Retrospective Study

Prognostic value of vascular endothelial growth factor receptor 1 and class III β-tubulin in survival for non-metastatic rectal cancer

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Abstract

AIM
To assess the long-term prognostic value of vascular endothelial growth factor receptor 1 (VEGFR1) and class III β-tubulin (TUBB3) mRNA expression in non-metastatic rectal cancer.

METHODS
A total of 75 consecutive patients with non-metastatic rectal cancer from March 2004 to November 2008 were analyzed retrospectively at our institute. The mRNA expressions of VEGFR1 and TUBB3 were detected by multiplex branched DNA liquid-chip technology. The Cut-off Finder application was applied to determine cutoff point of mRNA expression. SPSS software version 22.0 was used for analysis.
RESULTS
The median follow-up was 102.7 mo (range, 6-153.6). The $\chi^2$ and Fisher’s exact tests showed that VEGFR1 expression was related to lymph node metastasis ($P = 0.013$), while no relationships between TUBB3 and clinicopathological features were observed. Univariate analysis showed that T stage, lymph node metastasis, tumor differentiation, VEGFR1 and TUBB3 mRNA expression were correlated to overall survival (OS) ($P = 0.048, P = 0.003, P = 0.052, P = 0.003$ and $P = 0.015$, respectively). Also, lymph node metastasis and VEGFR1 expression independently influenced OS by multivariate analysis ($P = 0.027$ and $P = 0.033$). VEGFR1 expression was positively correlated with TUBB3 ($P = 0.024$). The patients with low expression of both TUBB3 and VEGFR1 presented a better OS ($P = 0.003$). In addition, the receiver operating characteristic analysis suggested that the combination of lymph node metastasis and VEGFR1 had a more favorable prognostic value ($P < 0.001$).

CONCLUSION
VEGFR1 expression and lymph node metastasis independently and jointly affect survival. Moreover, low expression of VEGFR1 and TUBB3 presented a better OS in patients with non-metastatic rectal cancer, which might serve as a potential prognostic factor.

Key words: Rectal cancer; Class III $\beta$-tubulin; Vascular endothelial growth factor receptor 1; Overall survival

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Core tip: Nowadays, personalized and precision medicine becomes vital in cancer treatment. Herein, we focus on the long-term prognostic value of vascular endothelial growth factor receptor 1 (VEGFR1) and class III $\beta$-tubulin (TUBB3) mRNA expression in non-metastatic rectal cancer. In the 75 consecutive patients enrolled, we found that VEGFR1 expression and lymph node metastasis were independent factors influencing overall survival, and the combination of them showed a favorable prognostic value. Also, VEGFR1 expression was significantly related to lymph node metastasis. In addition, VEGFR1 expression was positively correlated with TUBB3 expression.

INTRODUCTION
Rectal cancer is one of the most diagnosed malignancies among both males and females worldwide with worse outcomes than colon cancer. Clinically, patients showed various outcomes to multimodality therapies. Nowadays, personalized and precision medicine has become essential in the treatment of rectal cancer. Recent studies conducted gene expression profiling to predict the response and long-term prognosis of malignancies; however, no consensus was achieved on prognostic gene profiling for rectal cancer.

Vascular endothelial growth factor (VEGF) possesses a significant role in angiogenesis by binding to VEGFR1 and VEGFR2, which is required for cancer progression and metastasis. A phase II trial indicated that VEGF could predict the pathological response to locally advanced rectal cancer patients treated with neoadjuvant cetuximab-based chemoradiation. In addition, class III $\beta$-tubulin (TUBB3) has been reported to play a critical role in tumor development and malignant transformation as a $\beta$-tubulin isotype. The variable levels of expression of the gene have been reported in colon, lung, ovary, kidney, prostate, and throat cancer with worse outcomes than colon cancer. Clinically, patients showed various outcomes to multimodality therapies.
Table 1  Patient characteristics

| Characteristics                        | Data, n (%) |
|----------------------------------------|-------------|
| Gender                                 |             |
| Female                                 | 36 (48)     |
| Male                                   | 39 (52)     |
| Age (yr) median (range)                |             |
| ≤ 60                                   | 52 (29.74)  |
| > 60                                   | 58 (77.3)   |
| Pre-CEA (ng/mL) ≤ 5                   | 36 (63.2)   |
| > 5                                    | 21 (36.8)   |
| Pre-Hb (g/L) ≤ 120                    | 26 (34.7)   |
| > 120                                  | 49 (65.3)   |
| Distance to verge (cm) ≤ 5             | 46 (61.3)   |
| > 5                                    | 29 (38.7)   |
| T stage                                |             |
| T1 + T2                                | 13 (17.3)   |
| T3 + T4                                | 63 (82.6)   |
| Lymph node metastasis                  |             |
| Negative                               | 22 (29.3)   |
| Positive                               | 53 (70.6)   |
| Venous invasion                        |             |
| Negative                               | 68 (90.7)   |
| Positive                               | 7 (9.3)     |
| Tumor differentiation                  |             |
| Poorly differentiated                  | 20 (26.7)   |
| Moderately-well differentiated          | 55 (73.3)   |
| Chemotherapy                           |             |
| No                                     | 9 (12)      |
| Yes                                    | 66 (88)     |
| TUBB3 expression                       |             |
| Low-expression                         | 39 (52)     |
| High-expression                        | 36 (48)     |
| VEGFR1 expression                      |             |
| Low-expression                         | 53 (70.7)   |
| High-expression                        | 22 (29.3)   |
| TUBB3 and VEGFR1                       |             |
| Both low expression                    | 32 (42.6)   |
| Others                                 | 43 (57.3)   |

Pre-CEA: Preoperative carcino-embryonic antigen; Pre-Hb: Preoperative hemoglobin.

was defined as the duration from the date of diagnosis to the last follow-up or the date of death due to any cause, which was obtained from the medical records and telephonic interviews.

**Multiplex branched DNA liquidchip technology**

The formalin-fixed and paraffin embedded (FFPE) tumor tissue specimens containing more than 70% of tumor cells were selected. The Multiplex branched DNA liquidchip (MBL) technology (Guangzhou SurExam BioTech Co., Ltd., China) was implemented to determine the mRNA expression levels of VEGFR1 and TUBB3. The FFPE tissue samples were lysed in the presence of proteinase K, at 56°C for 2 h. Then, the lysate was transferred to a 96-well plate containing the blocking reagent, capture beads with probes for VEGFR1 and TUBB3, and target gene-specific probe sets. The sandwich nucleic acid hybridization was carried out for 16 h. The unbound RNA was removed by three washes with buffer under a vacuum system. The signal bound to the target mRNA was amplified with a streptavidin-conjugated phycoerythrin solution at 50°C for 30 min. The fluorescence values of the samples were identified and analyzed using Luminex 200 system (Luminex, Austin, TX, United States), which were regarded as the RNA expression levels of each gene. The cutoff point of mRNA expression affecting the survival was determined by the Cutoff Finder application.

**Statistical analysis**

The end point of our analysis was OS. The association of gene expression level and clinicopathological features was studied by the χ² and Fisher’s exact tests. The association between the mRNA expressions of VEGFR1 and TUBB3 was studied by the Spearman correlation test. The Kaplan–Meier test was used to analyze the OS, and Cox regression model (LR forward) was employed for univariate and multivariate analysis. Receiver operating characteristic (ROC) analysis was employed for assessing the specificity as well as the sensitivity of predicting OS by specific parameters. The statistical significance of area under the ROC (area under curve, AUC) was calculated by Delong’s test. P-values < 0.05 were deemed significant. The statistical analysis was conducted by SPSS version 22.0 (IBM Corporation, Armonk, NY, United States). The statistical methods of our study were reviewed by Qian-yu Ni from The First Affiliated Hospital of Fujian Medical University.

**RESULTS**

**Patient characteristics**

A total of 75 patients were enrolled in the present study. The characteristics of non-metastatic patients are summarized in Table 1. Median follow-up time was 102.7 mo (range: 6.0–153.6). The cohort comprised of 39 (52%) male and 36 (48%) female cases with the median age 52 years (range, 29–74). Among these patients, 21 (36.8%) cases presented pre-CEA records that were higher than 5 ng/mL, while they could not be accessed for 18 cases. In the case of pre-Hb, 26 (34.7%) patients were ≤ 120 g/L, and the remaining were > 120 g/L. In terms of the tumor location, 46 (61.3%) patients had low rectal cancer (0–5 cm distance to verge), while the other 29 (38.7%) patients were > 5 cm. In all, 22 (29.3%) with lymph node metastasis positive and 53 (70.6%) were negative. Twenty (26.7%) patients were identified as poorly differentiated and 55 (73.3%) as moderate-to-well differentiated. According to the cutoff Finder software, 0.0575 and 0.2025 were regarded as the optimal cutoff point for the VEGFR1 and TUBB3 expression value, respectively (Figure 1). In addition, 36 (48%) and 22 (29.3%) patients showed a high expression of VEGFR1 and TUBB3, respectively.

**Associations between mRNA expression and clinicopathological features**

The correlations between VEGFR1/TUBB3 mRNA expre-
Moreover, lymph node metastasis (HR = 3.042, 95%CI: 1.137-8.142, \( P = 0.027 \)) and VEGFR1 (HR = 2.151, 95%CI: 1.062-4.355, \( P = 0.033 \)) were independent factors influencing OS, as evaluated by the multivariate Cox regression model.

Prognostic value of different combinations on survival

VEGFR1 and TUBB3 expression were positively correlated (\( P = 0.006, r = 0.315 \)) by the Spearman's correlation test. Both low expression of VEGFR1 and TUBB3 were observed in 32 (42.6%) cases. Moreover, the Kaplan-Meier analysis showed that the 1-, 3-, and 5-year OS of both low-expression patients vs others were 96.9% vs 93.0%, 84.4% vs 53.5%, and 78.1% vs 46.5%, respectively (\( P = 0.003 \), Figure 3C). Meanwhile, the Kaplan-Meier analysis showed that the rates of 1-, 3-, and 5-year OS in positive lymph node metastasis patients with high expression of VEGFR1 vs others were 90.0% vs 98.2%, 35.0% vs 78.2%, and 30.0% vs 70.9%, respectively (\( P < 0.001 \)) (Figure 3D).

Finally, we combined the two independent prognostic factors, lymph node metastasis and VEGFR1 expression, to construct a prognostic model and supplemented the VEGFR1 expression to the lymph node metastasis and clinicopathological features were analyzed (Table 2). A majority of the patients displayed positive lymph node metastasis in the high-expression group of VEGFR1 (\( P = 0.013 \)). However, no significant difference was found between the expression level of TUBB3 expression and clinicopathological features (gender, age, pre-CEA, pre-Hb, distance to the verge, T stage, lymph node metastasis and venous invasion, all \( P > 0.05 \)).

Impact of VEGFR1 and TUBB3 on OS

The Cox regression analysis of OS influencing factors was shown in Table 3. Univariate analysis showed that T stage, lymph node metastasis, tumor differentiation, and VEGFR1 and TUBB3 expression were significantly related to OS (\( P = 0.048, P = 0.003, P = 0.052, P = 0.003 \) and \( P = 0.015 \), respectively) (Figures 2, 3 A and B). Moreover, Kaplan-Meier analysis showed that the rates of 1-, 3-, and 5-year OS in the TUBB3 low- and high-expression groups were 94.9% vs 94.4%, 76.9% vs 52.8%, and 71.8% vs 47.2%, respectively (\( P = 0.017 \)). The rates of OS in the VEGFR1 low- and high-expression groups were 98.1% vs 86.4%, 77.4% vs 36.4%, and 69.8% vs 36.4%, respectively (\( P = 0.003 \)).

Moreover, lymph node metastasis (HR = 3.042, 95%CI: 1.137-8.142, \( P = 0.027 \)) and VEGFR1 (HR = 2.151, 95%CI: 1.062-4.355, \( P = 0.033 \)) were independent factors influencing OS, as evaluated by the multivariate Cox regression model.

**Figure 1** Distribution-based cutoff optimization of vascular endothelial growth factor receptor 1 and class III \( \beta \)-tubulin expression value in 75 non-metastatic rectal cancer patients. A: Histograms of vascular endothelial growth factor receptor 1 (VEGFR1) expression value; B: Waterfall plot of optimal dichotomization for VEGFR1 expression value; C: Histograms of class III \( \beta \)-tubulin expression value; D: Waterfall plot of optimal dichotomization for VEGFR1 expression value.
by ROC analysis to assess the improvement of the model for OS. The lymph node metastasis (AUC: 0.688, 95%CI: 0.567–0.808, \( P = 0.005 \)) showed a better prognostic value than VEGFR1 expression (AUC: 0.635, 95%CI: 0.507–0.764, \( P = 0.045 \)). Furthermore, a better prognostic value was shown when combining the lymph node metastasis and VEGFR1 expression (AUC: 0.748, 95%CI: 0.637–0.859, \( P < 0.001 \)) (Figure 4).

DISCUSSION

Firstly, we evaluated the long-term prognostic value of VEGFR1 and TUBB3 expression after the diagnosis of non-metastatic rectal cancer with a median follow-up of 102 mo. Here, we found that VEGFR1 and TUBB3 expression affected OS in non-metastatic rectal cancer by univariate analysis. Moreover, a favorable OS in both low expression of VEGFR1 and TUBB3 was noted as compared to others. In addition, the association between VEGFR1 expression and lymph node metastasis was also assessed. The combination of lymph node metastasis and VEGFR1 expression might also provide a promising tool for the prognosis of non-metastatic rectal cancer.

Reportedly, VEGFR correlates with poor prognosis, metastasis, and recurrence in various tumor types, including breast and lung cancers\[^{14,15}\]. Moreover, previous studies demonstrated that VEGF plays a crucial role as a potent angiogenic factor in both experimental and human studies with respect to colorectal cancer progression and metastasis\[^{16-18}\]. The co-expression of VEGF and VEGFR1/2 in the nucleus stimulates the proliferation and migration of endothelial cells, thereby providing nutrition for growing tumors and establishing a continuity between tumor cells and host vasculature\[^{19}\].

VEGFR1 is primarily localized in the nucleus of endothelial cells; As the predominant receptor of the tumor microenvironment, it is essential for the survival of endothelial cells\[^{20}\]. Tsai et al\[^{21}\] reported that the overexpression of VEGF is a significant positive predictor for early postoperative relapse in stage I - III colorectal cancer patients, leading to poor OS (\( P = 0.002 \)). Similarly, Nriagu et al\[^{22}\] reported that the overexpression of VEGF mRNA was an independent factor affecting OS as assessed by multivariate analysis (HR = 1.94, \( P = 0.005 \)). Herein, we found that the low expression of VEGFR1 might positively affect OS with a 5-year OS of 69.8% for low

| Parameter                        | TUBB3   | VEGFR1  |
|----------------------------------|---------|---------|
|                                  | Low (n) | High (n) | P   | Low (n) | High (n) | P   |
| Gender                           |         |         |     |         |         |     |
| Female                           | 17      | 19      | 0.425 | 22      | 14      | 0.081 |
| Male                             | 22      | 17      | 0.31  | 31      | 8       |      |
| Age (yr)                         |         |         |     |         |         |     |
| \(< 60\)                         | 32      | 26      | 0.023 | 41      | 17      | 0.244 |
| \(> 60\)                         | 7       | 10      | 1    | 12      | 5       |      |
| Pre-CEA                          |         |         |     |         |         |     |
| \(\leq 5\)                       | 20      | 16      | 0.203 | 26      | 10      | 0.005 |
| \(> 5\)                          | 8       | 13      | 0.013 | 12      | 9       |      |
| Pre-Hb                           |         |         |     |         |         |     |
| \(\leq 120\)                     | 13      | 13      | 0.608 | 16      | 10      | 0.744 |
| \(> 120\)                        | 26      | 23      | 0.007 | 37      | 12      |      |
| Distance to verge (cm)           |         |         |     |         |         |     |
| \(\leq 5\)                       | 25      | 21      | 0.883 | 32      | 14      | 0.792 |
| \(> 5\)                          | 14      | 15      | 0.013 | 21      | 8       |      |
| T stage                          |         |         |     |         |         |     |
| T1 + T2                          | 7       | 6       | 0.754 | 10      | 3       | 0.939 |
| T3 + T4                          | 32      | 30      | 0.051 | 43      | 19      |      |
| Lymph node metastasis            |         |         |     |         |         |     |
| Negative                         | 15      | 7       | 0.156 | 20      | 2       |      |
| Positive                         | 24      | 29      | 1    | 33      | 20      |      |
| Tumor thrombus                   |         |         |     |         |         |     |
| Negative                         | 37      | 31      | 0.25  | 48      | 20      |      |
| Positive                         | 2       | 5       | 1    | 5       | 2       |      |
| Tumor differentiation            |         |         |     |         |         |     |
| Poorly                           | 11      | 9       | 0.024 | 14      | 6       |      |
| Moderately-well                  | 28      | 27      | 0.051 | 39      | 16      |      |
| Chemotherapy                     |         |         |     |         |         |     |
| No                               | 7       | 2       | 0.156 | 9       | 0       |      |
| Yes                              | 32      | 34      | 1    | 44      | 22      |      |

Pre-CEA: Preoperative carcino-embryonic antigen; Pre-Hb: Preoperative-hemoglobin.
that lymph node metastasis (positive vs negative, \(P < 0.001\)) and TNM stage (stage III vs I/II, \(P < 0.001\)) were related to increased VEGF expression. Moreover, the mean number of metastatic nodes was significantly associated with VEGF expression (1.06 ± 2.84 for low expression vs 2.45 ± 4.03 for high expression, \(P = \ldots\)

Pre-CEA: Preoperative carcino-embryonic antigen; Pre-Hb: Preoperative hemoglobin.

| Variables                      | Univariate |             |       | Multivariate |             |       |
|-------------------------------|------------|-------------|-------|--------------|-------------|-------|
|                               | HR         | 95%CI       | \(P\) | HR           | 95%CI       | \(P\)  |
| Gender                        |            |             |       |              |             |       |
| Female/male                   | 1.018      | 0.519-1.997 | 0.958 |              |             |       |
| Age                           |            |             |       |              |             |       |
| ≤ 60/> 60                     | 1.175      | 0.548-2.518 | 0.679 |              |             |       |
| Pre-CEA                       |            |             |       |              |             |       |
| ≤ 5/> 5                       | 1.067      | 0.496-2.298 | 0.868 |              |             |       |
| Pre-Hb                        |            |             |       |              |             |       |
| ≤ 120/> 20                    | 0.651      | 0.328-1.290 | 0.219 |              |             |       |
| Distance to verge (cm)        |            |             |       |              |             |       |
| ≤ 5/> 5                       | 1.265      | 0.642-2.491 | 0.497 |              |             |       |
| T stage                       |            |             |       |              |             |       |
| T1 + T2/T3 + T4               | 4.221      | 1.011-17.632 | 0.048 | 4.05         | 0.968-116.93 | 0.055 |
| Lymph node metastasis         |            |             |       |              |             |       |
| Negative/positive             | 6.247      | 1.905-20.491 | 0.003 | 3.042        | 1.137-8.142 | 0.027 |
| Tumor thrombus                |            |             |       |              |             |       |
| Negative/positive             | 1.303      | 0.458-3.705 | 0.62  |              |             |       |
| Tumor differentiation         |            |             |       |              |             |       |
| Poorly/moderately-well        | 0.503      | 0.251-1.006 | 0.052 |              |             |       |
| Chemotherapy                  |            |             |       |              |             |       |
| No/yes                        | 1.407      | 0.430-4.605 | 0.572 |              |             |       |
| TUBB3 expression              |            |             |       |              |             |       |
| Low/high                      | 2.407      | 1.188-4.877 | 0.015 |              |             |       |
| VEGFR1 expression             |            |             |       |              |             |       |
| Low/high                      | 2.817      | 1.424-5.570 | 0.003 | 2.151        | 1.062-4.355 | 0.033 |

A previous study evaluated VEGF expression in 117 colorectal adenocarcinoma patients, and confirmed that lymph node metastasis (positive vs negative, \(P < 0.001\)) and TNM stage (stage III vs I/II, \(P < 0.001\)) were related to increased VEGF expression. Moreover, the mean number of metastatic nodes was significantly associated with VEGF expression (1.06 ± 2.84 for low expression vs 2.45 ± 4.03 for high expression, \(P = \ldots\)

Figure 2 Kaplan-Meier survival curves of overall survival. A: T stage (T1 + T2 vs T3 + T4, \(P = 0.031\)); B: Lymph node metastasis (negative vs positive, \(P = 0.003\)); C: Tumor differentiation (poorly differentiated vs moderately-well differentiated, \(P = 0.052\)).

vs 36.4% for the high-expression group (HR = 2.151, \(P = 0.033\)). These results indicated that VEGFR1 functions as a positive regulator of angiogenesis\(^{[23]}\), which might lead to poor survival in cancer patients.

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VEGFR1 expression (low stage was 50% for VEGF-positive TUBB3 and non-metastatic rectal cancer are limited. The however, studies focusing on the relationship between patients treated with taxane-based chemotherapies was related to low response rate and poor survival in angiogenesis, and VEGFR1 is known to be involved in an adaptive response to low oxygen levels and poor nutrient supply in solid tumors. Therefore, we speculate that the underlying mechanism of the two correlations might be related to anoxic environments. Notably, this study was limited to a small-sample retrospective analysis. Thus, additional mRNA expression data might help to establish a superior predictor. Finally, prospective data and large sample size are essential for further substantiation of the results. We confirmed that the increased expression of VEGFR1 and TUBB3 might be negatively correlated with long-term prognosis of non-metastatic rectal cancer.
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Research background
Rectal cancer is one of the most common forms of cancer in both men and women. Gene expression profiles in predicting the response and long-term prognosis of malignancies has been reported in recent decades. Vascular endothelial growth factor receptor 1 (VEGFR1) and class III β-tubulin (TUBB3) have been reported to play a vital role in cancer progression. However, few studies focused on their role in rectal cancer.

Research motivation
We try to explore the potential prognostic value of VEGFR1 and TUBB3 for long-term survival in non-metastatic rectal cancer.

Research objectives
A total of 75 patients diagnosed with primary rectal adenocarcinoma without metastases were retrospectively analyzed.

Research methods
Multiplex branched DNA liquidchip technology was applied to detected mRNA expressions of VEGFR1 and TUBB3. The cutoff point of mRNA expression was determined by Cut-off Founder.

Research results
VEGFR1 expression was positively correlated to TUBB3. Patients with both low expression of TUBB3 and VEGFR1 presented a better overall survival (OS). In addition, VEGFR1 and lymph node metastasis had potential as prognostic factors for OS in non-metastatic rectal cancer patients, and the combination of them showed a favorable prognostic value.

Research conclusions
We confirmed that the increased expression of VEGFR1 and TUBB3 might be negatively correlated with long-term prognosis of non-metastatic rectal cancer. Furthermore, VEGFR1 expression and lymph node metastasis affected the survival independently, as well as synergistically. These results might provide additional prognostic information compared to the conventional tumor histopathological factors.

Research perspectives
VEGFR1 has the potential to contribute to decision making regarding individual treatment in rectal cancer. A larger sample size and additional mRNA expression data are warranted to establish a superior prognosis model.

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