Expression of the RNA-binding protein VICKZ in normal hematopoietic tissues and neoplasms

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ABSTRACT

Background and Objectives

VICKZ family members are RNA-binding regulatory proteins expressed during embryogenesis but not usually found in normal adult tissue. The presence of VICKZ in normal germinal centers (GC) prompted us to characterize the expression pattern of this protein in lymphoid and hematopoietic tissues.

Design and Methods

We generated a pan-VICKZ antibody that recognized all three isoforms of VICKZ protein and screened 889 patients' samples by immunohistologic methods. We also analyzed the expression of VICKZ in normal hematopoiesis tissue by staining samples of tonsils, lymph nodes

Results

VICKZ protein expression was documented for the first time in normal human GC and in follicular (126/165), mediastinal large B-cell (9/10), Burkitt (2/2), diffuse large B-cell (DLBCL, 155/200), lymphocyte-predominant Hodgkin's (12/13), classical Hodgkin's (101/108), and anaplastic large cell (6/8) lymphomas and in lymphoid and myeloid leukemias. Since DLBCL may derive from GC or non-GC B cells we performed hierarchical cluster analysis for VICKZ, HGAL, BCL6, CD10, MUM1/IRF4 and BCL2 which showed that VICKZ is expressed in both subtypes. In addition, VICKZ mRNA isoforms were differentially expressed in lymphoma subtypes and over 40% of DLBCL expressed hVICKZ2, an isoform not usually present in normal GC B cells.

Interpretation and Conclusions

We show that in normal lymphoid tissues VICKZ is expressed in GC lymphocytes but in lymphoid neoplasms its expression is not limited to GC-derived lymphoma subtypes. However, VICKZ exhibits differential expression in lymphoma subtypes and thus may be a marker of potential value in the diagnosis and study of hematopoietic neoplasia. The aberrant expression of its isoforms in DLBCL raises the possibility that these isoforms may be associated with different functions and suggests that further study of their role in normal and neoplastic lymphoid cells is warranted.

Key words: IMP, germinal center, diffuse large B-cell lymphoma, tissue microarray, immunohistochemistry.

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The VICKZ (Vg1 RBP/Vera, IMP1, 2, 3, CRD-BP, KOC, ZBP-1) family of RNA-binding proteins is highly conserved in vertebrates.[1-3] These proteins are involved in the regulation of RNA and orchestrate diverse cellular functions including subcellular localization, translational repression and stability, and the control of cell proliferation.[4-12] Humans elaborate three isoforms termed hVICKZ1, 2, and 3 (also known as IMP1, 2, and 3), encoded on separate chromosomes. VICKZ proteins are expressed during embryogenesis and are required for cell migration.[5,13,14] They are not usually found in adult tissue although one or more isoforms of VICKZ are overexpressed in colon, lung and ovarian carcinomas.[13,15-19] The expression in neoplastic conditions but not in normal adult tissue suggests a potential role for VCKZ in tumorigenesis.

To study VICKZ proteins further we generated a rabbit polyclonal antibody against recombinant *Xenopus laevis* VICKZ (xVICKZ3) protein that recognizes all three human isoforms. In our initial screening of human tissue samples we observed that VICKZ proteins were expressed in normal germinal centers (GC), but not in other lymphoid compartments. In the present study we conducted the first immunohistologic characterization of VICKZ protein in hematopoietic tissue by screening normal hematopoietic tissues and neoplasms. We further studied VICKZ protein expression in 200 cases of diffuse large B-cell lymphoma (DLBCL) to determine whether VICKZ expression correlates with prognostic subgroups of this type of lymphoma. Lastly, we studied the expression pattern of each of the VICKZ isoforms by reverse transcriptase polymerase chain reaction (RT-PCR) analysis to test whether there is differential expression of VICKZ mRNA isoforms in lymphoma subtypes.

Design and Methods

Generation of anti-VICKZ antibody

The His-tagged full-length xVICKZ3 (Vg1-RBP) recombinant protein used in our study was prepared as previously described using a pET21d expression vector system.[13] After purification using nickel column chromatography following the manufacturer's protocol (QIAGEN Inc., Valencia, CA, USA), an aliquot of the purified protein batch was subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis. The gel was stained by Coomasie blue to confirm the purity and length of the recombinant xVICKZ3 protein. Rabbit anti-xVICKZ3 serum was raised by immunization with the recombinant full length xVICKZ3, following a standard protocol.[20] The affinity of the antisera was tested by western blot analysis. Antiserum was then purified using xVICKZ3 protein column chromatography.[20] The polyclonal anti-VICKZ3 antibodies were released from the column using 0.1M glycine pH 4, and immediately titrated with 1M Tris pH 7.4. Bovine serum albumin was added to reach a final concentration of 1%. Dialysis against 1X-phosphate-buffered saline was performed and sodium azide (0.02%) was added. The affinity-purified antibody was used for all experiments described in this report.

Tissue samples and cell lines

Paraffin tissue

A total of 889 hematolymphoid tumors obtained from the Departments of Pathology, Stanford University Medical Center, Stanford, California, and Aarhus University Hospital, Aarhus, Denmark formed the material of the current study. Expression of VICKZ in normal hematopoietic tissue was analyzed by staining two examples each of tonsils, lymph nodes, thymus, spleen and bone marrow. Tissue microarrays or conventional sections of a broad spectrum of hematopoietic tumors diagnosed on the basis of histologic and immunohistologic criteria according to the current World Health Organization (WHO) classification[21] were studied; the tissue microarray construction has been described elsewhere.[22] Institutional Review Board (IRB) approval was obtained for these studies from all participating institutions.

Frozen lymphoma/leukemia specimens

Biopsy specimens from a total of 30 non-Hodgkin's lymphoma patients with either follicular lymphoma (12 patients) or with DLBCL (18 patients), as well as peripheral blood mononuclear cells from 9 patients with chronic lymphocytic leukemia (>80% leukemic cells) isolated by Ficoll-Hypaque density centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used in this study. All specimens had been stored in liquid nitrogen as viable cell suspensions from the initial biopsies. Tumor samples were immunophenotyped by flow cytometry for expression of immunoglobulin (Ig) light chains and B- and T-cell markers.

Cell lines

Ten B-lymphoid cell lines (Raji, Ramos, Daudi, SUDHL6, SUDHL4, HF1, OCI-Ly3, OCI-Ly10, RC-K8, and VAL), three non B-cell leukemia cell lines (Jurkat, K562 and HL-60), and three non-hematologic cell lines (HeLa, HEK293 and 3T3), were selected for this study. All cell lines, except OCI-Ly10 and OCI-LY3, were grown in RPMI 1640 medium (Fisher Scientific Co., LLC, Santa Clara, CA, USA), supplemented with 10% fetal calf serum, 2 mM/L glutamine (GIBCO BRL, Grand Island, NY, USA), and penicillin/streptomycin (GIBCO BRL). The OCI-Ly10 and OCI-LY3 cell lines were grown in IMDM essential medium (Fisher Scientific Co.), supplemented with 20% fresh human plasma and 50 mM 2-β mercaptoethanol. Germinal center B cells were purified from three human tonsils and pooled, as previously described.[23]

Immunohistochemistry

Serial 4 mM-thick sections from paraffin–embedded lymphoid tissue or tissue microarray blocks were deparaffinized in xylene and hydrated in a series of graded alcohol. Heat-induced antigen retrieval was carried out by

microwave pretreatment in EDTA buffer (1 mM pH 8.0) for 15 minutes. Affinity purified rabbit polyclonal pan-VICKZ antibody was used at a dilution of 1:150. Detection was carried out using the DAKO Envision method (DAKO Corporation, Carpinteria, CA, USA). Staining was optimized on normal paraffin-embedded tonsil sections and a cut-off of greater than 30% of lymphoma cells was chosen for a positive score. This cut-off was based on the need to use a non-ambiguous threshold for scoring tissue microarrays and does not reflect differences in intensity between normal and neoplastic tissue. Negative cases had less than 5% stained lymphoma cells. The materials and methods for HGAL, BCL6, CD10, BCL2 and MUM1/IRF4 immunostaining have been described previously.[22]

Data analysis and visualization

The stained lymphoma tissue microarray slides were scanned and stored as high resolution images using an automated scanner (Bacus Laboratories, Inc., Slide Scanner (BLISS) *http://www.bacuslabs.com*). A total of 2080 tissue microarray images generated from the current study are displayed on the following open-access website: *http://tma.stanford.edu/tma_portal/VICKZ*. The staining results of previously characterized antibodies (HGAL, BCL6, CD10, MUM1/IRF4 and BCL2) performed on the same 143 cases of DLBCL were assigned numerical scores based on the following scoring scale: staining in > 30% of lymphoma cells was scored positive and given the numerical score *2*; lack of staining in > 30% of lymphoma cells was scored negative and given the numerical score *0*. Stains that were not interpretable due to absence of diagnostic tissue in the core or due to loss of the core during processing were considered equivocal and given the numerical score *1*.

The *Deconvoluter* algorithm (custom WBS macro, Excel, Microsoft) with appropriate layout for use in the cluster software was used for hierarchical clustering to integrate all immunohistologic staining results as previously described (*http://genome-www.stanford.edu/TMA/*).[24] Positive staining is represented as red, lack of staining as green and non-interpretable staining as white.

Immunoblot analysis

Whole cell extracts for immunoblot analysis were prepared by lysing cells (5×10^6) with RIPA buffer [1X phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM phenylmethylsulfonyl fluoride, 1 mg/mL Aprotinin, 100 mM sodium orthovanadate], on ice for 30 min. After centrifugation, the supernatant was assayed for protein concentration by the BCA assay (Pierce Biotechnology Inc. Rockford, IL, USA). For immunoblotting, 20 mg of whole cell lysate were separated on 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (BioRad Labaratories Inc. Hercules, CA, USA) and probed with anti-VICKZ at a 1:10,000 dilution and anti β-actin antibodies at a 1:10,000 dilution (Sigma, St. Louis, MO, USA), overnight at 4°C. These antibodies were detected using a goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies at a 1:10,000 dilution (Jackson Immuno Research Laboratories, Inc; West Grove, PA, USA), respectively, and visualized by the Super Signal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology Inc, Rockford, IL, USA).

VICKZ mRNA quantification

Total cellular RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Expression of the hVICKZ 1, 2, and 3 isoforms was measured by real-time polymerase chain reaction (PCR) using the Applied Biosystems Assays-on-Demand Gene Expression Products (Hs00198023, Hs00538956 and Hs01122363, respectively) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), as we previously reported.[25] As an endogenous control of RNA quantity and quality, we used PGK1 Human TaqMan pre-developed assay reagent, as reported previously.[25] The quantification of relative gene expression was calculated by the 2^{-ddC_T} method, as reported previously.[25] C_T is the cycle threshold. As a calibrator we used normalized expression of the hVICKZ 1,2 and 3 isoforms in the Ramos cell line.

Results

Specificity of the anti-VICKZ antibody

The affinity purified pan-VICKZ antibody specifically recognizes all three human VICKZ isoforms. Following transfection of the HEK-293 cells with GFP-hVICKZ1, GFP-hVICKZ2, GFP-hVICKZ3, and GFP-xVICKZ3 (green fluorescent protein fused to *Xenopus* Vg1 RBP), and YFP-C1 control, immunoblot of cellular lysates demonstrated the presence of two specific bands. The 94kDa band represents the GFP chimeric hVICKZ proteins (GFP-xVICKZ3 is approximately 97 kDa), and the 70kDa band represents the endogenous hVICKZ (the latter were used as loading controls). The high specificity of the antibody is evident from the virtual absence of other detectable bands. All lanes have similar signal intensity, showing equal affinity for the members of the VICKZ protein family, regardless of species or isoform (Figure 1A). Purified VICKZ protein blocked the anti-VICKZ antibody immunoreactivity, thus confirming the specificity of the generated antibody (*data not shown*).

Expression of VICKZ protein in normal lymphoid tissue

VICKZ protein was highly expressed in lymphocytes within the GC of normal tonsils, lymph nodes and spleen (Figure 1B). The staining was localized to the cytoplasm. The mantle and marginal zones and interfollicular and paracortical regions of peripheral lymphoid tissue lacked staining with the exception of occasional scattered lym-

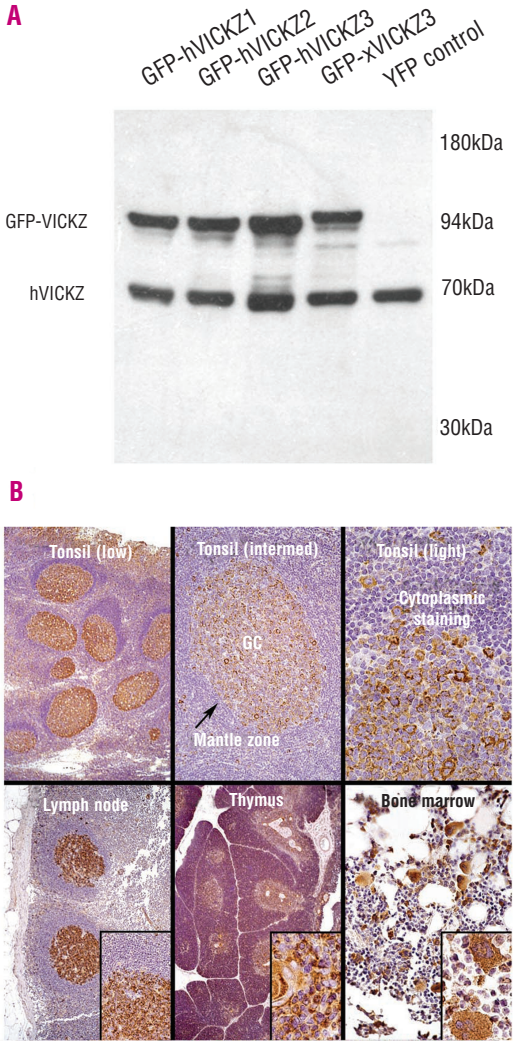

Figure 1. (A) Specificity of the pan-VICKZ antibody. Immunoblot analysis shows that the pan-VICKZ antibody specifically recognizes all three VICKZ isoforms. Total cell extracts from HEK-293 cells transfected with GFP-hVICKZ1, GFP-hVICKZ2, GFP-hVICKZ3, and GFP-xVICKZ3 (GFP fused to *Xenopus* Vg1 RBP), and YFP-C1 control were used. Two specific bands at 94kDa representing the GFP chimeric hVICKZ protein and at 70kDa representing the endogenous hVICKZ proteins can be recognized. (B) VICKZ protein staining in normal hematolymphoid tissue. Low, intermediate and high magnification images of a normal tonsil show germinal centers (GC) highlighted by VICKZ staining; mantle zones (indicated by arrow) and interfollicular areas lack staining. VICKZ staining in a normal lymph node, thymus and bone marrow are also shown. The high magnification image of the tonsil and the insets show localization of staining to the cytoplasm of B cells. Cortical thymocytes show weak staining while the medulla shows intense staining. Bone marrow megakaryocytes and myeloid precursors show staining for VICKZ protein.

phoid cells. In the thymus staining was present in cortical and medullary lymphoid cells. Splenic GC and occasional splenic marginal zone cells showed positive staining. In the normal bone marrow VICKZ staining was present in megakaryocytes, myeloid precursors and occasional lymphocytes and plasma cells. Staining was absent in mature myeloid elements including neutrophils.

Table 1. Immunohistologic analysis of VICKZ protein expression in hematolymphoid neoplasia.

Diagnosis	*Total positive**	*% positive*
B-cell lymphoma [n=439]		
Follicular Lymphoma	126/165	76%
Grade 1	30/42	71%
Grade 2	45/53	85%
Grade 3	51/70	73%
Diffuse large B-cell lymphoma	155/200	78%
Mediastinal large B-cell lymphoma	9/10	90%
Burkitt lymphoma	2/2	100%
Extranodal marginal zone lymphoma	2/25	8%
Splenic marginal zone lymphoma	1/5	20%
Nodal marginal zone lymphoma	1/5	20%
Mantle cell lymphoma	2/18	11%
Small lymphocytic lymphoma/CLL	3/38	8%
Lymphoplasmacytic lymphoma	0/5	0%
Hairy cell leukemia	7/10	70%
Precursor B-lymphoblastic lymphoma	4/13	25%
T-cell lymphoma [n=134]		
Precursor T-lymphoblastic lymphoma	4/14	29%
Peripheral T-cell lymphoma	3/21	14%
Anaplastic large cell lymphoma	6/8	75%
NK lymphoma	2/91	2%
Plasma cell neoplasms [n=174]		
Multiple myeloma	7/153	5%
Plasma cell leukemia	0/13	0%
Monoclonal gammopathy of undetermined significance (MGUS)	0/8	0%
Hodgkin's lymphoma [n=121]		
Lymphocyte predominant	12/13	92%
Classical Hodgkin's lymphoma	101/108	94%
Nodular sclerosis	82/85	96%
Mixed cellularity	19/23	83%
Myeloid Neoplasms [n=11]		
Acute myeloid leukemia	10/10	100%
Chronic myeloid leukemia	1/1	100%

**Cases were scored positive if >30% of lymphoma cells stained for VICKZ.*

Expression of VICKZ protein in hematopoietic neoplasia

The results of immunohistologic staining in hematolymphoid neoplasia are summarized in Table 1, and specific examples are illustrated in Figure 2. VICKZ staining was present in several subtypes of non-Hodgkin's and Hodgkin's lymphomas as well as in myeloid leukemias and exhibited an intensity of staining similar to that seen in normal germinal center B cells. Widespread immunoreactivity for VICKZ was observed in follicular lymphomas of all three histologic grades (126/165), Burkitt's lymphoma (2/2), hairy cell leukemia (7/10), lymphocyte predominant Hodgkin's lymphoma (12/13), mediastinal large B-cell lymphoma (9/10), and DLBCL (155/200). In addition, a small minority of marginal zone lymphoma (4/30), mantle cell lymphoma (2/18), small lymphocytic lymphoma/chronic lymphocytic leukemia (CLL/SLL, 3/38) and plasma cell myeloma (7/153), also expressed VICKZ protein. There was no particular correlation between VICKZ expression and ZAP70 expression in CLL/SLL cases that expressed the

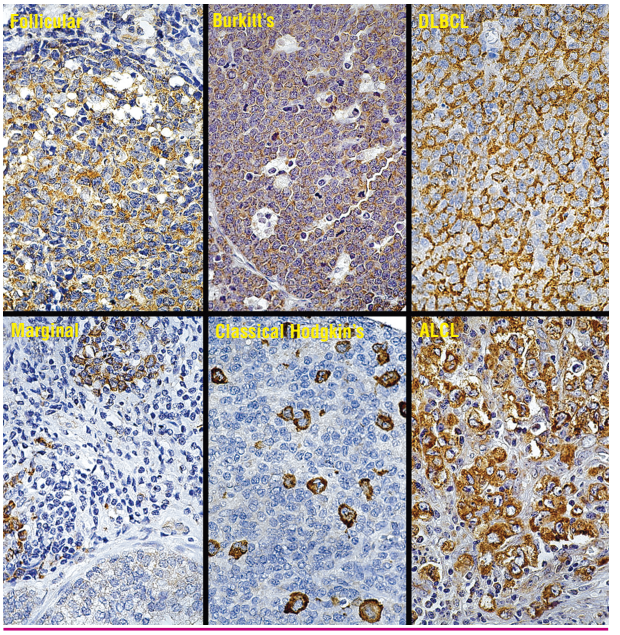

Figure 2. Expression of VICKZ protein in lymphomas. VICKZ staining in typical examples of follicular lymphoma, Burkitt's lymphoma, DLBCL, marginal zone lymphoma, classical Hodgkin's lymphoma, and anaplastic large cell lymphoma are shown. Although marginal zone lymphoma cells lack VICKZ staining entrapped germinal centers stain for VICKZ.

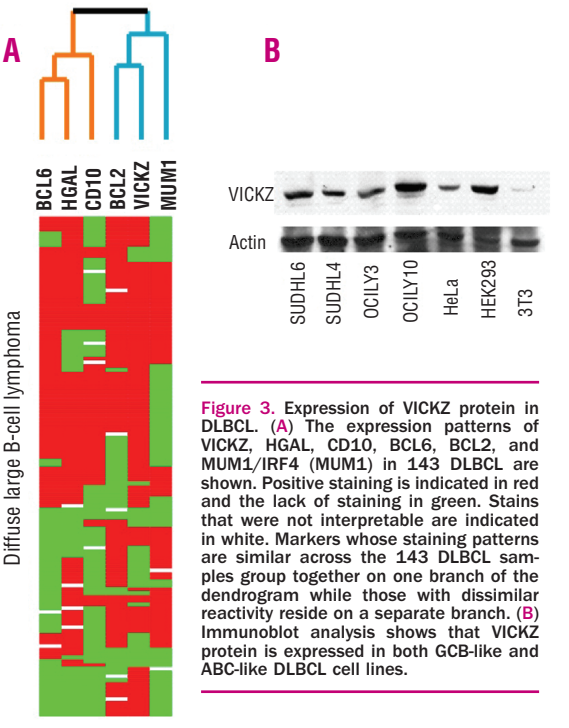

Figure 3. Expression of VICKZ protein in DLBCL. (A) The expression patterns of VICKZ, HGAL, CD10, BCL6, BCL2, and MUM1/IRF4 (MUM1) in 143 DLBCL are shown. Positive staining is indicated in red and the lack of staining in green. Stains that were not interpretable are indicated in white. Markers whose staining patterns are similar across the 143 DLBCL samples group together on one branch of the dendrogram while those with dissimilar reactivity reside on a separate branch. (B) Immunoblot analysis shows that VICKZ protein is expressed in both GCB-like and ABC-like DLBCL cell lines.

former protein. A subset of precursor B (4/13) and precursor T (4/14) acute lymphoblastic lymphoma/leukemias showed staining. Only rare peripheral T- and NK-cell lymphomas (5/112) stained for VICKZ with the exception of ALK1-positive anaplastic large cell lymphoma (6/8). Classical Hodgkin's lymphoma showed staining for VICKZ in 101 of 108 cases of nodular sclerosis and mixed cellularity subtypes.

Since early myeloid precursors and megakaryocytes in the normal bone marrow expressed VICKZ, we stained bone marrow core biopsies from ten patients with acute myeloid leukemia (AML) and one with chronic myeloid leukemia (CML). All of these cases were uniformly positive for VICKZ. Staining for VICKZ was seen in the immature blasts of all AML samples, regardless of the subtype, which included three with multilineage dysplasia, one with 11q23 abnormality, one with megakaryocytic differentiation (AML, M7), and one blast crisis transformation of CML harboring the BCR-ABL translocation. The case of CML (chronic phase) showed staining in immature myeloid precursors and in numerous micromegakaryocytes but not in mature myeloid lineage cells corroborating our finding in the normal bone marrow.

Expression of VICKZ protein in DLBCL

Since VICKZ protein was expressed in normal GC cells and because DLBCL are known to derive from either GC or non-GC B-cells, we further studied VICKZ expression in DLBCL. We also correlated VICKZ protein expression with five additional GC and non-GC markers (HGAL, BCL6, CD10, BCL2 and MUM1/IRF4) in 143 of the DLBCL cases for which immunostaining data were available from our prior work.[22] Hierarchical cluster analysis showed that VICKZ protein expression was different from the expression of the GC-specific markers HGAL, BCL6 and CD10 (Figure 3A). Using the immunohistologic algorithm proposed by Hans *et al.*[26] to classify DLBCL into GC and non-GC subtypes, we found that VICKZ staining was present not only in 50 of 62 cases classified as GC type but also in 47 of 66 cases classified as non-GC type. We previously reported that HGAL staining was also present in a significant proportion of cases classified as non-GC type by the Hans algorithm.[22] However, we found no clear correlation between the staining patterns of HGAL and VICKZ among the cases classified as non-GC type.

To examine whether the VICKZ protein is differentially expressed in GC-like and non-GC-like DLBCL cell lines, immunoblot analysis of four DLBCL cell lines was carried out. This analysis demonstrated that the VICKZ protein is expressed in all four cell-lines at levels similar to that found in a control cervical carcinoma cell line, HeLa, and in the human embryonic kidney cell line, HEK293. No difference in the levels of VICKZ protein was observed between the GC-like (SUDHL4 and SUDHL6) and the non-GC-like (OCI-LY3 and OCI-LY10) DLBCL cell lines (Figure 3B).

Expression of VICKZ mRNA and its isoforms in lymphoid cell lines and lymphomas

To ascertain whether the human VICKZ isoforms show differential expression in lymphoid cell lines, their expression was assessed using quantitative real time RT-PCR in four GC-like DLBCL cell lines (SUDHL4, SUDHL6, VAL, HF-1), two non-GC-like DLBCL cell lines (OCI-LY3 and OCI-LY10), three Burkitt cell-lines (Ramos, Raji and Daudi),

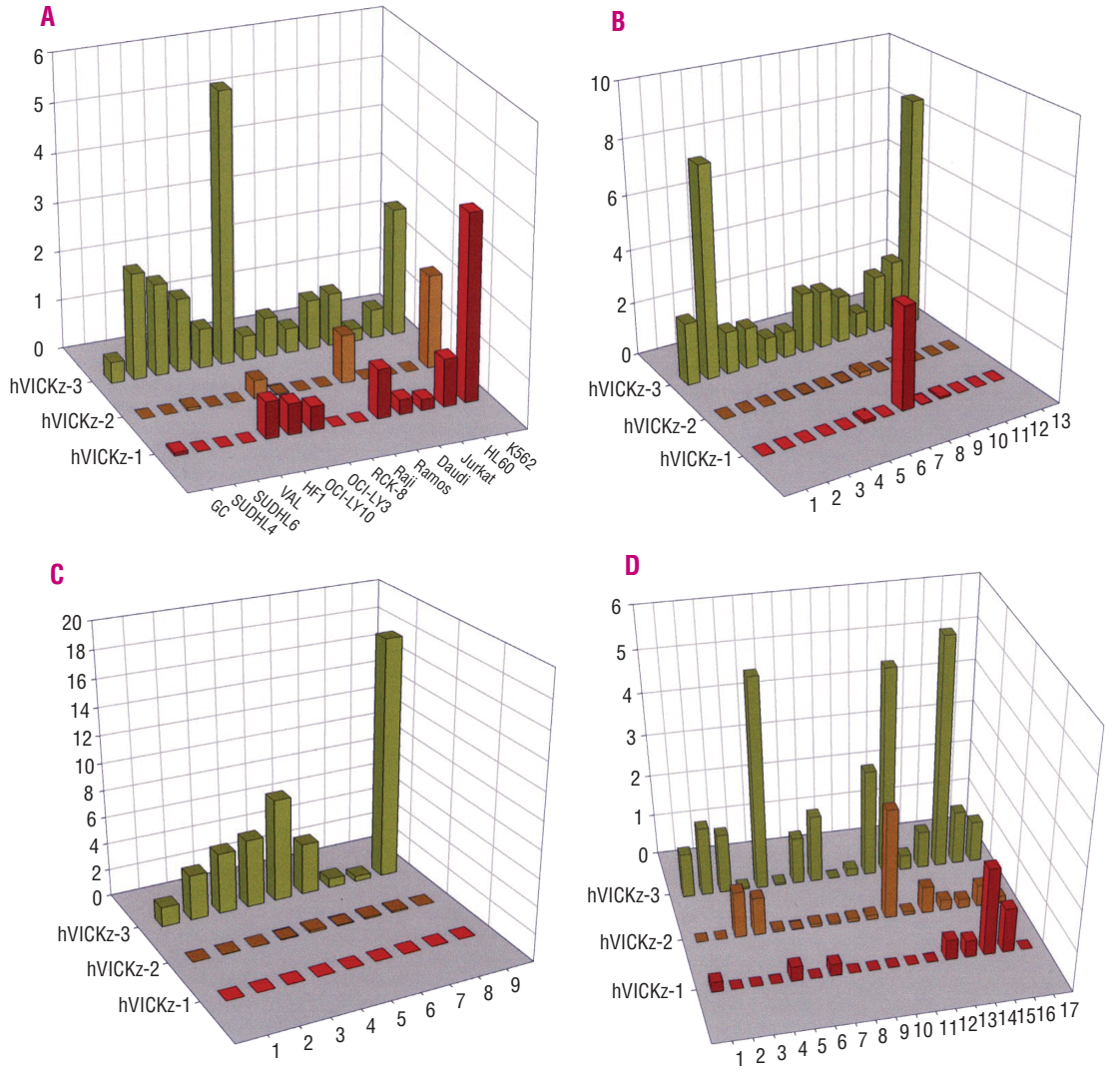

Figure 4. Expression of VICKZ mRNA and its isoforms. (A) assessed by quantitative RT-PCR in isolated normal GC B-cells, four GCB-like DLBCL cell-lines (SUDHL4, SUDHL6, VAL, HF-1), two ABC-like DLBCL cell-lines (OCI-LY3 and OCI-LY10), three Burkitt cell-lines (Ramos, Raji and Daudi), and one T-lymphoblastic leukemia cell-line (Jurkat); (B) expression of the VICKZ mRNA isoforms in follicular lymphoma; (C) expression of the VICKZ mRNA isoforms in chronic lymphocytic leukemia; (D) expression of the VICKZ mRNA isoforms in DLBCL.

one T-lymphoblastic leukemia cell-line (Jurkat), and in enriched pooled GC lymphocytes. The most frequently expressed VICKZ isoform in B-lymphoid cell-lines was hVICKZ3, while hVICKZ1 was the most abundant isoform in the Jurkat T-cell line and in non-lymphoid cell lines (Figure 4A). In comparison to isoforms hVICKZ1 and hVICKZ3, hVICKZ2 accounted for only a small proportion of VICKZ mRNA expression in all analyzed cell lines. Next, mRNA expression of VICKZ isoforms was evaluated in 12 follicular lymphoma, 9 chronic lymphocytic leukemia, and 18 DLBCL. All three types of lymphoma expressed mRNA of at least one of the VICKZ isoforms. In follicular lymphoma, the most abundant isoform was hVICKZ3 and only four of 12 analyzed cases expressed the hVICKZ1 isoform (Figure 4B). In comparison to normal GC lymphocytes, two thirds of the follicular lymphoma cases lacked expression of hVICKZ1 and almost exclusively expressed hVICKZ3. Similarly, all analyzed cases of chronic lympho-

Table 2. Comparative immunohistologic studies in 143 cases of DLBCL.

Score	*VICKZ*	*HGAL*	*CD10*	*BCL6*	*MUM1*	*BCL2*
Positive	108	97	51	78	65	95
Negative	34	41	80	54	66	32
Equivocal	1	5	12	11	12	16

Classification using the model of Hans et al.[26]	*Total*	*VICKZ Positive*	*VICKZ Negative*
GC	62	50	12*
Non-GC	66	47*	19
Cannot classify	15	–	–

Positive, staining in >30% lymphoma cells; Negative, lack of staining in >30% lymphoma cells; Equivocal, lack of core for scoring; GC, germinal center; cannot classify, indicates that because the CD10 core was uninformative, the Hans algorithm 26 could not be applied to these cases. Asterisk (*) indicates cases showing a different VICKZ staining score than predicted by the Hans algorithm.

cytic leukemia expressed hVICKZ3 (Figure 4C). In contrast, the expression of VICKZ isoforms in DLBCL tumors was very heterogeneous. Interestingly, many DLBCL tumors demonstrated expression of hVICKZ2, an isoform not usually found in GC lymphocytes (Figure 4D). The expression patterns of none of the isoforms, including hVICKZ2, correlated with either the GCB-like or the ABC-like categories of DLBCL in the small number of cases analyzed (*data not shown*).

Discussion

The VICKZ family of RNA-binding proteins is implicated in many aspects of RNA regulation, cell migration and control of cell proliferation.[5-12,27] Their multifaceted regulatory role, together with their oncofetal expression pattern, suggests that VICKZ proteins are likely to mediate steps in embryogenesis and carcinogenesis. Our findings show for the first time that VICKZ proteins are highly expressed in normal human germinal centers and in a subgroup of hematopoietic neoplasms that include subsets of Hodgkin's and non-Hodgkin's lymphomas and lymphoid and myeloid leukemias. In addition, we have produced a robust pan-VICKZ antibody that is suitable for use on routinely processed paraffin-embedded tissue sections, which together with the differential expression pattern of VICKZ protein, affords a marker of potential value in the diagnosis and study of hematopoietic neoplasia.

Staining for VICKZ protein in normal lymphoid tissue shows a site-specific expression pattern confined to GC of tonsils, lymph nodes and spleen with lack of staining in mantle, marginal and paracortical T-zones. In keeping with this pattern of expression, VICKZ staining was highly correlated (71-100%) with lymphoma subtypes derived from GC B cells such as follicular lymphoma, Burkitt's lymphoma, DLBCL, and lymphocyte-predominant and classical Hodgkin's lymphoma. However, rare cases of marginal zone, mantle cell, CLL/SLL, T- and NK-cell lymphoma and myeloma, and the majority of mediastinal large B-cell lymphomas showed positive staining. A subset of lymphoid and all myeloid leukemias also expressed VICKZ protein. In addition, although rarely expressed in most T- and NK-cell lymphomas, anaplastic large cell lymphoma expressed VICKZ protein. Consistent with its expression in the normal thymus VICKZ protein was found in a subset of precursor T-lymphoblastic lymphoma/leukemia.

Gene expression profiling studies have shown that distinct B-cell differentiation stages are typified by the expression of specific gene expression signatures that are maintained in lymphoid neoplasia.[23] DLBCL with a genetic signature exemplified by normal GC B cells (GCB-like DLBCL) distinguishes a subtype of patients with better overall survival than that of patients with a subtype showing a gene expression signature similar to that of activated peripheral blood B cells (ABC-like DLBCL).[23] The expression of VICKZ in normal GC B cells led us to explore whether this could distinguish lymphoma subtypes based on the cell of origin. Overall, our data demonstrate that while VICKZ protein is primarily expressed in normal GC B cells, VICKZ protein expression in lymphomas is not limited to lymphomas derived from GC B cells as demonstrated, for example, by its expression in primary mediastinal large B-cell lymphomas.

In our analysis of DLBCL we found that the majority of cases assigned to the GC type as well as a substantial proportion of cases assigned to the non-GC type by the algorithm proposed by Hans *et al.*[26] expressed VICKZ protein. This finding is further illustrated by hierarchical cluster analysis of DLBCL in which VICKZ clustered on a separate branch from the other GC markers, HGAL, BCL6 and CD10. In addition, all four DLBCL cell lines (two GCB-like and two non-GC-like) also showed VICKZ protein expression in immunoblot analysis. Of note, in our prior work on HGAL we found that HGAL protein was also expressed in a significant proportion of DLBCL cases lacking CD10 and BCL6.[22] VICKZ protein expression is similar in that it is also expressed in a subset of DLBCL cases that lack CD10 and BCL6. However the patterns of VICKZ and HGAL expression showed no correlation with each other, probably making redundant any speculation about these proteins identifying subgroups of non-GC type DLBCL.

One possible explanation for this observation may be that the VICKZ protein is expressed at all stages of the GC reaction while BCL6 expression is down-regulated in the late stages of the GC reaction once MUM1/IRF4 and BLIMP1 expression is upregulated. As such, VICKZ cannot discriminate between DLBCL tumors originating from lymphocytes at different stages of the GC reaction. Several other recently characterized GC-associated markers (AID, HGAL, PAG, JAW1/LRMP and LCK) also show a tissue distribution pattern that does not match their expression in the normal cellular counterpart.[22,28-31] These observations argue that although gene expression programs related to the cell of origin are maintained in neoplastic states, the transformation process also influences the final repertoire of genes and proteins elaborated in the neoplasm. Another explanation for the apparent lack of correlation of the VICKZ protein expression pattern with that of other GC markers may be due to our use of an antibody that recognizes all three isoforms of VICKZ protein. It is possible that the overall staining in different lymphoma subtypes is due to the recognition of different proportions of expressed VICKZ isoforms. Although the three VICKZ isoforms, encoded on three separate chromosomes in mice and man, show a high degree of homology, it is unknown whether they are functionally similar. Liao and colleagues recently showed that hVICKZ1 and hVICKZ3 may have opposing activity in the regulation of IGF2 mRNA translation and cell proliferation.[11,12] Our results show that hVICKZ1 and 3 (but not isoform 2) are expressed in normal GC B cells and are frequently expressed VICKZ isoforms in B-cell lymphomas. In contrast, over 40% of DLBCL cases showed expression of hVICKZ2. We did not find any correlation

with GCB-like and ABL-like DLBCL subtypes and the expression of different VICKZ mRNA isoforms in the small numbers of cases studied by this method. However, since isoform-specific VICKZ antibodies are unavailable, further analysis in lymphoma subtypes cannot yet be done. The patterns of expression of VICKZ mRNA isoforms suggest that isoform expression may be a highly regulated process and that each isoform may be functionally unique. It would be of interest to determine whether specific VICKZ isoforms have distinct functions that may contribute to the pathogenesis of DLBCL.

In conclusion, our results show that in normal lymphoid tissue VICKZ is expressed in germinal centers and can be detected in routine paraffin-embedded tissue biopsies. Although VICKZ protein is expressed in normal GC it is also variably expressed in subcategories of lymphomas and in acute leukemias indicating that its induction may be due to specific activation signals that are as yet undetermined. Moreover, some hematopoietic neoplasms also show deregulation of VICKZ isoforms suggesting that each isoform is likely to serve a different function. The potential contribution of deregulated expression of VICKZ or its isoforms to lymphomagenesis and leukemogenesis is of interest and warrants further investigation.

Authors' Contributions

YN: designed research, analyzed data and wrote paper; GV; generated VICKZ antibody and analyzed data; JC: performed research and analyzed data; SZ: performed research and analyzed data; RJM: designed web page and analyzed data; ASH: provided lymphoma cases; SH-D: provided lymphoma cases; EP: reviewed immunostaining; GA: reviewed immunostaining; RL: contributed to study design and revised the paper; JKI: contributed to study design, analyzed data and revised the paper; ISL: designed research, analyzed data and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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