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# Notch 1 and NF-κB Expression and Clinical Correlation in Chinese Patients with Lymphoblastic Lymphoma

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## ABSTRACT

T-cell acute lymphoblastic leukemia/lymphoma (T-ALL/T-LBL) is commonly associated with Notch 1 mutations. There is limited data on the relationship between Notch l and NF-κB expression and clinical features in LBL. We evaluated the expression of Notch l and NF-κB in LBL using immunohistochemistry and analyzed their relationship with clinical characteristics, treatment results, and survival. From October 2000 to August 2008, 34 untreated patients with LBL were enrolled in the study. Median age was 11.8 years (range, 1 - 25 years). Twenty-five patients were diagnosed with T-LBL and 9 patients with B-LBL. Most patients received chemotherapy consisting of modified ALL-BFM-90. Notch l showed high expression in 68% of T-LBL and low expression in 100% of B-LBL ($p = 0.015$). High expression of Notch l positively correlated with presence of a mediastinal mass but not with 5-year event free survival (EFS) in T-LBL. NF-κB showed high expression in 65% of all patients with LBL, with no difference between T- and B-LBL. NF-κB expression was higher in T-LBL patients with bulky disease and B symptom; it did not correlate with 5-year EFS in T-LBL. Expression of Notch 1 and NF-κB strongly correlated ($p = 0.014$) in T-LBL. Notch 1 is highly expressed in T-LBL. NF-κB is highly expressed in all patients with LBL with no difference between T-LBL and B-LBL. Notch 1 expression was significantly associated with NF-κB expression in T-LBL. Notch l and NF-κB may play an important role in the development of T-LBL; further investigation is warranted.

**Keywords:** Lymphoblastic Lymphoma; Notch l; NF-κB

## 1. Introduction

Lymphoblastic lymphoma (LBL) is a highly invasive malignancy originating from immature precursor lymphocytes. It is divided into precursor T cell lymphoblastic lymphoma (T-LBL) and precursor B cell lymphoblastic lymphoma (B-LBL). LBL and acute lymphoblastic leukemia (ALL) share common morphologic and immunophenotypic features as well as a favorable outcome after ALL-type chemotherapy; they are thought to represent a spectrum of a single disease entity, which is termed T (or B) lymphoblastic leukemia/lymphoma in the World Health Organization (WHO) [1] classification schemes. Children and adolescents with LBL have better survival when treated with intensive chemotherapeutic regimens used in ALL, with a 5-year event free survival of 70% - 90% [2,3]. However, 5-year event free survival is less than 50% in high risk and refractory patients in spite of intensive chemotherapy. New biological markers and targeted therapies are need for these refractory LBL patients.

In 1991, human Notch 1 was identified at the chromosomal breakpoint of a subset of T lymphoblastic neoplasm at the t (7;9) (q34;q34.3) chromosomal translocation [4]. Mammals have four Notch receptors (Notch 1 - 4) [5]. Notch l is a member of the Notch transmembrane bound receptor family. Being a signaling pathway, Notch 1 not only plays an important part in differentiation and development of normal cells, but also in the germination and growth of neoplastic lesions, and as a suppressor and/or promoter in various tumors [5,6]. Many studies report Notch 1 mutations in greater than 50% of human T-ALL cases [7-10]. G-secretase inhibitors may inhibit the growth of T-ALL through blockage of the Notch 1 signal pathway [11]. Activation of Notch 1 leads to the development of T-ALL in a mouse model [12].

NF-kappa B (NF-κB) was identified in B lymphocytes in 1986. It acts as a regulator of κB light chain expression in B cells, and was thus termed nuclear transcription factor-κB (NF-κB) [13]. NF-κB can promote the germination, proliferation, invasion, and metastasis of tumors

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through regulation of gene transfer [14]. The NF-κB pathway is a major downstream target of Notch 1 in T-cell leukemia and is highly active in established human T-ALL. Inhibition of this pathway can efficiently restrict tumor growth both *in vitro* and *in vivo*. Thus NF-κB is one of the major mediators of Notch 1-induced transformation, suggesting that the NF-κB pathway is a potential target for future T-ALL therapies [15].

The above mentioned studies focused on T-ALL and less on LBL. Although ALL and LBL are the same disease entity, they show some differences in clinical characteristics. The expression of Notch-1 and NF-kappa B in precursor lymphoid neoplasms has been widely explored. However, the data regarded mainly leukemic presentation while studies on lymphoblastic lymphoma are scarce. Our research aimed to detect expression of Notch l and NF-κB in tissues of LBL and normal lymph nodes using immunohistochemistry. We also analyzed the relationship between expression of these markers and clinical characteristics and prognosis, aiming to provide evidence for their potential use as biological markers.

## 2. Materials and Methods

### 2.1. Materials

From October 2000 to August 2008, the tumor samples from 34 untreated patients with lymphoblastic lymphoma at Sun Yat-Sen University Cancer Center were obtained. Patients included 30 males and 4 females with a median age of 11.8 years (range, 1 - 25 years; 1 - 18 years: 29 cases; 18 - 25 years: 5 cases). Twenty-five patients were diagnosed with T-LBL and 9 with B-LBL. According to the St. Jude staging system, our patient sample include 1 stage I, 4 stage II, 12 stage III, and 17 stage IV patients. Nine patients had B symptoms, 9 had large masses, 17 had bone marrow involvement, 22 had an anterior mediastinal mass, 9 had extranodal lesions, and 20 had increased serum lactate dehydrogenase (LDH) levels. Thirty-one patients were treated according to the modified ALL-BFM-90 protocol, 2 patients received CHOAP, and 1 patient abandoned treatment. Five normal lymph nodes were obtained from October 2007 to October 2008 to serve as a control group. The informed consent of patients or their guardian had been obtained.

### 2.2. Immunohistochemistry

The tissue was stained by a two-step immunohistochemistry staining method. The first antibody for Notch 1 staining was goat anti-human antibody from Santa Cruz (working concentration 1:100) and the first antibody for NF-κB staining was mouse anti-human antibody from Santa Cruz (working concentration 1:150). The second antibody for both stains was derived from an instant detection kit (PV-6003) from Zhongshan Goldenbridge Biotechnology Co., Ltd.

### 2.3. Microscopic Evaluation

Using the semi-quantitative evaluation method by Carcangiu *et al*. [16], membrane, plasma, and nuclear details of tumor cell were observed microscopically and evaluated for staining degree and number of positive cells. The staining degree of cells was defined as colorless, light tan, tan, or brown and was assigned a score of 0, 1, 2, or 3, respectively. The number of positive cells was defined as <5%, 5% - 35%, 36% - 70%, or >70% and was assigned a score of 0, 1, 2, or 3, respectively. Results were assessed by the product of the two scores as follows: ≤1 as negative (−), 2 - 3 as weak positive (+), 4 - 5 as median positive (++), and ≥6 as strong positive (+++). Negative and weak positive (+) were considered low expression, and median positive (++) and strong positive (+++) were considered high expression.

### 2.4. Follow-Up

Follow-up studies were performed at three-month intervals after ending maintenance therapy during the first year, six-month intervals during the second year, and one-year intervals during the following three years. Event free survival (EFS) was calculated from the first day of chemotherapy to death due to any cause, relapse, progression, second malignancy, or the date of the last follow-up contact for patients who were alive.

### 2.5. Statistical Analysis

Differences between two sample rates were analyzed with the Fisher exact test two-sided for discrete variables. The correlation between two nonparametric samples was analyzed with spearman correlation test. Kaplan-Meier test was implemented in survival analysis. The statistical analysis was performed using SPSS statistics (version 16.0; SPSS). P values were considered significant at less than 0.05.

## 3. Results

### 3.1. Expression of Notch-1 and NF-Kappa B in T-LBL Compared to B-LBL

Notch 1 protein was expressed in the cytomembrane and cytoplasm. High expression of Notch-1 was found in 50% (17/34) of all LBL cases. In T-LBL, high expression was seen in 68% (17/25) and low expression in 32% (8/25). In B-LBL, 100% (9/9) showed low expression. Five normal lymph nodes also showed low expression. Expression of Notch l was significantly different between T-LBL and B-LBL (p = 0.001); expression was also sig-

 

nificantly different when comparing T-LBL and normal lymph nodes (p = 0.009). B-LBL and normal lymph nodes all showed low expression of Notch 1 (**Table 1** and **Figure 1**).

NF-κB protein was expressed in the cytoplasm and nucleus. Low expression was seen in 35% (12/34) and high expression in 65% (22/34) of all LBL cases. In T-LBL, low expression occurred in 32% (8/25) and high expression in 68% (17/25). In B-LBL, low expression was seen in 44% (4/9) and high expression in 56% (5/9). There was no difference in NF-κB expression between T-LBL and B-LBL, p = 0.687 (**Table 2** and **Figure 2**).

We analyzed correlation of Notch-1 and NF-κB expression in T-LBL and found 14 cases with high expression of Notch1 also presented with high expression of NF-κB. Notch 1 expression correlated significantly with NF-κB expression in T-LBL, p = 0.014, no correlated was seen in B-LBL (**Table 3**).

### 3.2. Association of Notch-1 and NF-κB Expression with Clinical Characteristics of T-LBL

We analyzed T-LBL by one factor correlation analysis and found that high Notch l expression correlated significantly with the presence of a mediastinal mass (p = 0.046). However, Notch l expression did not significantly correlate with gender, age, extranodal lesions, bulky disease, LDH level, or presence of B symptoms (p > 0.05). NF-κB expression was positively related to bulky disease and B symptom (p = 0.013 and 0.000, respectively). NF-κB expression did not correlate with other clinical

**Table 1. Expression of Notch 1 in LBL and normal lymph nodes.**

| Samples | Case (n) | Low expression | | High expression | | p-value |
|---|---|---|---|---|---|---|
| | | Case (n) | Percent | Case (n) | Percent | |
| T-LBL | 25 | 8 | 32 | 17 | 68 | 0.001 |
| B-LBL | 9 | 9 | 100 | 0 | 0 | |
| T-LBL | 25 | 8 | 32 | 17 | 68 | 0.009 |
| Normal lymph nodes | 5 | 5 | 100 | 0 | 0 | |
| B-LBL | 9 | 9 | 100 | 0 | 0 | No |
| Normal lymph nodes | 5 | 5 | 100 | 0 | 0 | |

**Table 2. Expression of NF-κB in LBL and normal lymph nodes.**

| | Case (n) | Low expression | | High expression | | p-value |
|---|---|---|---|---|---|---|
| | | Case (n) | Percent | Case (n) | Percent | |
| T-LBL | 25 | 8 | 32 | 17 | 68 | 0.687 |
| B-LBL | 9 | 4 | 44 | 5 | 56 | |
| T-LBL | 25 | 8 | 32 | 17 | 68 | 0.009 |
| Normal lymph nodes | 5 | 5 | 100 | 0 | 0 | |
| B-LBL | 9 | 4 | 44 | 5 | 56 | 0.086 |
| Normal lymph nodes | 5 | 5 | 100 | 0 | 0 | |

**Table 3. Correlation of Notch-1 and NF-κB expression in T-LBL and B-LBL.**

| **Notch 1 expression** | **NF-κB expression** | | | | R value | p value |
|---|---|---|---|---|---|---|
| | − | + | ++ | +++ | | |
| T-LBL − | 0 | 2 | 2 | 0 | 0.485 | 0.014 |
| + | 1 | 2 | 1 | 0 | | |
| ++ | 3 | 0 | 1 | 3 | | |
| +++ | 0 | 0 | 6 | 4 | | |
| B-LBL − | 2 | 0 | 3 | 0 | 6.3 | 0.098 |
| + | 1 | 1 | 0 | 2 | | |
| ++ | 0 | 0 | 0 | 0 | | |
| +++ | 0 | 0 | 0 | 0 | | |

 

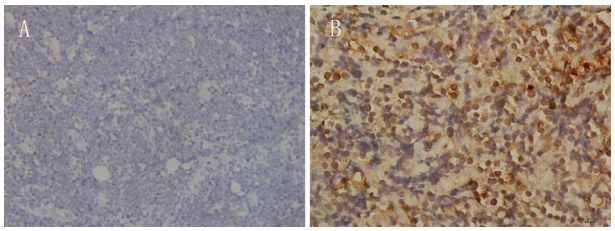


**Figure 1. Notch 1 expression in lymphoblastic lymphoma (400×). (A) Low expression of Notch1; (B) High expression of Notch1.**

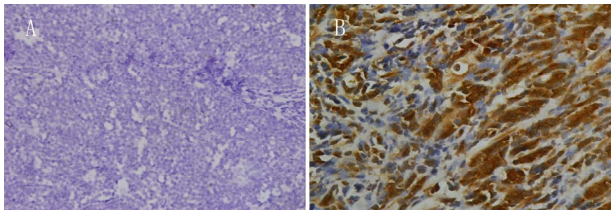


**Figure 2. NF-κB expression in lymphoblastic lymphoma (400×). (A) Low expression of NF-κB; (B) High expression of NF-κB.**

characteristics (**Table 4**).

### 3.3. Relationship between Notch 1 and NF-κB Expression and Survival in T-LBL

In the 25 patients with T-LBL, median follow up was 55 months (range, 11 - 142 months). Twenty-one patients were alive and 4 had died from disease. Five-year event free survival was 82% ± 8.1%. In the Notch l high expression and low expression groups, 5-year event free survival rates were 80.2% ± 10% and 87.5% ± 11%, respectively (p = 0.80) (**Figure 3**). The 5-year event free survival in the NF-κB high expression and low expression groups were 86.3% ± 9% and 75.0% ± 15%, respectively (p = 0.32) (**Figure 4**).

## 4. Discussion

Deregulated Notch signaling has been implicated in many diseases, but the clearest example of a pathogenic role is found in T-cell lymphoblastic leukemia/lymphoma, where the majority of human and murine tumors have acquired mutations that lead to aberrant increase in Notch 1 signaling. Many studies report Notch 1 mutations in greater than 50% of human T-ALL/T-LBL cases [7-10]. Most of these studies detected Notch 1 mutation by gene sequencing. Notch l gene mutation may result in overexpression of Notch 1 protein [17]. Immuohistochemical detection of Notch 1 protein is a very common and simple method in clinical practice. Notch 1 is a transmembrane bound receptor expressed in the cytomembrane and cytoplasm. Notch 1 antibody, which targets the Notch 1 protein, can be used to detect expression of Notch 1 by immunohistochemistry. Franziska identified Notch 1 protein expression in 90 cases of lymphoma by immunohistochemistry and found a 100% positivity rate in classic Hodgkin lymphoma and anaplastic large cell lymphoma but no expression in B cell lymphoma [18]. Kamstrup also detected the Notch1 expression in cutaneous T-cell lymphoma by using immunohistochemistry and found the prominent expression in 21/40 cases, the expression of Notch increased with the more advanced stage [19]. In the present study we found high expression of Notch l in 68% of T-LBL and low expression in 100% of B-LBL. Although we did not evaluate for Notch 1 gene mutation in our patients, our finding of high Notch 1 protein expression in T-LBL is similar to the results of Notch 1 mutation analysis in many studies reporting mutations in more than 50% of T-ALL/LBL cases [7-10]. These results confirm that Notch l may play an important role in the germination and proliferation of T-LBL but not B-LBL. Detection of Notch 1 expression by immunohistochemistry may be supplementary to understanding deregulated Notch l signaling in T-LBL.

We analyzed the relationship between Notch 1 expression and clinical features in patients with T-LBL and found that Notch l expression positively correlated with the presence of a mediastinal mass. Most T-LBL patients with an anterior mediastinal mass showed high expression of Notch 1. Zhu [20] reported that Notch l mutations were significantly associated with higher blast counts in the peripheral blood at diagnosis in T-ALL. In spite of the similarities between T-ALL and T-LBL, there are also obvious differences in their clinical features. It is interesting that the predominant manifestation in T-LBL patients is an anterior mediastinal mass, while in patients with T-ALL bone marrow involvement is the predominant site of disease. Thus both findings, *i.e*. Notch 1 mutation correlating with higher blast counts in the peripheral blood in T-ALL and with mediastinal mass in T-LBL, show that Notch 1 signaling may play an important role in development of T-ALL/T-LBL.

Multiple clinical series confirm that Notch 1 mutations in T-ALL/LBL are frequent in all genetic and clinical subtypes of human T-ALL/LBL, leading investigators to ask if mutational status is a useful biomarker. However, associations between mutation status and outcome are inconsistent. While a few series suggest that Notch 1 mutations are associated with worse outcomes [20], some studies have shown no association [21,22], and the BFM groups studies reported that Notch 1 mutations correlate with better treatment response and overall survival in T-ALL [8,10]. Callens reported that Notch 1 and/or FBXW7 mutations were associated with improved event-free survival and overall survival in pediatric T-lymphoblastic lymphoma, patients with NOTCH1 mutation demonstrated a significantly better outcome than NOTCH1 wild type patients with 96% (SE, 0.04) versus 53% (SE,

 

**Table 4. Notch 1 and NF-κB expression and clinical characteristics in T-LBL.**

| | | n | Notch 1 Expression | | p value | NF-κB Expression | | p value |
|---|---|---|---|---|---|---|---|---|
| | | | Low | High | | Low | High | |
| Sex | Male | 22 | 4 (18%) | 18 (82%) | 0.420 | 4 (18%) | 18 (82%) | 0.420 |
| | Female | 3 | 0 (0%) | 3 (75%) | | 0 (0%) | 3 (100%) | |
| Age | ≤18 | 21 | 4 (23%) | 17 (77%) | 0.341 | 4 (18%) | 17 (82%) | 0.341 |
| | >18 | 4 | 0 (0%) | 4 (100.0%) | | 0 (0%) | 4 (100%) | |
| Mediastinal mass | No | 5 | 3 (60%) | 2 (40%) | 0.046 | 0 (0%) | 5 (100%) | 0.275 |
| | Yes | 20 | 1 (5%) | 19 (95%) | | 4 (20%) | 16 (80%) | |
| LDH | Normal | 8 | 0 (0%) | 8 (100%) | 0.134 | 2 (25%) | 6 (75%) | 0.400 |
| | High | 17 | 4 (23%) | 13 (77%) | | 2 (12%) | 15 (88%) | |
| Extralymph node | 0 | 20 | 4 (20%) | 16 (80%) | 0.275 | 4 (20%) | 16 (80%) | 0.275 |
| | ≥1 | 5 | 0 (0%) | 5 (100%) | | 0 (0%) | 5 (100%) | |
| Stage | III | 11 | 2 (18%) | 9 (82%) | 0.386 | 4 (36%) | 7 (64%) | 0.659 |
| | IV | 13 | 5 (39%) | 8 (62%) | | 3 (23%) | 10 (77%) | |
| B symptoms | No | 20 | 4 (20%) | 16 (80%) | 0.275 | 0 (0%) | 20 (100%) | 0.000 |
| | Yes | 5 | 0 (0%) | 5 (100%) | | 4 (80%) | 1 (20%) | |
| Bulky | No | 17 | 2 (12%) | 15 (88%) | 0.088 | 0 (0%) | 17 (100%) | 0.013 |
| | Yes | 8 | 2 (25%) | 6 (75%) | | 4 (50%) | 4 (50%) | |

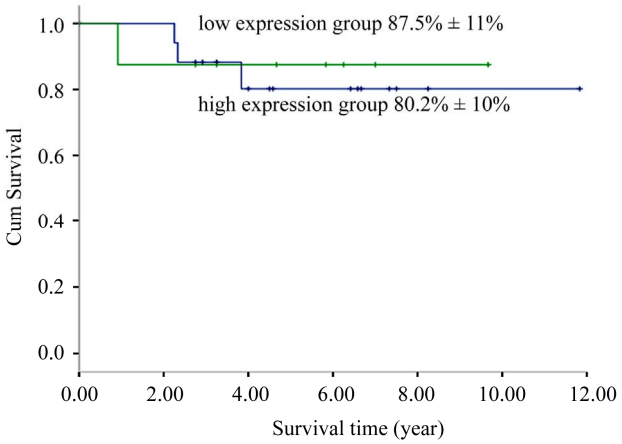


**Figure 3. Survival curve and expression of Notch 1 in T-LBL.**

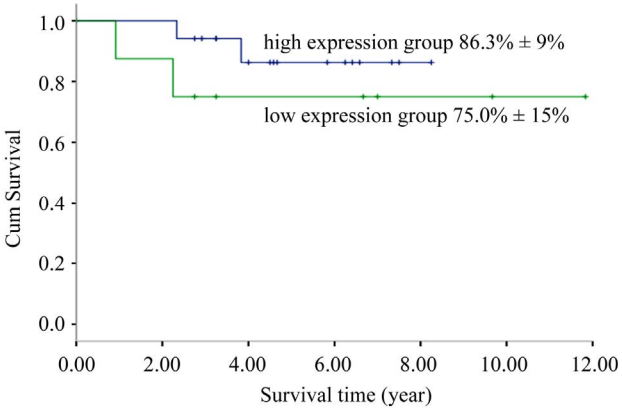


**Figure 4. Survival curve and expression of NF-κB in T-LBL.**

0.11) 5-year EFS, respectively, (p = 0.002) [23], all these patients received the BFM protocol treatment. It showed the Notch1 mutation status were associated with survival in T-ALL/LBL patients who received BFM protocol. So far the BFM protocol is one of best protocols in treatment of pediatric ALL/LBL. In our study, we only detected the expression of Notch 1 in T-LBL, no detecting the Notch1 mutation, and found that Notch l expression did not relate to 5-year event free survival in T-LBL. Through high expression of Notch1 were found in our patients, but the sample was small and it did not get a well evaluation when comparing the association between the expression and survival. Moreover, the relationship between Notch l mutation and expression needs to further study.

Notch-related T-ALL/LBL is also associated with constitutive activation of NF-κB [24,25]. NF-κB is one of the important nuclear transcript regulating factors and plays a role in the onset of T-ALL as a downstream regulator of Notch 1. Blocking this pathway can suppress carcinoma growth, implying that NF-κB may be a future treatment target in T-ALL [26]. To produce these therapeutic agents, the relationship between NF-κB and Notch 1 expression in LBL should be understood. Up to now, only a few reports on the expression of NF-κB in LBL exist. In our research, we found that high expression of NF-κB occurred in 65% of all LBL cases. There was no difference in expression of NF-κB between T-LBL and B-LBL. High expression of NF-κB was significantly related to bulky disease and B symptom. These findings show that NF-κB is highly activated in LBL and closely related to the germination and proliferation of LBL. We

 

analyzed the relationship between expression of NF-κB and Notch 1 in LBL and found there was a significant positive correlation between the expression of Notch 1 and NF-κB in T-LBL ($p = 0.014$). Our results showed that high expression Notch 1 was accompanied by high expression of NF-κB in T-LBL, and both have well correlation. This supports the hypothesis that the Notch 1 signaling pathway is associated with constitutive activation of NF-κB and demonstrates that NF-κB may be a signal transduction factor downstream of the Notch 1 pathway active in the germination of T-LBL. In contrast, we found low expression of Notch1 and high expression of NF-κB in B-LBL, and no correlation between the expression of Notch 1 and NF-κB in B-LBL, It suggesting that NF-κB might be activated via other pathways in B-LBL to result in tumor proliferation.

When analyzing the relationship between NF-κB expression and clinical characteristics of T-LBL, we found NF-κB expression to be higher in T-LBL patients with bulky disease and B symptoms. On the other hand, no correlation was found between NF-κB expression and sex, age, or extranodal disease. Bavi detected expression of NF-κB in 203 cases of diffuse large B cell lymphoma (DLBCL) using immunohistochemistry and reported that NF-κB expression occurred in 25.6% (52/203) of DLBCL tumors, was associated with activated B cell (ABC) phenotype, and showed a significantly poorer overall survival as compared to those without NF-κB expression [27]. A study in laryngeal squamous cell carcinoma also showed that overexpression of NF-κB was associated with worse overall survival and was an independent prognostic factor [28]. In our study we found that NF-κB expression did not correlate with 5-year event free survival in T-LBL. However, our sample size was small and further investigation is warranted.

This study showed that Notch 1 is highly expressed in T-LBL and weakly expression in B-LBL. NF-κB was highly expressed in LBL with no difference between T-LBL and B-LBL. Notch 1 expression was significantly associated with NF-κB expression in T-LBL. Notch l and NF-κB may play important roles in the germination and development of T-LBL and are potential therapeutic targets worthy of additional investigation.

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