distinguishing L-HLH from non-HLH in patients with lymphoma.

sVSIG4 levels in different populations

Further verification of sVSIG4 quantification was performed using ELISA on 57 serum samples from patients with lymphoma. The median value of sVSIG4 concentration was 209 pg/ml (range, 0–3258 pg/ml) and 5528 pg/ml (range, 1037–13017 pg/ml) and the mean value of sVSIG4 concentration was 662·3 pg/ml (95% CI, 402·8–921·7 pg/ml) and 6072 pg/ml (95% CI, 4358–7786 pg/ml) in the serum of lymphoma patients with non-HLH and those with L-HLH respectively (both $P < 0{\cdot}0001$). To determine the potential specificity of sVSIG4 in the diagnosis of L-HLH in patients with lymphoma, we then detected the sVSIG4 levels from the serum of the 120 healthy controls, and of 40 breast cancer, 40 gastric cancer and 39 lung cancer cases collected in our hospital. The median values of sVSIG4 concentration in the serum of healthy controls, breast cancer, gastric cancer and lung cancer cases were 0 pg/ml and the mean values of sVSIG4 concentration in the serum of healthy controls, breast cancer, gastric cancer and lung cancer cases were 3·50, 6·475, 13·33 and 28·31 pg/ml respectively. Both median values and mean values in healthy controls and non-lymphoma patients were much lower than those in lymphoma patients with non-HLH or L-HLH. In a one-way ANOVA analysis, the difference in sVSIG4 levels in the serum was significant not only for lymphoma patients with L-HLH compared with those with non-HLH or the healthy control, breast cancer,

Table I. Characteristics of patients with lymphoma.

Characteristics	non-HLH	L-HLH	*P*-value
Number of patients, *n*	39	18	
Male, *n* (%)	21 (53·8)	15 (83·3)	0·04
Age (years), median (range)	50 (23–83)	55 (26–84)	0·7867
Fever, *n* (%)	7 (18)	18(100)	<0·0001
Splenomegaly, *n* (%)	5 (13)	14 (78)	<0·0001
White blood cell count, median (range) ($\times 10^9$/l)	5·90 (0·25–43·05)	3·55 (0·03–12·75)	0·0086
Lymphocyte count, median (range) ($\times 10^9$/l)	1·25 (0·15–40·47)	0·39 (0·07–2·34)	0·0003
Neutrophil count, median (range) ($\times 10^9$/l)	3·23 (0·44–11·4)	2·54 (0·03–8·98)	0·2518
Monocyte count, median (range) ($\times 10^9$/l)	0·49 (0·01–1·5)	0·32 (0–1·27)	0·2715
Red blood cell count, median (range) ($\times 10^{12}$/l)	3·89 (1·61–5·49)	2·88 (1·74–4·11)	0·0017
Haemoglobin, median (range) (g/l)	108 (47–164)	81·5 (49–107)	0·0017
Platelet count, median (range) ($\times 10^9$/l)	219 (20–696)	70 (3–177)	<0·0001
Triglyceride, median (range) (mmol/l)	1·28 (0·61–2·54)	2·14 (0·92–7·17)	0·0094
Fibrinogen, median (range) (g/l)	3·74 (0·47–6·59)	3·16 (0·95–6·34)	0·0397
Ferritin, median (range) (μg/l)	395 (30–3174)	2341 (630–125320)	<0·0001
sIL-2Rα, median (range) (U/ml)	1479 (311–37488)	20354 (4834–35696)	<0·0001
Haemophagocytosis, *n* (%)	0 (0)	4 (22)	
Number of HLH-2004 criteria fulfilled, median (range)	1 (0–4)	5 (5–7)	<0·0001
B-cell lymphoma, *n* (%)	21 (54)	8 (44)	0·5765
T/NK-cell lymphoma, *n* (%)	11 (28)	9 (50)	0·2378
Hodgkin lymphoma, *n* (%)	5 (13)	0 (0)	
Mixed or undefined, *n* (%)	2 (5)	1 (6)	
EBV^+ in blood, *n* (%)	11 (28)	7 (38)	0·3568
Bacterial infection, *n* (%)	2 (5)	4 (22)	0·9185
Time of follow-up, median (range) (days)	332 (72–526)	34·5 (1–527)	<0·0001
Overall mortality, *n* (%)	11 (28)	17 (94)	<0·0001

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Table II. Differentially expressed proteins in L-HLH detected by iTRAQ.

Accession no.	Protein names	Ratio*	FDR
A8K5T0	Complement factor H	27·45	0·015373901
P02741	C-reactive protein	18·55	0·0017742
H7C062	V-set and immunoglobulin domain-containing protein 4	11·18	0·000000000842
A0A087WXI2	IgG Fc-binding protein	4·88	0·000670416
P01034	Cystatin-C	4·5	0·00004
E5RIR0	Fatty acid-binding protein, adipocyte	4·23	0·047561702
B4E1I8	Leucine-rich alpha-2-glycoprotein	4·23	0·000343188
E7ENL6	Collagen alpha-3(VI) chain	4·2	0·032199188
Q5H9A7	Metalloproteinase inhibitor 1	3·93	0·021974873
P41222	Prostaglandin-H2 D-isomerase	3·84	0·016578961
H0YLF3	Beta-2-microglobulin	3·71	0·0000269
Q7Z4G9	HBxAg-binding protein	3·64	0·00000198
A9X9L1	Desmocollin 2	3·6	0·000000603
D3DQX7	Serum amyloid A protein	3·37	0·015747224
Q5TA01	Glutathione S-transferase omega-1	3·33	0·021974873
H0YD13	CD44 antigen	3·28	0·0000161
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1	3·19	0·041304601
Q9UN20	Fc gamma receptor III-A	3·15	0·0000287
Q9UNH2	MC51L-53L-54L homolog	3·01	0·0000183
Q9UEW3	Macrophage receptor MARCO	2·99	0·000541688
C9J6H2	Insulin-like growth factor-binding protein 1	2·97	0·021974873
E9PR54	Cathepsin B	2·97	0·003023285
D3DUU0	CD163 antigen, isoform CRA_a	2·92	0·000319118
P22692	Insulin-like growth factor-binding protein 4	2·89	0·000661156
A0A024R8C9	ADAMTS-like 2, isoform CRA_a	2·86	0·001934285
Q8TCF0	LBP protein	2·84	0·020244771
Q4LDE5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	2·83	0·00000383
E9PNW4	CD59 glycoprotein	2·78	0·001666006
P18065	Insulin-like growth factor-binding protein 2	2·75	0·000058
P0DJI8	Serum amyloid A-1 protein	2·68	0·016577571
P05362	Intercellular adhesion molecule 1	2·65	0·0000485
A2VDG3	CSF1R protein	2·59	0·006976951
K7EMR1	Granulins	2·58	0·006213416
A1L4H1	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	2·51	0·003220358
P19320	Vascular cell adhesion protein 1	2·45	0·00000251
L8E853	von Willebrand factor	2·39	0·0000799
H7C427	IQ domain-containing protein E	2·39	0·006668591
P0DJI9	Serum amyloid A-2 protein	2·35	0·012078245
F5H5I5	ATP-binding cassette sub-family B member 9	2·32	0·038868364
J3QKR4	Intercellular adhesion molecule 2	2·3	0·00093563
P01833	Polymeric immunoglobulin receptor	2·26	0·008720629
B9EJA8	Mannose receptor, C type 1-like 1	2·26	0·001094472
Q9ULI3	Protein HEG homolog 1	2·24	0·00074161
A0A024R6I7	Alpha-1-antitrypsin	2·21	0·0000206
P35527	Keratin, type I cytoskeletal 9	2·19	0·002064868
K7EP52	Intercellular adhesion molecule 3	2·18	0·006076212
Q59GX5	L-plastin variant	2·15	0·001419501
B4DE78	cDNA FLJ52141, highly similar to 14-3-3 protein gamma	2·14	0·033316634
H6VRG2	Keratin 1	2·14	0·047564525
B7Z5Q2	cDNA FLJ58075, highly similar to ceruloplasmin	2·09	0·000126815
A0A0A0N0L2	IL6ST isoform 4	2·07	0·036766487
B4DE80	cDNA FLJ52255, highly similar to angiotensinogen	2·02	6·84E-08
V9GYE7	Complement factor H-related protein 2	1·96	0·018885139
Q8NBJ4	Golgi membrane protein 1	1·96	0·002220793
H2B4M3	LILRA3 protein	1·96	0·033004753

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Table II. (*Continued*)

Accession no.	Protein names	Ratio*	FDR
B4DSV9	cDNA FLJ56632, moderately similar to target of Nesh-SH3	1·95	0·001811254
Q9NPR2	Semaphorin-4B	1·94	0·022590271
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	1·92	0·000109973
P63104	14-3-3 protein zeta/delta	1·92	0·012599945
P02461	Collagen alpha-1(III) chain	1·91	0·007955334
P35908	Keratin, type II cytoskeletal 2 epidermal	1·91	0·013937366
P55058	Phospholipid transfer protein	1·9	0·000890632
C9JEE7	WD repeat-containing protein 43	1·89	0·018079585
P34096	Ribonuclease 4	1·86	0·002267311
F5GXJ9	CD166 antigen	1·84	0·000111916
Q8WVW5	Putative uncharacterized protein	1·84	0·02351326
B2RMS9	Inter-alpha (globulin) inhibitor H4	1·83	0·000016
Q07954	Prolow-density lipoprotein receptor-related protein 1	1·83	0·006976951
E5RIA2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	1·82	0·012599945
X6RLJ0	Complement C1q subcomponent subunit A	1·82	0·01862898
P05155	Plasma protease C1 inhibitor	1·8	0·000142055
B2R888	Monocyte differentiation antigen CD14	1·8	0·0000031
O00187	Mannan-binding lectin serine protease 2	1·79	0·00000625
Q14126	Desmoglein-2	1·74	0·00022776
Q5EFE6	Anti-RhD monoclonal T125 kappa light chain	1·72	0·000193974
A0A0U1RQV3	EGF-containing fibulin-like extracellular matrix protein 1	1·72	0·001094472
Q9H804	cDNA FLJ14022 fis, clone HEMBA1003538, weakly similar to complement C1R component	1·65	0·000000973
Q59EZ3	Insulin-like growth factor 2 receptor variant	1·65	0·00004
Q8NBP7	Proprotein convertase subtilisin/kexin type 9	1·61	0·00000625
P16930	Fumarylacetoacetase	1·6	0·000661156
G3V0E5	Transferrin receptor	1·59	0·01337998
P11021	78 kDa glucose-regulated protein	1·57	0·0000409
A0A0X9TD88	GCT-A3 heavy chain variable region	1·56	0·041444691
Q92782	Zinc finger protein neuro-d4	1·54	0·003220358
Q6FHW3	DF protein	1·53	0·000699185
A0A024R035	Complement component 9, isoform CRA_a	1·53	0·006976951
B3KSS4	cDNA FLJ36858 fis, clone ASTRO2015185, highly similar to poliovirus receptor	1·52	0·001094472
V9GYL7	Neuropilin-1	1·5	0·000961068
A0A172Q3A0	Fibroblast activation protein	1·5	0·022164431
A0A0S2Z4F5	Selectin P isoform 3	0·4	0·000000165
A8K335	Gamma-glutamyl hydrolase	0·45	0·0000518
O75753	Surf5	0·47	0·000774695
A0A024R9Q1	Thrombospondin 1, isoform CRA_a	0·5	1·41E-08
Q5SRP5	Apolipoprotein M	0·51	2·77E-08
P02776	Platelet factor 4	0·52	0·000550378
P08567	Pleckstrin	0·54	0·002137534
A0A024CIM4	Carboxylic ester hydrolase	0·55	0·0000012
Q53HF2	Heat shock 70kDa protein 8 isoform 2 variant	0·56	0·023278522
B2R5G8	Serum amyloid A protein	0·58	0·00000028
P06396	Gelsolin	0·62	0·00004
Q584P1	Prenylcysteine oxidase	0·63	0·00000983
I3L0A1	Cysteine-rich secretory protein 3	0·66	0·000550378
P23142	Fibulin-1	0·66	0·004344317
P15924	Desmoplakin	0·66	0·003245892

L-HLH, lymphoma-associated haemophagocytic lymphohistiocytosis; iTRAQ, isobaric tags for relative and absolute quantitation.
*Equal to L-HLH/non-HLH.

gastric cancer and lung cancer groups, but also in lymphoma patients with non-HLH compared with the healthy control or other cancer groups (Fig 1). To validate the current result, we measured the sVSIG4 in plasma from another cohort of HLH patients (from Professor Zhou's group), that was made up of 10 EBV, seven aggressive NK cell leukaemia (ANKL)

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and seven other subtype lymphoma patients. The median value of sVSIG4 concentration was 1440 pg/ml (range, 423–2202 pg/ml) and 3635 pg/ml (range, 1352–6765 pg/ml) and the mean value of sVSIG4 concentration was 1343 pg/ml (95% CI, 825–1860 pg/ml) and 3629 pg/ml (95% CI, 3160–4097 pg/ml) in the plasma of HLH patients with lymphoma or EBV infection and healthy controls respectively (both $P < 0{\cdot}0001$).

Diagnostic value of sVSIG4 in patients with L-HLH

To define the optimal cut-off point of sVSIG4 concentration in the serum for discriminating L-HLH from non-HLH patients with lymphoma, we studied its sensitivity and specificity with ROC analysis. The ROC curve indicated that the sVSIG4 concentration had good to excellent performance in diagnosing L-HLH in patients with lymphoma, with an AUC of 0·98 (95% CI, 0·94–1·01; Fig 2A). On the basis of this curve, the highest Youden index was 0·99, which yielded an optimal cut-off point of 2195 pg/ml. At this cut-off point, the corresponding sensitivity was 94·44% (95% CI, 72·71–99·86%) and the corresponding specificity was 94·87% (95% CI, 82·68–99·31%) for the diagnosis of L-HLH in patients with lymphoma. Simultaneously, we also examined the ROC curve of sIL-2Rα for the diagnosis of L-HLH in patients with lymphoma; the AUC was 0·94 and the optimal cut-off was 8033 U/ml, with a highest Youden index of 0·82. The corresponding sensitivity was 94·44% (95% CI, 72·71–99·86%) and the corresponding specificity, 87·18% (95% CI, 72·57–95·70%) (Fig 2A), values consistent with most other reported results (Lehmberg *et al.*, 2013; Hayden *et al.*, 2017). Pairwise comparison of the ROC curves demonstrated that there was no significant difference between VSIG4 and sIL-2Rα for the diagnosis of non-HLH and L-HLH in patients with lymphoma ($P = 0{\cdot}2706$, DeLong test). In addition, sVSIG4 and sIL-2Rα concentrations were highly correlated in patients with lymphoma ($P < 0{\cdot}0001$; $R^2 = 0{\cdot}456$, Fig 2B). The extended data demonstrated the same AUC of 0·98 (95% CI, 0·93–1·02) for discriminating HLH patients from healthy controls with the sVSIG4 concentration in the plasma. With an optimal cut-off point at 2206 pg/ml and a highest Youden index of 0·96, the corresponding sensitivity was 95·83% (95% CI, 78·88–99·89%) and the corresponding specificity was 100% (95% CI, 63·06–100%).

Prognostic value of sVSIG4 in patients with lymphoma

Based on the diagnostic significance of serum sVSIG4 in patients with lymphoma, we set out to investigate the prognostic role of sVSIG4 in the same population. Of the 57 patients with lymphoma we followed up until March 2018, one-year overall survivals for the 39 patients in the non-HLH group and for the 18 patients in the L-HLH group were significantly different (70·2% vs. 5·6%, $P < 0{\cdot}0001$); the median survival time of L-HLH was 34·5 days and non-HLH not reached (Fig 3A). When the sVSIG4 cut-off point of 2195 pg/ml was exploited to analyze the 57 patients, the result demonstrated that one-year overall survivals were also significantly different in the 38 patients below 2195 pg/ml and in the 19 patients above 2195 pg/ml (72·2% vs. 5·3%, $P < 0{\cdot}0001$) and the median survival time of the patients above 2195 pg/ml was 35 days and below 2195 pg/ml not reached (Fig 3B). When the survival of the 57 patients was analyzed with sIL-2Rα, if setting 8033 U/ml as the cut-off point, the median survival time of the 22 patients above 8033 U/ml was 59 days (Fig 3C), and one-year survival of patients under and above 8033 U/ml was 75·1% and 9·1% respectively ($P < 0{\cdot}0001$). However, if using the HLH-2004 criterion of 2400 U/ml as the sIL-2Rα cut-off point, the median survival time of the 32 patients above 2400 U/ml was 131·5 days (Fig 3D) and one-year survival of patients under and above 2400 U/ml was 91·8% and 15·1% respectively ($P < 0{\cdot}0001$). Prognostic analysis stratified with the sVSIG4 cut-off point seemed closer to that when separating non-HLH and L-HLH; however, there was no significant difference in prognostic analysis between sVSIG4 and sIL-2Rα in the enrolled patients with lymphoma.

Discussion

Most studies focus on esablishing the underlying triggers in patients with HLH, which is crucial for the treatment of HLH and the triggering conditions (Riviere *et al.*, 2014; Al-

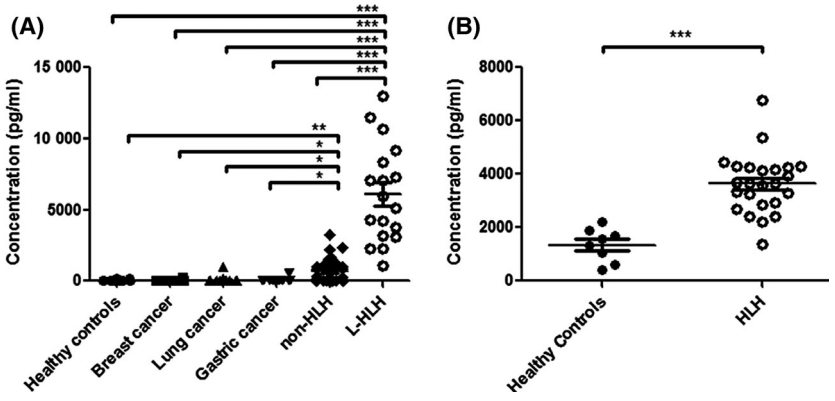

Fig 1. Levels of soluble V-set and immunoglobulin domain-containing protein-4 (sVSIG4) in the serum of healthy controls and of patients with breast cancer, lung cancer, gastric cancer, lymphoma without haemophagocytic lymphohistiocytosis (non-HLH) and lymphoma with HLH (L-HLH) (A). Levels of sVSIG4 in the plasma of healthy controls and of patients with lymphoma or Epstein–Barr virus (EBV)-associated HLH (B). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$, $^{***}P < 0{\cdot}0001$.

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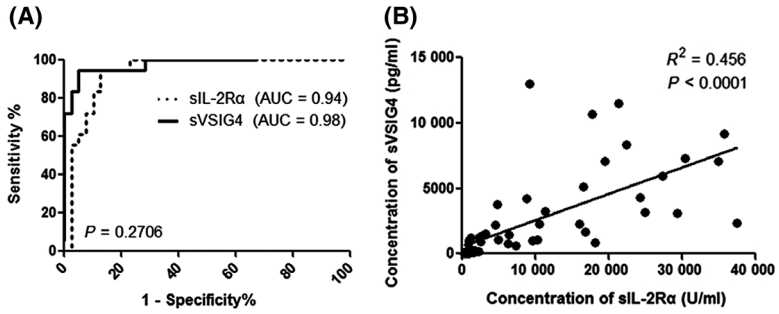

Fig 2. Receiver operating characteristics curves (ROC) of soluble V-set and immunoglobulin domain-containing protein-4 (sVSIG4) and soluble interleukin-2 receptor alpha (sIL-2Rα) for the diagnosis of lymphoma-associated haemophagocytic lymphohistiocytosis in patients with lymphoma (A). Correlation between sIL-2Rα and sVSIG4 in patients with lymphoma (B). AUC, area under the ROC curve.

Samkari & Berliner, 2018). Given the high incidence and poor prognosis of lymphoma patients with L-HLH in comparison with those without HLH (Maruoka *et al*., 2014; Ramos-Casals *et al*., 2014), the primary goal of this study was initially to look for more specific diagnostic serum marker(s) with potential use in discriminating L-HLH from lymphoma patients. For HLH diagnosis, up to now, there is no broad consensus on diagnostic markers. sIL-2Rα is a marker for activated T cells, which could be elevated in many conditions other than HLH. We therefore set out to investigate markers other than sIL-2Rα, particularly those related to activated macrophages, another important cellular player other than T lymphocytes in L-HLH.

In the present study, we first conducted a quantitative proteomics analysis of serum samples with eight-plex iTRAQ technology on lymphoma patients with L-HLH and those without HLH. A series of receptor proteins specifically expressed on the surface of macrophages have been identified as being elevated in the serum of lymphoma patients with L-HLH, some of which, such as soluble receptors CD163 and CD14, have been reported as being highly expressed in the serum of patients with HLH (Bleesing *et al*., 2007; Koh *et al*., 2017). Beside sCD163 and sCD14, other receptors initially expressed on the surface of macrophages, such as VSIG4, MARCO, FcγRIIIA and CSF1R were also found to be highly expressed in the serum of lymphoma patients with L-HLH. Consistent with previous reports, these soluble receptors present in the serum may reflect the degree of activation and expansion of haemophagocytic macrophages in L-HLH (Bleesing *et al*., 2007; Koh *et al*., 2017), but sVSIG4 has the highest ratio of L-HLH/non-HLH among all of these macrophage-associated receptors identified by iTRAQ analysis. Of note, the parallel expression of VSIG4 and CD163 in activated macrophages has been confirmed in previous publications (Walker, 2002; van Lookeren Campagne & Verschoor, 2018). Although some studies demonstrated IP-10/CXCL10 and MIG/CXCL9 as novel markers for the diagnosis of L-HLH patients, the major source of IP-10/CXCL10 and MIG/

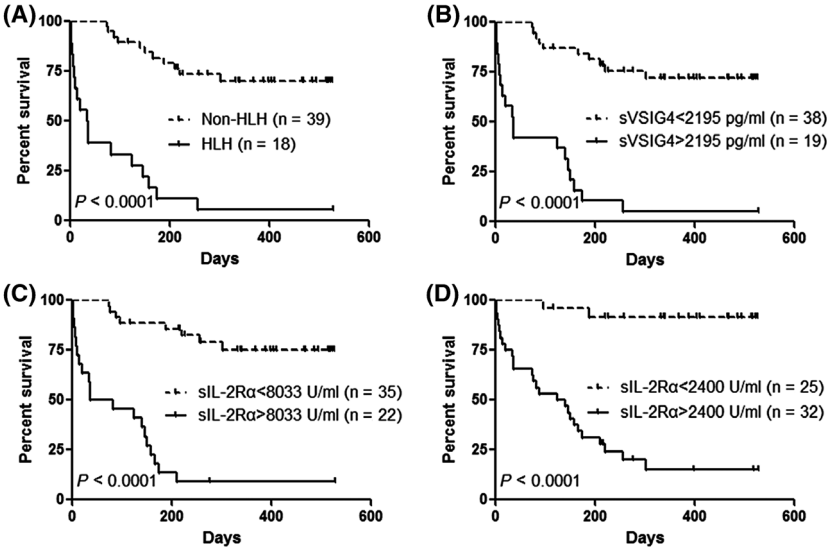

Fig 3. Analyses of overall survival in patients with lymphoma according to HLH and non-HLH subgroups (A), with serum soluble V-set and immunoglobulin domain-containing protein-4 (sVSIG4) concentration above and below 2195 pg/ml (B), with serum soluble interleukin-2 receptor alpha (sIL-2Rα) concentration above and below 8033 U/ml (C) and with sIL-2Rα above and below 2400 U/ml (D).

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CXCL9 produced by macrophages or lymphoma cells remains to be elucidated (Maruoka *et al*., 2014; Grom *et al*., 2016). Therefore, the diagnostic role of sVSIG4 (which is specifically produced by macrophages) in the diagnosis of L-HLH merits further investigation.

Discovered in 2000, VSIG4 is a member of the immunoglobulin-like superfamily and is predominantly expressed on the surface of tissue-resident macrophages, particularly Kupffer cells in the liver (Helmy *et al*., 2006; Vogt *et al*., 2006). To date, *in vitro* and *in vivo* studies have consistently implicated VSIG4 as a receptor of complement C3 on the surface of macrophages playing a vital role in phagocytosis and the clearance of pathogens in the circulation (Helmy *et al*., 2006; Vogt *et al*., 2006). Most studies have focused on the function of VSIG4 in phagocytosis (Gorgani *et al*., 2011; Broadley *et al*., 2016; Zeng *et al*., 2016; Nagre *et al*., 2018), although several recent studies have found that VSIG4 is associated with tumour progression (Liao *et al*., 2014; Xu *et al*., 2015; Byun *et al*., 2017). Normally, VSIG4 is expressed on resting macrophages and expression is lost upon activation, and VSIG4^{hi} macrophages plays an anti-inflammatory and immunosuppressive role *in vivo* (Vogt *et al*., 2006; Irvine *et al*., 2016). Thus, we suppose that free sVSIG4 showing up in the serum may be shed by activated macrophages during the development of HLH, which might be the potential reason macrophages lose VSIG4 expression upon activation. From this perspective, the present observations suggest that sVSIG4 may be considered a critical marker of macrophage activation in the pathogenesis of L-HLH in patients with lymphoma.

Since 1989, serum sIL-2Rα as an indicator of activated CTLs gradually became recognized as an important biomarker for HLH diagnosis, culminating in the HLH-2004 criteria (Komp *et al*., 1989; Hayden *et al*., 2016; Lin *et al*., 2017). Although a few diagnostic guidelines adopted by certain centres exclude the measurement of sIL-2Rα for the diagnosis of HLH, the HLH-2004 guideline is still the most widely cited one (Hayden *et al*., 2016; Lin *et al*., 2017). However, high sIL-2Rα levels are found not only in HLH and/or lymphoma, but also in autoimmune lymphoproliferative disorders, cancers and other conditions associated with T-cell activation such as transplantations (Adams *et al*., 1989; Foley *et al*., 1998; Bien & Balcerska, 2008; Hayden *et al*., 2017). In particular, several studies have revealed that IL-2Rα positive cells exist in some cancer tissues such as breast cancer, gastric cancer and colorectal cancer and metastases to lymph nodes (Murakami, 2004). In addition, a high concentration of serum sIL-2Rα has been demonstrated to be associated with lymph node metastasis and poor prognosis in cancer patients (Kuhn & Dou, 2005; Nukui *et al*., 2017). These data suggest that sIL-2Rα is a good marker for activated CTLs but not an exclusive biomarker for the diagnosis of HLH. Hence, a specific biomarker is still an unmet need to help directly assess patients with L-HLH.

In our case cohort, we also found that sVSIG4 has AUC, specificity and sensitivity values similar to those of sIL-2Rα in the diagnosis of lymphoma patients with HLH. The predictive power for prognosis in these populations was also very similar whether analyzed by sVSIG4 or sIL-2Rα levels. However, unlike sIL-2Rα, which is expressed by various cell types including primed activated CTLs in activated immune states, VSIG4 was predominantly expressed by macrophages (Andrew *et al*., 1984; Rubin *et al*., 1985; Jacques *et al*., 1986; Walker, 2002; Helmy *et al*., 2006; Vogt *et al*., 2006). The results presented herein support the hypothesis that sIL-2Rα and sVSIG4 can be used to evaluate activated CTLs and macrophages in L-HLH respectively. Furthermore, the correlation between sVSIG4 and sIL-2Rα levels in patients with lymphoma appeared to reflect the interaction between activated macrophages and activated CTLs, two parallel and complementary components in the pathogenesis of L-HLH. In particular, sVSIG4 level in the plasma also corroborated its diagnostic value in patients with HLH triggered by lymphoma or EBV infection. Hence, sVSIG4 can be regarded as an alternative biomarker for sIL-2Rα in the diagnosis and prognosis of lymphoma patients with or without HLH.

Several limitations in our present study merit discussion. We recognize that the overall number of patient serum specimens was relatively small and the patient cohort was heterogeneous. Hence, the results must be interpreted with caution. Further larger studies are clearly warranted that may help to translate sVSIG4 into the clinical arena as a serum biomarker for distinguishing L-HLH from non-HLH lymphoma patients and also lymphoma patients from patients with other cancers. In addition, although EBV and bacterial infections are comparable between lymphoma patients with and without HLH in the current study, studies involving non-lymphoma settings, such as infection-associated HLH and rheumatoid disease-associated macrophage activation syndrome, should be carried out to determine whether the diagnostic and prognostic role of sVSIG4 in HLH is still applicable. Owing to the limited serum sample, the significance of other macrophage-related surface receptors, such as MARCO, CD163 and CD14 detected by iTRAQ in the serum, as candidates in the diagnosis and prognosis of L-HLH has not been investigated in this study, nor has their correlation with sVSIG4. Several other cytokines or chemokines such as interferon-gamma, IP-10/CXCL10 and MIG/CXCL9 have been found to be elevated in HLH patients (Maruoka *et al*., 2014; Ramos-Casals *et al*., 2014; Grom *et al*., 2016). However, in the current iTRAQ screening test, they did not differ significantly between L-HLH and non-HLH lymphoma patients, and this requires further investigation. It should also be noted that in this retrospective study, a test for NK cell function was not available, triglyceride and fibrinogen values were missing in some patients and haemophagocytosis-positive results were also fewer than in other publications; all these factors might result in an underestimate of the number of true HLH cases as judged by HLH-2004 criteria in our cohort. Indeed, all six patients without HLH but fulfilling 3–4 indices of the HLH-2004 criteria with sVSIG4 concentration above 2195 pg/ml died

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within five months after initial diagnosis (data not shown). Therefore, the role of sVSIG4 in the differentiation between L-HLH and non-HLH in patients with lymphoma deserves further investigation.

For the first time, our study demonstrated serum sVSIG4 as a useful biomarker in distinguishing L-HLH from non-HLH and the corresponding subgroups with good and poor prognoses in patients with lymphoma respectively. Our results also showed that VSIG4 was more likely to be detected in the serum of patients with lymphoma relative to healthy controls or patients with other commonly occurring cancers. Thus, if used in future studies as a marker to screen for patients with potential lymphoma with HLH or substantial 'pre-HLH' immune dysregulation without fully fulfilling the HLH-2004 criteria, sVSIG4 will be of clinical diagnostic value.

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The Fifth Medical Center of PLA General Hospital has filed a provisional patent application with the China National Intellectual Property Administration (CNIPA) (application no. 201811422840.1). The application is directed toward the clinical application of sVSIG4 for the diagnosis of patients with potential HLH. The authors would like to thank all members of the Department of Lymphoma, the Department of Laboratory Medicine, and the Department of Oncology Laboratory in the Former 307th Hospital of PLA (now re-named The Fifth Medical Center, Chinese PLA General Hospital) for their contributions in patient management, sample collection and data processing and Drs Weiran Lin and Peng Wu (Beijing Proteome Research Center, National Center for Protein Sciences Beijing) for technical assistance in the iTRAQ analysis. We sincerely appreciate the work of Drs Brian Eyden (Manchester, UK) and Wenda Gao (Boston, USA) for final English language editing.

Author contributions

Shunzong Yuan and Shiping Song designed and coordinated the study; Yanqing Wang, Hui Luo, Jianfeng Zhou, Bing Qiao, Yan Jiang, Yaning Hu and Yang Cheng gathered samples, collected clinical parameters and generated experimental data; Yanqing Wang, Jianfeng Zhou, Shiping Song and Zheng Jiang gathered clinical history details of patientsand analyzed and reviewed data; Weihua Gong, Weipeng Zhao and Deyan Luo performed the statistical analysis; Yanqing Wang, Xilin Chen, Shunzong Yuan and Hang Su contributed to patient care; Yong Huang and Shunzong Yuan contributed to pathological diagnosis; Shunzong Yuan and Bing Liu wrote the manuscript, and all co-authors reviewed and approved the submitted manuscript.

Conflict of interest

The authors declare no competing financial interests.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. Characteristics of patients used for differential protein screening by an iTRAQ test.

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