**Original Article**

**Canonical and non-canonical Wnt signal pathway in classic Hodgkin lymphoma and the prognostic significance of LEF1, β-catenin, FZD6 and Wnt5a/b**

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**Abstract:** Aberrant Wnt signaling has been found in many solid organ cancers, as well as hematological malignancies. However, its role in classic Hodgkin lymphoma (CHL) remains unclear. We evaluated the expression of Wnt signaling components LEF1, β-catenin, FZD6 and Wnt5a/b and their correlation with the prognosis in 50 CHL patients by immunohistochemical stains. The neoplastic Hodgkin/Reed-Sternberg (HRS) cells showed expression of LEF1, FZD6, and Wnt5a/b but absent nuclear β-catenin. Wnt5a/b expression was seen in a significantly higher percentage of stage IV patients (67%) compared to other stages (p=0.03). However, there was no correlation between the expression of LEF1, FZD6 and Wnt5a/b and patients’ stage or survival. In summary, our results confirmed decreased β-catenin expression in HRS. Non-canonical Wnt pathway may play a role in the microenvironment that facilitates HRS migration, however, it is not sufficient to consider LEF1, β-catenin, FZD6 and Wnt5a/b as prognostic factors for CHL.

**Keywords:** Classic Hodgkin lymphoma, Wnt signal pathway, prognosis

**Introduction**

Wnt signaling is a critical regulator in many physiological processes, including tissue development, differentiation, and cell homeostasis [1, 2]. Classically, Wnt signaling is divided into canonical and non-canonical pathways. The key mediator of canonical pathway is β-catenin. In the absence of Wnt ligands, a destruction complex is formed, which includes APC (adenomatous polyposis coli), Axin (axis inhibitor), and GSK-3β (glycogen synthase kinase-3 beta). The destruction complex leads to phosphorylation and ubiquitination-mediated degradation of β-catenin. When Wnt ligand binds to the Frizzled (FZD) receptor, some components of the destruction complex are recruited to the cell membrane, which leads to dissociation of the destruction complex and accumulation of β-catenin. Accumulated β-catenin translocates to the nucleus, interacts with transcription factors TCF (T-cell factor)/LEF (lymphocyte-enhancer-binding factor), and promotes the transcription of Wnt target genes including VEGF, CCND1, and cMYC [3].

Non-canonical Wnt signaling pathways are further divided into Wnt/planar cell polarity (PCP) signaling and Wnt-cGMP/calcium signaling, which are involved in the process of polarity and cell motility. Wnt proteins are grouped as canonical Wnts such as Wnt1, Wnt2, Wnt3 and Wnt8a, and non-canonical Wnts, including Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt11, etc. [4].

Dysregulated Wnt signaling has been associated with the pathogenesis of many solid organ cancers such as colorectal cancer, breast cancer and hepatocellular carcinoma, as well as hematological malignancies including classic Hodgkin lymphoma (CHL) [5]. CHL is a B cell lymphoma characterized by mononuclear Hodgkin cells and multinucleated Reed-Sternberg cells. Hodgkin/Reed-Sternberg (HRS) cells usually comprise a minority of the total cellularity and are embedded in a background of non-neo-
plastic reactive immune cells. Morrison et al. reported expression of nuclear and cytoplasmic β-catenin in 15% of Epstein-Bar virus (EBV)-positive and 7% of EBV-negative Hodgkin lymphomas [6]. Sohlbach et al. investigated the expression of the components of Wnt signaling in a CHL cell line, L1236 cells, and found overexpression of Wnt5a, Wnt5b, Wnt8a, β-catenin, FZD6, LEF1, and cMYC. However, β-catenin expression was not identified in the HRS cells of 28 cases of CHL, but instead was found in the dendritic processes of stromal cells to varying degrees [7]. As one of the major components of the destruction complex, an activated form of GSK-3β, pY216 GSK-3β, was found to be present in 100 of 100 (100%) CHL cases, while β-catenin was detected in the cytoplasm of only 12% of the cases. The accumulation of cytoplasmic β-catenin was associated with the co-existence of an inhibitory form of GSK-3β, pS9 GSK-3β [8]. Linke et al. reported overexpression of a non-canonical Wnt protein, Wnt5α, stimulated migration of CHL cell lines and promoted lymphoma engraftment and vascularization in chick chorioallantoic membrane (CAM) assay [9]. The same research group also studied the role of canonical Wnt signaling in the interactions between CHL cells and vascular endothelial cells (EC). LEF1 and β-catenin were found to be required for CHL cell migration toward ECs and promoted angiogenesis in vitro. Low levels of nuclear β-catenin were detected in CHL cell lines, such as L428 cells and KM-H2 cells, indicating a basal canonical Wnt signaling activity. Nuclear LEF1-positive HRS cells were found in 3 of 9 (33%) mixed-cellularity CHL cases and 1 of 18 (6%) nodular sclerosis CHL cases [10].

Although the canonical Wnt signaling pathway was reported to be activated at basal levels in CHL cell lines [10], nuclear β-catenin accumulation in CHL cases has been reported in variable ratios, ranging from 0% to 15% in different patient groups [6-8]. A non-canonical Wnt protein, Wnt5α, and canonical Wnt signaling components, LEF1 and β-catenin, were demonstrated to promote cell migration in vitro [9, 10], but their expression levels in relation to prognosis in CHL patients has not been evaluated to date. In our study, we evaluated the expression of multiple Wnt signaling components including β-catenin, LEF1, FZD6 and Wnt5α/b in 50 CHL patients and correlated their expression with the stage and survival of patients.

Materials and methods

Case selection

We selected a total of 50 patients diagnosed with CHL in the University of Kansas Health System and available paraffin tissue from 2008 to 2016. The cases that did not have paraffin embedded tissue blocks in our archive were excluded. All cases were diagnosed according to the current World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissue (2017). Clinical, morphologic and immunophenotypic data were reviewed. This retrospective study was approved by the institutional review board of University of Kansas Medical Center.

Immunohistochemical methods

Immunohistochemical analyses were performed using formalin-fixed, paraffin-embedded tissue sections. The antibodies used were as follows: anti-phospho-β-catenin Ser 552 rabbit polyclonal antibody (Stowers Institute for Medical Research, Kansas City, MO, USA) [11]; Wnt5α/b rabbit polyclonal antibody (1:200; Thermo Fisher Scientific, Waltham, MA, USA); LEF1 rabbit monoclonal antibody (1:50; Cell
Figure 1. Expression of LEF-1, β-catenin, FZD6 and Wnt5a/b in CHL patients. A. Prominent Hodgkin/Reed-Sternberg (HRS) cell in classic Hodgkin lymphoma (CHL), H&E, 200×; B. Positive nuclear staining of LEF1 in HRS cells, 200×; C. Negative LEF1 staining in HRS cells, 200×; D. Negative β-catenin staining in HRS cells, 200×; E. Positive membranous staining of FZD6 in HRS cells, 200×; F. Positive cytoplasmic staining of Wnt5a/b in HRS cells, 200×.

Figure 2. Scatterplots of expression for LEF-1, FZD6 and Wnt5a/b.
Marque, MilliporeSigma, Burlington, MA, USA), and FZD6 rabbit polyclonal antibody (1:100; Thermo Fisher Scientific, Waltham, MA, USA). Immunostaining procedures were performed using the Biocare IntelliPath autostainer. Epitope retrieval was performed using the Biocare Decloaking Chamber. Slides were counterstained with hematoxylin and permanently mounted.

LEF1 positivity was defined as at least 10% of the HRS cells having nuclear staining [12]. FZD6 positivity was defined as at least 10% of the HRS cells having membranous staining and Wnt5a/b was defined as at least 10% of the HRS cells having cytoplasmic staining. β-catenin positivity was defined as positive nuclear stained cells per 40x high power filed [11]. Expression of β-catenin, LEF1, FZD6 and Wnt5a/b was evaluated manually by 3 pathologists, the percentage of positive cells per 100 HRS cells was counted, and a score of the average count was assigned to each case.

Statistical analysis

Overall survival (OS) was defined as date of diagnosis to date of death from any cause. Patients who were lost to follow-up were censored on their last follow-up date. Fisher’s exact test was used to evaluate the relationship between the expression of LEF1, FZD6, Wnt5 and stage and OS. Regression analysis was performed using the statistical software R-4.0, and ggplot2 to correlate the expression of proteins with the patients’ overall survival (OS). Wilcoxon rank sum test was used to assess their expression in alive versus dead patients. Two-tailed Fisher exact test was performed using the statistical software R-4.0. P value < 0.05 was considered statistically significant.

Results

Clinicopathologic characteristics of CHL patients

The study included 25 female and 25 male CHL patients with a mean age of 38 years (range, 18-84 years). The clinicopathologic characteristics of CHL patients are summarized in Table 1, which includes age, gender, histologic subtypes, Ann arbor stage, bulky disease, number of involved lymph nodes, extranodal disease, bone marrow involvement and erythrocyte sedimentation rate (ESR). The main histologic sub-type was nodular sclerosis (80%), and the other subtypes were mixed cellularity (10%) and lymphocyte-rich (10%). According to the Ann Arbor staging system, 23 patients (46%) had stage I/II disease, while 27 patients (54%) had stage III/IV disease. A total of 36 patients (72%) had more than 2 lymph nodes involved by lymphoma and 13 (26%) had bulky mediastinal mass. Extranodal disease was detected in 17 patients (34%) and bone marrow involvement was found in 8 cases (16%). A total of 19 patients (38%) had an ESR that is over 50 mm/h.

Expression of LEF1, β-catenin, FZD6 and Wnt5a/b in CHL patients

Figure 1A demonstrates the typical features of mononucleated Hodgkin cells and multinucleated Reed-Sternberg cells with large nuclei and prominent eosinophilic nucleoli. Thirty eight out of 50 (76%) CHL patients had positive nuclear LEF1 staining in HRS cells as well as in some of the surrounding nonneoplastic cells (Figure 1B). Figure 1C shows that the HRS cells were negative for LEF1, while the surrounding lymphocytes were LEF1-positive. None of the 50 CHL patients (0%) had β-catenin expression in HRS cells or nonneoplastic cells (Figure 1D). A total of 12 patients (24%) showed membranous staining of FZD6 in HRS cells only (Figure 1E) and 22 patients (44%) showed cytoplasmic staining of Wnt5a/b in HRS cells only (Figure 1F). To evaluate the correlation between LEF1, FZD6, and Wnt5a/b, Spearman’s rank correlation was used. The scatterplots and near-zero coefficients indicated that there was no correlation between LEF1, FZD6, and Wnt5a/b (Figure 2 and Table 2).

Prognostic impact of LEF1, FZD6 and Wnt5a/b expression in CHL patients

The number of patients with and without expression of LEF1, FZD6 and Wnt5a/b at different Ann Arbor stages were showed in Figure 3. Fisher’s exact test was used to determine if there was any association between the clinical stage and the expression of these 3 proteins. Wnt5a/b expression was seen in a higher per-
percentage of stage IV patients (10/15 cases, 67%) compared to other stages (p=0.03). However, there was no difference in LEF1 and FZD6 expression in patients at different stages.

We also compared the expression of LEF1, FZD6 and Wnt5a/b in alive versus dead patients using Fisher exact test and Wilcoxon rank sum test (data not shown), and no significant difference was identified between the two groups in terms of LEF1, FZD6 and Wnt5a/b expression (Table 3). We further evaluated whether the expression of these 3 proteins correlated with the patients’ overall survival (OS). The coefficients of determination (R²) derived from regression analysis were close to zero, which indicated there was no correlation between OS and expression of LEF1, FZD6 and Wnt5 (Figure 4).

Discussion

The roles of Wnt signaling in many cancers are well established. However, in the case of CHL, the impact of aberrant Wnt signaling is less clear. As the indicator of canonical Wnt signaling activation, the expression of β-catenin in CHL cases has been reported inconsistently. β-catenin expression in HRS cells were variable [6-8]. In this study, we investigated 50 CHL cases and did not identify β-catenin expression in the HRS cells, indicating β-catenin-dependent canonical Wnt signaling was unlikely to play an important role in CHL tumorigenesis or progression. Consistent with the low or absent expression of β-catenin in mature lymphocytes [13], β-catenin was not detected in the lymphocytes surrounding the HRS cells either.

LEF1 was found to be consistently overexpressed in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and was usually absent in other small B-cell lymphomas, which made LEF1 a highly specific marker for CLL/SLL [12]. LEF1 expression was also identified in a subset of cases of Burkitt lymphoma, follicular lymphoma and diffuse large B-cell lymphoma [14, 15]. In the case of CHL, LEF1 expression was reported with variable rates in several studies. One study observed positive LEF1 expression in 6% (1/18) of nodular sclerosis and 33% (3/9) of mixed-cellularity CHL cases [9]. Another study found that LEF1 was expressed in 28% (12/43) of de novo CHL cases and 60% (12/20) of CHL associated with Richter transformation [16]. Rea et al. reported LEF1 positivity in 15 of 19 (70%) cases of CHL and all 4 cases of CHL with Richter transformation [17]. Our investigation of LEF1 revealed that 76% (38/50) of CHL cases had positive nuclear LEF1 staining in HRS cells. High LEF1 expression was reported to be a favorable prognostic factor in patients with cytogenetically normal acute myeloid leukemia [18]. However, the expression of LEF1 in the CHL cases included in our study did not correlate with the patients’ stages or survival.

The functions of Wnt5a in solid organ cancers remain controversial. Some studies dem-
onstrated that Wnt5a promoted metastasis in lung cancer and thyroid cancer, while other studies indicated its function as a tumor suppressor in colon cancer and thyroid cancer [4]. Knowledge of the roles of Wnt5a in CHL is limited. One study proved Wnt5a to be a motility-promoting factor as overexpression of Wnt5a stimulated migration of Hodgkin lymphoma cells in culture and promoted lymphoma engraftment and vascularization [10]. In our study, a total of 22 patients (44%) were positive for Wnt5a/b in HRS cells and Wnt5a/b expression was seen in a significantly higher percentage of stage IV patients (67%) compared to other stages, which was consistent with the above proposed function of Wnt5a. CHL is unique for its scattered tumor cells and complex microenvironment, which consists of non-malignant T and B lymphocytes, plasma cells, histiocytes, eosinophils, neutrophils, mesenchymal stromal cells and endothelial cells that surround the HRS cells. Tumor microenvironment can promote cancer progression through its influences on cell migration and angio-

Figure 4. Correlation of LEF-1 (A), FZD6 (B) and Wnt5a/b (C) expression with overall survival.
sis. The Vascular endothelial growth factor (VEGF) family is one of the downstream target genes of Wnt signaling. Linke et al. reported that LEF1 and β-catenin promoted angiogenesis in vitro by regulating the expression of VEGF-A. Analyses of VEGF-A expression in CHL patients demonstrated that low VEGF-A expression was associated with a better overall survival of CHL patients [9]. In a large cohort study of 194 CHL cases, Dimtsas et al. performed immunohistochemical evaluation of VEGF-A, VEGFR1 and VEGFR2 and found the HRS cells expressed VEGF-A, VEGFR1 and VEGFR2 in over 90% of cases. Although no correlation with patients’ outcome was observed, all 3 molecules were positively correlated with microvessel branching and VEGFR-2 was positively correlated with serum albumin levels ≥ 4 g/dL [19]. Touati et al. reported CD68+ tumor-associated macrophages were associated with the progression of CHL and affected response to treatment and OS [20]. However, in our study, the low CD68 expression group (< 25%) and the high CD68 expression group (≥ 25%) did not show statistical significance related to the stage or OS (data not shown). The correlation between LEF1, Wnt5a, CD68 and the VEGF family may be of interest for future studies.

In conclusion, multiple factors may be involved with the dissemination or migration of HRS cells, and our study further confirms the high levels of expression of LEF1 in the HRS cells in CHL. All the CHL cases included in this study lack β-catenin expression, indicating the β-catenin-dependent canonical Wnt signaling may not play a critical role in the tumorigenesis migration of HRS cells or progression of CHL. Wnt5a/b expression was correlated with a higher stage but not overall survival of the patients. Our case study is small; however, these findings indicate the non-canonical Wnt pathway may play a role as part of microenvironment that contributes to migration or dissemination of HRS cells. Based on our results, it is not sufficient to consider LEF1, β-catenin, FZD6 and Wnt5a/b or CD68 alone as prognostic factors for CHL.

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Disclosure of conflict of interest

None.

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