Research Article

Increased LEF1 Expression and Decreased Notch2 Expression Are Strong Predictors of Poor Outcomes in Colorectal Cancer Patients

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Background/Objective. We aimed to examine the expression of lymphoid enhancer factor 1 (LEF1) and Notch2 in colorectal cancer (CRC) and their association with clinicopathologic variables and CRC patients’ prognosis. Methods. Immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blot analysis were performed to assess the expression of LEF1 and Notch2 in 184 patients with CRC. Results. We observed a strong negative correlation between LEF1 expression and Notch2 expression ($P < 0.001$). Both LEF1 mRNA and protein expression increased while the Notch2 mRNA and protein expression decreased in tumor specimens compared with the matched paratumorous normal tissue ($P < 0.001$). An increase in LEF1 protein expression was significantly associated with lymph node metastases, distant metastasis, advanced TNM (tumor-node-metastasis) stage, and shorter overall survival. A decrease in Notch2 protein expression was associated with poorly differentiated tumors, lymph node metastases, distant metastasis, advanced TNM stage, and shorter overall survival of patients. In the multivariate Cox regression analysis, the LEF1 protein expression ($P < 0.001$), Notch2 protein expression ($P < 0.001$), TNM stage ($P < 0.001$), and the combination of increased LEF1 protein coexpression and decreased Notch2 protein coexpression ($P < 0.001$) were found to be independent prognostic indicators in CRC. Conclusion. Our results suggest that increased LEF1 coexpression and decreased Notch2 coexpression represent a risk factor for poor overall survival of CRC patients.

1. Introduction

Over the last few decades, a significant decline in cancer-related mortality of colorectal cancer (CRC) has been observed due to the considerable progress in the diagnosis and treatment, but CRC still remains a major public health problem throughout the world. Colorectal cancer is ranked second within cancer-related deaths in the United States [1, 2] and the fourth in China [3]. Therefore, finding new molecular biomarkers is necessary to improve the prognosis of CRC, as well as to create new treatment strategies and improve clinical outcome.

The Wnt and Notch signaling pathways have been shown to play a major role in intestinal morphogenesis and homeostasis [4–7]. The Wnt signaling pathway primarily regulates the self-renewal of the intestinal epithelium, and a high incidence of gastrointestinal malignancies might be induced by deregulation of this self-renewal processes [8]. Moreover, several members of the Wnt signaling pathway, either tumor suppressors APC and Axin2 or oncogene β-catenin and lymphoid enhancer factor-1 (LEF1), are often aberrantly activated in CRC development [9]. The inappropriate activation of the Wnt signaling pathway and the subsequent formation of nuclear LEF1/TCF/β-catenin complexes lead to uncontrolled downstream target gene activation and ultimately result in the malignant transformation of cells [10]. In addition, transcription factor LEF1 is a potential candidate biomarker for CRC since it serves as a key role in the regulation of many important cellular functions, including proliferation [11], growth [12], survival [13], mobility [14], and angiogenesis [15].

The Notch signaling pathway maintains the balance between cell proliferation, differentiation, and apoptosis [16]. Furthermore, it has been reported that the Notch signaling pathway promotes cell survival, angiogenesis, and resistance
Table 1: Results of LEF1 and Notch2 immunohistochemical analysis in tumor tissue in relation to the clinicopathologic characteristics of CRC patients and their tumors.

| Characteristic               | n   | LEF1 expression | P    | Notch2 expression | P    |
|-----------------------------|-----|-----------------|------|-------------------|------|
|                             |     | Low            |      | High              |      |
| Total                       | 184 | 58             |      | 126               | 0.330|
| Age (years)                 |      |                | 0.634|                   |      |
| <60                         | 72  | 26             |      | 46                |      |
| ≥60                         | 112 | 32             |      | 80                |      |
| Gender                      |      |                | 0.339|                   | 0.640|
| Male                        | 104 | 36             |      | 68                |      |
| Female                      | 80  | 22             |      | 58                |      |
| Tumor location              |      |                | 0.858|                   | 0.200|
| Right colon                 | 40  | 12             |      | 28                |      |
| Left colon                  | 46  | 16             |      | 30                |      |
| Rectum                      | 98  | 30             |      | 68                |      |
| Histology (differentiation) |      |                | 0.071|                   | <0.001|
| Well                        | 86  | 24             |      | 62                |      |
| Moderate                    | 68  | 28             |      | 40                |      |
| Poor                        | 30  | 6              |      | 24                |      |
| Node metastasis             |      |                | 0.001|                   | <0.001|
| N₀                          | 80  | 36             |      | 44                |      |
| N₁₋₃                       | 104 | 22             |      | 82                |      |
| Distant metastasis          |      |                | <0.001|                  | <0.001|
| No                          | 144 | 56             |      | 88                |      |
| Yes                         | 40  | 2              |      | 38                |      |
| TNM stage                   |      |                | <0.001|                  | <0.001|
| I                           | 14  | 6              |      | 8                 |      |
| II                          | 56  | 12             |      | 44                |      |
| III                         | 74  | 36             |      | 38                |      |
| IV                          | 40  | 4              |      | 36                |      |

Based on these findings, the Wnt and Notch signaling pathways have been established as important key mediators of intestinal tumorigenesis. Nevertheless, the relationship between the combination of LEF1 status and Notch2 status and their prognostic relevance in CRC has yet to be established. Therefore, the aim of this study was to examine the potential crosstalk between these two pathways and their possible synergistic effects in tumorigenesis. We have evaluated the significance of LEF1 expression and Notch2 expression and their crosstalk in CRC. In addition, we have examined the association of these two potential biomarkers with the clinicopathologic characteristics and survival of patients.

2. Materials and Methods

2.1. Ethics Statement. Tissue collection was compliant with the agreement of the Conduct of Human Ethics Committee of the First Affiliated Hospital, College of Medicine of Xi’an Jiaotong University. Written informed consent was obtained from each patient.

2.2. Patients and Specimens. One-hundred and eighty-four patients with colorectal cancer who underwent curative surgery without chemotherapy and/or radiotherapy treatment at the First Affiliated Hospital, College of Medicine of Xi’an Jiaotong University from 2007 to 2008 were enrolled in this study. The study included 104 men and 80 women aged between 30 and 78 years (mean, 54 years). In addition to tumor tissue, 184 samples of matched paratumorous normal colorectal tissue (at 5 cm distance from the tumor) were taken from the same patients.

The main clinicopathologic data are presented in Table 1. The pathological types of all the specimens were confirmed by two independent pathologists under double-blinded conditions. Tumors were classified according to the criteria from the TNM Union for International Cancer Control (UICC), while tumor cellular differentiation (TCD) was defined by Edmondson’s classification. The followup for all cases was terminated in February of 2013. During survival analysis, cases were regarded as censored data when patients were either lost to followup or died of causes other than CRC.

2.3. Immunohistochemistry. Formalin fixed paraffin-embedded sections were deparaffinized, rehydrated, and incubated
with 3% hydrogen peroxide. Next, the sections were heated in a microwave oven for 3 min at 100°C for antigen retrieval. Slides were then incubated with blocking serum and primary antibodies for LEF1 (1:100, C12A5; Cell Signaling Technology, Danvers, MA) and Notch2 (1:200, D76A6; Cell Signaling Technology, Danvers, MA), overnight at 4°C. The immunohistochemical reaction was visualized with 0.05% 3’,3’-diaminobenzidine tetrahydrochloride (DAB) followed by a counterstaining with hematoxylin. Finally, the sections were examined and analyzed using a microscope (Q550CW; Leica, Mannheim, Germany). For the negative controls, the sections were incubated with preimmune rabbit serum instead of the primary antibodies. Immunostaining was defined independently by two pathologists blinded to the clinical data and scored by multiplying the intensity of staining and the percentage of the stained tumor cells. The intensity of staining was graded as follows: 0 (colorless), 1 (pallide-flavens), 2 (yellow), and 3 (brown). The percentage of the stained tumor cells was graded as 0 (<5%), 1 (5%–25%), 2 (26%–50%), 3 (51%–75%) and 4 (>75%). The final score was ranged from 0 to 12. A score of 0 was defined as negative expression, scores 1–4 as “weak expression,” scores 5–8 as “moderate expression,” and scores 9–12 as “strong expression” [21]. For the purpose of further analysis, the samples with score 0–4 were defined as low expression, while the samples with scores 5–12 were grouped and defined as high expression [22]. Few specimens with inconsistent score were re-evaluated again by two pathologists until the agreement was reached.

2.4. Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis. The total mRNA was extracted from fresh tissue samples with TRIzol reagent according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA). Less than 500 ng of total RNA were used for complementary DNA synthesis with an SYBR ExScript RT-PCR kit (Takara, Dalian, China). The reaction was performed using the iQ5 Multicolour Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and SYBR Premix Ex Taq TM II (Takara). The primer sequences used for LEF1 and Notch2 analysis were as follows:

LEF1 forward 5’-AGCGAATGTGGTGGTGTCTGGA-3’, reverse 5’-CTCTTGCAGACCAGCTTGGCTAA-3’, Notch2 forward 5’-CTACAGTCTGCTGCCTGTC-3’, reverse 5’-GTGGAGAGGGCAGCTTGGA-3’, respectively.

GAPDH was used as the internal housekeeping gene control, and the primer sequences were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5’-ATGGGGAAGGTGATGC3’, reverse 5’-GGGTATCTGATGGAACAATATC-3’. For each real-time RT-PCR reaction, a dissociation curve analysis was performed with each reaction in triplicate. The data were acquired as a threshold cycle ($\Delta C_t$) value. The $\Delta C_t$ values were determined by subtracting the average internal housekeeping gene $C_t$ value from the average target gene $C_t$ value. Since the amplification efficiency of the target genes and internal control gene was equal, the relative gene expression in the CRC tissues compared with paratumorous normal colorectal tissues was calculated using the $2^{-\Delta\Delta C_t}$ method, where $\Delta C_t = C_t$ (cancer tissue) − $C_t$ (paratumorous normal tissue).

2.5. Western Blot. The tissue samples used in Western blot analysis were lysed with cell lysis buffer as previously described [23]. Equal amounts of protein were separated by 6%–12% SDS-PAGE and were electroblotted onto polyvinylidene difluoride membranes (Millipore, Danvers, MA), which were blocked with 5% blocking buffer and subsequently incubated with the following primary antibodies: anti-LEF1 antibody (1:800, C12A5; Cell Signaling Technology, Danvers, MA, USA), anti-Notch2 (1:800, D76A6; Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin antibody (1:1000, sc-130301; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the blots were visualized using an ECL detection system (Millipore, Danvers, MA, USA). Qualitative analysis was used to define the negative expression (protein band, absent) or positive expression (protein band, present). Each experiment was performed three times.

2.6. Statistical Analysis. The associations between the LEF1 expression and Notch2 expression and each clinicopathologic parameter were examined using either the $\chi^2$ test or Fisher’s exact test (two sided). Student’s $t$-tests (independent samples $t$-test) or a $u$-test were adopted to determine the difference between two sample means. One-way analysis of variance (ANOVA) was used to assess the difference among three or four sample means, and Spearman’s rank test was used to assess the correlation between LEF1 expression and Notch2 expression. The survival rates were assessed by the Kaplan-Meier method and compared using the log-rank test. Multivariate analysis, using the Cox proportional hazards regression model, was performed to assess the prognostic value of the marker expression and clinicopathologic factors. All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, USA). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Expression of LEF1 in CRC Tissues. We have examined the LEF1 expression in 184 primary CRC tissues and paired paratumorous normal colorectal tissues. Among these 184 primary CRC tissues, 126 (68.5%) cases showed high LEF1 expression, while only 40 (21.7%) cases in matched paratumorous normal colorectal tissues. Immunohistochemical staining revealed a predominantly nuclear localization of LEF1 (Figure 1(a)). The results of immunohistochemical analysis showed that LEF1 expression was significantly higher in CRC tissue than LEF1 expression in the paratumorous normal colorectal tissue ($P < 0.001$). In addition, significant differences in LEF1 expression in tumor tissue were observed between tumors with node metastasis, distant metastasis and different TNM stages ($P = 0.001$, $<0.001$, $<0.001$, resp.) (Table 1). There was no significant association observed between the LEF1 expression and age, gender of patients, tumor location, or histology (Table 1). In addition to immunohistochemical analysis, real-time PCR analysis was used to
assess the LEF1 mRNA expression in 184 pairs of CRC tissues and paratumorous normal colorectal tissues. Our results showed that LEF1 mRNA was significantly increased in most CRC tissues compared with the paratumorous normal colorectal tissues \((P < 0.001)\), and the association between LEF1 mRNA expression and clinicopathologic factors was in accordance with the results of the immunohistochemical analysis (Table 2).

In addition, Western blot was used to confirm these results in the examined 184 paired tumor and corresponding normal tissues. The rate of positive LEF1 expression was 64.1% (118 out of 184) in CRC tissues and 20.1% (37 out of 184) in the matched paratumorous normal colorectal tissues. LEF1 positive expression was significantly higher in CRC tissues than that in the matched paratumorous normal colorectal tissues \((P < 0.01)\) (Figures 2(a) and 2(c)).

3.2. Expression of Notch2 in CRC Tissues. As shown in Table 1, only 64 out of 184 (34.8%) analyzed cancer tissues showed high Notch2 protein expression. In contrast, high Notch2 expression was detected in 142 (77.2%) out of 184 of paratumorous normal colorectal tissues, and Notch2 protein expression was observed in the membrane and/or cytoplasm of tissue cells (Figure 1(b)).

In our study, Notch2 expression presented a negative association with colorectal carcinomas \((P < 0.001)\). Low Notch2 expression was strongly correlated with poor differentiation status, node metastasis, distant metastasis, and TNM stage \((P < 0.001, \text{resp.})\) (Table 1).

In addition, Notch2 mRNA levels were significantly decreased in most CRC tissues compared with paratumorous normal colorectal tissues \((P < 0.001)\) (Table 2). In Western blot analysis, positive Notch2 expression was detected in only 40 out of 184 (21.7%) analyzed cancer tissues compared with 73.9% (136 out of 184) in paratumorous normal colorectal tissues. Notch2 positive expression was significantly lower in cancer tissues than that in matched paratumorous normal colorectal tissue \((P < 0.01)\) (Figures 2(b) and 2(c)). Furthermore, Notch2 mRNA expression was decreased in samples from patients with less differentiated tumors, node metastasis, distant metastasis, and of an advanced TNM stage \((P < 0.05)\) (Table 2), consistent with the results from immunohistochemical analysis.

3.3. Correlation between LEF1 Expression and Notch2 Expression in CRC. We analyzed the correlation between LEF1 expression and Notch2 expression in CRC at the protein level and mRNA level. Among the 184 analyzed CRC samples, 58 (31.5%) were LEF1-low whereas 126 (68.5%) were LEF1-high tumors. In Notch2 immunohistochemical analysis, 120 (65.2%) tumors were found to be Notch2 low, whereas 64 (34.8%) were found to be Notch2 high (Table 1). A significant negative correlation between the LEF1 expression and Notch2 protein expression in the CRC samples was observed \((r = -0.315, P < 0.001, \text{Spearman's rank test})\) (Figure 3). When analyzing Western blot data, we found a negative correlation between the LEF1 expression and Notch2 expression in the CRC tissues \((r = -0.430, P < 0.001, \text{Spearman's rank test})\). A similar correlation between the LEF1 and Notch2 mRNA expression in the CRC samples was also observed \((r = -0.571, P < 0.001, \text{Spearman's rank test})\).

3.4. Survival Analysis. All 184 patients were included in the survival analysis (with followup period of 5 years) to assess LEF1 expression and Notch2 expression as potential prognostic factors in CRC. The survival time of patients included in
Table 2: Results of LEF1 and Notch2 mRNA real-time PCR analysis in tumor tissue in relation to the clinicopathologic characteristics of CRC patients and their tumors.

| Characteristic         | n    | LEF1, mean (SD)  | P      | Notch2, mean (SD) | P      |
|------------------------|------|------------------|--------|-------------------|--------|
| Tissue type            |      |                  |        |                   |        |
| CRC tissues            | 184  | 3.7270 (1.7635)  | <0.001 | 0.5263 (0.3320)   | <0.001 |
| Paratumorous normal tissues | 184  | 0.8239 (0.2624)  |        | 1.8675 (0.4230)   |        |
| Age (years)            |      |                  |        |                   |        |
| <60                    | 72   | 3.6757 (1.5221)  | 0.245  | 0.5613 (0.4012)   | 0.292  |
| ≥60                    | 112  | 3.8617 (1.1551)  |        | 0.5038 (0.2875)   |        |
| Gender                 |      |                  |        |                   |        |
| Male                   | 104  | 3.7410 (1.6041)  | 0.879  | 0.5241 (0.4057)   | 0.712  |
| Female                 | 80   | 3.7088 (1.2707)  |        | 0.5417 (0.2362)   |        |
| Tumor location         |      |                  |        |                   |        |
| Right colon            | 40   | 3.6889 (1.3662)  | 0.611  | 0.5579 (0.2331)   | 0.365  |
| Left colon             | 46   | 3.5371 (1.4057)  |        | 0.5784 (0.4124)   |        |
| Rectum                 | 98   | 3.8317 (1.8993)  |        | 0.4992 (0.3346)   |        |
| Histology (differentiation) |     |                  | 0.609  | <0.001            |        |
| Well                   | 86   | 3.6022 (1.9496)  | <0.001 | 0.6760 (0.4661)   | <0.001 |
| Moderate               | 68   | 3.7840 (1.6342)  |        | 0.4514 (0.2112)   |        |
| Poor                   | 30   | 3.9557 (1.5231)  |        | 0.3002 (0.2214)   |        |
| Node metastasis        |      |                  |        |                   |        |
| N₀                     | 80   | 2.2058 (1.7023)  | <0.001 | 0.6510 (0.4138)   | <0.001 |
| N₁₋₃                   | 104  | 4.8971 (1.8106)  |        | 0.4304 (0.2691)   |        |
| Distant metastasis     |      |                  | <0.001 | <0.001            |        |
| No                     | 144  | 3.1840 (1.7412)  | <0.001 | 0.6084 (0.1059)   | <0.001 |
| Yes                    | 40   | 5.6818 (1.8334)  |        | 0.2557 (0.5460)   |        |
| TNM stage              |      |                  | <0.001 | <0.001            |        |
| I                      | 14   | 2.1012 (1.7731)  |        | 0.8526 (0.2113)   | <0.001 |
| II                     | 56   | 2.9875 (1.2111)  |        | 0.6752 (0.2547)   |        |
| III                    | 74   | 3.3415 (1.8521)  |        | 0.4369 (0.1821)   |        |
| IV                     | 40   | 6.0445 (1.9694)  |        | 0.3940 (0.6921)   |        |

This study ranged from less than four months to more than 60 months, and the median survival time was 51 months. The analysis of prognostic factors for survival is summarized in Table 3.

3.5. Kaplan-Meier Univariate Survival Analysis of Clinicopathologic Factors and LEF1 Protein Expression and Notch2 Protein Expression. In order to assess the prognostic significance of the LEF1 and Notch2 protein expression, Kaplan-Meier survival curves were established (Figures 4(a) and 4(b)), and the results of the log-rank tests for the clinicopathologic factors and LEF1 protein expression and Notch2 expression in CRC patients are summarized in Table 3. In the univariate analysis, a statistically significant association with shorter survival time was observed for patients with poor tumor differentiation status, advanced TNM stage of tumors, and the combination of increased LEF1 coexpression and decreased Notch2 coexpression (P < 0.05). The high LEF1 protein expression [Exp(B), 2.31; 95% CI, 1.15–4.64; P = 0.016] and low Notch2 protein expression [Exp(B), 2.26; 95% CI, 1.15–4.43; P = 0.017] were also proved to be associated with shorter survival and higher risk of death in patients with CRC. However, gender (P = 0.909), age (P = 0.698) or tumor location (P = 0.644; P = 0.587) had no prognostic value on survival of patients with CRC.

3.6. Multivariate Survival Analysis of Clinicopathologic Factors and LEF1 Protein Expression and Notch2 Protein Expression. In multivariate analysis, the Cox proportional hazards model was adjusted for gender, age, differentiation status, TNM stage, and LEF1 expression and Notch2 expression. As a result, LEF1 and Notch2 protein levels proved to be independent predictors of survival for patients with CRC, indicating that patients with a high LEF1 expression and low Notch2 expression had a higher risk of death than those with LEF1 (low)/Notch2 (high) tumors. Moreover, TNM stage and the combination of increased LEF1 coexpression and decreased Notch2 coexpression also proved to be prognostic factor for CRC patients (P < 0.05) (Table 3). There was no significant association found with the overall survival for other analyzed clinicopathologic factors.

3.7. Prognostic Significance of the Combined LEF1 Protein Expression and Notch2 Protein Expression Profile. The present results showed that LEF1 expression was higher in CRC
tissues compared to the corresponding paratumorous normal tissue, whereas Notch2 expression was lower in the tumor tissue than in the matching normal colorectal tissue. These results indicated that LEF1 and Notch2 might present an opposite function during the development of CRC. In addition, the Spearman’s rank test showed that there was a significant negative correlation between LEF1 expression and Notch2 expression in these tumors.

In order to estimate the prognostic significance of the combined LEF1 and Notch2 expression profile and to detect the LEF1 expression and Notch2 expression associations with overall survival, we have reclassified patients into the following four groups: LEF1 (low)/Notch2 (high) \((n = 33)\), LEF1 (high)/Notch2 (high) \((n = 31)\), LEF1 (low)/Notch2 (low) \((n = 25)\), and LEF1 (high)/Notch2 (low) \((n = 95)\). The subgroup analysis showed that patients with an LEF1 high expression and Notch2 low expression had a shorter overall survival time than all other combined status patients (Figures 4(c) and 4(d)). Multivariate analysis revealed that patients with high LEF1 expression and low Notch2 expression tumors had a significantly worse overall survival compared with patients of other combined LEF1/Notch2 expression groups (Table 3).
and Notch2 may be used to predict the clinical outcome.

high and low levels of LEF1 and Notch2 suggested that LEF1 significantly different outcomes between patients expressing 

nosis of CRC patients, consistent with our data. Nevertheless, with unfavorable clinicopathologic features and poor prognosis, patients’ survival, and tumors with high LEF1 expression had a shorter survival time [27]. In the study of Chu et al. [28], low Notch2 expression was associated with unfavorable clinicopathologic features and a poor prognosis of CRC patients, consistent with our data. Nevertheless, they did not compare it with the LEF1 expression. The significantly different outcomes between patients expressing high and low levels of LEF1 and Notch2 suggested that LEF1 and Notch2 may be used to predict the clinical outcome.

These findings are valuable in the providing potential therapeutic targets for the future treatment of CRC.

It has been shown that LEF1 is critical for the adhesion and/or migration of tumor cells, indicating that LEF1 is probably involved in tumor invasion and metastasis [29]. LEF1 protein belongs to a high mobility group (HMG) family, which has been implicated in DNA binding [30, 31]. In addition, it plays a pivotal role in carcinogenesis and the progression of CRC partly due to its involvement in the LEF1/β-catenin complex, a crucial effector of the Wnt signaling pathway. Our study established that increased LEF1 expression was correlated with node metastasis, distant metastasis, and the advanced TNM stage of tumors. These results have proven that LEF1 is involved in the invasion and metastasis of CRC, consistent with some early findings of other authors [32–34].

The Notch signaling pathway plays an essential role in the differentiation of the gastrointestinal tract [6, 35, 36] and can either have oncogenic or tumor suppressor functions in different cancers. Notch2 is one of the Notch receptors, which interacts with the DSL (Delta/Serrate/Lag-2) family of ligands to regulate the cell differentiation [37]. Notch2 has the highest homology with Notch1, but unlike Notch1, it can present a tumor-suppressive action in breast cancer [38, 39]. It has recently been reported that Notch2 is a novel target for β-catenin-dependent Wnt signaling [40]. In our study, low Notch2 mRNA and protein expression correlated with poor differentiation status, node metastasis, distant metastasis, and the advanced TNM stage in CRC. Therefore, a loss of Notch2 is a general feature during CRC progression, indicating a potentially conflicting role of LEF1 and Notch2 in this type of cancer.

It is known that alterations in the Wnt and Notch signaling pathways play a significant role in numerous cancers. Studies have shown that these two pathways are aberrantly activated in CRC [21, 28, 29]; however, the possible crosstalk between these two pathways in cancer development is unknown. Although an increasing body of evidence indicates that Wnt and Notch pathways crosstalk and transactivate each other in the normal tissue development as well as in cancer [41], such as in the survival of murine T-cell lymphomas [42], the mechanism by which Wnt pathway transactivates Notch pathway has not yet been fully determined. Therefore, further studies are necessary to disclose the possible convergent points of these two pathways in order to completely understand the relationship between the Wnt and Notch pathways in tumorigenesis.

In our study, we have examined the LEF1 and Notch2 mRNA and protein expression levels in a collection of 184 CRC patients stratified according to their outcomes. We showed the first, direct evidence of a negative correlation between LEF1 expression and Notch2 expression, as well as a high LEF1 expression and low Notch2 expression in relation with malignant CRC transformation. Moreover, high LEF1 protein expression and low Notch2 protein expression indicated a poorer prognosis in CRC. In viewing these results, it is understood that LEF1 has an oncogenic and Notch2 a tumor suppressor role during the development of CRC, consistent with the results of previous studies [42–44]. The possible reason for the functional diversity of LEF1 and Notch2 might

4. Discussion

In this study, we aimed to examine the expression of LEF1 and Notch2 in CRC and their association with clinicopathologic variables and CRC patients’ overall survival; therefore, an appropriate IHC scoring system and statistical method were very important for our study. We semiquantified the IHC staining for LEF1 and Notch2 ranging from 0 to 12, then classified score 0–4 as low expression and 5–12 as high expression and used these re-converted enumeration data for statistical analyses. We thought that the enumeration data were more suitable for the comparison between kinds of groups, especially for statistical analysis with respect to overall survival. There are several similar literature reports which use the evaluation of the converted IHC staining results before starting the statistical analysis, compared with the use of final score [24–26]. Our results indicate that LEF1 mRNA and protein expression were increased in CRC tissue and were correlated with the node metastasis, distant metastasis and the TNM stage of tumors. Most importantly, a statistically significant relation was observed between the LEF1 protein expression and patients’ survival, and those whose tumors were high LEF1 expression had a shorter survival time. Opposite to our findings, in the study by Kriegl et al., higher LEF1 expression was associated with longer patient survival time [27]. In the study of Chu et al. [28], low Notch2 expression was associated with unfavorable clinicopathologic features and a poor prognosis of CRC patients, consistent with our data. Nevertheless, they did not compare it with the LEF1 expression. The significantly different outcomes between patients expressing high and low levels of LEF1 and Notch2 suggested that LEF1 and Notch2 may be used to predict the clinical outcome.

The size of the points reflected the number of data points in each location (i.e., larger points reflect a greater number of data points at a given location).

**Figure 3:** A correlation between LEF1 immunohistochemical expression and Notch2 immunohistochemical expression in CRC. The dot figure showed a negative correlation between LEF1 expression and Notch2 expression (Spearman’s rank test $r = -0.315, P < 0.001$). X-axis: Notch2 expression level of each patient; Y-axis: LEF1 expression level of each patient. Some of the data was overlapping.
be in the regulation of different upstream proteins. Ungerbäck et al. [40] have reported increased Notch2 promoter activity upon the cotransfection of colon cancer cells with high expression recombinant LEF1. Based on these findings, we assumed that LEF1 either directly regulates Notch2 or another protein acting as a bridge, which then activates LEF1 and inhibits Notch2 activity. Nevertheless, the exact molecular mechanism of these processes will need to be determined in future studies.

We further examined whether the expression of LEF1 and Notch2 was associated with the survival of CRC patients. Although both LEF1 and Notch2 protein expression were independent prognostic factors, they had completely opposite effects on survival. Patients with a high LEF1 protein expression had a worse outcome prognosis than those with a low LEF1 expression, consistent with the oncogenic role of LEF1.

We have also investigated the protein expression pattern of Notch2 and opposite to LEF1 findings, patients with a loss of Notch2 expression had a higher risk of death than those with a higher Notch2 expression. Based on our results, it seems that either low LEF1 expression or high Notch2 expression might be predictors of good survival outcomes in CRC patients. Furthermore, we showed that the abnormal coexpression of LEF1/Notch2 was a more efficient predictor than any LEF1 expression or Notch2 expression when separately analyzed. The abnormal coexpression of LEF1 (high)/Notch2 (low) was correlated with the advanced TNM stage of tumors. Also, a worse disease outcome and an extremely poor survival rate were observed in patients with LEF (high)/Notch2 (low) tumors when compared to patients whose tumors showed either LEF1 high expression or Notch2 low expression. These findings imply that a combination of LEF1 expression and Notch2 expression could be an effective molecular prognostic marker in CRC. To our knowledge, this is the first study to demonstrate these findings.

In conclusion, we have shown the possible coregulation of Wnt and Notch signaling pathways through the association of LEF1 expression and Notch2 expression with some of the CRC clinical characteristics and their opposite prognostic roles in CRC. This combined status could potentially be used as an even more efficient prognostic predictor in CRC patients. Based on our results, we conclude that LEF1 and Notch2 play key roles in CRC tumorigenesis and could potentially represent new targets for the design of new antitumor therapies.

### Table 3: Association of LEF1/Notch2 and clinical factors with overall survival.

| Clinicopathologic characteristics | n   | Risk ratio\(^a\) (95% CI) | P    | Risk ratio\(^b\) (95% CI) | P    |
|-----------------------------------|-----|--------------------------|------|--------------------------|------|
| **LEF1**                          |     |                          |      |                          |      |
| Low                               | 58  | —                        |      | —                        |      |
| High                              | 126 | 2.31 (1.15–4.64)         | 0.016| 2.67 (1.31–4.85)         | <0.001|
| **Notch2**                        |     |                          |      |                          |      |
| High                              | 64  | —                        |      | —                        |      |
| Low                               | 120 | 2.26 (1.15–4.43)         | 0.017| 2.48 (1.22–4.80)         | <0.001|
| **Age**                           |     |                          |      |                          |      |
| <60                               | 72  | —                        |      | —                        |      |
| ≥60                               | 112 | 0.44 (0.23–0.87)         | 0.698| 0.35 (0.12–1.01)         | 0.053 |
| **Gender**                        |     |                          |      |                          |      |
| Female                            | 104 | —                        |      | —                        |      |
| Male                              | 80  | 0.98 (0.55–1.71)         | 0.909| 1.01 (0.97–1.06)         | 0.532 |
| **Tumor location**                |     |                          |      |                          |      |
| Right colon                       | 40  | —                        |      | —                        |      |
| Left colon                        | 46  | 1.17 (0.59–2.37)         | 0.644| 0.65 (0.13–1.27)         | 0.604 |
| Rectum                            | 98  | 0.79 (0.34–1.83)         | 0.587| 0.63 (0.18–1.25)         | 0.476 |
| **Histology (differentiation)**   |     |                          |      |                          |      |
| Well                              | 86  | —                        |      | —                        |      |
| Moderate                          | 68  | 1.45 (1.20–1.98)         | 0.001| 0.57 (0.19–1.69)         | 0.313 |
| Poor                              | 30  | 2.72 (2.11–3.42)         | <0.001| 0.55 (0.20–1.47)         | 0.231 |
| **TNM stage**                     |     |                          |      |                          |      |
| I                                 | 14  | —                        |      | —                        |      |
| II                                | 56  | 1.32 (0.29–6.14)         | 0.719| 1.19 (0.72–1.72)         | 0.256 |
| III                               | 74  | 2.88 (1.06–7.28)         | 0.038| 2.85 (1.13–7.44)         | 0.030 |
| IV                                | 40  | 2.76 (1.38–5.51)         | 0.004| 2.83 (1.68–4.74)         | <0.001|
| **LEF1 and Notch2**               |     |                          |      |                          |      |
| Other combinations                | 89  | —                        |      | —                        |      |
| LEF1 (H)/Notch2 (L)               | 95  | 3.40 (1.90–7.25)         | 0.006| 2.66 (1.98–3.32)         | <0.001|

\(^a\)Unadjusted risk ratio in univariate models.  
\(^b\)Adjusted risk ratio in multivariate models.  
CI: confidence interval.  
L: low expression; H: high expression.
Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contribution

Wen-Juan Wang and Yu Yao are equal contributors.

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