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Utility of LRF/Pokemon and NOTCH1 Protein Expression in the Distinction of Nodular Lymphocyte-Predominant Hodgkin Lymphoma and Classical Hodgkin Lymphoma

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Abstract

Classical Hodgkin lymphoma (CHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) are considered separate entities with different prognosis and treatment. However, morphologic features can be similar and immunohistochemical studies are essential in the distinction; thus, determination of additional biomarkers is of utmost importance. LRF/Pokemon is a protooncogene, an interacting partner co-expressed with BCL6 in germinal centers and highly expressed in diffuse large B-cell lymphoma and follicular lymphoma. Conversely, loss of the LRF gene in mouse hematopoietic stem cells results in complete block of early B cell development with concomitant Notch derepression, indicating its critical role in B versus T cell fate decision at the hematopoietic stem cell stage. For the first time, we show that LRF/Pokemon is predominantly expressed in NLPHL cases as is BCL6 with low to absent NOTCH1 protein expression; while Hodgkin Reed-Sternberg (HRS) cells in CHL show low to absent BCL6 and LRF/Pokemon expression with higher NOTCH1 expression. We illustrate a potential functional interaction between LRF and BCL6 in NLPHL pathogenesis, and differential expression of LRF/Pokemon and NOTCH1 proteins in CHL thus showing differential expression, making for an additional diagnostic marker and therapeutic target.

Keywords

LRF; Pokemon; Hodgkin lymphoma; nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL); classical Hodgkin lymphoma (cHL)

Introduction

The distinction between classical Hodgkin lymphoma (CHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is essential for determination of accurate therapy. Morphologic criteria alone can misclassify the entities. Although some

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immunophenotypic markers have been used, some cases remain difficult to classify using our current antibody battery.

Several POZ and *Kruppel* Zinc Finger (POK) proteins have been identified so far in the human genome. POK proteins are composed of an amino terminal POZ domain associated with homo and heterodimerization and recruitment of histone deacetylases and a carboxy terminal zinc finger domain mediating specific DNA recognition and binding.^{1,2} Leukemia/lymphoma-related factor/POK erythroid myeloid ontogeneic factor (LRF/Pokemon) is a transcription factor, and belongs to the POK protein family, which is also known in the literature as OCZF (osteoclast-derived zinc finger), FBI-1 (factor that binds IST, HIV inducer of short transcripts), and ZBTB7A (zinc finger and BTB domain containing 7A). Although LRF/Pokemon has been found to be upregulated in several forms of cancer, including lymphomas, gliomas, breast, lung, and ovary^{3,4} and in vitro and in vivo studies based on transgenic mice have clearly demonstrated its potent protooncogenic activity especially in hematopoietic tissue^{5,6} and new data are intriguingly redefining this concept describing LRF/Pokemon as a key oncosuppressor in specific tissues and context.^{2,7,8} LRF/Pokemon has critical and pleiotropic functions in cellular differentiation² and can physically interact with BCL6.⁹ Importantly, LRF is necessary for germinal center formation in mice, as germinal center B cells are significantly reduced in B cell-specific LRF conditional knockout mice after immunization with T cell-dependent antigen.¹⁰

Notch1 is found in bone marrow, thymus, fetal liver hematopoietic cells, and the developing yolk sac vas-culature.^{11,12} Notch1 is a gene encoding a transmembrane receptor that is integrally involved in normal T-cell development and regulation.¹³ NOTCH1 receptor is a ligand activating transcription factor, which transduces extracellular signals into the nucleus.¹⁴ Activating NOTCH1 gene mutations are found in chronic lymphocytic leukemia (CLL) and T-acute lymphoblastic leukemia (T-ALL) and have been identified as a prognostic factor.¹⁵ NOTCH1 is a novel therapeutic target in T-ALL.

Hodgkin lymphoma (HL) is the most common lymphoid neoplasm in population younger than 40 years.¹⁶ CHL is a malignant proliferation characterized by the presence of a minority of Hodgkin Reed-Sternberg (HRS) cells, derived from B-cell germinal center cells admixed with a predominantly CD4+ helper and regulatory T-cell infiltrate.¹⁶⁻¹⁸ CHL encompasses 90% to 95% of HL and shows a bimodal distribution, with a first peak in early adulthood and a second peak in patients older than 55 years.¹⁹ NLP HL is recognized as a separate entity in the World Health Organization classification. NLP HL is an indolent germinal center B-cell neoplasm, characterized by the presence of nodules with large cells with multilobated nuclei, also known as popcorn or lymphocyte predominant (LP) cells surrounded by small lymphocytes and histiocytes.¹⁹ NLP HL accounts for approximately 5% of HL cases; although occurs in all age-groups, there is a peak incidence in the fourth decade.¹⁹ NLP HL is another hematopoietic lymphoid tumor that expresses BCL6 and shows localization to neoplastic LP cells.²⁰ Although Notch1 and Notch2 family members expression have been aberrantly expressed in HRS cells in CHL promoting proliferation and protecting from apoptosis,^{21,22} it has neither been extensively studied in NLP HL nor ever correlated with LRF/Pokemon expression.

We therefore sought to compare LRF/Pokemon and NOTCH1 protein expression in NLP HL and CHL cases and determine their differential expression and diagnostic utility.

Materials and Methods

The study cohort included a total of 50 patients with HL, including 20 patients with NLP HL and 30 patients with CHL, all of nodular sclerosis subtype, that were consecutively

ascertained at Memorial Sloan-Kettering Cancer Center from 1985 to 2005. Biopsies were reviewed and classified based on hematoxylin and eosin staining and immunophenotyping according to the World Health Organization classification.²³ This was a retrospective review and a waiver of authorization from our Institutional Review Board and Human Tissue Utilization Committee was obtained. For the NLPHL cases, age ranged from 28 to 92 years, with 2 females and 18 males. All cases of NLPHL showed classic morphology and LP cells that were CD20+ CD15- CD30-; 4 cases did not have workup. For the CHL cases, age ranged from 13 to 62 years, with 14 females and 16 males. CHL cases were all of the nodular sclerosis subtype with classic morphology, RS cells were CD15+ CD30+ CD20- Pax5 dim, with 3 cases showing weak CD20 expression.

Pretreatment conditions for the following primary antibodies included the following: LRF (hamster monoclonal, clone 13E9, citric acid, pH 6.0, dilution of 1:8000) using steam or heat induced epitope retrieval as previously described⁵; BCL-6 (monoclonal, Novocastra Laboratories, Ltd, Newcastle, UK; EDTA, pH 8.0, 1:100); and Notch1 (Epitomics, Burlingame, CA; EDTA pH 8.00 at 1:100 dilution, with secondary anti-rabbit, at 1:500 dilution, 60 minutes at room temperature, and tertiary antibody, DAKO, Glostrup, Denmark; streptavidin at 1:500 dilution, 60 minutes at room temperature). Please note, the LRF antibody is commercially available through Santa Cruz Biotechnology (Santa Cruz, CA), however, for this project and article, we used the internal antibody generated in the Monoclonal Antibody Core Facility, which was characterized for paraffin-fixed embedded sections in the Immunohistochemistry Laboratory of the Pathology Core Facility of the Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center.

Sections of reactive human tonsil were stained as controls for LRF and showed expression highlighting nuclei in squamous epithelium as well as centrocytes and centroblasts in reactive germinal centers (not shown). NOTCH1 positive control of a T-ALL showed diffuse strong nuclear localization and reactivity (not shown). For NLPHL, the cohort was composed of 19 males and 2 females and for CHL, 16 males and 14 females. Twenty NLPHL and 30 CHL tissue biopsies were stained using whole sections. Intensity and percent positivity of malignant LP cells and HRS cells were scored as well as surrounding reactive lymphocytes, histiocytes, and endothelial cells of blood vessels evaluated. Tumor cells in HL were scored for BCL6, LRF/Pokemon, and NOTCH1 expression and defined as 0 (negative), 1 (scattered <50%, weak positive), and 2 (diffuse >50%, strong positive) with a nuclear or cytoplasmic localization pattern. Statistical correlations were performed by χ^2 test and Fisher's exact test analyses.

Results

In NLPHL, 18/20 (90%) cases showed diffuse strong nuclear positivity in >50% of neoplastic LP cells whereas in CHL cases, 6/30 (20%) cases showed diffuse strong nuclear positivity in >50% of neoplastic HRS tumor cells for LRF protein expression. There was no significant correlation with LRF expression and age (<60 vs >60 years; $P = .54$) or gender ($P = .55$) in CHL. Correlation with immune status was not performed in this limited study. In sharp contrast, NOTCH1 was significantly expressed in CHL compared with NLPHL tumor cells ($P < .0001$), and significantly inversely correlated with LRF/Pokemon expression ($P < .00001$; Figures 1 and 2). Of note, expression was predominantly cytoplasmic and further work is being carried out on automated platforms. BCL6 protein expression was positive in 12/20 (60%) cases, which showed weak, scattered to diffuse strong nuclear positivity in LP tumor cells and 1/30 (3%) with scattered weak reactivity in CHL malignant HRS cells. The staining was performed in the Immunohistochemistry Laboratory of the Pathology Core Facility, which uses a Novocastra clone and was

performed manually at the time of this project. Therefore, neoplastic LP cells co-express BCL6 and LRF proteins but show low to negative NOTCH1 expression.

Discussion

Notch signaling pathway has been implicated in differentiation, proliferation, apoptosis, and cancer progression.^{21,24} In CHL, up to 40% of cases can express Epstein-Barr virus, and activation of the Notch-related signaling pathways may represent a key mechanism by which EBNA2 contributes to Epstein-Barr virus-induced immortalization²⁵; however, EBNA2 upregulates genes in B lymphoblasts that are different from those that Notch IC upregulates in thymocytes.²⁶ The effect of Notch signaling can also be mediated by the early transcription factor GATA-2. GATA-2 expression was found in HRS cells in 50% of CHL cases, while NLPHL cases were negative.²⁷ In addition, in a study, Stanelle et al²⁸ found that GATA3, an essential transcription factor for early T-cell development is overexpressed in HRS cells in CHL. In CHL, increased LRF expression in reactive lymphocytes may be speculated to be associated with CD4 lineage commitment similar to the Th-POK, a close homologue of LRF, as recently reported.²⁹ Of note, LRF mRNA is moderately expressed in CD4/8 single positive mouse thymic T cells, while its expression is downregulated in double positive T cell population.⁶ CHL and NLPHL have been reported to have more CD4 compared with CD8 reactive surrounding background T lymphocytes since HL patients display a variety of T-cell immune defects, including the absence of CD8-positive T cells surrounding the neoplastic cells.^{30,31} NLPHL has recently been shown to express a double positive CD4+CD8+ T-cell population as well as increased CD57+ T-cells, which may be cytokine driven.^{32,33} We observed LRF/Pokemon staining in surrounding lymphocytes in NLPHL and also observed increased staining for CD3 and CD4 over CD8 on a few limited cases (data not shown). Of note, NOTCH1 showed predominantly cytoplasmic localization in HRS cells in CHL (Figure 1, lower right) and anaplastic large cell lymphoma (data not shown) as previously reported.²² In contrast, predominantly variably strong nuclear localization was seen in positive control cases of acute T-cell precursor lymphoblastic lymphoma (data not shown).

We have shown that LRF is a novel protooncogene and a specific ARF transcriptional repressor.⁵ Loss of LRF causes aberrant ARF upregulation, resulting in premature senescence and unresponsiveness to oncogenic stimuli in mouse embryonic fibroblasts. LRF null embryos demonstrate defects in fetal B-cell development, and LRF is co-localized and expressed in the germinal center of lymphoid tissues with BCL6.⁹ LRF/Pokemon overexpression leads to overt oncogenic transformation both in vitro and in vivo in transgenic mice. We initially found markedly high LRF expression in diffuse large B-cell lymphoma and follicular lymphoma. Co-expression of BCL6 and LRF correlated with higher proliferation and predicted for better overall survival in diffuse large B-cell lymphoma.⁵

Recently, however, LRF/Pokemon has been described as a potent oncosuppressor in prostate cancer through the repression of SOX9 oncogenic transcriptional activity and by supporting fundamental pro-apoptotic pathways during androgen deprivation therapy.^{7,8}

Abrogation of the *Zbtb7a* gene that encodes LRF in mice, results in embryonic lethality and impaired cellular differentiation in multiple tissues, especially in the hematopoietic lineage cells. LRF was found to be indispensable for normal B cell development by opposing Notch signaling at the hematopoietic stem cell/progenitor levels.⁶ Loss of the LRF gene in mouse hematopoietic stem cells resulted in CD4/CD8 double positive T-cell accumulation in the bone marrow at the expense of normal B-cell development indicating its critical role in B versus T lymphoid fate decision.⁶

Classical Hodgkin lymphoma constitutively expresses nuclear factor (NF)- κ B, which is induced by LMP1, CD30, CD40, tumor necrosis factor- α , and Notch interactions, which ultimately result in upregulation of chemokines, cytokines, apoptotic and intracellular signaling molecules.^{25,34–36} In addition, Notch is an essential upstream regulator of NF- κ B, relevant for survival of HRS cells.²¹ Furthermore, in a recent study, Steidl et al¹⁶ using microdissected HRS cells for gene expression profiling studies found downregulation of B-cell lineage genes and upregulation of NF- κ B and JAK-STAT pathway activators.¹⁶

In summary, this is the first report showing dominant LRF/Pokemon expression in NLP HL and inverse expression of LRF and NOTCH 1 between NLP HL and CHL that makes for an additional diagnostic and attractive therapeutic target in HL.

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Figure 1.

Representative hematoxylin and eosin (H&E)–stained images of nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) and classical Hodgkin lymphoma (cHL) (H&E, 600×, upper left and right, respectively). LRF/Pokemon protein expression in a representative case of NLPHL with reactivity to nuclei in malignant lymphocyte predominant (LP) cells (middle left) compared with a representative case of nodular sclerosis CHL with no reactivity to malignant Hodgkin Reed-Sternberg (HRS) cells (middle right) but reactivity to nuclei of surrounding reactive lymphocytes and histiocytes seen in NLPHL and CHL. (Not shown) LRF protein expression in reactive human tonsil localized to nuclei in squamous epithelium as well as centrocytes and centroblasts in reactive germinal centers. Negative NOTCH1 protein expression in a representative case of NLPHL (lower left), which had strong LRF/Pokemon and BCL6 expression in malignant LP cells (not shown); positive NOTCH1 protein expression in HRS cells with predominant cytoplasmic localization from a representative case of CHL, which had negative POK and BCL6 protein expression in HRS cells (lower right). In general, surrounding reactive lymphocytes and endothelial cells were also more positive for NOTCH1 in CHL compared with NLPHL cases. NOTCH1 protein expression in a positive control case of acute precursor T-cell lymphoblastic lymphoma showed variably strong nuclear localization (not shown).

Figure 2.

Inverse LRF/Pokemon and NOTCH1 protein expression in neoplastic Hodgkin Lymphomas (HL) cells. Significant inverse correlation with LRF+ NOTCH1– LP cells compared with LRF– NOTCH1+ Hodgkin Reed-Sternberg (HRS) cells was seen ($P < .00001$) (A). When comparing LRF+/BCL6+ malignant cells in nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) versus classical Hodgkin lymphoma (CHL), there were significantly more cases of NLPHL (12 cases), compared with 1 case of CHL, showing LRF+/BCL6+ malignant LP cells versus HRS cells ($P < .001$) (B). The number of cases showing LRF+/BCL6+ positive surrounding reactive lymphocytes were increased for both NLPHL and CHL but without a significant difference compared with LRF–/BCL6– surrounding reactive lymphocytes.