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Glioma Associated Oncogene Homologue 3 (GLI3), a Hedgehog Transcription Factor, is Highly Expressed in Hodgkin and Reed-Sternberg Cells of Classical Hodgkin Lymphoma

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Abstract

The hedgehog signaling pathway has been shown to play a pathogenic role in diffuse large B-cell lymphoma and anaplastic large cell lymphoma, but has not been assessed in classical Hodgkin lymphoma. GLI1, GLI2, and GLI3 are transcriptional effectors of the hedgehog pathway. In this study, we first used real-time quantitative PCR to investigate expression of *GLI1*, *GLI2*, and *GLI3* in 3 classical Hodgkin lymphoma cell lines. *GLI1* and *GLI2* were variably expressed, but *GLI3* was highly expressed in all cell lines. We then used immunohistochemistry to assess GLI1, GLI2, and GLI3 in 39 classical Hodgkin lymphoma patient samples. GLI1 and GLI2 were weakly to variably expressed in a subset of classical Hodgkin lymphoma patient samples. In contrast, GLI3 showed strong, uniform nuclear expression in virtually all Hodgkin/Reed-Sternberg cells. We then performed an immunohistochemical survey of GLI3 expression in 13 cases of nodular lymphocyte predominant Hodgkin lymphoma and 218 non-Hodgkin lymphomas. Most other lymphoma types showed variable or no expression of GLI3, with a minor subset of cases of nodular lymphocyte predominant Hodgkin lymphoma, ALK-positive and ALK-negative anaplastic large cell lymphoma, and B-cell lymphoma, unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma showing a GLI3 staining pattern indistinguishable from classical Hodgkin lymphoma. Our data provide a rationale to further investigate the biological significance of GLI3 in classical Hodgkin lymphoma biology.

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DISCLOSURES/CONFLICTS OF INTEREST

There are no disclosures to declare.

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Keywords

GLI3; hedgehog signaling; classical Hodgkin lymphoma; immunohistochemistry

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INTRODUCTION

Hodgkin lymphoma (HL) represents approximately 30% of all lymphomas and can be divided into classical HL (CHL) and nodular lymphocyte predominant (NLPHL) types, representing 95% and 5% of cases, respectively (1). CHL can be further divided into four histologic types: nodular sclerosis (NS), lymphocyte-rich (LR), lymphocyte-depleted (LD) and mixed cellularity (MC). The pathogenesis of CHL is not well defined and recent studies have shown that constitutive activation of multiple signaling pathways contributes to survival and proliferation of Hodgkin/Reed-Sternberg (HRS) cells. In particular, constitutive activation of the NF κ B, PI3K/AKT and JAK/STAT pathways have been implicated (2). Hodgkin/Reed-Sternberg cells also commonly express Bcl-2 protein suggesting that evasion of apoptotic mechanisms plays a role in HRS cell survival (3, 4). In addition, there is evidence that the microenvironment is essential for survival of HRS cells. It is difficult to grow HRS cells in culture or to generate cell lines and it is rare to find circulating HRS in peripheral blood. When CHL does disseminate to non-lymphoid organs, HRS cells are accompanied by a typical microenvironment (2). It appears that the composition of the microenvironment is regulated by a range of cytokines and chemokines produced by HRS cells and the microenvironment (2).

The Hedgehog (HH) protein family is a group of secreted signaling molecules critical for normal mammalian development and homeostasis of adult renewable tissues (5, 6). Recent studies have shown that secreted HH ligands protect non-neoplastic germinal center B-cells as well as of low-grade B-cell lymphoma cells from apoptosis (7, 8). The HH signaling pathway is incompletely understood. Briefly, the HH ligands, Sonic (SHH), Indian (IHH) and Desert (DHH), interact with a membrane-bound receptor complex composed of two proteins, patched (PTCH) and smoothened (SMO) (9). PTCH is the HH ligand-binding subunit and SMO is the signal transduction component. In the absence of HH ligands, PTCH inhibits SMO. Once HH ligands bind to PTCH, this inhibition is released allowing SMO to transduce the HH signal mediated by the transcription factors GLI1, GLI2 and GLI3 (10). While both GLI2 and GLI3 have transcriptional activation and repression properties, GLI1 is a strong positive regulator of HH transcriptional targets and is, itself, a transcriptional target of HH (11). GLI3 is a bifunctional transcriptional regulator that, in the absence of HH, is proteolytically cleaved to form the truncated GLI3 protein which acts as a repressor of target gene transcription (GLI3R) (12). On the other hand, in the presence of HH signals, the full-length form of GLI3 acts as a transcriptional activator (GLI3A). The functions of GLI3 in the hematopoietic system are not well characterized.

Inappropriate activation of the HH pathway has been shown in many cancers (13, 14), but has not been assessed in CHL. In this study, we investigated the expression of HH transcription factors in a series of CHL patient samples. We found that GLI3 is highly expressed in CHL and shows a distinctive immunohistochemical expression pattern in HRS cells. Expression of GLI3 protein by HRS cells provides a rationale for further investigation of the biological role of GLI3 in CHL. Furthermore, as GLI3 is expressed by thymic stroma and is described to regulate T-cell differentiation, GLI3 may be involved in regulating the composition of the microenvironment in CHL.

MATERIALS AND METHODS

Cell Lines

The CHL-derived cell lines used included KMH2 and HDMYZ (purchased from DSMZ, Germany), and L428. We also used a DLBCL-derived cell line, BJAB (purchased from ATCC, Manassas, Virginia). All cell lines were maintained at 37°C in RPMI 1640 (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO) in a humidified atmosphere containing 5% CO₂.

Real-Time Quantitative (q) Reverse Transcriptase (RT)-PCR

Total mRNA from cell lines was extracted using RNeasy Minikit (Qiagen, Valencia CA) as per the manufacturer's protocol. After DNase digestion, first-strand cDNA was synthesized from total RNA using random primers and SuperScript II^{TM} Reverse Transcriptase (Invitrogen, Carlsbad CA). Gene expression of *GLI3* was analyzed by RT-qPCR assay using respective primers (Applied Biosystems, CA) and Taqman probes. Reactions were performed using the ABI PRISM 7500HT Sequence Detection system (Applied Biosystems, CA) and the expression of each gene was measured in duplicate and then normalized with 18s rRNA endogenous gene. Relative quantification of gene expression levels was assessed by using the delta CT (Δ CT) method. BJAB cells were used as a negative reference control.

Patients Samples and Immunohistochemistry

The study group included 33 cases of nodular sclerosis (NS) and 6 cases of mixed cellularity (MC) CHL. Either routine histologic sections or tissue microarrays were used. We then performed a survey of 13 cases of nodular lymphocyte predominant HL and 218 non-Hodgkin lymphomas as follows: 54 DLBCL, not otherwise specified (NOS), 28 follicular lymphoma (FL), 21 ALK-positive ALCL, 16 ALK-negative ALCL, 16 chronic lymphocytic lymphoma/small lymphocytic lymphoma (CLL/SLL), 13 B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and CHL (BCLU), 12 T cell/histiocyte rich large B-cell lymphoma (TCHRLBCL), 12 Burkitt lymphoma (BL), 7 mantle cell lymphoma (MCL), 7 primary mediastinal large B-cell lymphoma (PMLBCL), 7 marginal zone B-cell lymphomas (MZL), 10 natural killer/T-cell lymphoma (NKTCL), 9 peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS) and 6 angioimmunoblastic T-cell lymphoma (AITL). The cases of FL and DLBCL in this series include those reported in a previous study plus additional cases of DLBCL (15). All cases were diagnosed at The University of Texas M. D. Anderson Cancer Center between (1987 and 2009) except for 9 previously reported gray-zone lymphomas provided by the Centro Oncológico MD Anderson International España (Madrid, Spain). The clinicopathologic features of the BCLU cases used in this study were published previously by one of the authors JFG (16). The diagnosis of all the neoplasms was based on morphological and immunohistological criteria according to the World Health Organization 2008 classification criteria and reviewed by three of the authors (LJM, FV and WOG). The diagnosis of PMLBCL was based on clinicopathologic correlation and included only patients with an anterosuperior mediastinal mass, with no disease outside the mediastinum at the time of the diagnosis, and with morphologic and immunophenotypic features consistent with PMLBCL. The diagnosis of ALK-negative ALCL was established for those lymphomas morphologically within the spectrum of ALK-positive ALCL with strong and uniform expression of CD30, but lacking ALK protein expression.

Immunohistochemical methods were performed using routinely processed paraffin embedded tissue specimens as previously described (17). Antibodies and staining conditions are listed in Table 1. All tissue sections underwent heat-induced epitope retrieval in pH 6.0

citrate buffer (Dako, Carpinteria CA). Endogenous peroxidase reaction was blocked by 3% H₂O₂ solution for 10 minutes. To avoid nonspecific binding of primary antibodies, serum free blocking solution (Dako) was applied for 40 minutes at room temperature. Detection was performed using the LSAB plus-streptavidin-HRP system (Dako). For this study, we analyzed GLI3 protein expression in all 270 cases of lymphoma and 3 thymus specimens. In addition, other HH signaling proteins (HH, GLI1 and GLI2) were assessed in a subset of CHL cases that were available including HH (n=24), GLI1 (n=25) and GLI2 (n=17). The baseline expression of HH signaling proteins in normal lymphoid cells was previously assessed and described (15). Appropriate external controls were used for each antibody.

Cases were considered positive if 10% or more of the tumor cells exhibited cytoplasmic staining for HH or nuclear staining for GLI1, GLI2 and GLI3 as previously reported (18). Protein expression was scored as negative, weakly positive (1+) and strongly positive (2+) depending on the staining signal intensity. In addition, the percentage of GLI3-positive cells was calculated by manually counting at least 200 tumor cells from representative fields in each case. We performed double immunostaining for GLI3 and CD30 or CD20 in a subset of cases of CHL, NLPHL and TCHRLBCL and for GLI3 and cytokeratin, CD68 or CD20 in benign thymus using the EnVision™ G2 System [DakoCytomation, CA] according to the manufacturer's recommendations.

Statistical Analysis

All statistical analyses were two sided and performed using SPSS 16.0 (SPSS Inc., Chicago, IL) considering p values less than 0.05 (two-sided) as significant. Comparison of GLI3 expression frequencies among the different lymphoma types was done by Kruskal-Wallis and U Man Whitney non-parametric tests.

RESULTS

Real time-qPCR analysis of CHL cell lines

Real time qPCR analysis showed high expression of *GLI3* in all three CHL cell lines assessed. In contrast, *GLI1* and *GLI2* were variably expressed by CHL cell lines (Fig. 1). BJAB cells, a human germinal center derived DLBCL cell line, and CLL cells isolated from the peripheral blood of a patient were used as control references. Studies in our laboratory have shown that expression of GLI3 is usually not detected in DLBCL cell lines and is frequently detected in circulating CLL cells.

Baseline expression of GLI3 in normal thymus and reactive lymph nodes as detected by immunohistochemistry

In the thymus, GLI3 was expressed by cortical and medullary stromal cells including epithelial cells, endothelial cells, and histiocytes (Fig. 2). Double immunostaining for CD68 and cytokeratin confirmed the identity of histiocytes and epithelial cells. Thymocytes were negative and there was no co-expression of GLI3 in CD20-positive B cells (not shown).

In a previous study, we assessed the baseline protein expression of HH ligands, GLI1, GLI2 and GLI3 in benign reactive lymph nodes using immunohistochemistry (15). Briefly, HH ligands were expressed by follicular dendritic cells and macrophages. GLI1 was not detected in centrocytes or centroblasts and GLI2 was weakly positive in paracortical T-cells and in a subset of centroblasts and centrocytes. GLI3 was expressed by histiocytes, follicular dendritic cells and endothelial cells but not by lymphoid cells.

GLI3 is characteristically and distinctively expressed in classical Hodgkin lymphoma

The expression of the HH signaling proteins in CHL as tested by immunohistochemistry is summarized in Table 2. HH, GLI1 and GLI2 showed variable expression in HRS cells. HH ligands were expressed in 20 of 24 (83%) cases (Fig. 3A). Most positive cases (12 of 20, 60%) showed weak HH staining while 8 cases showed strong (2+) expression. GLI1 was expressed in 7 of 25 (28%) cases and was weakly positive, and GLI2 was weakly positive in 9 of 17 (53%) cases. In contrast, all 39 CHL tumors showed strong and uniform expression of GLI3 in virtually 100% of the tumor cells (Fig. 3B and C). GLI3 in HRS cells was expressed in a nuclear pattern with nuclear membrane accentuation. Double IHC with CD30 performed in a subset of cases confirmed that the CD30-positive HRS cells were uniformly positive for GLI3 (Fig. 3D).

Survey of other lymphoma types

We assessed GLI3 expression by immunohistochemistry in a large series of B- and T-cell non-Hodgkin lymphomas. The findings are summarized in Table 3 and examples of the staining pattern of GLI3 in lymphomas other than CHL are shown in Fig. 4. Our data show that GLI3 is more frequently expressed in CHL tumors compared with all other lymphoma types studied ($p < 0.0001$).

Four tumor types showed variable GLI3 expression. ALK-negative ALCL, ALK-positive ALCL, BCLU with features intermediate between DLBCL and CHL, and NLPHL showed heterogeneous GLI3 expression with a variable percentage positive tumor cells with a median of 20%, 65%, 40% and 55%, respectively (Fig. 5A, B, C and D). In most of these cases only a subset of tumor cells were positive for GLI3; however, a subset of cases showed strong nuclear staining of GLI3 in =90% of tumor cells in a pattern indistinguishable from that seen in CHL (Fig. 5E, F and G). These cases included 2 of 13, (15%) NLPHL, 5 of 16, (31%) ALK-negative ALCL, 8 of 21, (38%) ALK-positive ALCL, and 3 of 13, (23%) BCLU with features intermediate between DLBCL and CHL.

Among B-cell non-Hodgkin lymphomas, there was no expression of GLI3 in SMZBCL, MZL, MCL and BL whereas 2 cases of FL showed positive staining for GLI3 in a subset of centroblasts. Large B-cell lymphomas including all 7 PMLBCL, a subset of DLBCL, NOS and TCHRLBCL showed relatively low levels of GLI3 expression (median <30% positive tumor cells per case). In contrast with CHL, which showed uniformly strong GLI3 staining in all tumor cells, the intensity of GLI3 staining in the tumor cells of B-cell non-Hodgkin lymphomas, if positive, was always weak and lacked the distinctive nuclear membrane accentuation seen in CHL. Although the cytologic features as well as the immunostaining pattern of GLI3 allowed the distinction between histiocytes and tumor cells in most cases, we performed double immunostaining with GLI3 and CD20 in some cases of TCHRLBCL with a high content of histiocytes to highlight the tumor cells. These studies confirmed that the tumor cells were predominantly negative for GLI3 (not shown).

Interestingly, cases of CLL/SLL with significantly increased large cells showed positive GLI3 staining in the large cells (median 83% positive large tumor cells). These tumor cells showed weak nuclear staining with no nuclear membrane accentuation, a pattern easily distinguishable from that seen in CHL (Fig. 4D). Cases of CLL/SLL without increased large cells did not express GLI3.

Of the T- and NK-cell lymphomas, NKTCL and AILT showed no GLI3 expression and 1 of 9 (11%) PTCL, NOS showed weak nuclear GLI3 expression in a subset of neoplastic cells.

DISCUSSION

This is the first study showing high expression of the HH transcription factor GLI3 in HRS cells of CHL. We also show that HRS cells express relatively low-levels of other HH related molecules including HH ligands and the transcription factors GLI1 and GLI2. GLI3 is universally expressed in HRS cells and is either not expressed or significantly less frequently expressed in other lymphomas. However, a small subset of other lymphomas also express GLI3 including ALK-positive ALCL, ALK-negative ALCL, BCLU with features intermediate between DLBCL and CHL, and NLPHL. A subset of tumors in these four categories, one third of cases or less, showed a pattern of GLI3 staining indistinguishable from that seen in CHL. The reason for the expression of GLI3 in some of these cases is unclear; however, these findings provide further support for the existence of a subset of lymphomas with intermediate/hybrid features between CHL and non-HL.

Inappropriate activation of the HH signaling pathway has been shown in many cancers including ALK-positive ALCL and DLBCL (18–20). However, this aberrant activation is mediated by the transcription factors GLI1 and GLI2 and not by GLI3. In fact, GLI3 mainly functions *in vivo* as a transcriptional repressor. Furthermore, in most systems studied, GLI3 limits HH pathway activation and absence of GLI3 causes up-regulation of HH signaling. Thus, the biological significance of the expression of GLI3 in HRS is uncertain at this time.

Constitutive activation of the PI3K/AKT and NFκB pathways has been implicated in CHL (2) and crosstalk between these pathways with expression of HH ligands have been described. It has been shown that some growth factors such as platelet-derived growth factor, epidermal growth factor and insulin-like growth factor 1 increase expression of HH ligands and that this increase is dependent of PI3K and AKT activation (21, 22). We and others have shown that adenoviral delivery of activated AKT up-regulates HH ligand expression and that recombinant HH induces activation of PI3K/AKT (18, 22, 23). Similarly, it has been shown that NFκB regulates and contributes to HH ligand expression (24, 25). The mode of action of HH signaling in cancers appears to be complex and may be tumor dependent. Several studies suggest an autocrine action from HH signaling in some tumors and others favor a paracrine model. In the paracrine model, neoplastic cells secrete HH ligands that may stimulate stromal cells near the tumor (endothelial cells, fibroblasts, and immune cells) to support tumor growth through secreting of growth factors. It is possible that the secretion of HH ligands by HRS contributes in the interactions between tumor cells and surrounding stroma in HL. However, the relationship between PI3K/AKT and NFκB signaling pathways with expression of GLI3 is uncertain at this time.

One of the historic challenges to a more complete understanding of the pathobiology of CHL is the lineage infidelity frequently demonstrated by HRS cells. For instance, for many years, CHL was thought to be derived from histiocytic/dendritic cells because they frequently express histiocytic/dendritic cell markers like fascin (26). Other lineage-associated markers variably expressed by HRS cells include ID2 (dendritic cell marker), CD3 and CD4 (T-cell markers) and CD15 and colony-stimulating factor 1 receptor (myeloid markers) (27–29). Some authors have shown that the unique phenotype of HRS cells results from disruption or imbalances in the B-cell lineage transcriptional program and inhibition of the function of B-cell-determining transcription factor E2A (28, 30). Currently, HRS cells are considered to be of germinal center B-cell origin because they undergo B-cell receptor gene rearrangement and express B-cell specific transcription factors like PAX-5 (2, 31). We suggest that the expression of GLI3 in HRS cells is another example of their lineage infidelity given that GLI3 is normally expressed by stromal cells in the lymph node (including follicular dendritic cells, histiocytes and endothelial cells) and thymus

(histiocytes, endothelial cells and epithelial cells), but not by mature or immature lymphocytes.

Additionally, CHL is characterized by a rich inflammatory milieu including T- and B-cells admixed with variable numbers of plasma cells, macrophages, eosinophils and mast cells (32, 33). Regulatory T (Treg) cells comprise a considerable fraction of infiltrating T-cells in CHL (34, 35). Tanijiri and colls showed that the co-culture of CHL cell lines with naïve CD4⁺ T-cells promotes the bidirectional differentiation of naïve T-cells toward CD25⁺Foxp3⁺T-cells and CD4⁺ cytotoxic T-cells without the cooperation of any other factor and without requiring additional cell types. This would explain, at least in part, the predominance of Treg cells in CHL (36). Furthermore, Hager-Theodorides and colls recently showed that GLI3 in thymic stromal cells regulates T-cell selection and differentiation (37, 38). Whether GLI3 plays a regulatory role in the T-cell population in CHL remains to be studied, but we think that it is an interesting hypothesis and our findings provide a rationale for further investigation of this potential regulatory role.

From a practical point of view, our findings are also of particular interest because we found that lack of GLI3 staining is a strong negative predictor against the diagnosis of CHL. Thus, GLI3 can be used in conjunction with the currently available extensive list of immunohistochemical markers to help distinguish CHL from possible mimics.

In summary, we have shown that GLI3 is strongly expressed by virtually 100% of the HRS cells of CHL. These data provide a rationale for further investigation of the biological significance of GLI3 in CHL tumor biology, in particular, a potential regulatory role in the composition of the CHL microenvironment.

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Figure 1. Real-time RT-qPCR analysis for *GLI* molecules in CHL

RT-qPCR analysis in CHL cells shows the presence of mRNAs for *GLI1*, *GLI2* and *GLI3*. Overall, there is variable expression of *GLI1* and *GLI2* mRNAs in the three CHL cell lines tested. However, consistently elevated expression of *GLI3* mRNA is seen in all three CHL cell lines. BJAB, a DLBCL cell line, and one CLL patient sample are used as control references.

Figure 2. Immunohistochemical expression of GLI3 in normal adult thymus

(A) Benign thymus showing cortex and medulla with stromal cells including epithelial cells, histiocytes and endothelial cells. (B) GLI3 immunostain highlights cortical and medullary stromal cells in the thymus, including Hassall's corpuscles. Thymocytes are negative. (Original magnification $\times 100$ –200.)

Figure 3. Immunohistochemical expression of HH ligand and GLI3 in HRS cells

(A) Cytoplasmic staining for HH ligand in HRS cells. Lymphocytes are negative for HH ligand (B and C) GLI3 shows characteristic strong nuclear staining with nuclear membrane accentuation in virtually all HRS cells. Note in (C) that the histiocytes and endothelial cells show weaker staining for GLI3 than the HRS cells. The neoplastic cells are easily recognized by their large size, irregular nuclear contours and, in some cases, multinucleation. (D) Double immunostaining with GLI3 (brown chromogen) and CD30 (red) confirms the expression of GLI3 in neoplastic HRS cells. (Original magnification $\times 100$ –1000.)

Figure 4. GLI3 expression is negative in most non-Hodgkin lymphomas

Examples of non-Hodgkin lymphomas in which the tumor cells were negative or focally positive for GLI3; FL (A), DLBCL, NOS (B), BL (C), MCL (D), TCHRLBCL (E) and PTCL (F). In these cases, histiocytes, follicular dendritic cells and/or endothelial cells provide an internal positive control as they show positive nuclear staining for GLI3. (Original magnification $\times 100$ –400.)

Figure 5. GLI3 is heterogeneously expressed in cases of ALCL, ALK-positive and ALK-negative, BCLU with features intermediate between DLBCL and CHLs and NLPHL with most cases showing focal or no expression in the tumor cells

The tumor cells in most cases of ALK-negative ALCL (A), BCLU with features intermediate between DLBCL and CHL (B), NLPHL (C) and CLL/SLL (D) show negative or focally positive staining for GLI3. For those cases with focal positivity, GLI3 was expressed in a subset of the tumor cells with weaker expression than that seen in histiocytes and endothelial cells (black arrow in C). However, a small subset of ALK-negative ALCL (E), BCLU with features intermediate between DLBCL and CHL (F) and NLPHL (G) show a GLI3 expression pattern indistinguishable from CHL, i.e. strong nuclear staining with nuclear membrane accentuation in virtually 100% of the tumor cells. The pattern of staining in CLL with increased large cells (H) was characterized by weak nuclear expression of GLI3, as compared to intrinsic histiocytes and endothelial cells, without nuclear membrane accentuation. Note the stronger expression of GLI3 in histiocytes in comparison with the positive tumor cells.

Table 1

Antibodies used for immunohistochemistry

Antibody	Clone	Manufacturer	Dilution	Incubation
HH	H-160	Santa Cruz	1:150	RT, 1h
GLI1	H-300	Santa Cruz	1:200	RT, 1h
GLI2	NA	Abcam	1:200	RT, 1.5h
GLI3	H-280	Santa Cruz	1:150	RT, 1h
CD20	L26	Dako	1:1000	RT, 1h
CD30	Ab36749	Abcam	1:150	RT, 1.5h

NA, Not available; RT, Room temperature

Table 2

Expression of HH signaling proteins by immunohistochemistry in classical Hodgkin lymphoma

	# of cases	Intensity of expression		Total Positive	% Positive
		1+	2+		
HH	24	12	8	20	83%
GLI1	25	7	0	7	28%
GLI2	17	9	0	9	53%
GLI3	39	0	39	39	100%

Table 3

Immunohistochemical expression of GLI3 in lymphoid neoplasms

Diagnosis	N	Positive (%)	* Median % positive tumor cells per case (range)	Number of cases with % positive tumor cells			
				0–25%	26–50%	51–75%	76–100%
CHL	39	39 (100%)	798 (80–100)	0	0	0	39
NLPHL	13	6 (46%)	55 (10–100)	9	1	1	2
CLL/SLL	16	7 (44%)	83 (80–90)	9	0	0	7 [‡]
FL	28	2 (7%)	10 (10)	28	0	0	0
MZL	4	0	0	4	0	0	0
SMZBCL	3	0	0	3	0	0	0
MCL	7	0	0	7	0	0	0
PMLBCL	7	7 (100%)	25 (10–40)	6	1	0	0
DLBCL	54	21 (39%)	15 (10–50)	51	3	0	0
Gray zone	13	13 (100%)	40 (10–100)	5	3	0	5
TCHRLBCL	12	6 (50%)	27 (10–80)	9	2	0	1
BL	12	0	0	12	0	0	0
PTCL, NOS	9	1 (11%)	25	9	0	0	0
AIT	6	0	0	6	0	0	0
ALCL, ALK negative	16	12 (75%)	20 (10–100)	11	0	0	5
ALCL, ALK positive	21	21 (100%)	65 (10–100)	5	3	5	8
NKTCL	10	0	0	10	0	0	0
Total	270						

* Only cases which showed positive staining are used to calculate the median

[‡] Three cases of CHL showed <100%, but more than 75%, GLI3 positive tumor cells[‡] Only CLL/SLL cases with increased large cells showed GLI3 expression

Gray zone lymphoma refers to BCLU with features intermediate between DLBCL and CHL